

RESEARCH PAPER

# Cloning, functional characterization, and co-expression studies of a novel aquaporin (FaPIP2;1) of strawberry fruit

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## Abstract

In strawberry, the putative participation of aquaporins should be considered during fruit ripening. Furthermore, the availability of different firmness cultivars in this non-climacteric fruit is a very useful tool to determine their involvement in softening. In a previous work, the cloning of a strawberry fruit-specific aquaporin, FaPIP1;1, which showed an expression profile associated with fruit ripening was reported. Here, FaPIP2;1, an aquaporin subtype of PIP2 was cloned and its functional characterization in *Xenopus* oocytes determined. The FaPIP2;1 gene encodes a water channel with high water permeability ( $P_f$ ) that is regulated by cytosolic pH. Interestingly, the co-expression of both FaPIP subtypes resulted in an enhancement of water permeability, showing  $P_f$  values that exceeds their individual contribution. The expression pattern of both aquaporin subtypes in two cultivars with contrasting fruit firmness showed that the firmer cultivar (Camarosa) has a higher accumulation of FaPIP1 and FaPIP2 mRNAs during fruit ripening when compared with the softer cultivar (Toyonoka). In conclusion, not only FaPIP aquaporins showed an expression pattern associated with fruit firmness but it was also shown that the enhancement of water transfer through the plasma membrane is coupled to the presence/absence of the co-expression of both subtypes.

**Key words:** Aquaporin, fruit ripening, PIP, strawberry, water transport.

## Introduction

In recent years, more attention has been paid to the possible role played by aquaporins in fruit water status and its relevance for fruit physiology.

Water movements are crucial during ripening for at least two key events: (i) the rapid expansion of fruit achieved by the accumulation of large amounts of water during the developmental process (Coombe, 1976), and (ii) the loss of turgor associated with fruit ripening after solute accumulation in the apoplast (Wada *et al.*, 2008, 2009). Knowledge about the participation of water movements in these events gives good reasons for the current investigation on the specific involvement of aquaporins in ripening (Chervin *et al.*, 2008; Fouquet *et al.*, 2008; Mut *et al.*, 2008). The

relevance of water channels in cell physiology arise from the fact that aquaporins can give to the cell rapid and reversible changes in its hydraulic conductance by modulating membrane water permeability. For instance, anoxia modifies cytosolic pH with the consequent closure of plasma membrane aquaporin activity, causing a fast reduction of the hydraulic conductance of Arabidopsis roots (Tournaire-Roux *et al.*, 2003). The plasma membrane water permeability is likely to allow cells to equilibrate within seconds in response to changes in apoplastic water potential, in contrast to a fruit development process, that takes days to weeks. However, it has been proposed that, at the tissue and organ levels, many non-steady-state physiological processes

involve water transport through membranes and that even when the kinetics of water equilibration of whole organs can be of the order of hours or days, these time constants would be even longer if aquaporins did not contribute to transmembrane water flow (Tyerman *et al.*, 1999).

Many studies on fruit ripening have focused on the analysis of aquaporin gene expression and their participation at different stages of fruit development has been suggested (Hu *et al.*, 2003; Chervin *et al.*, 2008; Fouquet *et al.*, 2008). However, the functional characterization of fruit aquaporins is still scarce in comparison with data available from gene expression analysis.

In a previous work, a full-length sequence of a PIP1 subtype aquaporin was cloned (FaPIP1;1, accession number GQ390798.1) whose expression increased during strawberry fruit ripening and was negatively regulated by auxins (Mut *et al.*, 2008). However, overexpression of FaPIP1;1 in *Xenopus* oocytes failed to contribute to water transport through the plasma membrane unless it was co-expressed with AtPIP2;3 (Mut *et al.*, 2008). This particular result could be explained by the mechanism of PIP trafficking (Chaumont *et al.*, 2000; Zelazny *et al.*, 2007, 2009). Two different aquaporin subtypes are involved in water transport through the plasma membrane: PIP1 and PIP2. These subtypes displayed different transport activity when expressed alone in *Xenopus* oocytes: while PIP2 increased oocyte membrane water permeability, PIP1 displayed no or low water permeability (Chaumont *et al.*, 2000). This differential behaviour 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 (Zelazny *et al.*, 2007, 2009). These functional features of PIP1 and PIP2 seem to be shared by all plant PIPs studied to date, with the exception of some *Arabidopsis* PIP1s that show moderate water transport (Tournaire-Roux *et al.*, 2003) and a few PIP2s that seems to be unable to increase water permeability when expressed alone in the oocyte plasma membrane (Zhou *et al.*, 2007; Azad *et al.*, 2008).

The fact that FaPIP1;1 is able to reach the oocyte membrane only if a PIP2 aquaporin (in our case AtPIP2;3) is also present (Mut *et al.*, 2008), suggested that a FaPIP2 should be expressed in the same tissue as FaPIP1;1. Unfortunately, the *Fragaria×ananassa* complete genome sequence is not available yet, so the full number and types of aquaporins present in strawberry are still unknown. EST libraries show that PIP2 aquaporins are expected to be expressed in strawberry fruit (<http://www.bioinfo.wsu.edu/gdr/index.php>).

In this work, the cloning of the first-full length sequence of a PIP2 from strawberry fruit is reported, and the expression pattern of FaPIP subtypes during ripening of two cultivars with contrasting softening rate has been analysed. The study also includes the functional characterization of FaPIP2;1 and its interaction with FaPIP1;1.

## Materials and methods

### Plant material

Strawberry (*Fragaria×ananassa*, Duch.) fruit were obtained from local producers (La Plata, Buenos Aires Province, Argentina).

Fruit from Camarosa (high firmness) and Toyonoka (low firmness) cultivars were harvested at different ripening stages and classified according to the external coloration degree: large green (LG), white (W), 25% red (25% R), 50% red (50% R), and 100% red (100% R). Fruits were washed, drained, and after calyx and peduncle removal, they were cut apart, frozen in liquid nitrogen, and stored at  $-20^{\circ}\text{C}$  until use.

### Cloning of FaPIP2;1

The first cloning step of FaPIP2;1 was made with degenerated primers designed by means of CODEHOP online program (Rose *et al.*, 1998) using PIP2 subtype aquaporin sequences available in the GenBank. A cDNA library (Stratagene, La Jolla, CA, USA) constructed from 25–75% R strawberry fruit (cv. Chandler) was used as a template (Civello *et al.*, 1999). Amplification products were cloned into pGEM-T Easy vector (Promega) according to the manufacturer's instructions and sequenced on both strands (Macrogen, Inc., Seoul, Korea). Specific primers designed within the 5' and 3' end of cDNAs corresponding to several PIP2 were used in combination with T3 and T7 primers, respectively. PCR products were cloned into pGEM-T Easy vector and sequenced to obtain the full PIP2 sequence. Finally, the specific primers (5'-GGGAGATCTATGGCGAAAGACGTTG-3') and (5'-GG-GACTAGTTTAAGCATTGCTCCTGAAAGACC-3'), both including *Bgl*III and *Spe*I restriction sites, were used for the last cloning step, and the ORF of FaPIP2;1 was inserted into the *Bgl*III and *Spe*I sites of a pT7s derived vector carrying 5' and 3' untranslated sequences of a  $\beta$ -globin gene from *Xenopus* (Agre *et al.*, 1999). The FaPIP2;1 sequence was deposited in GenBank under the accession number GQ390799.

### Sequence analysis

Analysis of FaPIP2;1 sequence and its comparison with known sequences was carried out using NCBI Blast server (Altschul *et al.*, 1997). The ClustalW program (Thompson *et al.*, 1997) was used for sequence alignment. ESPript was used to generate a PostScript output from aligned sequences (Gouet *et al.*, 1999). Phylogenetic analyses and trees were done using MEGA version 3.0 (Tamura *et al.*, 2007).

### In vitro synthesis and translation

Capped complementary RNAs (cRNA) encoding for FaPIP2;1 and FaPIP1;1 were synthesized *in vitro* using the mMESSAGE mMACHINE T7 High Yield Capped RNA Transcription Kit (Ambion, Austin, Texas, USA) and using *Eco*RI linearized pT7s derived vector carrying the corresponding PIP as the template. AtPIP2;3 (Daniels *et al.*, 1994) was synthesized using mMESSAGE mMACHINE T3 Kit (Ambion Austin, Texas, USA). The synthesized products were suspended in RNase-free water for performing the oocyte microinjection. The quantification of cRNA was done by means of ethidium bromide staining after 0.8% agarose gel electrophoresis. Comparison of band intensities was performed with a marker previously measured by spectrophotometry. The absence of unincorporated nucleotides in all cRNA was also checked by agarose gel electrophoresis and ethidium bromide staining.

### Oocyte transport studies

$P_f$  assays: Defollicled *Xenopus* oocytes were injected with 3–50 ng of cRNA dissolved in RNase free water. Injected oocytes were incubated for 3 d at  $18^{\circ}\text{C}$  in ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM  $\text{MgCl}_2$ , 1.8 mM  $\text{CaCl}_2$ , and 5 mM HEPES pH 7.5;  $\sim 200\text{ mOsmol kg}^{-1}\text{ H}_2\text{O}$ ) supplemented with  $1\ \mu\text{g ml}^{-1}$  gentamicin sulphate. Osmotic water permeability ( $P_f$ ) was determined by measuring the rate of oocyte swelling induced by a hypo-osmotic shock of  $160\text{ mOsm kg}^{-1}$ . Changes in cell volume were video-monitored

by a VX-6000 colour video-camera (Microsoft, CA, USA) attached to a zoom stereo-microscope (Olympus SZ40, Olympus Co., Tokyo, Japan).

The cell swelling was video-captured in still images (each 20 s during 180 s) using the AMCaP version 9.20 (<http://noeld.com/programs.asp?cat=video#AMCap>) and then the images were analysed by treating each oocyte image as a growing sphere whose volume could be inferred from its cross-sectional area (Image Tool version 3, <http://ddsdx.uthscsa.edu/dig/itdesc.html>). The osmotic water permeability ( $P_f$ ) was calculated according to Zhang and Verkman (1991) and Agre *et al.* (1999).

As a negative control, non-injected oocytes were used after checking that they did not show significant differences with water-injected oocytes (data not shown). AtPIP2;3 was used as the positive control in every experiment.

**$P_f$  inhibition assays:** For pH inhibition experiments, the oocyte internal (cytosolic) or external pH was modified as previously described (Tournaire-Roux *et al.*, 2003). Briefly, the oocyte internal or external pH was acidified by pre-incubating them for 15 min in 50 mM sodium acetate (for internal pH modification) or NaCl (for only external pH modification), 20 mM MES pH 6.0 and mannitol until the desired osmolality was achieved (~200 mOsmol kg<sup>-1</sup> H<sub>2</sub>O). In order to reach pH 7.5, MES was replaced by HEPES in the solution described above. The swelling response was performed by transferring the oocyte to the same solution diluted 5-fold with distilled water.

In all treatments, negative controls were performed by submitting non-injected oocytes to the same protocol and the percentage inhibition was calculated using the formula:

$$\text{Inh}(\%) = [1 - (\text{Treatment } P_f - \text{NIP}_f) / (\text{Control } P_f - \text{NIP}_f)] \times 100$$

**Solute transport measurement:** Solute transport measurement was performed as previously described in Soto *et al.* (2008). Briefly, *Xenopus* oocytes were transferred from ND96 solution (200 mosmol kg<sup>-1</sup>) to a 5-fold diluted medium supplemented with the corresponding solute to be tested (boric acid, glycerol, ammonia or urea) up to 200 mOsmol kg<sup>-1</sup> (Hansen *et al.*, 2002; Beitz *et al.*, 2004). Thus, this final solution behaves as an isotonic solution unless the aquaporin is permeable to the solute to be tested. Under these conditions, the increase in oocyte volume is expected to be a consequence of water influx driven by the osmotic gradient caused by the initial solute uptake (chemical gradient).

Solute permeabilities were compared by analysing the initial swelling rates ( $d(V/V_0)/dt$ ). As a control, the correct expression and functional activity of the aquaporin (as a water channel) was previously tested in the same batch of oocytes used in the experiments described above.

#### RNA isolation and Northern blotting assays

Approximately 30 fruits at each ripening stage were harvested, cut in quarters, and immediately frozen. Two independent isolates were made of total RNA from each pool of frozen fruits using the hot borate method (Wan and Wilkins, 1994). For Northern blotting assays, total RNA (10 µg) was electrophoresed on 1.2% (w/v) formaldehyde denaturing agarose gel. To ensure that equal amounts of RNA per lane were loaded, samples were stained with ethidium bromide and individual lanes were evaluated for comparable fluorescence levels upon exposure to a UV light source. After running, the RNA was transferred to Hybond-N<sup>+</sup> nylon membrane (Amersham-Pharmacia Biotech, UK), and cross-linked with an UV-Stratalinker Model 1800 (Stratagene, Texas, USA). Membranes were prehybridized with 25 ml of a solution containing 50% (v/v) formamide, SSPE buffer (50 mM NaCl, 12 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 7.4), 5× Denhart's solution, 150 µg ml<sup>-1</sup> denatured salmon sperm DNA, and 0.5% (w/v) SDS at 42 °C for 4 h and then hybridized overnight at 42 °C with the denatured

radiolabelled probe. The membranes were washed once at 42 °C and twice at 50 °C for 30 min in 25 ml of SSC buffer (15 mM sodium citrate, 150 mM NaCl, pH 7.0) with 0.1% (w/v) SDS. The blot was exposed to X-ray film (X-OMAT AR, Kodak) with an intensifying screen at -80 °C.

Bands corresponding to FaPIP1 and FaPIP2 expression from each ripening stage of both cultivars were analysed by densitometry (Gel Pro Analyzer v 3.0). Relative expression (RE) from two independent samples was analysed as follows: RE = [(FaPIP1 BIX/rRNA BIX)/(FaPIP1 BITLG/rRNA BITLG)] and [(FaPIP2 BIX/rRNA BIX)/(FaPIP2 BITLG/rRNA BITLG)], where BIX represents the band intensity for each ripening stage, and BITLG represents the band intensity for the Toyonoka LG ripening stage.

#### Probe preparation

Probes were prepared by restriction of plasmids T7Ts containing FaPIP1;1 and FaPIP2;1 ORFs with endonucleases *Bgl*III and *Spe*I (Promega, USA). After restriction, the inserts (873 bp and 858 bp for FaPIP1;1 and FaPIP2;1, respectively) were used as templates in a random primer labelling reaction using [<sup>32</sup>P] dATP. After checking probe specificity by dot-blot assays (see Supplementary Fig. S1 at *JXB* online), they were used for Northern blotting experiments.

#### General analytical methods

Osmolarities of all solutions were determined using a vapour pressure osmometer (5520C Wescor, Logan, UT). Chemicals were purchased from Sigma (St Louis, MI, USA) unless otherwise indicated.

#### Statistical analysis

Data for  $P_f$  values were analysed by Student's *t* test at a significance level of 0.05. The heterologous expression of FaPIP1;1 and FaPIP2;1 was performed at least three times with independent batches of oocytes. The figures show one typical experiment, indicating in the legend the number of oocytes employed for each treatment.

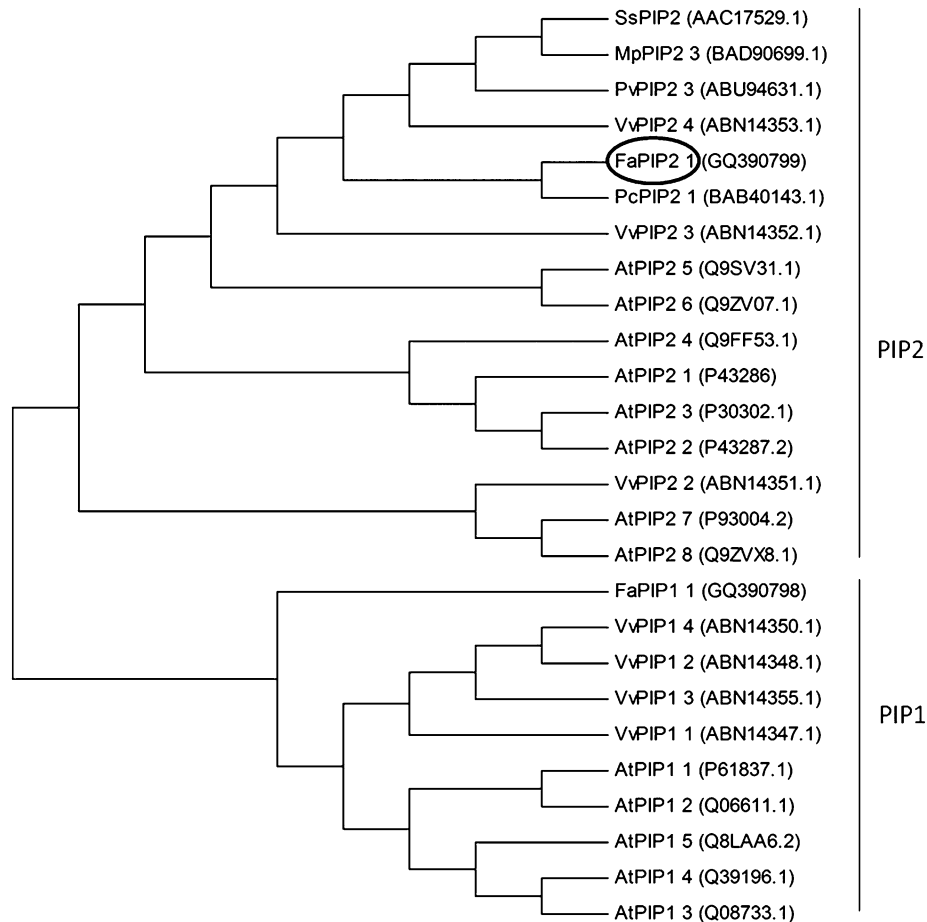
## Results

### Cloning and analysis of FaPIP2;1

An aquaporin was cloned from a library of *Fragaria* × *ananassa* fruit and named FaPIP2;1. Its nucleotide sequence (858 bp) code a protein of 285 amino acids with theoretical PI and MW of 8.6 and 30.2 kDa, respectively, similar to PIP aquaporins from other species (Gomes *et al.*, 2009).

The cloned aquaporin was classified as a PIP2 based on BLASTN sequence analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and named FaPIP2;1 following the nomenclature proposed for plant aquaporins (Johanson *et al.*, 2001). A phylogenetic tree was constructed by including FaPIP2;1 and PIPs from *Arabidopsis thaliana*, *Vitis vinifera*, and other species. As shown in Fig. 1, FaPIP2;1 is clustered with all PIP2.

The predicted amino acid sequence of FaPIP2;1 was aligned and compared with aquaporins from different sources (Fig. 2). The criteria used to select sequences for alignment was to choose not only PIPs with the highest identity to FaPIP2;1, but also PIPs from *Arabidopsis thaliana* and *Zea mays* reported in functional studies.



**Fig. 1.** Phylogenetic analysis of full-length deduced amino acid sequences of plant aquaporins, including our clones, FaPIP2;1 and FaPIP1;1. Deduced amino acid sequences from full-length plant aquaporins encoding genes from *Arabidopsis thaliana*, *Vitis vinifera*, and other plant PIP2s with high identity to FaPIP2;1 were compared using Clustal W. Phylogenetic analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007).

According to BLASTP analysis of sequence identity, the higher hit (91% identity) for FaPIP2;1 was obtained with *Pyrus communis* PIP2;2 (BAB40143.1), that belongs to the same taxonomic family than *Fragaria×ananassa* (Rosaceae) and that is also expressed in its fruit. FaPIP2;1 also presents high identity percentage (>89%) with other PIP2 aquaporins, for example, with *Samanea saman* (PIP2, AC17529.1), *Vitis vinifera* (VvPIP2;4, ABN14353.1), and *Phaseolus vulgaris* (PvPIP2;3, ABU94631.1).

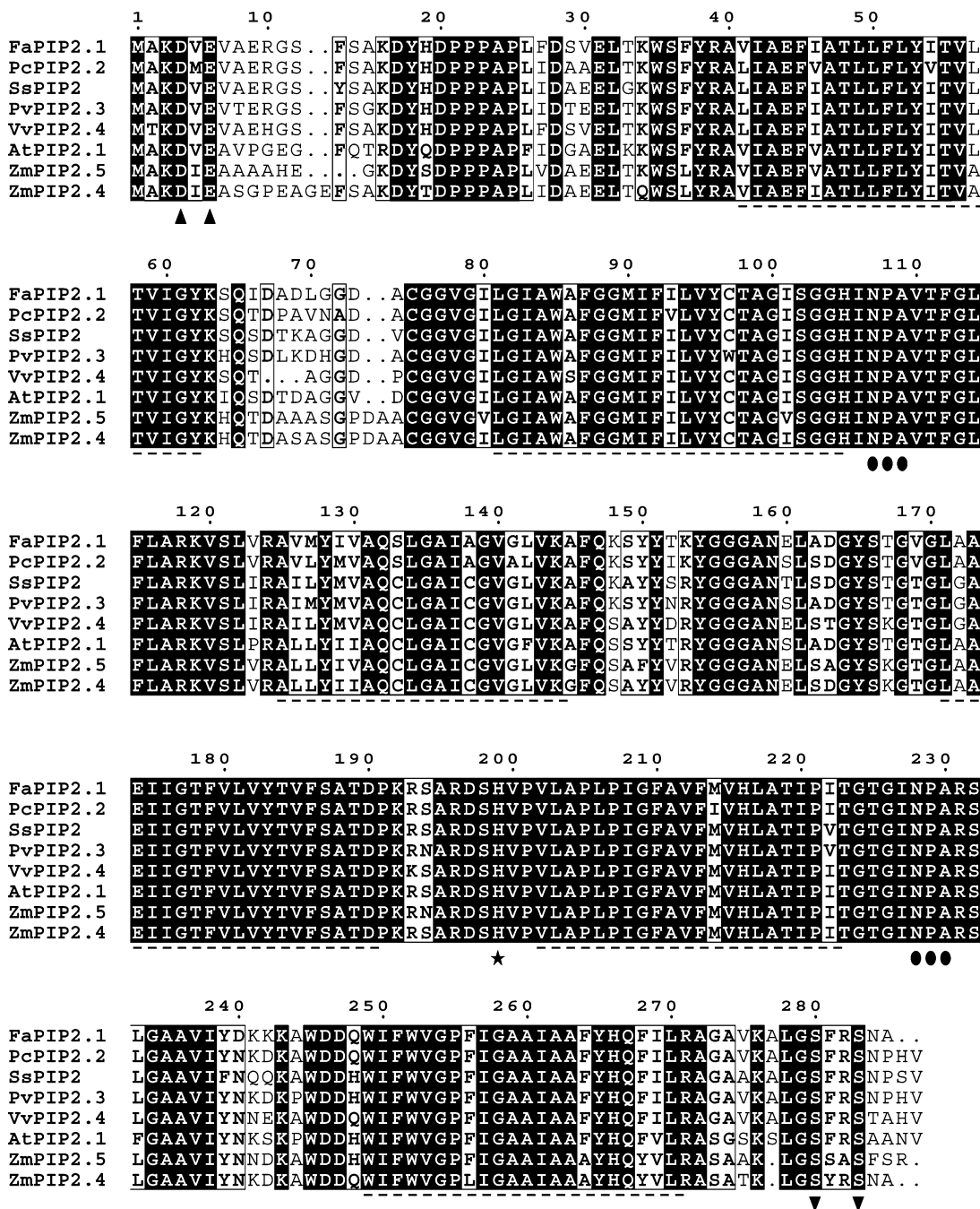
The analysis of the FaPIP2;1 amino acid sequence shows that the protein shares the following features with other reported aquaporins: (i) a hydrophobic profile consistent with six alpha-helical transmembrane domains and five inter-helical loops (predicted by TMHMM, <http://www.cbs.dtu.dk/services/TMHMM/>), (ii) two highly conserved NPA (Asn-Pro-Ala) and the ar/R (Phe-His-Thr-Arg) motifs, both proposed as defining the specificity of the water pore (Forrest and Bhawe, 2007), (iii) the Lys3 and Glu6 amino acids, that were shown to undergo methylation in *At*PIP2;1 (Santoni *et al.*, 2006), (iv) the Ser280 and Ser283 determined as phosphorylated in *At*PIP2;1 (Prak *et al.*, 2008), (v) the pH sensor, in this case His199 (Tournaire-Roux *et al.*, 2003), and (vi) the motif DIE (Asp-Ile-Glu) in position 4–6,

recently proposed to have a putative role in endoplasmic reticulum export signalling (Zelazny *et al.*, 2009).

#### Functional studies of FaPIP2;1 in *Xenopus laevis* oocytes

To analyse water transport capacity, *Xenopus* oocytes expressing FaPIP2;1 were exposed to a hypo-osmotic shock of 160 mOsm kg<sup>-1</sup>. Expression of this aquaporin in *Xenopus* oocytes led to an almost 10-fold increase of the swelling rate compared with the negative control oocytes (Fig. 3). From the calculated  $P_f$  values ( $130 \pm 43 \times 10^{-4}$  cm s<sup>-1</sup>), FaPIP2;1 can be characterized as a water channel with a high water permeability, like the well-known aquaporin *At*PIP2;3 (positive control;  $P_f$   $180 \pm 43 \times 10^{-4}$  cm s<sup>-1</sup>). As expected, when FaPIP1;1 was expressed alone in the same batch of oocytes, the osmotic water permeability remained very low ( $P_f$   $21 \pm 8 \times 10^{-4}$  cm s<sup>-1</sup>).

The transport of small, uncharged solutes across the oocytes expressing FaPIP2;1 was also tested. Urea, glycerol, boric acid, and ammonia permeability were analysed in iso-osmotic swelling assays with an inwardly directed gradient of solute (Beitz *et al.*, 2004; Soto *et al.*, 2008). Oocytes



**Fig. 2.** Alignment of predicted amino acid sequence of *Fragaria x ananassa* aquaporin (FaPIP2;1) with other aquaporins (ClustalX). The predicted amino acid sequence of FaPIP2;1 was compared with aquaporins from different sources (aquaporins with the higher identity with FaPIP2;1 or very well studied in the literature). Transmembrane domains are shown with a dashed line below the alignment; triangles indicate a potential diacidic motif (putative ER signal); circles indicate the NPA selectivity filter; a star indicates His199, and inverted triangles putative phosphorylated Ser residues.

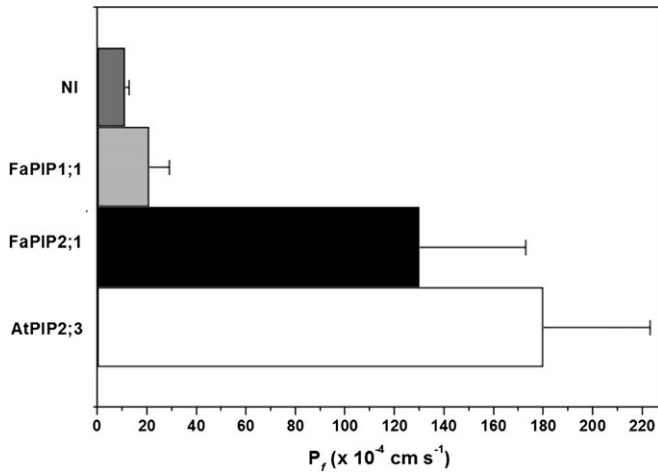
injected with 25 ng of FaPIP2;1 cRNA showed no significant swelling rates for any of the solutes tested (Table 1).

*Co-expression of FaPIP in Xenopus laevis oocytes*

Our next step was to test if FaPIP co-expression enhances water permeability. This approach requires a small amount of the PIP with high  $P_f$  (in our case FaPIP2;1) to be co-injected with a larger amount of the PIP1. Figure 4A clearly

shows that small amounts of FaPIP2;1 cRNA co-injected with FaPIP1;1 cRNA (mass ratio 1:4, in mass values 3 ng:12 ng of FaPIP2;1:FaPIP1;1) increased  $P_f$  to  $181 \pm 23 \times 10^{-4} \text{ cm s}^{-1}$ . This result represents an increment of 79% compared with the  $P_f$  obtained by the injection of 3 ng of FaPIP2;1 alone ( $39 \pm 11 \times 10^{-4} \text{ cm s}^{-1}$ ).

It is interesting to note that, while there is a correlation between the cRNA mass of FaPIP2;1 injected alone and the resulting oocyte  $P_f$ , this correlation is lost when



**Fig. 3.** Functional expression of FaPIP2;1 in *Xenopus* oocytes. Calculated mean water permeabilities ( $P_f \pm \text{SEM}$ ) of oocytes under hypo-osmotic conditions for NI (non-injected, negative control) or expressing 25 ng of FaPIP1;1, FaPIP2;1, and AtPIP2;3 (used as positive control) are shown. The number of measured oocytes for each condition is 5 to 8.  $P_f$  of oocytes expressing both FaPIP2;1 and AtPIP2;3 present significant differences from negative control ( $p < 0.05$ ).

**Table 1.** FaPIP2;1 does not transport solutes when expressed in *Xenopus* oocytes

Most common solutes transported by aquaporins have been tested to be transported through FaPIP2;1. Values are mean  $\pm$ SEM of the time-course volume changes of oocytes for different solute challenges; the number of tested oocytes is in brackets. No transport was detected for any of the assayed solutes. Two independent oocyte batches were used with similar results.

Solute tested	$d(V/V_0)/dt$ ( $10^{-4} \text{ s}^{-1}$ )
Urea	$-0.15 \pm 0.20$ ( $n=7$ )
Boric acid	$-0.44 \pm 0.12$ ( $n=6$ )
Ammonia	$-0.26 \pm 0.14$ ( $n=7$ )
Glycero 1	$0.10 \pm 0.19$ ( $n=10$ )

FaPIP2;1:FaPIP1;1 are co-expressed. Figure 4B shows that oocyte  $P_f$  remained high and similar for all injected cRNA mass ratios.

#### $P_f$ inhibition assays

Highly conserved histidines have been described to trigger a pH inhibitory response, shutting down PIPs when cytosolic medium is acidified (Tournaire-Roux et al., 2003). In order to test if FaPIP2;1 also shows a functional blockage of its activity, oocytes expressing FaPIP2;1 were exposed to different pH values. The results shown in Fig. 5A confirms that FaPIP2;1 partially shuts down water permeability when the oocyte internal pH was acidified. The percentage inhibition when three independent inhibition experiments from different batches of oocytes were pooled is  $\sim 52\%$ . External pH acidification does not modify FaPIP2;1 activity.

The pH inhibitory response on FaPIP co-expression was also tested and, interestingly, in this condition the inhibition

was complete (98%; Fig. 5B). Thus, the partial blockage displayed by FaPIP2;1 alone is replaced by a complete reduction in water transport when both types of aquaporins are present in the oocyte plasma membrane. A similar observation has recently been reported in *Beta vulgaris* PIP (Bellati et al., 2010).

#### Expression of FaPIP2 and FaPIP1 during strawberry fruit ripening

After checking probe specificity, by dot-blot assays (see Supplementary Fig. S1 at *JXB* online), complete FaPIP1;1 and FaPIP2;1 ORFs were used as probes in Northern blot experiments. Due to the high identity between different PIP1 isoforms from a single species, it cannot be confirmed that FaPIP1;1 is distinguishable from other unknown PIP1; then, this probe was considered as a general probe against PIP1. The same was considered regarding the PIP2 subtype and its isoforms.

The expression of both mRNAs was analysed in fruit at different ripening stages from two cultivars: one that produces firm fruit (Camarosa) and another whose fruit are very delicate and soft (Toyonoka) (Fig. 6; see Supplementary Fig. 2 at *JXB* online). The expression of both aquaporins was clearly lower in Toyonoka than in Camarosa during fruit ripening.

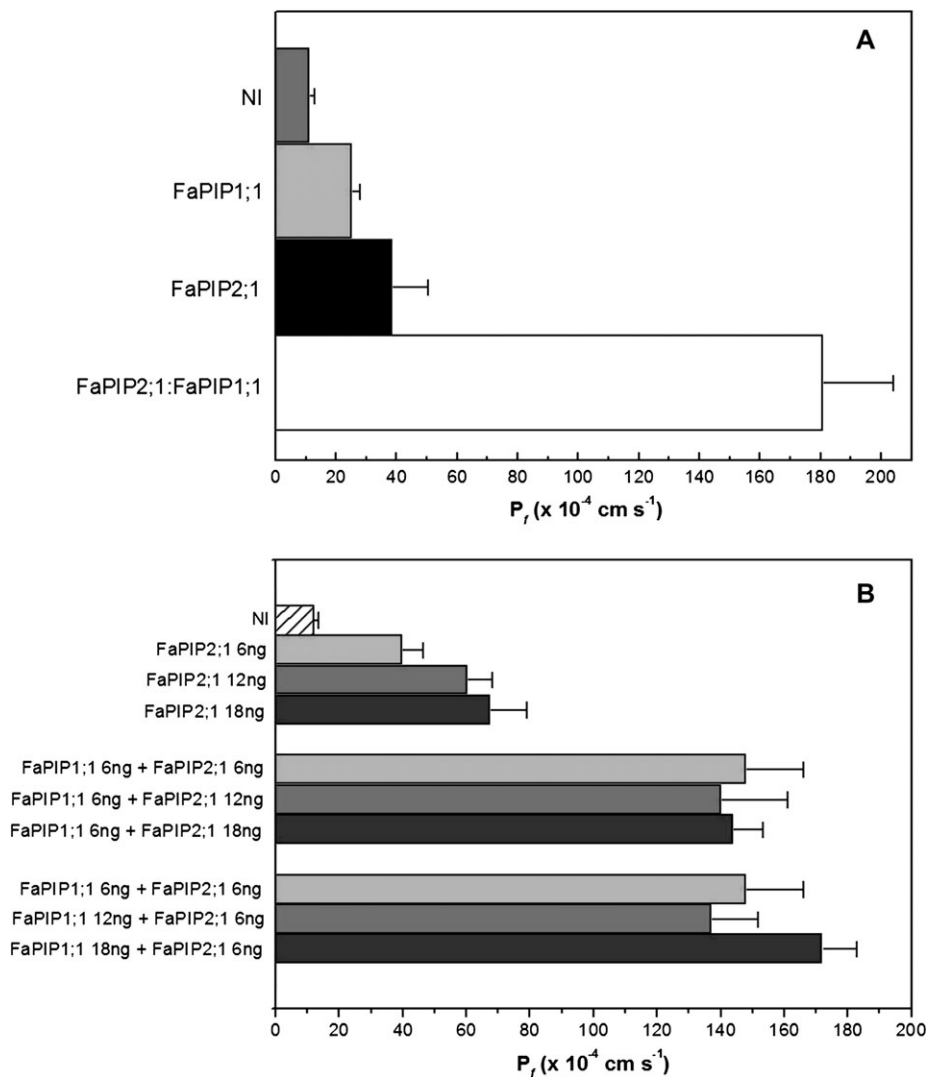
The expression of FaPIP1 in Camarosa was low in the LG stage, increased at W and kept increasing until the end of ripening (100% R). In the case of Toyonoka, the expression of this gene was low in LG, W, and 50% R stages, and increased at 25% R and 100% R stages (Fig. 6A, B).

In the case of FaPIP2, the expression in Camarosa increased markedly from the LG to the W stage, reaching the highest level, and decreasing progressively until the end of ripening (Fig. 6A, B). In Toyonoka, the expression increased slightly in the W stage and then remained approximately constant during ripening (Fig. 6C, D).

## Discussion

To our knowledge, FaPIP2;1 is the first full-length PIP2 aquaporin described in *Fragaria x ananassa*, after the characterization of a root-specific TIP (Vaughan et al., 2006) and FaPIP1;1 (Mut et al., 2008). FaPIP2;1 is a water channel which promotes very high membrane  $P_f$  (Fig. 3), and is unable to transport some solutes already described for other plant aquaporins: urea, ammonia, boric acid or glycerol (Table 1). Although it cannot be dismissed that FaPIP2;1 might be involved in the transport of other non-tested solutes or even ions, this aquaporin seems to be mainly involved in water transport.

The co-injection of cRNA of FaPIP2;1 and FaPIP1;1 significantly raised the oocyte membrane  $P_f$  (Fig. 4A). Despite a correlation between oocyte  $P_f$  and the FaPIP2;1 cRNA mass injected (Fig. 4B), this relationship is not observed when FaPIP2;1 and FaPIP1;1 are co-expressed, and  $P_f$  values remain high and constant in all the different



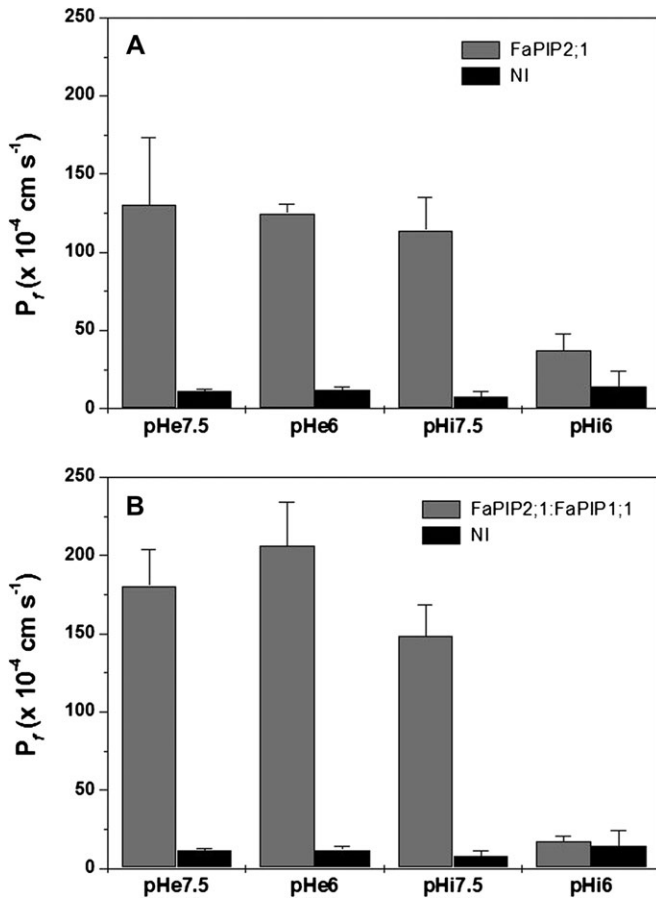
**Fig. 4.** Co-expression of FaPIP2;1 and FaPIP1;1. (A) Co-expression of 3 ng of cRNA of FaPIP2;1 with 12 ng of cRNA of FaPIP1;1 is shown (white bar). As a control, 3 ng of FaPIP2;1 (black bar) and 12 ng of FaPIP1;1 (light grey bar) were injected separately. The co-expression increased significantly the water permeability six times compared with the expression of FaPIP2;1 alone ( $p < 0.05$ ). Data are expressed as mean  $P_f \pm \text{SEM}$ ,  $n=5$  or 6 oocytes. NI are non-injected oocytes (grey bar). (B) Increasing cRNA mass of FaPIP2;1 injected alone in oocytes (from 6 ng to 18 ng) shows an increasing  $P_f$ . This relationship is not observed in FaPIP2;1-FaPIP1;1 co-expressing oocytes.  $P_f$  remains high but constant no matter the cRNA mass ratio injected. Data are shown as mean  $P_f \pm \text{SEM}$ ,  $n=6-8$  oocytes.

mass ratios assayed. This result could be reflecting ER processing of aquaporins when their cRNA are co-injected in the same oocyte (Zelazny *et al.*, 2007). Co-expressed aquaporins from grape berries show a similar response to the one reported here (Vandeleur *et al.*, 2009).

PIP1-PIP2 interaction has been reported both in *Xenopus* oocytes and in living plant cells (Fetter *et al.*, 2004; Zelazny *et al.*, 2007) and as a consequence, if PIP2 is not expressed, PIP1 alone cannot enhance water permeability. It is therefore expected that the modification of FaPIP1;1 and FaPIP2;1 expression profiles during ripening reported here could be another mechanism of controlling plasma membrane water transport. That is, high levels of FaPIP1;1 at the red stage (as in the case of Camarosa) does not guarantee the enhancement of water transport unless FaPIP2;1 is present. On the other hand, although FaPIP2;1 can concede high water permeability to the membrane, the

presence of FaPIP1;1 can co-operatively trigger much higher values. Isolated vesicles of an enriched fraction of plasma membrane from the 100% red stage fruit show very high water permeability values (Mut *et al.*, 2008), which supports our hypothesis.

When oocytes expressing FaPIP2;1 are subjected to cytosolic acidification, membrane  $P_f$  is partially shut down (Fig. 5A). These results are in accordance with the presence of the highly conserved His199 in the FaPIP2;1 sequence, a residue shown to be responsible for water transport blockage under cytosolic acidification in other PIP2 aquaporins (Tournaire-Roux *et al.*, 2003). It is interesting to analyse the reduction of water transport under cytosolic acidification detected for FaPIP2;1 (partial inhibition) compared with FaPIP2;1-FaPIP1;1 co-expression (total inhibition). This differential response of water channels, also found for *Beta vulgaris* aquaporins (Bellati *et al.*, 2010),



**Fig. 5.**  $P_f$  inhibitory response after cytosolic acidification. (A) FaPIP2;1 expressing oocytes were exposed to different external ( $pH_e$ ) or internal ( $pH_i$ ) pH conditions. Negative controls (NI) are non-injected oocytes.  $P_f$  for oocytes expressing FaPIP2;1 exposed to internal acidification is statistically different from its control (i.e.  $pH_i=7.5$ ), while treatment with  $pH_e=6.0$  does not result in a significant inhibition ( $p < 0.05$ ) when compared with its control ( $pH_e=7.5$ ). (B) FaPIP2;1-FaPIP1;1 was co-expressed in *Xenopus* oocytes and exposed to different external ( $pH_e$ ) or internal ( $pH_i$ ) pH conditions. Negative controls (NI) are non-injected oocytes. Data are shown as mean  $P_f \pm \text{SEM}$ ,  $n=8-10$  oocytes.

could reflect the faculty of the cell to turn partial water transport to zero water transport through aquaporins under cytosolic acidification depending on the PIP2-PIP1 relative expression in the plasma membrane. So, not only the co-expression of aquaporins, but also the pH response of the assembly formed by this co-expression widens interestingly the modulating possibilities of water transport cell control.

Aquaporin gene expression during ripening might be associated with the main mechanisms involved in cell division and cell expansion. The water channel expression pattern during ripening has been studied in grape berry, a non-climacteric fruit (Fouquet et al., 2008). In a previous work, it was shown that the expression of FaPIP1;1 varies with the ripening stages of the strawberry cultivar Selva (Mut et al., 2008). Four genes annotated as aquaporins (according to the putative function of its closest NCBI database homologues) have been detected as expressed

preferentially in strawberry fruit receptacles, with higher expression in the red stage (three out of four genes studied) compared with the green stage or with a higher expression in turning/white stage (one of four genes studied) (Aharoni et al., 2002).

In ripening, despite cell division and cell expansion, fruit softening is also an important feature. Cell wall disassembly is a key element determining softening; however, softening has also been postulated as a physical consequence of a reduction in cell turgor (Thomas et al., 2006; Saladiè et al., 2007; Wada et al., 2008, 2009). Turgor diminution can be attributed to the water loss that follows apoplastic solute accumulation occurring during ripening (Wada et al., 2008). All this evidence supports the idea that a decline of fruit turgor, in addition to cell wall degradation, could contribute to fruit softening.

In strawberry it has been reported that cell turgor values decline from 250 kPa in green-white fruit to 50 kPa in pink fruit mainly due to high solute accumulation in the apoplast (Pomper and Breen, 1995). In this sense, the osmotic gradient generated by solute accumulation in the apoplast could lead to fruit turgor reduction and aquaporins (if expressed mainly in the receptacle tissue as shown by Aharoni et al., 2002) could speed up water outflow from cells.

Here FaPIP1 and FaPIP2 mRNA accumulation during ripening of firm (Camarosa) and soft (Toyonoka) strawberry cultivars has been evaluated. Gene expression of FaPIP1 and FaPIP2 increase in W or 25% R stages in both cultivars, coincidentally with the main firmness decrease that occurs at the early ripening stages (Rosli et al., 2004, 2009; Villarreal et al., 2008) (Fig. 6).

Higher levels of both FaPIP1 and FaPIP2 were detected in the firmer cultivar (Camarosa) than the softer one (Toyonoka). It is worth mentioning that, in strawberry fruit, the extension of cell wall disassembly is cultivar-dependent (Rosli et al., 2004, 2009; Bustamante et al., 2006; Villarreal et al., 2008). Some correlations between a higher expression of genes related to cell wall degradation and a higher fruit softening have been found. Among them, the cultivar Toyonoka shows a higher and earlier expression of a polygalacturonase and two expansin genes, compared with the Camarosa cultivar (Dotto et al., 2006; Villarreal et al., 2008). Assuming that both cell wall disassembly and cell turgor contribute to fruit softening, it is possible to hypothesize that loss of cell turgor mediated by aquaporins had a higher contribution to Camarosa softening, while in the Toyonoka cultivar the main source of fruit softening would be cell wall disassembly.

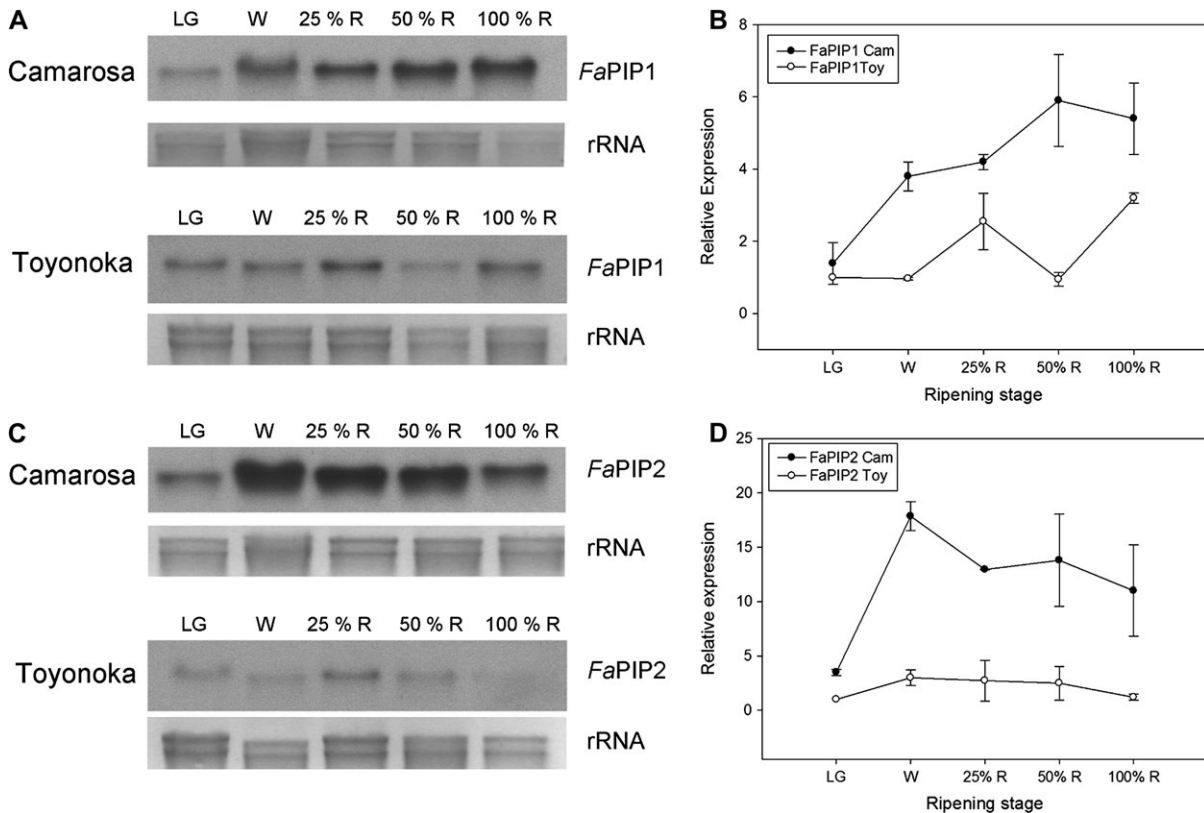
In this work it is speculated that, if the main target tissue of PIPs expression in strawberry fruit is the receptacle parenchyma, fruit softening could be associated not only with cell wall disassembly but also to loss of cell turgor mediated by water flow through aquaporins (Fig. 7).

In conclusion, oocyte experiments proved (i) that FaPIP2;1 is a highly active water channel able to reduce its water transport activity by cytosolic acidification but insensitive to external (in plants, apoplastic) acidification,

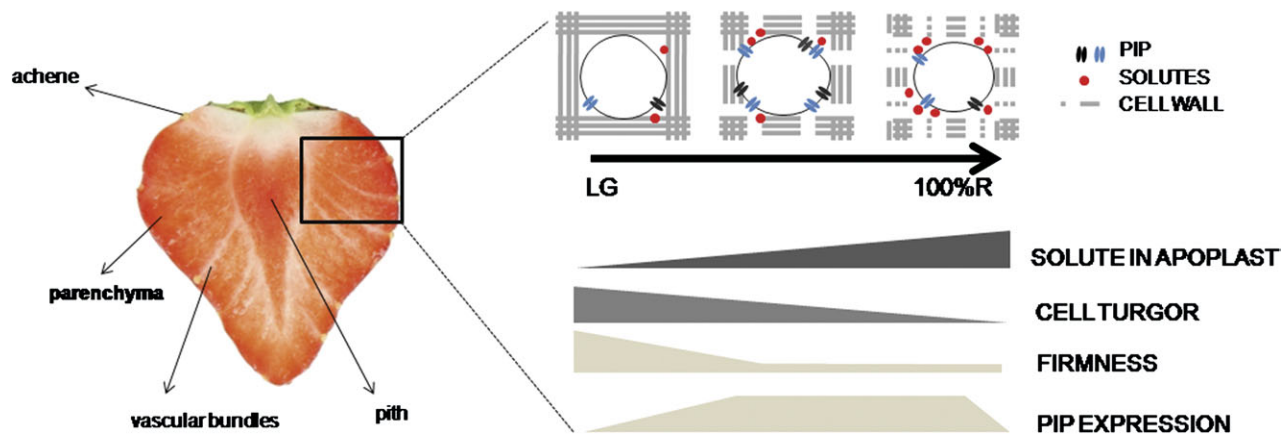


and (ii) that FaPIP2;1 could interact with FaPIP1;1 not only modifying final plasma membrane  $P_f$  but also pH response. In addition, both aquaporins are expressed (at different levels) through all the ripening stages of strawberry in two cultivars with different firmness. These results suggest that a complex regulation of water transport depending on the PIP1 and PIP2 rate expression profile could play a role in fruit physiology. Our results open the

field of aquaporin participation in specific ripening events and their coupling with fruit softening. In this context, cell wall degradation and water exchange mediated by aquaporins could be juxtaposed and even integrated events during ripening. Further work will help to clarify how specific target tissues request changes in plasma membrane water transport activity at the cell level to impact on fruit water balance.



**Fig. 6.** FaPIP1 and FaPIP2 expression pattern during strawberry fruit ripening. (A, C). Northern blot showing the accumulation of FaPIP mRNA in different ripening stages of strawberry fruits: large green (LG), white (W), 25% red (25%R), 50% red (50%R), and 100% red (100%R) for two different cultivars, a firm (Camarosa) and a softer one (Toyonoka). (B, D) Quantification of FaPIP1 and FaPIP2 expression relative to Toyonoka LG stage is shown for Camarosa and Toyonoka cultivars (Gel Pro Analyzer v 3.0 was used). Data are shown as mean  $\pm$  SEM,  $n=2$ .



**Fig. 7.** Hypothetical and schematic representation of the role of aquaporins in fruit ripening. Section of a strawberry fruit showing fibrovascular strands (vascular bundles), achenes, the interior of the receptacle (pith), and main parenchyma; all marked tissues are possible targets of PIP expression due to their participation in water and solute balance in strawberry fruit.

## Supplementary data

Supplementary data can be found at *JXB* online.

**Supplementary Fig. S1.** Dot blot assays after hybridization with the *FaPIP1;1* and *FaPIP2;1* probes.

**Supplementary Fig. S2.** The expression of both mRNAs in fruit at different ripening stages from the two cultivars, Camarosa and Toyonoka.

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