RESEARCH PAPER

Role of vacuolar H\(^+\)-inorganic pyrophosphatase in tomato fruit development

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Abstract
cDNA corresponding to two type-I vacuolar H\(^+\)-inorganic pyrophosphatases (V-PPases) \((\text{SVIP}1, \text{SVIP}2)\) and one type-II V-PPase \((\text{SVIP}3)\) was isolated from tomato fruit to investigate their role in fruit development. Southern analysis revealed that type-I V-PPase genes form a multigene family, whereas there is only one type-II V-PPase gene in the tomato genome. Although \text{SVIP}1 and \text{SVIP}2 were differentially expressed in leaves and mature fruit, the highest levels of both \text{SVIP}1 and \text{SVIP}2 mRNA were observed in fruit at 2–4 days after anthesis. The expression pattern of type-II \text{SVIP}3 was similar to that of \text{SVIP}2, and the highest levels of \text{SVIP}3 mRNA were also observed in fruit at 2–4 days after anthesis, thus suggesting that \text{SVIP}3 plays a role in early fruit development. Because \text{SVIP}1 and \text{SVIP}2 mRNA was more abundant than \text{SVIP}3 mRNA, expression of type-I V-PPases was analysed further. Type-I V-PPase mRNA was localized in ovules and their vicinities and in vascular tissue at an early stage of fruit development. Tomato RNAi lines in which the expression of type-I V-PPase genes was repressed using the fruit-specific promoter \(\text{TPRP-F1}\) exhibited fruit growth retardation at an early stage of development. Although the major function of V-PPases in fruit has been believed to be the accumulation of materials such as sugars and organic acids in the vacuole during cell expansion and ripening, these results show that specific localization of V-PPase mRNA induced by pollination has a novel role in the cell division stage.

Key words: Fruit development, tomato, vacuolar H\(^+\)-inorganic pyrophosphatase, V-PPase.

Introduction

Two major electrogenic proton pumps exist in the plant vacuolar membrane: \(\text{H}^+\)-ATPase (V-ATPase) and \(\text{H}^+\)-inorganic pyrophosphatase (V-PPase). These pumps acidify vacuoles and generate an electrochemical gradient in the plant cell. This proton electro motive force allows the secondary active transport of inorganic ions, sugars, and organic acids (Maeshima, 2000). V-ATPase, a multisubunit enzyme, is a universal component of eukaryotic organisms. In tomato \((\text{Solanum lycopersicum})\) fruit, the antisense suppression of the V-ATPase A subunit using a fruit-specific promoter results in small fruit with few seeds and increased sucrose concentrations (Amemiya et al., 2006). In contrast, V-PPase, which consists of a single polypeptide, is distributed among most land plants, some algae, and in protozoa, bacteria, and archaeabacteria (Maeshima, 2000). Transgenic plants overexpressing the \(\text{Arabidopsis} \text{V-PPase} \text{AVP1}\) displayed enhanced drought and salt tolerance associated with increased solute content (Gaxiola...
et al., 2001), increased cell division at the onset of organ formation, hyperplasia, and increased auxin transport (Li et al., 2005). The avp1-I null mutant exhibited severely disrupted root and shoot development and reduced auxin transport (Li et al., 2005).

cDNA of the type-I V-PPase has been isolated from fruit, and the expression of their mRNA has been investigated in grape (Venter et al., 2006), pear (Suzuki et al., 1999), and peach (Etienne et al., 2002a). In grape, at least two type-I V-PPase genes are expressed during fruit development, showing increased expression after veraison, when sugars and organic acids accumulate (Venter et al., 2006). In pear, the mRNA level of the type-I V-PPase gene PVP1 increases during fruit expansion and sugar accumulation (Suzuki et al., 1999). In peach fruit, the expression of two type-I V-PPase genes has been shown to be related to the accumulation of organic acids in the vacuole (Etienne et al., 2002a). These findings suggest that fruit V-PPases play a role in the accumulation of sugars and organic acids in the vacuole. In fact, the peach V-PPase gene is a candidate quantitative trait locus that controls the accumulation of sugars and organic acids (Etienne et al., 2002b).

Type-I and -II V-PPases, which have divergent primary structures, coexist in the plant cell (Drozdowicz et al., 2000). The Arabidopsis genes encode the type-I V-PPase AVP1 and the type-II V-PPase AVP2, which show inorganic pyrophosphate (Pi) hydrolysis and H\(^+\) translocation activities, although the K\(^+\) sensitivity of the two types differs (Drozdowicz et al., 2000). AVP1 and AVP2 are located in different subcellular organelles (Mitsuda et al., 2001). As far as is known, AVP2 is the only type-II V-PPase that has been reported previously, and its physiological role is unknown.

In this study, cDNA corresponding to two type-I V-PPases (SIV1P1, SIV1P2) and one type-II V-PPase (SIV1P3) was cloned from tomato fruit and analysed. Interestingly, their expression patterns and the phenotype of the type-I V-PPase RNAi lines reveal a new aspect of the physiological role of V-PPases in fruit development.

**Materials and methods**

**Plant materials**

Tomato (*S. lycopersicum* ‘Ailsa Craig’) plants were grown in a greenhouse. Leaves, roots, stems, flowers, and fruit were sampled and stored at −80°C for further analysis. To investigate the effect of pollination and auxin on mRNA levels, stamens were removed 2 days before anthesis to prevent self-pollination, and the flower buds were covered with paper bags. Pollination was performed 2 days after emasculation. The emasculated flowers were treated with p-chlorophenoxyacetic acid (4-CPA, 15 ppm), a synthetic auxin that induces parthenocarpy in the absence of pollination. Ovaries were sampled at 6 days after emasculation.

**Molecular cloning of tomato V-PPase cDNA**

The SIV1P2 cDNA containing the entire coding region and partial SIV1P1 and SIV1P3 cDNA was amplified by RT-PCR using total RNA extracted from the fruit as a template. The primers were used designed based on a tomato expressed sequence tag (EST; accession numbers JX561343.1. (Takara). The amplified fragments were fused into the pSTBlue AccepTor Vector (Novagen) and sequenced. 5′-Rapid amplification of cDNA ends (RACE) was performed to isolate the 5′ region of the SIV1P1 cDNA. A cDNA library was constructed from RNA of the young tomato fruit using the ZAP-cDNA Synthesis Kit and the ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene), and an SIV1P3 cDNA containing the entire coding region was isolated by screening the cDNA library. To isolate additional V-PPase genes from tomato fruit, degenerate primers were designed based on highly conserved regions of the amino acid sequences of the V-PPases of several plant species. The primer sequences used for the type-I V-PPases were 5′-CCGTTMTCAAACTGCG3′- and 5′-CATNCNCAGCGCGCGC-3′, respectively. Reverse transcription (RT) was performed using an oligo VP1, SlVP2, SlVP3, and SlVP3

**Southern and Northern blot analyses**

Southern blot analysis was performed according to the method of Kanayama et al. (1997). Tomato genomic DNA was digested, electrophoresed on an agarose gel, and blotted onto Hybond N* membrane (GE Healthcare). Hybridization was performed at 37°C in DIG Easy Hyb solution (Roche Diagnostics) containing a digoxigenin (DIG)-labelled DNA probe. The blots were washed twice at 65°C in 0.2 × SSC containing 0.1% (w/v) sodium dodecyl sulphate (SDS) and exposed to an X-ray film.

RNA was extracted using the SDS-phenol method (Nakajima et al., 1988) or the RNeasy Plant Mini Kit (Qiagen). Northern blot analysis was performed according to the method of Odanaka et al. (2002). Five µg of total RNA extracted from various tomato organs was separated on 1.2% (w/v) agarose gels containing 1.85% (w/v) formaldehyde and blotted onto Hybond N* membrane. Hybridization was performed at 65°C in DIG Easy Hyb solution containing a DIG-labelled RNA probe. The blots were washed twice at 65°C in 0.2 × SSC containing 0.1% (w/v) SDS and exposed to an X-ray film.

For Southern and Northern blot analysis, the 3′-untranslated region (UTR) of the SIV1P1 cDNA between nucleotides +2271 and +2524 (from the start codon), the 3′-UTR of the SIV1P2 cDNA between nucleotides +2269 and +2499, and the coding region of the SIV1P3 cDNA between nucleotides +1570 and +2024 were used as gene-specific probes. The coding region of the SIV1P1 cDNA between nucleotides +1693 and +2291 was used as a probe to detect the type-I V-PPase gene family.

**In situ hybridization**

*In situ* hybridization was carried out by a method modified from those of Kanayama et al. (1998) and Kato et al. (2003). Ovaries at 2 days after anthesis (DAA) were fixed with 4% (w/v) paraformaldehyde overnight at 4°C. They were then dehydrated in a graded ethanol series, substituted with xylene, and embedded in histoparaffin. Microtome sections (7-µm thick) were placed on silane-coated glass slides. The tissues were deparaffinized with xylene and rehydrated through a graded ethanol series. They were incubated with 0.2 N HCl for 20 min and then 5 µg ml\(^{-1}\) proteinase K in 100 mM sodium phosphate buffer (pH 7.4) overnight at 4°C. They were then dehydrated in a graded ethanol series, substituted with xylene, and embedded in histoparaffin. Microtome sections (7-µm thick) were placed on silane-coated glass slides. The tissues were deparaffinized with xylene and rehydrated through a graded ethanol series. They were incubated with 0.2 N HCl for 20 min and then 5 µg ml\(^{-1}\) proteinase K in 100 mM Tris-HCl (pH 7.5) containing 50 mM EDTA for 30 min at 37°C to digest the proteins. The sections were treated with 0.1 M triethanolamine (pH 8.0) for 5 min and then with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min to acetylate any remaining positive charges, and incubated in 2 × SSC. Hybridization was performed in a solution containing 50% (v/v) formamide, 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 1 × Denhardt’s solution, 125 µg ml\(^{-1}\) herring sperm DNA, 125 µg ml\(^{-1}\) yeast tRNA, 0.25% (w/v) SDS, 10% (w/v) dextran sulphate, and DIG-labelled RNA probes at 1 µg ml\(^{-1}\). An RNA probe corresponding to the coding region of the SIV1P1 cDNA between nucleotides +1693 and +2291 was synthesized and restrictively hydrolysed to approximately 100 bases. Hybridization was carried out at 48°C for 16 h, after which the slides were washed four times in 4 × SSC at 50°C for 10 min. The excess probe was removed by incubation in a solution containing 10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM EDTA, and 10 µg ml\(^{-1}\) RNase A at 37°C for 30 min. The slides were then washed twice in the same solution without RNase A for 10 min at 37°C, 2 × SSC for 30 min.
at 50 °C, and 0.1 × SSC for 30 min at 50 °C. Hybridization signals were detected using anti-DIG-alkaline phosphatase (Roche Diagnostics). After colour development with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, the slides were washed in a solution containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA and mounted in CrystalMount (Biomedica). A DIG-labelled sense RNA probe of the SiVP1 cDNA was used as a control.

Plant transformation

The coding region of SiVP2 cDNA between nucleotides +1220 and +1559 was amplified by PCR using primers 5′-CCGGAATTCATATGAGTGTGGCCGTGTGTTCT-3′ and 5′-CCGGATCTCTAGTGCAGGCCCATTCTCAG-3′, and the PCR fragment was used as an inverted-repeat construct. The β-glucuronidase (GUS) gene in the binary vector pBI121 was replaced with the inverted fragments separated by a spacer containing approximately 1 kb of the coding region of the GUS gene. The cauliflower mosaic virus 35S promoter was replaced with a fragment from approximately 2.6 kb upstream from the start codon of TPRP-F1 (Carmi et al., 2003) that was amplified by PCR using the primers 5′-CCCAGCTTTATGACTCCCATCAATAAGGGCAGA-3′ and 5′-CCGGATCTCTAGTGCAGGCCCATTCTCAG-3′. Together, the primers were amplified by PCR using the primers 5′-CCGGATCTCTAGTGCAGGCCCATTCTCAG-3′ and 5′-GAGACTTTAACGCGCGAGAC-3′ for SiVP1, 5′-AAAAGGGCAATGACAAGAAGG-3′ for SlVP1, and 5′-ACGTCCCTGACAATTTTCTCG-3′ for actin.

Expression analysis of the V-PPase gene in transgenic plants

Total RNA was extracted from fruits of the TPR and control lines 2 weeks after anthesis by the SDS–phenol method. Removal of genomic DNA, RT, and real-time PCR were performed using the method of Hori et al. (2011). The following gene-specific primer sets were designed using the Primer 3 software: 5′-TGCAACCAACTCCTATGACTG-3′ and 5′-CGGGATCCCTCATGCCAGCCATCTCAG-3′ for SiVP2, 5′-TGCAACCAACTCCTATGACTG-3′ and 5′-CGGGATCTCTAGTGCAGGCCCATTCTCAG-3′ for SiVP3, 5′-CCGGAATTCATATGAGTGTGGCCGTGTGTTCT-3′ and 5′-CCGGATCTCTAGTGCAGGCCCATTCTCAG-3′, and the PCR fragment was used as an inverted-repeat construct. The β-glucuronidase (GUS) gene in the binary vector pBI121 was replaced with the inverted fragments separated by a spacer containing approximately 1 kb of the coding region of the GUS gene. The cauliflower mosaic virus 35S promoter was replaced with a fragment from approximately 2.6 kb upstream from the start codon of TPRP-F1 (Carmi et al., 2003) that was amplified by PCR using the primers 5′-CCCAGCTTTATGACTCCCATCAATAAGGGCAGA-3′ and 5′-CCGGATCTCTAGTGCAGGCCCATTCTCAG-3′. Together, the primers were amplified by PCR using the primers 5′-CCGGATCTCTAGTGCAGGCCCATTCTCAG-3′ and 5′-GAGACTTTAACGCGCGAGAC-3′ for SiVP1, 5′-AAAAGGGCAATGACAAGAAGG-3′ for SlVP1, and 5′-ACGTCCCTGACAATTTTCTCG-3′ for actin.

Results

Isolation of tomato V-PPase cDNA

Partial SiVP1 and SiVP3 cDNA and SiVP2 cDNA containing the entire coding region were obtained from tomato ESTs. 5′-RACE was performed to isolate the entire coding region of SiVP1. The full-length SiVP3-coding region was obtained by screening a cDNA library prepared from tomato fruit at 15 DAA. To isolate additional type-I and -II V-PPase genes, degenerate primers were designed based on conserved regions of higher-plant type-I and -II V-PPases, respectively, and V-PPase cDNA fragments were amplified by PCR. Approximately 50 type-I and 30 type-II V-PPase clones were sequenced. Three V-PPase-like sequences that differed from SiVP1 and SiVP2 were obtained and identified as type-I V-PPases. The sequences of all the type-II clones were identical to SiVP3.

Analysis of the above cDNA from the EST database and the cDNA isolated by PCR with degenerate primers revealed that at least five type-I V-PPase genes and one type-II V-PPase gene are expressed in tomato fruit. Because RT-PCR using RNA from leaf and fruit at 15 DAA showed very low expression of the three type-I V-PPase genes other than SiVP1 and SiVP2 (data not shown), only SiVP1, SiVP2, and SiVP3 were analysed more extensively.

Type-I V-PPases have been isolated from several higher plants, and their amino acid sequences are highly conserved. The amino acid sequences of SiVP1 and SiVP2 had 87 and 89% identity, respectively, to Arabidopsis AVP1, and the amino acid sequence of SiVP1 had 89% identity to SiVP2 (Fig. 1). In contrast to the type-I enzymes, information was lacking on a plant type-II V-PPase gene. The SiVP3 amino acid sequence had 84% identity to Arabidopsis AVP2. The type-I V-PPases SiVP1 and SiVP2 had only 41% identity to the type-II V-PPase SiVP3. The similarities between the amino acid sequences of the Arabidopsis type-I and -II V-PPases were also very low. However, the putative PPI-binding sequence AADVGADLVGKVE was conserved in both types (Fig. 1). In contrast, the putative 14-3-3 protein ligand-binding sequence RQFNTIP (Venter et al., 2006) was conserved only in the type-I V-PPase. The phylogenetic analysis showed that type-I and -II V-PPases formed different groups (Fig. 2). SiVP2 was grouped into a clade that included AVP1. SiVP1 and SiVP2 could be orthologous to Nicotiana tabacum 31 and N. tabacum 5, respectively (Fig. 2).

Southern and Northern blot analyses

Southern hybridization was carried out using PCR fragments of the coding regions of SiVP1 and SiVP3 as probes (Fig. 3A, 3D). Several bands were detected when the SiVP1 probe was used (Fig. 3A). In contrast, a single band was detected in the blot hybridized with the SiVP3 probe (Fig. 3D). These results suggest that type-I V-PPase genes form a gene family and the type-II V-PPase gene exists as a single copy in the tomato genome. Southern hybridization was also carried out using the 3′-UTR regions of SiVP1 and SiVP2 as probes. A single band was detected by using either SiVP1 or SiVP2, indicating that these probes are gene specific (Fig. 3B, 3C). Therefore, these probes were used to investigate the expression of SiVP1 and SiVP2.

In vegetative tissues, the levels of the SiVP2 and SiVP3 transcripts were higher in young leaves than in mature leaves, whereas the level of the SiVP1 mRNA was similar in young and mature leaves (Fig. 4). The three SiVPs showed differential expression patterns in each organ. In fruit, the level of the SiVP1 transcript was high until 35 DAA, corresponding to the cell-expansion stage, but was low at the mature green stage and later. In contrast, the level of SiVP2 transcripts increased during ripening.

RT-PCR was performed using fruit collected at 2 DAA to compare the transcript levels of the members of the V-PPase gene family (Fig. 5). The level of SiVP1 transcripts was as high as that of SiVP2 transcripts, but the level of SiVP3 transcripts was much lower. Thus, this study focused on the role of the type-I V-PPases SiVP1 and SiVP2 during fruit development. The transcript levels of SiVPs, including type II, peaked from 2–4 DAA, after which they decreased rapidly until 15 DAA (Fig. 6). These results show that the SiVPs are highly expressed during the very early developmental stage of fruit after pollination, much more highly than during the other fruit developmental stages.
The effect of pollination on the accumulation of SlVP transcripts was investigated using emasculated, pollinated, and auxin-treated ovaries (Fig. 6). The expression of SlVP1, SlVP2, and SlVP3 was induced by pollination, indicating that the peaks in the levels of these transcripts at 2–4 DAA are pollination-specific. The levels of SlVP1 and SlVP3 mRNA increased in ovaries treated with 4-CPA, which is a synthetic auxin used in the induction of parthenocarpy, independent of pollination in tomato.

Spatial expression patterns

Because the type-II V-PPase SlVP3 mRNA was not abundant (Fig. 5), this study investigated the in situ localization of mRNA of the type-I V-PPases at 2–4 DAA are pollination-specific. The levels of SlVP1 and SlVP3 mRNA increased in ovaries treated with 4-CPA, which is a synthetic auxin used in the induction of parthenocarpy, independent of pollination in tomato.

Fig. 1. Multiple alignment of deduced amino acid sequences of tomato V-PPase genes. SlVP1 (accession number AB300442) and SlVP2 (LES278019) were found to encode similar amino acid sequences but showed only 41% identity to the deduced amino acid sequence of SlVP3 (AB300443). The putative PPI-binding sequence AADVGADLVGKVE is underlined, and the putative 14-3-3 protein ligand-binding sequence RQFNTIP is underlined with a dashed line.

Fruit growth in V-PPase RNAi lines

Type-I V-PPase gene expression was suppressed in RNAi lines using the coding region of the type-I V-PPase gene with the young-fruit-specific promoter TPRP-F1 (Salts et al., 1991). In this study, expression of both type-I V-PPase genes, SlVP1 and SlVP2, was suppressed because functional redundancy of the type-I V-PPase was expected in gene-specific suppression of SlVP1 or SlVP2, which showed the same expression pattern during early fruit development. Three normal fertile diploid RNAi lines, TPR43, TPR79, and TPR81, were grown independently with control plants. The observed morphological differences were limited to fruit, as expected, and fruit of the RNAi lines in which the expression of V-PPase genes was decreased (Fig. 8) showed fruit growth retardation from the early developmental stage (Figs. 9 and 10). The final fruit sizes in RNAi and control lines were comparable. However, the fruits of RNAi lines continued to grow even after most of the fruits in control lines were mature and ripe. Because the fruit-specific promoter, TPRP-F1,
is highly expressed in young fruits (Salts et al., 1991), the fruits of RNAi lines likely grew to normal sizes at a later stage.

Discussion

The type-II V-PPase gene was found to exist as a single copy in the tomato genome, whereas the type-I V-PPase genes formed a gene family. The high level of identity among members of the type-I V-PPase gene family and low level of identity between type-I and -II V-PPase genes in tomato is similar to the case in Arabidopsis (Drozdowicz et al., 2000). Phylogenetic analysis confirmed the well-defined lineages of plant type-I and -II V-PPases. The putative 14-3-3 type motif RQFNTIP, which is conserved in the type-I V-PPases SlVP1 and SlVP2, is not conserved in the type-II V-PPase SlVP3. V-PPase genes are stress-responsive (Fukuda and Tanaka, 2006; Gao et al., 2006; Venter et al., 2006), and the 14-3-3-binding motif could be related to stress responsiveness (Venter et al., 2006). SlVP3 may not be regulated by 14-3-3 protein binding.

The expression of SlVP1 is constitutive, except in ripe fruit, whereas the level of SlVP2 transcripts is higher in young leaves. High V-PPase activity in young vegetative tissues has been reported in mung bean hypocotyls (Maeshima, 1990), pumpkin cotyledons (Suzuki and Kasamo, 1993), and radish taproots (Maeshima et al., 1996). These reports suggest that V-PPase utilizes and/or scavenges a large amount of PPI produced as a byproduct of the synthesis of RNA, proteins, and polysaccharides in young tissues. The higher level of SlVP2 transcripts in young leaves supports this hypothesis. SlVP2 mRNA levels increased during ripening, suggesting a physiological role for SlVP2 in fruit ripening. In addition, differential responses of SlVP1 and SlVP2 to ripening and auxin treatment were observed (Fig. 6). Differential responses to plant hormones have also been reported for the barley type-I V-PPase genes HVP1 and HVP10 (Fukuda and Tanaka, 2006).

Despite the divergent amino acid sequences, type-II V-PPases are not as different from type-I V-PPases as the H+-translocating PPI hydrolytic enzyme, although the type-II V-PPases are

Fig. 2. Phylogenetic tree based on deduced amino acid sequences of V-PPases from A. thaliana 2 (accession number AF182813), Prunus persica 2 (F367447), N. tabacum 31 (X83729), N. tabacum 5 (X77915), A. thaliana 1 (BT002481), Hordeum vulgare 1 (AB032839), and H. vulgare 10 (D13472). The tree was constructed using the neighbour-joining method after sequence alignment using CLUSTAL W. SIVP1 and SIVP2 are classified as type-I V-PPases and SIVP3 as a type-II V-PPase. Bar, 0.1 substitutions per amino acid.

Fig. 3. Southern blot analysis of tomato V-PPase genes. Genomic DNA extracted from tomato leaves was cut with the indicated restriction enzymes and separated on an agarose gel. Southern blot hybridization was performed using DIG-labelled probes: SIVP1-coding region (A), SIVP1 3′-UTR (B), SIVP2 3′-UTR (C), and SIVP3-coding region (D). The sizes of molecular weight markers are indicated on the left.
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K⁺-insensitive, unlike the type-I V-PPases (Drozdowicz et al., 2000). As far as is known, the present study is the first to show the detailed expression pattern of a type-II V-PPase gene, including the fruit developmental stages. Interestingly, the SlVP3 transcript level is high in developing tissues, including young leaves and fruit up to 8 DAA. In this respect, SlVP3 is similar to SlVP2, perhaps suggesting that SlVP3 plays a supplemental role as a type-I V-PPase if SlVP3 is localized to the vacuolar membrane. Alternatively, type-II V-PPases may function in the Golgi apparatus as described by Mitsuda et al. (2001) and Segami et al. (2010). The plant Golgi apparatus is involved in the modification and sorting of proteins destined for the cell surface and vacuoles (Nebenführ and Staehelin, 2001). The high expression level of SlVP3 after pollination, when the synthesis of various proteins increases because of requirements for fruit development, could be associated with the role of SlVP3 in the Golgi apparatus if SlVP3 is localized to that.

Expression of V-PPase genes during fruit expansion and ripening has been reported in pear (Suzuki et al., 1999), Japanese pear (Suzuki et al., 2000), peach (Etienne et al., 2002a), and grape (Venter et al., 2006). These studies indicated that V-PPases play a role as a proton pump in the accumulation of sugars and organic acids in the vacuole. In tomato fruit, cell expansion begins with the formation of large vacuolated cells at approximately 10 DAA and continues to the mature green stage, after which the fruit ripens with climacteric ethylene production (Tanksley, 2004). In the present study, the accumulation of V-PPase mRNA from 15 DAA to the ripening stage was related to the previously proposed roles of cell expansion and the accumulation of materials in fruit.

This study focused on the very early stage of fruit development. The levels of V-PPase mRNA were much higher at 2–4 DAA than at other stages. Because cell expansion has not yet begun at 2–4 DAA (Tanksley, 2004), when the SlVPs are highly expressed, it is unlikely that V-PPases play a role at that stage in the accumulation of materials in the vacuole and the consequent cell expansion, as described previously. Pollination-specific expression of the type-I V-PPase at this stage and fruit growth retardation from the early developmental stage in RNAi lines indicated a novel role of V-PPases at the cell division stage. It is unlikely that the type-I V-PPase directly promotes cell division in fruit pericarp tissue according to in situ localization of type-I V-PPase mRNA or that it promotes fruit growth through seed development, judging from the normal seed number in RNAi lines (data not shown). Li et al. (2005) reported a novel role for...
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Fig. 6. Expression of tomato V-PPase genes during the early stage of fruit development and the effect of pollination and auxin on their expression. Total RNA was extracted from fruit harvested at different developmental stages (−2, 0, 2, 4, 8, 15 DAA) and separated on agarose gels containing formaldehyde. Total RNA was also extracted from emasculated (E), emasculated and pollinated (P), and emasculated and auxin-treated (A) fruit at 4 DAA. Northern hybridization was performed using a DIG-labelled gene-specific probe. The blot was exposed to X-ray film for 30 min (SlVP1 and SlVP2) or 90 min (SlVP3). rRNA stained with ethidium bromide is shown as a control.

Fig. 7. In situ localization of type-I V-PPase mRNA in tomato fruit at 2 DAA. In situ hybridization was performed using DIG-labelled SlVP1-coding region as a probe. An antisense RNA probe was used to detect type-I V-PPase mRNA (A–C), and a sense RNA probe was used as a control (D–F). Signals were detected in the seeds and their surroundings (A, C) and in the vascular tissue of the pericarp (A, B). No signal was detected in the control.

The study describes the expression patterns of two type-I V-PPase genes, SlVP1 and SlVP2, and one type-II V-PPase gene, SlVP3, in tomato plants. The major function of V-PPases in fruit has been believed to be the accumulation of materials such as sugars and organic acids in the vacuoles during cell expansion and ripening. However, these results show that specific localization of V-PPase mRNA induced by pollination plays a novel role in the cell division stage.
Fig. 8. Expression analysis of type-I V-PPase genes, SlVP1 and SlVP2, in three RNAi lines (TPR43, TPR79, TPR81). Real-time PCR was performed with cDNA prepared from total RNA extracted from fruit at 2 weeks after anthesis. Relative expression was determined in triplicate measurements in three independent biological replicates. Data shows the relative expression levels normalized against rRNA (TPR43) and Actin (TPR79, TPR81) with the standard errors.

Fig. 9. Fruit growth retardation in three RNAi lines (TPR43, TPR79, TPR81). The RNAi lines were independently grown with control plants, and fruit diameter was measured in the second inflorescences every week after anthesis. Values shown are the average with the standard errors ($n = 4–6$).

Fig. 10. Fruit growth retardation at an early stage of fruit development by the suppression of type-I V-PPase gene expression. Fruits of the control and RNAi line TPR79 are shown 2 weeks after anthesis.
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