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Expression analysis of genes encoding plasma membrane aquaporins during seed and fruit development in tomato

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Abstract

Aquaporins facilitate water flux across biomembranes in plant cells, and are involved in various physiological phenomena in several plant tissues. To determine whether aquaporins have physiological roles during seed development, we analyzed the expression of genes encoding plasma membrane intrinsic proteins (PIPs) during seed and fruit development in tomato (*Lycopersicon esculentum* Mill.). Six genes encoding PIPs were detected in mature tomato seeds by RT-PCR using PCR primers corresponding to conserved transmembrane domains and NPA motifs. The expression of these genes in developing seeds and fruit was analyzed by RT-PCR using primers specific for nine tomato *PIP*s, including the six *PIP*s detected and an additional three *PIP*s from the tomato EST database. Increased expression of seven *PIP*s was detected during the earlier phase of seed development [12–32 days after flowering (DAF)], and the expression of these genes decreased during the later phase (36–56 DAF). Each tomato *PIP* showed a distinct expression pattern during fruit development. In addition, the water content of the cells was calculated. The seed water content decreased gradually in the earlier phase of seed development (12–32 DAF), and was subsequently maintained at 44–50% from 36 to 56 DAF, whereas the water content of the fruit remained at 90% throughout fruit development. These results suggest that plasma membrane aquaporins play a physiological role during seed and fruit development in tomato.

Keywords: Aquaporin; *Lycopersicon esculentum* Mill.; Seed development

1. Introduction

Aquaporins are transmembrane proteins that function as channel proteins, selectively allowing the flow of water molecules across biological membranes [1,2]. Genome projects have revealed that more than 30 aquaporins exist in higher plants [3,4]. The plant aquaporins are classified into plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs), and Nod26-like intrinsic proteins (NIPs). The genes encoding plant aquaporins are expressed in several plant tissues, and are regulated by certain environmental stress factors, such as cold, drought, and salinity [1,2,5]. In plants, water is indispensable for photosynthesis, growth, and development. Therefore, the various aquaporins are probably involved in the maintenance of adequate water levels in various tissues at different developmental stages.

Seed formation is a distinct developmental stage of higher plants. Seeds show dormancy with desiccation in the later phase of seed maturation [6]. Generally, dry seeds contain very low levels of water, with a water content below 10% [6]. When seeds desiccate, the water supply from the maternal plant may be blocked by an abscission zone, and the water in the cells diffuses into the air [6]. Little is known, however, about the roles of aquaporins in seed dehydration. It has been suggested that plasma membrane aquaporins (PIPs) are involved in rehydration during seed germination because their expression increases in germinating seeds, whereas very weak expression of *PIP*s is observed in dry seeds [7–9]. Therefore, we hypothesized that plasma membrane aquaporins are involved in water flux of developing seeds, as well as in their rehydration upon germination.

In this study, we analyzed the expression of genes encoding plasma membrane aquaporins in tomato seeds. Developing tomato seeds are not directly exposed to air because they are

Abbreviation: DAF, days after flowering

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Table 1
Primer pairs used for RT-PCR

Gene name	Forward primers (5′–3′)	Reverse primers (5′–3′)	T_m (°C)	Amplicon size (bp)
*PIP*s	AQ-S1 (5′-gtctactgcacggccggtat-3′)	AQ-A3 (5′-ggaatagtagccaaatgcac-3′)	58	380
*PIP*s	AQ-S2 (5′-ggtcacattaacccagctgtg-3′)	AQ-A4 (5′-cttctagctgggttgatacc-3′)	60	386
LePIP1-1	LAQ11-S (5′-aggatctgaattcaatcattt-3′)	LAQ11-A (5′-cggaaaaggcaaggtact-3′)	54	183
LePIP1-2	LAQ12-S (5′-agagttgaagagacctttta-3′)	LAQ12-A (5′-gaatctttcacttgatttgga-3′)	54	160
LePIP1-3	LAQ13-S (5′-aagagtctcaaaccatttttct-3′)	LAQ13-A (5′-catttaaagaagcttttacatac-3′)	58	125
LePIP1-4	LAQ14-S (5′-gacacacaaagcaaaaagctg-3′)	LAQ14-A (5′-tccaataggccaaggtgttc-3′)	60	937
LePIP1-5	LAQ15-S (5′-gttatcagtctttaaacaatg-3′)	LAQ15-A (5′-tcaaagataaaaataaagaacc-3′)	54	207
LePIP2-1	LAQ21-S (5′-caactttcattcacaagttca-3′)	LAQ21-A (5′-agaatagaccaccaactcta-3′)	56	181
LePIP2-2	LAQ22-S (5′-caataactaaagcattcaattg-3′)	LAQ22-A (5′-atatgaccaatactaataagga-3′)	56	170
LePIP2-3	LAQ23-S (5′-cagagcatactctgttttccc-3′)	LAQ23-A (5′-ccatatctattgtagtatgcact-3′)	62	538
LePIP2-4	LAQ24-S (5′-gctggtacccgggaattc-3′)	LAQ24-A (5′-attggaatagtggccaaatgaa-3′)	58	764
LeEF1	LeEF1-F (5′-tggccctactggtttgacaactg-3′)	LeEF1-R (5′-cacagttcacttccccttcttctg-3′)	55	550

covered with fruit tissues that contain a large amount of water. Consequently, developing tomato seeds were predicted to show changes in water content and gene expression based on a developmental program, but were thought to be unaffected by environmental factors. Previous studies have revealed the tomato aquaporins tomato ripening-associated membrane protein (TRAMP) and LeAqp2, but the expression of these genes in seeds has not been determined [10–12]. Furthermore, the expression of tomato *PIP*s in seeds has not been observed through expressed sequence tag (EST) analyses [13,14]. In this report, we present the first evidence of the expression of seven tomato *PIP*s during seed development.

2. Materials and methods

2.1. Plant materials

Mature leaves and developing fruit were collected from tomato (*Lycopersicon esculentum* Mill. cv. Sugarlamp) plants grown in the experimental field at Yokohama City University (Kanazawa-hakkei, Yokohama, Japan). The small fruits of this tomato cultivar (30 mm in diameter) are mature at 50–60 days after flowering (DAF). Pericarp (mesocarp and endocarp), loculus tissue, and seeds were dissected from fruits harvested on various DAF. The tissues were measured, weighed, and stored at −80 °C before use.

2.2. Calculation of water content

The weights of the pericarp (mesocarp and endocarp), loculus tissue, and seeds at various DAF were recorded both before (fresh weight) and after (dry weight) desiccation. The tissues were desiccated for 24 h in a freeze–dryer (Asahi Kagaku, Tokyo, Japan). At least 0.1 g of tissue was used for each sample. Water content (%) was defined as [(fresh weight − dry weight)/fresh weight] × 100.

2.3. Isolation of RNA from tomato tissues and reverse-transcription PCR (RT-PCR)

Total RNA was isolated from developing fruit (mesocarp, endocarp, and loculus tissue), developing seeds, and mature leaves using the phenol/SDS method [15]. The total RNA was treated with RNase-free DNase I. First-strand cDNA mixtures were prepared from 1 μg of total RNA with oligo(dT_{15}) primer and AMV reverse transcriptase XL (Takara Bio, Ohtsu, Japan). The cDNA synthesized from 30 ng of total RNA and 12 pmol of the PCR primers shown in Table 1 was used for PCR analysis.

To isolate partial cDNA fragments of tomato *PIP* genes, RT-PCR was performed using mRNA from mature seeds. PCR primers were constructed from the nucleotide sequences of 13 *Arabidopsis PIP*s (AGI codes At3g61430, At2g45960, At1g01620, At4g00430, At4g23400, At3g53420, At2g37170, At2g37180, At5g60660, At3g54820, At2g39010, At4g35100, and At2g16850) [4]. The PCR primers AQ-S1 and AQ-A3 were constructed to correspond to the second and fifth transmembrane domains, and AQ-S2 and AQ-A4 were designed to correspond to the NPA motifs (Table 1). DNA was amplified using the following conditions: 1 cycle of 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 40 °C for 30 s, and 72 °C for 90 s (Table 1). The amplified products were subcloned into the pCRII vector (Invitrogen, Carlsbad, CA, USA).

To analyze the expression of the tomato *PIP* genes, specific PCR primers were designed for *LePIP1-1*, *LePIP1-2*, *LePIP1-3*, *LePIP1-4*, *LePIP1-5*, *LePIP2-1*, *LePIP2-2*, *LePIP2-3*, *LePIP2-4*, and *LeEF-1* (Table 1) [16]. PCR amplification was performed using these primers and template cDNA from developing seeds, developing fruit, and mature leaves under the following conditions: 1 cycle of 94 °C for 2 min, followed by 25 or 30 cycles of 94 °C for 30 s, 54–62 °C for 30 s, and 72 °C for 60–90 s (Table 1). The primer pairs for each gene yielded an abundant single band.

2.4. Sequencing cDNA clones

To sequence the cDNA clones, double-stranded plasmid DNA was isolated and sequenced using the dye terminator cycle sequencing method following the protocol of the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

3. Results and discussion

Several reports describe the expression of genes encoding plasma membrane aquaporins in the seeds of some plant

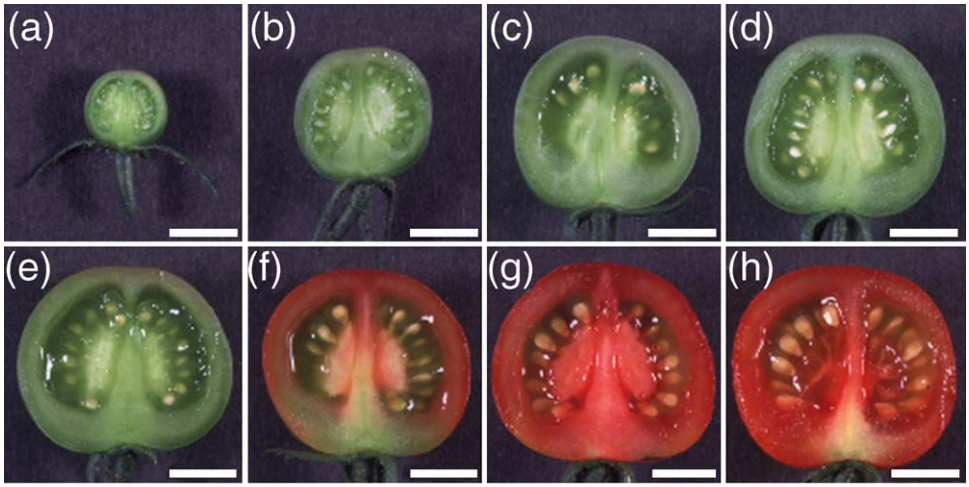
species [7,8,17,18]. In this study, we amplified seven *PIP* genes from mature tomato seeds using RT-PCR. This is the first report on the detection of tomato *PIP*s in seeds.

3.1. Water content during seed and fruit development

To better understand tomato seed development, the fresh and dry weights and the lengths of the long and short axes of developing seeds were measured. The ripening-associated change in fruit color began at 30–32 DAF, and proceeded for 30–40 DAF (Fig. 1a–f). The fruits were completely red after 44 DAF (Fig. 1g and h). The weight of fresh fruit increased gradually throughout development, indicating that maternal plants provide water and nutrients continuously to the developing fruit (Fig. 1i). Tomato seeds were dissected from fruit harvested at 12–56 DAF because the seeds could be dissected from fruit tissues after 12 DAF and mature fruit abscised from the maternal plant after 56 DAF.

The length of the short axis of seeds increased from 12 to 16 DAF, but not from 20 to 56 DAF (Fig. 2a). The length of the long axis of seeds increased gradually from 12 to 52 DAF, with a marked increase at 12–16 DAF (Fig. 2a). The fresh weight of seeds increased dramatically from 12 to 20 DAF, but stabilized from 20 to 56 DAF (Fig. 2b). Additionally, the fresh weight decreased transiently from 28 to 32 DAF (Fig. 2b). The dry weight of seeds increased steadily throughout seed development (Fig. 2b), indicating that the nutrients stored in seeds are produced continuously and are accumulated.

The water content of seeds decreased gradually during seed development from 95% (12 DAF) to 50% (32 DAF), and stabilized at 44–50% from 32 to 56 DAF (Fig. 3). A remarkable decrease in water content was observed from 28 to 32 DAF (Fig. 3). The water content of completely desiccated tomato seeds was $6.7 \pm 0.2\%$ (data not shown). In contrast, the pericarp and loculus tissue, which separated from the developing fruit, had high water content (>90%) throughout



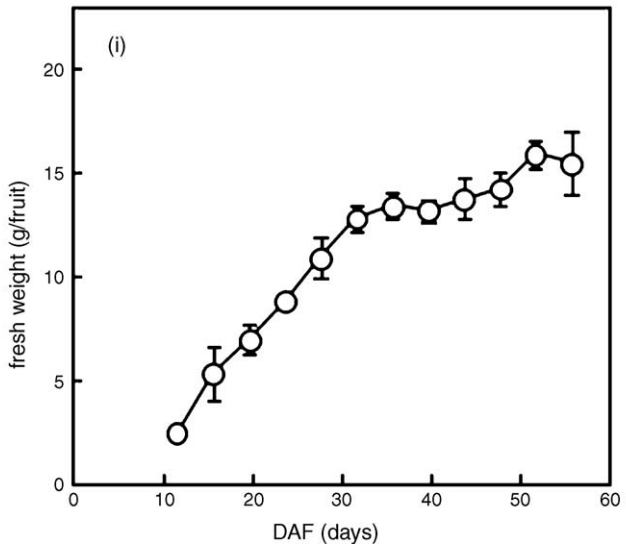


Fig. 1. Tomato fruit development. Morphological changes (a–h) in tomato fruit at different numbers of days after flowering (DAF): (a) 8 DAF; (b) 12 DAF; (c) 20 DAF; (d) 28 DAF; (e) 36 DAF; (f) 40 DAF; (g) 48 DAF; and (h) 52 DAF; bars: 10 mm. (i) Changes in the fresh weight of fruits. The average weight of developing fruits is shown ($n = 6$). Error bars indicate S.E.

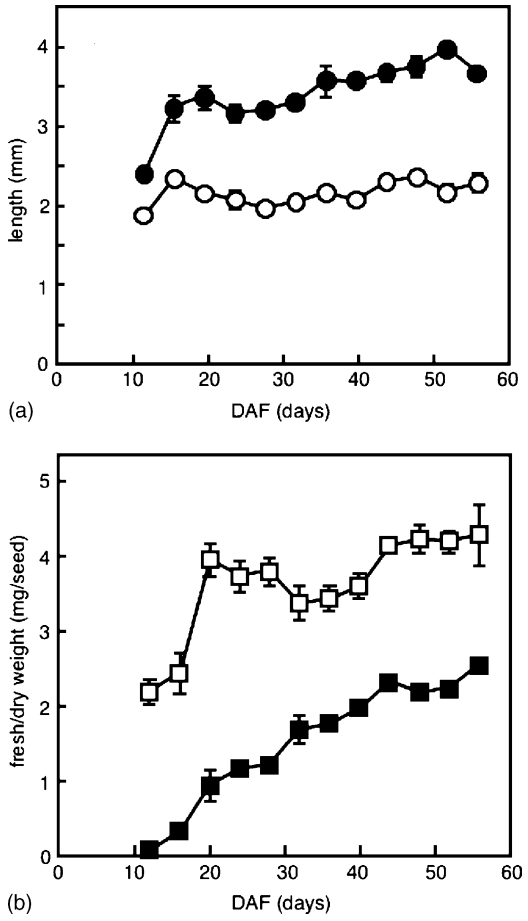


Fig. 2. Tomato seed development. (a) Changes in the length of the long (solid circles) and short (open circles) axes of seeds at different numbers of days after flowering (DAF). The average length is indicated ($n = 5$). (b) Changes in the fresh (open squares) and dry (solid squares) weights of seeds at different numbers of DAF. The average weight is indicated ($n = 40$). The values represent the average of three independent experiments. Error bars indicate S.E.

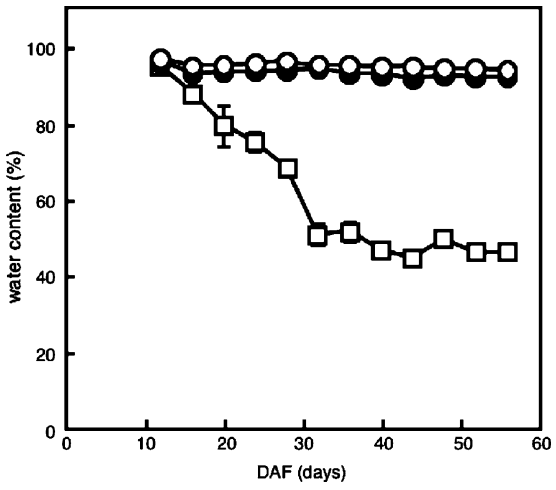


Fig. 3. Water content during seed and fruit development. Changes in water content during seed and fruit development are indicated. Seeds (open squares), pericarps (open circles), and loculus tissue (solid circles) were isolated from tomato fruits at different numbers of days after flowering (DAF). Water content was calculated as the ratio of the dry weight to the fresh weight of 40 seeds and at least 0.1 g of pericarp or loculus tissue. The values represent the average of three independent experiments. Error bars indicate S.E.

fruit development (Fig. 3). These results support the reports of Berry and Bewley [19] and Bradford et al. [20].

Generally, maturing seeds that are covered with scant fruit tissues, as in *Arabidopsis*, become desiccated by the arrest of the water supply and the diffusion of water into air [6]. In tomato seeds, which are covered with fruit tissues that contain a large amount of water, other mechanisms may be involved in the promotion and maintenance of seed desiccation.

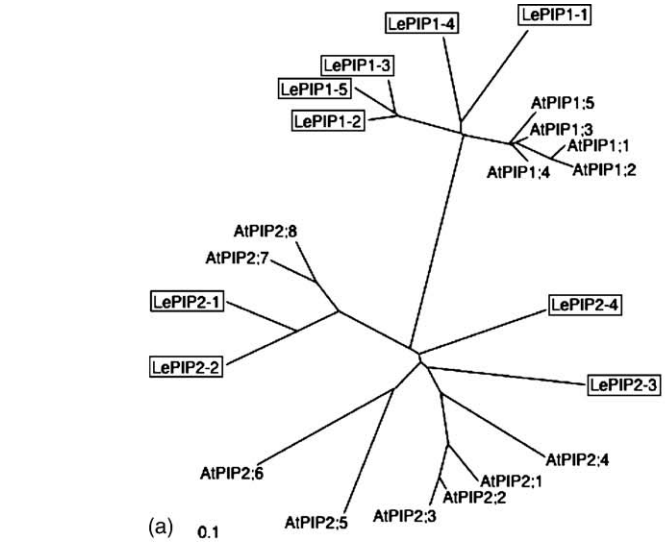
3.2. Detection of expression of PIPs in mature tomato seeds

Based on the results of genome projects, it has been proposed that *Arabidopsis* possesses five *PIP1*s and eight *PIP2*s, and maize possesses six *PIP1*s and seven *PIP2*s [3,4]. Therefore, as many as 13 *PIP*s may exist in the tomato genome. To detect *PIP*s in mature tomato seeds, PCR primers were constructed using the conserved nucleotide sequences of *Arabidopsis PIP*s. RT-PCR was performed using total RNA from mature tomato seeds isolated from ripened and abscised fruit.

The sequences of the amplified DNA fragments (approximately 400 bp) were used in database searches [the EMBL/GenBank/DDBJ database and the tomato EST database (E6203 or Micro-Tom)]. The fragments encode six *PIP1*s (designated *LePIP1-1*, *LePIP1-2*, *LePIP1-3*, *LePIP1-4*, *LePIP2-1*, and *LePIP2-2*; Fig. 4a). *LePIP1-1*, *LePIP1-2*, *LePIP1-3*, *LePIP2-1*, and *LePIP2-2* show identity with the tomato EST clones BP887068, BP884557, AW625013, BI929127, and BG128835, respectively (accession numbers BP887068, BP884557, AW625013, BI929127, and BG128835) [13,14,21]. The EST clone AW625013 (*LePIP1-3*) is expressed exclusively in roots, whereas the other EST clones are expressed in several plant tissues [13,14,21]. *LePIP1-4* shows similarity to *LeAqp2* (accession number AF218774), which may be involved in protection against plant–plant infection [12]. No expression of these tomato *PIP*s has been reported in seeds.

Fray et al. [10] reported the first tomato aquaporin, TRAMP (accession number X73848). TRAMP belongs to the PIP1 family and is expressed in fruit, roots, leaves, and flowers [10]. In this study, we analyzed *LePIP1-5*, which corresponds to *TRAMP* (Fig. 4a). We also analyzed two tomato *PIP2*s [*LePIP2-3* = EST clone AW224678 (accession number AW224678) and *LePIP2-4* = BP875728 (accession number BP875728)] that appear as full-length cDNA sequences in the tomato EST database (MiBASE; http://www.kazusa.or.jp/jsol/microtom/indexj.html) (Fig. 4a). No expression of these three tomato *PIP*s has been reported in seeds.

All nine of these tomato PIPs may function as water channels because they possess the six conserved transmembrane domains and the two conserved NPA motifs involved in the selective permeability of water (Fig. 4b). Generally, PIP2s show strong water-flux activity in *Xenopus laevis* oocytes and yeasts, whereas PIP1s do not [2,22]. In tomato, TRAMP/LePIP1-5 and LeAqp2/LePIP1-4 showed water-flux activity in *Xenopus* oocytes, although the activity was very weak [12]. It has also been suggested that PIP1s interact with other factors in

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LePIP1-1     1 -MEHREEDVRLGANKYSERQAIGIAAQSEDKDYKEPPPAPLFEPGELMSWSFYRAGIAEFV  60
LePIP1-4     1 -MEGKEEDVKVGANKYSERQPLGTSAQS--KDYKEAPPAPLFEAGELHSWSFWRAGIAEFM  58
LePIP1-5     1 MAENKEEDVKLGANKFRETQPLGTAAQT-DKDYKEPPPAPLFEPGELSSWSFYRAGIAEFM  60
LePIP1-3     1 MAENKEEDVNLGANKFREPQPLGTSAQT-DKDYKEPPPAPLYEPGELSSWSFYRAGIAEFM  60
LePIP1-2     1 MAENKEEDVNLGANKYRETQPLGTAAQT-DKDYKEPPPAPLFEPGELSSWSFYRAGIAEFM  60
LePIP2-3     1 -------------MTKEVEAVHEQAVEYSAKDYTDPPPAPLIDFEELTKWSLYRAAIAEFI  48
LePIP2-4     1 -------------MAKDMEYGNDQYAPS--KDYQDPPPAPLIDPEELGKWSFYRAIIAEFI  46
LePIP2-2     1 ---------------MSKEVIEEGQVQQHGKDYVDPPPAPLLDFAELKLWSFYRALIAEFI  46
LePIP2-1     1 ---------------MSKDVIEEGQAHHHGKDYVDPPPAPLLDMAELTKWSFYRALIAEFI  46
                                         .    *** :.***** :  **  **::** ****:

                          TM1                                  TM2
LePIP1-1    61 ATFLFLYITVLTGMGVSKSDSKCS------TGGIQGIAWAFGGMIFALVYCTAGISGGHIN 115
LePIP1-4    59 ATFLFLYITVLTVMGYSRANSKCS------TVGVQGIAWAFGGMIFALVYCTAGISGGHIN 113
LePIP1-5    61 ATFLFLYITILTVMGLKRSDSLCS------SVGIQGVAWAFGGMIFALVYCTAGISGGHIN 115
LePIP1-3    61 ATFLFLYITILTVMGLKRSDSLCS------SVGIQGVAWAFGGMIFALVYCTAGISGGHIN 115
LePIP1-2    61 ATFLFLYITILTVMGLKRSDSLCS------SVGVQGVAWAFGGMIFALVYCTAGISGGHIN 115
LePIP2-3    49 ATLLFLYITILTVIGYKHQADVKAGGDICGGVGLLGIAWAFGGMIFVLVYCTAGISGGHIN 109
LePIP2-4    47 ATLLFLYITVLTVIGYKSQSDGDQ----CGGVGILGIAWAFGGMIFVLVYCTAGISGGHIN 103
LePIP2-2    47 ATLLFLYVTVATVIGHKKLNGADK----CDGVGILGIAWAFGGMIFVLVYCTAGISGGHIN 103
LePIP2-1    47 ATLLFLYVTVATVIGHKKLNALDQ----CDGVGILGIAWAFGGMIFVLVYCTAGISGGHIN 103
                **:****:*: * :* .              *: *:*********.**************

                                        TM3
LePIP1-1   116 PAVTFGLFLARKLSLTRAVFYMVMQCLGAICGAALVKAFGKTLYQTKGGGANVVNVGYTKG 176
LePIP1-4   114 PAVTFGLFLARKLSLTRAVFYIVMQCLGAICGAGVVKGFQPSLFGTKGGGANVVAHGYTKG 174
LePIP1-5   116 PAVTFGLFLARKLSLTRAVFYMVMQCLGAICGAGVVKGFMVGPYQRLGGGANVVNPGYTKG 176
LePIP1-3   116 PAVTFGLFLARKLSLTRAVFYMVMQCLGAICGAGVVKGFMQGPYQRLGGGANVVQPGYTKG 176
LePIP1-2   116 PAVTFGLFLARKLSLTRAVFYIVMQCLGAICGAGVVKGFMVGPYERLGGGANVVNPGYTKG 176
LePIP2-3   110 PAVTFGLFLARKVSLIRAILYMVAQCLGAICGVGFVKAFQSAYYNRYGGGVNVMAGGHTKG 170
LePIP2-4   104 PAVTFGLLLARKVSLVRAIFYIVAQCLGAICGCGLVKLFQKAYYVKYGGGANELAVGYNIA 164
LePIP2-2   104 PAVTFGLFLARKVSLVRAVGYIIAQCLGAICGVGFVKAFMTHPYNALGGGANFVQSGYNNG 164
LePIP2-1   104 PAVTFGLFLARKVSLIRAVAYIIAQSLGAICGVGFVKAFMKHYYNTEGGGANFVNPGYNNG 164
                *******:****:** **: *:: *.****** ..** *    :   ***.* :  *:. .

                    TM4                                  TM5
LePIP1-1   177 DGLGAEIIGTFVLVYTVFSATDAKRSARDSHVPILAPLPIGFAVFLVHLATIPVTGTGINP 237
LePIP1-4   175 DGLGAEIIGTFVLVYTVFSATDAKRNARDSHVPILAPLPIGFAVFLVHLATIPITGTGINP 235
LePIP1-5   177 DGLGAEIIGTFVLVYTVFSATDAKRNARDSHVPILAPLPIGFAVFLVHLATIPITGTGINP 237
LePIP1-3   177 DGLGAEIIGTFVLVYTVFSATDAKRNARDSHVPILAPLPIGFAVFLVHLATIPITGTGINP 237
LePIP1-2   177 DGLGAEIIGTFVLVYTVFSATDAKRSARDSHVPILAPLPIGFAVFLVHLATIPITGTGINP 237
LePIP2-3   171 VGLAAEIIGTFVLVYVVFSATDPKRSARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINP 231
LePIP2-4   165 TGLGAEIIGTFVLVYTVFSATDPKRNARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINP 225
LePIP2-2   165 TALGAEIIGTFVLVYTVFSATDPKRSARDSHIPVLAPLPIGFAVFMVHLATIPITGTGINP 225
LePIP2-1   165 TALGAEIIGTFVLVYTVFSATDPKRSARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINP 225
                 .*.***********.******.**.*****:*:***********:*******:*******

                                        TM6
LePIP1-1   238 ARSLGAAVIYNNEQAWKDHWIFWVGPFIGAALAALYHQVVIRAIPFKSR---------  286
LePIP1-4   236 ARSLGAAIVYNKEHAWDDHWIFWVGPFIGAALAALYHQVIIRAIPFKSGN--------  275
LePIP1-5   238 ARSLGAAIIYNDEHAWNDHWIFWVGPMIGAALAAIYHQIIIRAMPFHRS---------  286
LePIP1-3   238 ARSLGAAIIFNQDQAWDDHWIFWFGPFIGAALAAIYHQIIIRAIPFKSRA--------  287
LePIP1-2   238 ARSLGAAIIFNKDEAWDDHWIFWVGPFIGAALAAVYHQIIIRAIPFKSSRS-------  288
LePIP2-3   232 ARSFGAAVIFNGDKAWDEHWIFWVGPFIGAFIAAVYHQYILRAGAIKALGSFRSNA--  287
LePIP2-4   226 ARSFGAAVIYGKNKSWDDQWIFWVGPFIGAAIAAIYHQYILRAGASKSINSFRSNA--  281
LePIP2-2   226 ARSFGAAVIADNKNVWDDQWIFWVGPFVGALLAAAYHQYILRAAAIKALGSFRSNATN  283
LePIP2-1   226 ARSFGAAVIYGNEKIWDDQWIFWVGPMVGAMAAAIYHQFILRAGAVKALGSFRSNQTN  283
                ***:***:: . .. *.::****.**::**  ** *** ::** . :
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(b)

Fig. 4. Comparison of the predicted amino acid sequences encoded by *PIP1*s and *PIP2*s in tomato and *Arabidopsis*. (a) A phylogenetic tree for the predicted amino acid sequences of *PIP1*s and *PIP2*s in tomato and *Arabidopsis*. An unrooted neighbor-joining (N-J) tree was constructed with CLUSTALW using predicted sequences from the database. (b) Multiple alignment of the predicted amino acid sequences of nine tomato *PIP*s. Common amino acid residues are indicated with asterisks. Similar amino acid residues are indicated with colons or periods. Lines and boxes indicate the transmembrane domains and NPA motifs, respectively. The accession

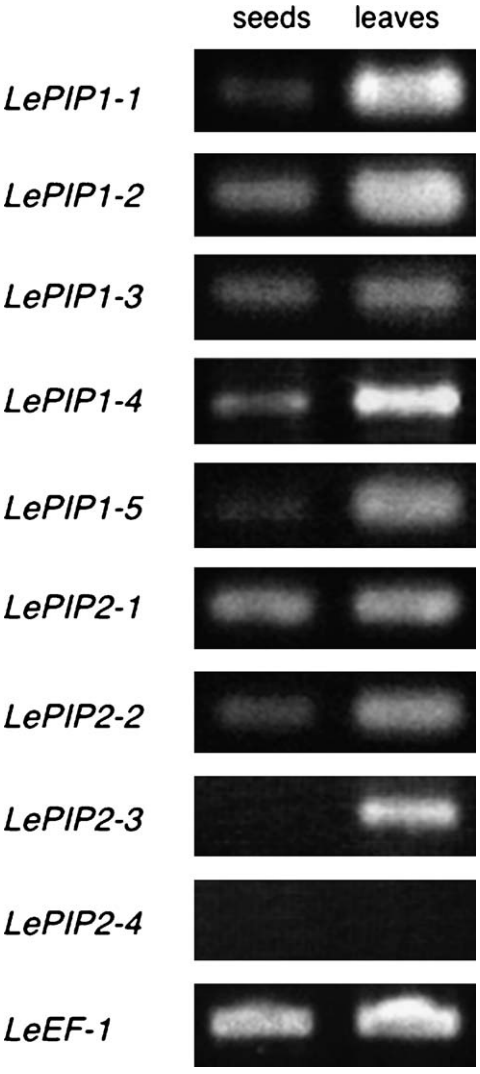

Fig. 5. Expression of tomato *PIP*s in mature seeds and leaves. The expression of tomato *PIP*s in mature seeds and leaves was analyzed by RT-PCR, which was performed using an equal volume of cDNA as template DNA and specific primers for the nine tomato *PIP*s. The products resulting from 30 PCR cycles were fractionated on a 1.5% agarose gel and visualized using ethidium bromide staining. *LeEF-1* was used as a transcriptional control. The data shown are representative of multiple experiments.

plant cells, but can act alone because increased water-flux activity is observed in transgenic plant cells that overexpress *PIP1*s [23,24]. Recently, Fetter et al. [25] postulated that PIP1 and PIP2 heterotetramers serve as water channels in plant cells. Thus, each tomato PIP1 may not show water-flux activity in *Xenopus* oocytes and yeasts.

In mature seeds, the expression of *LePIP1-1*, *LePIP1-2*, *LePIP1-3*, *LePIP1-4*, *LePIP1-5*, *LePIP2-1*, and *LePIP2-2* was detected with RT-PCR, using specific PCR primers (Table 1), but the expression of *LePIP2-3* and *LePIP2-4* was not detected (Fig. 5). In contrast, in mature leaves, the expression of eight *PIP*s was observed (excluding *LePIP2-4*; Fig. 5). The levels of *LePIP1-1*, *LePIP1-2*, *LePIP1-3*, *LePIP1-4*, *LePIP1-5*, and *LePIP2-2* expression in mature seeds were lower than in mature leaves, whereas the level of *LePIP2-1* expression in mature seeds was similar to that in mature leaves (Fig. 5). *LePIP2-4* expression was detected in roots (data not shown), but not in mature seeds or leaves (Fig. 5). Consequently, five *PIP1*s and two *PIP2*s were expressed in mature tomato seeds. These results suggest that *PIP1*s, rather than *PIP2*s, are mainly expressed in tomato seeds.

3.3. Analysis of expression of tomato PIPs during seed development

RT-PCR revealed the expression of five *PIP1*s and two *PIP2*s throughout seed development, with variations in the expression patterns (Fig. 6). Stronger and weaker expression of *LePIP1-1* was detected from 12 to 28 and 32 to 56 DAF, respectively (Fig. 6). The level of *LePIP1-2* expression was high from 12 to 32 DAF, and greater expression was detected from 20 to 28 DAF (Fig. 6). The level of *LePIP1-3* expression from 12 to 28 and 52 to 56 DAF was greater than that from 32 to 48 DAF (Fig. 6). Greater expression of *LePIP1-4* and *LePIP2-1* was detected from 12 to 28 DAF, with a transient peak at 20 DAF (Fig. 6). The expression of *LePIP1*-5 decreased gradually from 12 to 28 DAF, and remained steady from 28 to 56 DAF (Fig. 6). Therefore, each gene tended to show higher expression in the earlier phase of seed development (12–32 DAF) and lower expression in the later phase (36–56 DAF). In contrast, no expression of *LePIP2-3* or *LePIP2-4* was detected throughout seed development (data not shown).

During seed development, the water content of seeds decreased markedly in the earlier phase, and remained between 44% and 50% in the later phase (Fig. 3), indicating that water flux across the plasma membrane was high in the earlier phase, but was reduced in the later phase. In the earlier phase of seed development, plasma membrane aquaporins are expressed highly and may play some role in water flux; however, in the later phase, the expression of *PIP*s may remain low and the number of water channels may decrease, allowing the water content in seed cells to be maintained at a low level.

LePIP1-1, *LePIP1-3*, *LePIP2-1*, and *LePIP2-2* showed a second period of high expression at 52–56 DAF, following a period of lower expression from 32 to 48 DAF (Fig. 6). At 56 DAF, the seeds may be sufficiently mature because approximately 90% of the seeds harvested at this stage germinated (data not shown). This suggests that the number of aquaporin molecules may increase and be maintained at high levels in mature seeds in reserve for germination. Generally, the expression of aquaporins increases markedly upon germination, when water absorption causes the cellular volume to expand [7–9]. In pea, PIPs are involved in water absorption

numbers and AGI codes are as follows: BP887068 for *LePIP1-1*, BP884557 for *LePIP1-2*, AW625013 for *LePIP1-3*, AF218774 for *LePIP1-4*, X73848 for *LePIP1-5*, BI929127 for *LePIP2-1*, BG128835 for *LePIP2-2*, AW224678 for *LePIP2-3*, BP875728 for *LePIP2-4*, At3g61430 for *AtPIP1;1*, At2g45960 for *AtPIP1;2*, At1g01620 for *AtPIP1;3*, At4g00430 for *AtPIP1;4*, At4g23400 for *AtPIP1;5*, At3g53420 for *AtPIP2;1*, At2g37170 for *AtPIP2;2*, At2g37180 for *AtPIP2;3*, At5g60660 for *AtPIP2;4*, At3g54820 for *AtPIP2;5*, At2g39010 for *AtPIP2;6*, At4g35100 for *AtPIP2;7*, and At2g16850 for *AtPIP2;8*.

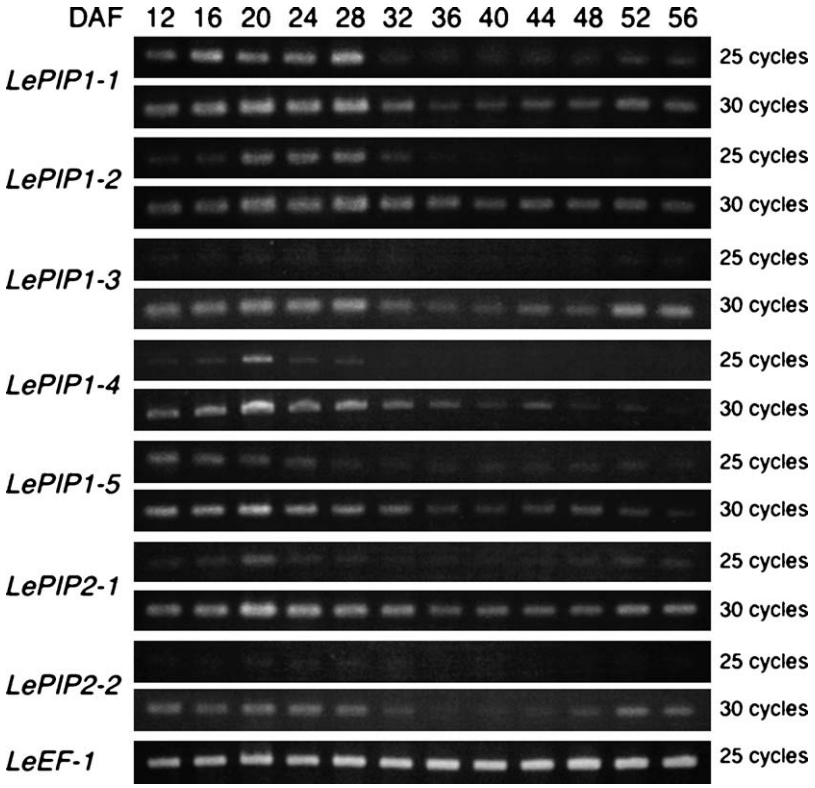

Fig. 6. Analysis of expression of tomato *PIP*s during seed development. The expression of tomato *PIP*s during seed development was analyzed by RT-PCR. Total RNA extracted from tomato seeds at different numbers of days after flowering (DAF) was used in RT-PCR analysis, which was performed using equal volumes of cDNA as template DNA and specific primers for seven tomato *PIP*s. The products resulting from 25 or 30 PCR cycles were fractionated on a 1.5% agarose gel and visualized using ethidium bromide staining. *LeEF-1* was used as a transcriptional control. The data shown are representative of multiple experiments.

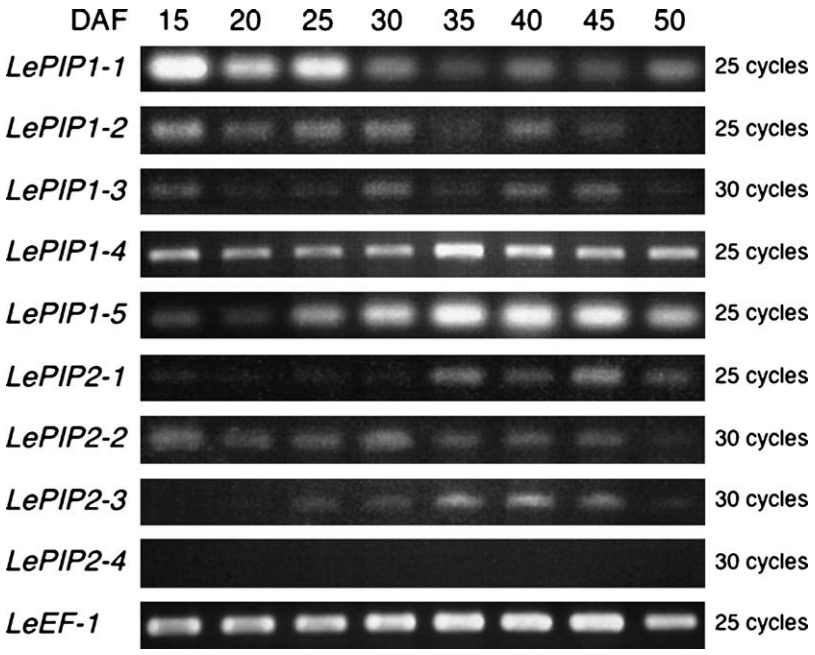

Fig. 7. Analysis of expression of tomato *PIP*s during fruit development. The expression of tomato *PIP*s during fruit development was analyzed by RT-PCR. Total RNA extracted from tomato fruit (mesocarp, endocarp, and loculus tissue) at different numbers of days after flowering (DAF) was used in RT-PCR analysis, which was performed using equal volumes of cDNA as template DNA and specific primers for the nine tomato *PIP*s. The products resulting from 25 or 30 PCR cycles were fractionated on a 1.5% agarose gel and visualized using ethidium bromide staining. *LeEF-1* was used as a transcriptional control. The data shown are representative of multiple experiments.

during germination because the expression of *PIP1* and *PIP2* increases during seed coat development [9]. Therefore, the four tomato PIPs may also play a role in the initiation of water absorption upon germination.

3.4. Analysis of expression of tomato PIPs during fruit development

The expression of the nine tomato *PIP*s was also analyzed in developing fruit, including mesocarp, endocarp, and loculus tissues, using RT-PCR (Fig. 7). Strong expression of *LePIP1-1*, *LePIP1-4*, and *LePIP1-5* was detected, whereas the expression levels of *LePIP1-2*, *LePIP1-3*, *LePIP2-1*, *LePIP2-2*, and *LePIP2-3* were low. In contrast, no *LePIP2-4* expression was detected throughout fruit development.

The eight tomato *PIP*s showed distinct expression patterns. The expression of *LePIP1-1*, *LePIP1-2*, and *LePIP2-2* was stronger in the earlier phase (15–30 DAF) than in the later phase (35–50 DAF) of fruit development (Fig. 7). In contrast, the expression of *LePIP1-5*, *LePIP2-1*, and *LePIP2-3* was stronger in the later phase of fruit development than in the earlier phase (Fig. 7). The level of *LePIP2-1* expression was higher after 35 DAF, and the levels of *LePIP1-5* and *LePIP2-3* expression were higher after 25 DAF, showing a transient peak 40 DAF (Fig. 7). Expression of *LePIP1-3* and *LePIP1-4* was observed throughout fruit development (Fig. 7). Therefore, more types of *PIP*s were expressed in developing fruit, and the levels of expression in fruit were higher than in developing seeds. In addition, the expression patterns of tomato *PIP*s in developing fruit were more diverse than those in developing seeds. Nevertheless, it seems likely that the total level of *PIP* expression is maintained at each stage of tomato fruit development. During fruit development, the fresh weight of the fruit increased gradually (Fig. 1i), whereas the water content of fruit tissues remained stable (Fig. 3). This indicates that cell growth continues throughout fruit development, requiring the transport of both water and nutrients across the plasma membrane, and suggests that eight tomato PIPs are involved in water flux during fruit development.

A previous report showed that *TRAMP*/*LePIP1-5* is expressed throughout fruit development, and that its expression increases gradually during fruit ripening [11]. Antisense expression analysis suggests that TRAMP is involved in the movement and accumulation of sugars and organic acids, rather than in water flux, upon fruit ripening [11]. It has also been reported that aquaporins, in addition to transporting water, also have the ability to transport glycerol, urea, ammonia, polyalcohol, CO_2, and H_2O_2 [1,2,26,27]. Therefore, in addition to TRAMP, tomato PIPs may be involved in the movement of both water and the above small compounds during fruit ripening. However, Kim and Grierson [28] recently suggested that TRAMP is not involved in the movement of sugars or organic acids because TRAMP proteins are located in the plasma membrane, not in the tonoplast membrane.

We analyzed the expression of five *PIP1*s and two *PIP2*s during seed development in tomato, and postulated that lower expression of *PIP*s is involved in the maintenance of lower water levels in maturing seeds based on the correlation between the expression patterns of *PIP*s and the water content in seeds. Groot et al. [29] reported that gibberellin affects the water content of developing tomato seeds. It is likely that the expression of *PIP*s is regulated by gibberellin in developing tomato seeds. To determine the physiological roles of aquaporins during seed development, it will be important to analyze the actual direction and level of water movement in developing seeds and to measure the water potential of the cells in developing seeds and fruit.

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