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## RESEARCH PAPER

# Genetic suppression analysis in novel vacuolar processing enzymes reveals their roles in controlling sugar accumulation in tomato fruits

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## Abstract

In plant cells, many vacuolar proteins are synthesized as precursors in the endoplasmic reticulum and are subsequently transported to the vacuole. These precursors are subject to post-translational modifications to allow the active mature forms to be produced. Vacuolar processing enzyme (VPE) has been identified as a family of cysteine proteases involved in protein maturation in the vacuole. In this study, novel VPE genes were isolated from tomato (*Solanum lycopersicum*), and they were designated *SIVPE1*–*SIVPE5*. Phylogenetic analysis suggested that *SIVPE1* and *SIVPE2* were categorized as the seed coat type, *SIVPE4* was categorized as the seed type, and both *SIVPE3* and *SIVPE5* were categorized as the vegetative type. Expression analysis demonstrated that these genes were expressed during fruit development, and that their expression profiles agreed with this classification. High VPE enzyme activity was observed during tomato fruit development; the enzyme activity was correlated with the *SIVPE* mRNA levels, indicating that the *SIVPE* encoded active VPE proteins. The total sugar content was higher in RNA interference (RNAi) lines compared with the control plants, suggesting negative roles for *SIVPE* in sugar accumulation. The quantitative expression analysis of each *SIVPE* gene in the RNAi lines suggested that the suppression of *SIVPE5* probably had the strongest effect on the sugar accumulation observed. The suppression of *SIVPE* did not influence the total amino acid content, suggesting that the molecular targets of *SIVPE* were mainly involved in sugar accumulation.

**Key words:** Fruit, RNA interference, sugar accumulation, tomato, vacuolar processing enzyme.

## Introduction

The precursors of many vacuolar proteins are synthesized in the endoplasmic reticulum (ER) via the Golgi apparatus before they are transported into the vacuole. These precursors are then processed to generate mature active proteins. This protein maturation process is generally achieved by the cleavage of asparagine or aspartic acid residues in the C-terminal region, and this reaction is catalysed by vacuolar processing enzyme (VPE) proteins (Hara-Nishimura *et al.*, 1985, 2005; Hara-Nishimura and Nishimura, 1987; Hiraiwa *et al.*, 1997, 1999; Kuroyanagi *et al.*, 2002). VPE protein was originally identified as a novel

cysteine proteinase involved in the maturation process of seed storage proteins (Hara-Nishimura *et al.*, 1991). The VPE proteins belong to a family of cysteine proteinases and are well conserved among a variety of organisms including many plant and animal species (Hara-Nishimura *et al.*, 1993; Hara-Nishimura, 1998; Shirahama-Noda *et al.*, 2003). It has been demonstrated that the VPE proteins are synthesized in the ER as inactive precursors before they are converted into the mature active forms by self-catalytic activity under acidic conditions; this implies that VPE maturation takes place in the vacuole (Kuroyanagi *et al.*,

Abbreviations: DTT, dithiothreitol; PCD, programmed cell death; PMSF, phenylmethylsulphonyl fluoride; RNAi, RNA interference; SGN, Solanaceae Genomics Network; Sl, *Solanum lycopersicum*; SIVPE, *Solanum lycopersicum* vacuolar processing enzyme; VPE, vacuolar processing enzyme; z-ANN-MCA, benzylloxycarbonyl-L-alanyl-L-asparagine-4-methyl coumaryl-7-amide.

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2002). Several lines of evidence have also demonstrated that the cleavage of specific amino acid residues within the VPE proteins is essential for the maturation of the VPE proteins (Hiraiwa *et al.*, 1997, 1999; Chen *et al.*, 1998). Consistent with this, artificial mutations in these amino acid residues fail to produce the active forms of the VPE proteins (Hiraiwa *et al.*, 1999).

Previous phylogenetic, as well as expression and sequence analyses of VPE proteins have indicated that they are classified into the three following categories: seed type, seed coat type, and vegetative type (Yamada *et al.*, 2005). Two vegetative-type VPE proteins have been identified in *Arabidopsis*, namely  $\alpha$ VPE and  $\gamma$ VPE, both of which are mainly expressed in vegetative tissues. The mRNA expression of these genes shows up-regulation upon various abiotic stresses, such as wounding, senescence, and treatment with hormones such as jasmonic acids, ethylene, and salicylic acid (Kinoshita *et al.*, 1995, 1999; Yamada *et al.*, 2004). It is likely that  $\gamma$ VPE is specifically localized in vacuoles (Kinoshita *et al.*, 1995, 1999), and  $\gamma$ VPE exhibits caspase-1-like activity, which is thought to be the critical factor initiating programmed cell death (PCD) in animals (Rojo *et al.*, 2004). Although studies on the direct targets of VPE proteins have not been very successful, the processing activity of the vegetative  $\gamma$ VPE on yeast carboxypeptidase Y (CPY) has been demonstrated by heterologous expression analysis (Kinoshita *et al.*, 1999). In addition, the *Arabidopsis* orthologous AtCPY protein appears to be targeted for processing by  $\gamma$ VPE (Rojo *et al.*, 2003). Furthermore,  $\gamma$ VPE is essential for the degradation of the senescence-induced AtFRUCT4 (*Arabidopsis*  $\beta$ -FRUCTOSIDASE4) protein encoding an *Arabidopsis* vacuolar invertase in senescent leaves, indicating that  $\gamma$ VPE exhibits proteolytic activity (Rojo *et al.*, 2003). Interestingly, a proteomics-based approach using tandem mass spectrometry analysis suggested that  $\gamma$ VPE targets various types of vacuolar hydrolases (such as  $\beta$ -glucosidase,  $\alpha$ -mannosidases, and  $\alpha$ -galactosidases) for degradation, thus suggesting the presence of a regulatory mechanism for sugar accumulation and degradation by VPE proteins (Rojo *et al.*, 2003).

The seed-type VPE proteins, such as *Arabidopsis*  $\beta$ VPE, are known to be involved in the processing of seed storage proteins (Gruis *et al.*, 2002, 2004; Shimada *et al.*, 2003; Wang *et al.*, 2009). Seeds from the *Arabidopsis*  $\beta$ vpe knockout mutant are hindered in the maturation of storage proteins such as globulin and albumin. In addition,  $\beta$ VPE is highly expressed not only in seeds, but also in flower buds and mature pollen grains, suggesting that  $\beta$ VPE has nutritional roles required for pollen germination (Kinoshita *et al.*, 1995; Noguchi, 2006). Moreover, some seed-type VPE proteins are involved in the activation of antibiotic peptides in maturing seeds (Hara-Nishimura *et al.*, 1991; Yamada *et al.*, 1999; Shimada *et al.*, 2003). The *Arabidopsis*  $\delta$ VPE protein is a representative of the seed coat-type VPE proteins.  $\delta$ VPE is specifically expressed in two cell layers of the inner integument within the seed coat, and the loss of function of the  $\delta$ VPE gene results in delayed cell death of the two layers (Nakaune *et al.*, 2005). Unlike vegetative-

type VPE proteins, the seed coat-type  $\delta$ VPE lacks vacuolar sorting signals in the C-terminal region. It is therefore secreted into the apoplastic compartment and act as a regulator for PCD of the inner seed coat during the seed coat formation.

In addition to *Arabidopsis*, orthologous VPE genes have been reported in other plants. For example, the processing activity of castor bean and pumpkin VPEs on several seed storage proteins has been demonstrated *in vitro* (Hara-Nishimura *et al.*, 1993; Yamada *et al.*, 1999). Furthermore, tobacco was reported to express a functional VPE protein which acts as a key factor initiating hypersensitive cell death in response to infection with tobacco mosaic virus (TMV) and *Pseudomonas syringae* pv *phaseolicola* (Woltering *et al.*, 2002; Hatsugai *et al.*, 2004). Mutations in the *Oryza sativa* VPE1 (*OsVPE1*) or *GLUTELIN PRECURSOR 3* (*GLUP3*) in rice resulted in decreased VPE enzyme activity in seeds (Wang *et al.*, 2009; Kumamaru *et al.*, 2010). The decreased VPE enzyme activity in rice seeds not only causes the overaccumulation of proglutelin in seeds, but also alters the structure and morphology of the protein storage vacuoles and is involved in the compartmentalization of the storage proteins. Several VPE genes have been also identified in fruit crops. A functional VPE gene has been identified in *Citrus sinensis*; its mRNA expression was high in fruit and gradually increased as fruit matured (Alonso and Granell, 1995). In tomato, the expressed sequence tag (EST) sequence of the VPE orthologue, *Lycopersicon esculentum* VPE (*LeVPE1* or *SIVPE1* in this study) was reported (Lemaire-Chamley *et al.*, 1999). *LeVPE* is specifically expressed in fruit; however, no biochemical and genetic evidence for this gene has been reported.

In this study, the expression and activity of the tomato VPE genes were examined during fruit development. First, four additional VPE genes (*SIVPE2*–*SIVPE5*) were identified in the tomato genome by BLAST database searches using *SIVPE1* as the query; the quantitative expression analysis of the five VPE genes was conducted. Next, expression analysis of the five *SIVPE* promoters using the *GUS* ( $\beta$ -glucosidase) reporter gene was performed; the spatial expression pattern of these *SIVPE* genes was examined during fruit development. The expression of the *SIVPE* genes was suppressed by the RNA interference (RNAi) strategy to examine the effect of decreased *SIVPE* mRNA expression on VPE enzyme activity. The results suggest that the levels of *SIVPE* mRNA expression were correlated to the levels of VPE activity; decreased VPE enzyme activity interfered with sugar accumulation in mature fruits.

## Materials and methods

### Plant materials and growth conditions

Seeds of *S. lycopersicum* cv. Micro-Tom (Scott and Harbaugh, 1989) were obtained from the National BioResource Project Tomato (NBRP-tomato, <http://tomato.nbrp.jp/indexEn.html>) (Yamazaki *et al.*, 2010) from the TOMATOMA database ([Downloaded from \[jxb.oxfordjournals.org\]\(http://jxb.oxfordjournals.org\) at UNIV OF TSUKUBA on June 26, 2011](http://</a></p>
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tomato.nbrp.jp). Seeds of the wild type, a transformant with the control vector (control), transformants in which each *SIVPE* gene is suppressed by RNAi (*RNAi-SIVPE*), and transformants in which each *SIVPE* promoter is transcriptionally fused to the *GUS* gene were used in this study. All seeds were imbibed in sterile water at 26 °C for 7 d under fluorescent light (16 h/8 h day/night). The seedlings were then transferred to rock wool and grown under the same conditions.

#### Protein structural analysis of the *SIVPE* proteins using publicly available databases

To identify orthologous *VPE* genes from the tomato genome, a BLAST database search analysis was performed at SGN (Solanaceae Genomics Network, <http://solgenomics.net/>) and KafTom (<http://www.pgb.kazusa.or.jp/kafTom/>) (Aoki *et al.*, 2010). The primary features of the *SIVPE* proteins were examined using the publicly available databases mentioned below. To predict the presence of the signal peptide and to postulate subcellular localization, the iPSORT (<http://ipsort.hgc.jp/>), SignalP (<http://www.cbs.dtu.dk/services/SignalP/>), and TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) programs were used. To predict the presence of functional motifs, the Pfam (<http://pfam.sanger.ac.uk/>) program was used. The MEGA4 (<http://www.megasoftware.net/mega.html>) program was used for phylogenetic analysis. To predict the molecular weight and isoelectric point (pI), the Compute pI/Mw tool ([http://au.expasy.org/tools/pi\\_tool.html](http://au.expasy.org/tools/pi_tool.html)) program was used.

#### Vector construction and transformation

RNAi constructs to suppress mRNA expression of each *SIVPE* gene were created under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. To create RNAi constructs targeted for *SIVPE1* suppression, the DNA fragment of *SIVPE1* was amplified as the RNAi-targeted region, by using the gene-specific primers shown in Supplementary Table S5 available at *JXB* online. The PCR fragment was directly cloned into the entry vector pCR8/GW/TOPO (Invitrogen) and was then transferred into the Gateway vector pBIDAVL-GWR1 (*In planta* innovation) by the Gateway LR Clonase enzyme (Invitrogen), resulting in *SIVPE1/pBIDAVL-GWR1*. This construct was designated *RNAi-SIVPE1*. The same strategy was used to create RNAi constructs for the suppression of other *SIVPE* genes using the primers shown in Supplementary Table S5. The RNAi constructs obtained targeted *SIVPE2*, *SIVPE3*, *SIVPE4*, and *SIVPE5* for down-regulation, and were designated *RNAi-SIVPE2*, *RNAi-SIVPE3*, *RNAi-SIVPE4*, and *RNAi-SIVPE5*, respectively.

For spatial expression analysis, transcriptional fusion constructs in which each *SIVPE* promoter was fused to the *GUS* reporter gene were created. To create the *SIVPE1* transcriptional fusion vector, ~2.0 kb of the promoter region of *SIVPE1* was amplified using promoter-specific primers (Supplementary Table S5 at *JXB* online) and directly cloned into the entry vector pCR8/GW/TOPO. The PCR fragment of the promoter region was transferred to the Gateway vector pWGB3 in which the *GUS* reporter gene is present at the C-terminus (Nakagawa *et al.*, 2007); the resulting construct was an *SIVPE1* promoter transcriptional vector designated *SIVPE1p-GUS*. The same strategy was used to create transcriptional fusion constructs for the other *SIVPE* genes, and the constructs were designated *SIVPE2p-GUS*, *SIVPE3p-GUS*, *SIVPE4p-GUS*, and *SIVPE5p-GUS*, respectively.

These constructs were then transformed into *Agrobacterium tumefaciens* GV2205 by the freeze-thaw method (An *et al.*, 1988). The constructs were transformed into WT Micro-Tom by the *Agrobacterium* method (Sun *et al.*, 2006). The pGWB3 empty vector was also introduced into Micro-Tom to create control plants. The transgenic plants were selected on Murashige and Skoog (MS) agar plates containing kanamycin (100 mg l<sup>-1</sup>). In this study, the transgenic RNAi lines with single copy insertions

were selected, and their T<sub>2</sub> generations were used for further studies.

#### Quantitative expression analysis

The mRNA expression of each *SIVPE* gene was analysed by quantitative reverse transcription-PCR (qRT-PCR) using total RNA extracted from the whole fruit, leaf, root, stem, flower, and anther tissues indicated in Fig. 2 and using an RNA easy kit (Qiagen). Genomic DNA contamination was removed using the RNase-free DNase Set (Qiagen). cDNA was generated from 5 µg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The cDNA was then used as a template for quantitative PCR and was amplified with gene-specific primers (Supplementary Table S5 at *JXB* online). The primer specificities were confirmed by sequencing the PCR fragments. The qRT-PCR experiments were performed using a Takara Thermal Cycler Dice Real Time System with SYBR Premix Ex Taq II. The PCR conditions were as follows: 10 s of denaturation at 95 °C, followed by 40 cycles of 5 s of denaturation at 95 °C and 30 s of annealing/extension at 55–60 °C, depending on the primer pairs (Supplementary Table S5). The transcript levels were analysed to determine the mRNA levels of each *SIVPE* relative to the control *UBIQ-UITIN* (*UBQ*) mRNA as performed by Kim *et al.* (2010).

#### Histochemical expression analysis

The spatial *GUS* expression pattern of each *SIVPEp-GUS* plant was analysed by a histochemical assay using fruit samples, as indicated in Figs 3 and 4. T<sub>1</sub> generations of each transgenic plant were used for histochemical analysis. These fruit samples were soaked in a *GUS* assay solution consisting of 100 mM sodium phosphate (pH 7.0), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 0.3% Triton X-100, 20% methanol, and 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-D-glucuronide). The samples were incubated at 37 °C for 24 h, washed with water several times, and bleached with 70% ethanol. The samples were mounted on slides and photographed using a light microscope.

#### Measurement of *VPE* enzyme activity

The *VPE* enzyme activity was determined using the fluorescent *VPE*-specific substrate z-ANN-MCA (benzyloxycarbonyl-L-alanyl-L-asparagine-4-methyl coumaryl-7-amide; Peptide Institute) to monitor the rate of substrate degradation by *VPE* enzyme activity, according to Shimada *et al.* (2003). The tissues listed in Figs 5 and 7, and in Supplementary Figs S2–S4 at *JXB* online, were ground with liquid N<sub>2</sub>, and the respective powders were resuspended in buffer A [50 mM Na-acetate buffer (pH 5.5), 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM E-64-d]. Then, the solution was mixed well and centrifuged at 10 000 g for 30 min. The supernatant was used to measure enzyme activity. Exactly 30 µl of the supernatant were resuspended in 130 µl of distilled deionized water and 40 µl of buffer B [buffer B is 5× solution; 500 mM Na-acetate buffer (pH 5.5), 500 mM dithiothreitol (DTT)], and the solution was mixed well. The mixture was incubated at 37 °C for 10 min, before 4 µl of 10 mM z-ANN-MCA was added. The solution was incubated at 37 °C, and the fluorescence intensity was determined using Wallac 1420 ARVO MX/Light (Perkin-Elmer). The fluorescence was measured at an excitation wavelength of 380 nm and at an emission wavelength of 460 nm.

#### Measurement of sugar content in fruits

Whole fruits were harvested from the control plants transformed with the empty vector and T<sub>2</sub> generations of transgenic RNAi plants at the mature green (MG) stage [27–30 days after flowering (DAF)] and mature red (RED) stage (DAF39–45); these samples were used for sugar analysis. The fruit samples were ground under

liquid N<sub>2</sub>, and 200 mg of the powder was resuspended in 200 µl of deionized water. The samples were boiled at 100 °C for 10 min to inactivate the sugar-degrading enzymes, placed on ice for 5 min, and centrifuged at 15 000 g for 30 min. Next, 200 µl of supernatant was resuspended in 600 µl of acetonitrile, centrifuged at 15 000 g for 15 min, and the supernatant was filtrated using a filter (PTFE 0.45 µl DISMIC 25HP, Millipore). The content of glucose, fructose, and sucrose was determined by high-performance liquid chromatography (HPLC). The soluble sugars were separated at 40 °C on a Shodex Asahi pack NH2P-50 4E column (250×4.6 mm, Showa Denko KK) installed in the LC system 8020 series (Tosoh Co.), and the refractive index (RI) was detected using an RI detector. The mobile phase was acetonitrile/water (75%/25%) at a flow rate of 1 ml min<sup>-1</sup> as described previously (Yin et al., 2010).

#### Measurements of amino acids and related metabolites

The whole fruits at the MG and RED stages were ground under liquid N<sub>2</sub>; 50 mg of the powdered tissues were resuspended in 500 µl of 8% trichloroacetic acid (TCA) solution and centrifuged at 15 700 g for 20 min. Then, 300 µl of supernatant were transferred to a 1.5 ml tube, and 400 µl of diethyl ether was added. The solution was mixed well for 10 min and was centrifuged at 13 400 g for 5 min. The lower phase was resuspended with 400 µl of diethyl ether, mixed well for 10 min, and centrifuged at 13 400 g for 5 min. The upper phase (diethyl ether) was removed, and the tubes were dried for 30 min at room temperature. The remaining solution was completely evaporated by a centrifugal evaporator at 60 °C for 30 min. The pellet was dissolved in 100 µl of deionized water, and evaporated by the centrifugal evaporator. This process was repeated once more, and the dried samples were dissolved in 300 µl of 0.1 N HCl. The metabolites were determined using an amino acid analyser (JEOL JLC-500/V2).

#### Cytological analysis

To observe seed coat formation, fruit samples at DAF19 were fixed overnight in FAA (formalin–acetic acid–alcohol: ethanol 50%, acetic acid 5.0%, and formaldehyde 3.7%), dehydrated in a graded ethanol series (50% ×2, 70%, 85%, and 100% ×3), and embedded in paraffin (Paraplast Plus, Oxford). Seed transverse sections (15–20 µm) were then observed under a light microscope.

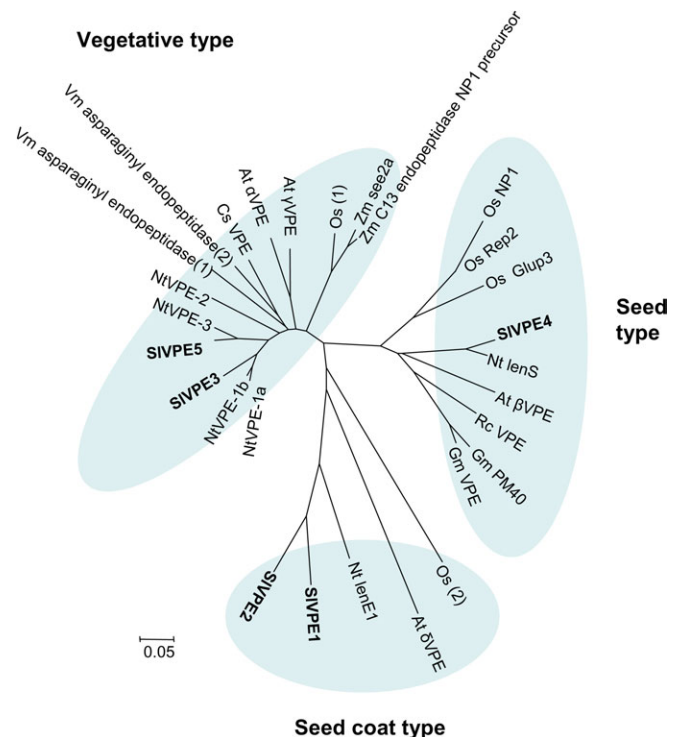
## Results

### Isolation of a family of SIVPE genes from tomato

A previous study isolated an orthologue of the *Arabidopsis* VPE, named *LeVPE* (*SIVPE1* in this study), which was originally identified as the EST sequence specifically expressed in tomato fruits (Lemaire-Chamley et al., 1999). To isolate its homologous genes in tomato, the *SIVPE1* sequence was subjected to a BLAST database search at the SGN. This analysis isolated four additional homologous genes, designated *SIVPE2*, *SIVPE3*, *SIVPE4*, and *SIVPE5*. *SIVPE5* appeared to be identical to *S. lycopersicum* CYSTEINE PROTEINASE (*SICp*), a gene which was previously identified as showing sequence similarity to cysteine proteinases (Matarasso et al., 2005). These five full-length coding sequences (CDS) were recovered from the SGN and Kaftom databases (Supplementary Table S1 at JXB online) (Aoki et al., 2010), and their chromosome locations were examined. Only *SIVPE5* was located on chromosome 12; the other four genes were located on

chromosome 8. Interestingly, *LeVPE1/SIVPE1* and *SIVPE2* were tandemly located within an 8 kb interval, implying that both might result from gene duplication. The sequence similarity among these proteins was generally high (77.6–91.1%). In addition, the five SIVPE proteins had primary protein features (e.g. length of CDS, protein length, and molecular weight) comparable with the *Arabidopsis* VPE proteins (Supplementary Table S2 at JXB online). Interestingly, *SIVPE1* and *SIVPE2* showed the highest similarity (91.1%) among the five SIVPE proteins. Consistently, phylogenetic analysis indicated that *SIVPE1* and *SIVPE2* were classified as seed coat-type VPEs, which also include *Arabidopsis* δVPE (Fig. 1). *SIVPE4* belonged to the seed-type VPEs containing the *Arabidopsis* βVPE, whereas *SIVPE3* and *SIVPE5* belonged to the vegetative-type VPEs containing the *Arabidopsis* αVPE and γVPE.

The presence of a signal peptide in the SIVPE proteins was predicted by the three representative databases, iPSORT, SignalP3.0, and TargetP. This analysis suggested that all SIVPEs contained signal peptides, like the *Arabidopsis* VPE proteins (Supplementary Table S2 at JXB online). Next, the sequences of these SIVPEs were subject to a Pfam motif analysis. Like the *Arabidopsis* VPE proteins, all the SIVPEs contained a motif for peptidase C13, which is well conserved in members of asparaginyl



**Fig. 1.** Phylogenetic analysis of VPE and VPE-like proteins in plants. Plant VPE proteins were classified based on their protein sequences into the three following categories: seed coat type, seed type, and vegetative type. Phylogenetic analyses were conducted using MEGA4. At, *Arabidopsis thaliana*; Cs, *Citrus sinensis*; Gm, *Glycine max*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Rc, *Ricinus communis*; Vm, *Vigna mungo*; Zm, *Zea mays*. The size bar shows the estimated evolutionary distance.

peptidases and VPE proteins (Hara-Nishimura *et al.*, 1993; Yamada *et al.*, 2005). In addition, these five SIVPE proteins contained one histidine and two cysteine residues, all of which are required for the VPE self-catalytic activity and were similar to the already characterized *Arabidopsis* and rice VPE proteins (Supplementary Fig. S1A–C) at *JXB* online (Chen *et al.*, 1998; Hiraiwa *et al.*, 1999). Activation of VPE requires processing of the VPE precursors in the C-terminal region, while proper protein folding may require cleavage in the N-terminal region (Kuroyanagi *et al.*, 2002). All SIVPE proteins contained conserved cleavage sites in both the N- and C-terminal regions (Supplementary Fig. S1D, E at *JXB* online). These results suggested that the five SIVPE genes identified in this study had primary protein features very similar to those of other VPE proteins, suggesting that these SIVPE proteins might encode proteins exhibiting VPE biochemical activity.

#### Quantitative temporal expression analysis of SIVPE genes

To examine the temporal expression pattern of the SIVPE genes, qRT-PCR analysis was carried out using mRNA extracted from leaf, stem, root, and different developmental stages of anther and fruit as depicted in Fig. 2A. The mRNA accumulation of SIVPE1 and SIVPE2 was specifically observed in fruit, especially during early fruit development (i.e. after DAF9), and expression of SIVPE1 and SIVPE2 was specifically high at DAF15 and DAF21, whereas their expression was significantly decreased after DAF27 (Fig. 2B). Although these two seed coat-type SIVPE genes showed similar temporal patterns of mRNA expression, the other members showed different expression patterns. The mRNA expression of SIVPE3 and SIVPE5, classified as vegetative-type VPEs, was elevated around DAF15–27, and the highest expression was observed at DAF39 and DAF33, respectively, followed by a decrease at DAF45 (Fig. 2D, F). In addition, the mRNA expression of SIVPE3 and SIVPE5 was observed in vegetative tissues, and strong expression was evident in anthers at anthesis. The mRNA expression of SIVPE4, classified as a seed coat-type VPE, was observed during fruit development, especially after DAF15, and the highest expression was evident at DAF33 (Fig. 2E). The mRNA expression of SIVPE4 was decreased after DAF39. In addition, SIVPE4 expression was detected in vegetative and flower tissues. These results suggested that a family of SIVPE genes might function throughout many plant tissues.

#### Spatial expression analysis of SIVPE genes using the GUS reporter gene

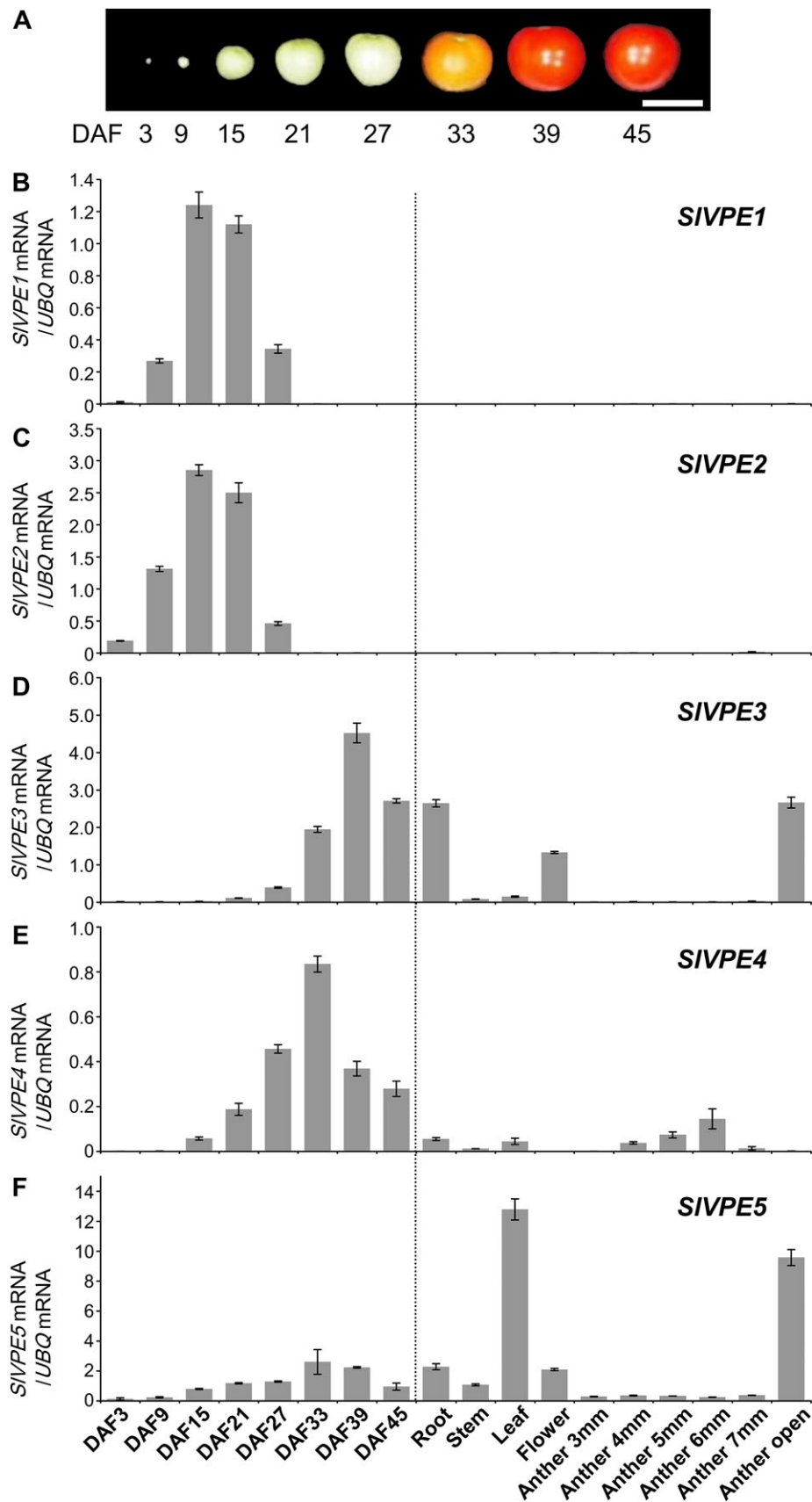
Next the spatial expression pattern of the SIVPE genes during fruit development was explored using a 2 kb promoter region of each SIVPE gene, which was fused to the GUS reporter gene (SIVPE1p–SIVPE5-GUS). Histological staining using developing fruits from the SIVPE1p-GUS lines showed that the majority of GUS

activity was evident in the seeds within fruits at the MG (DAF27–30) stage (Fig. 3A). The SIVPE2p-GUS plants showed GUS activity in the seeds and in the vascular bundles at the MG stage (Fig. 3B). Weak GUS activity was observed within the placenta at the yellow (YL; DAF30–33) stage in SIVPE1p-GUS and SIVPE2p-GUS. Clear GUS activity was observed in vascular bundles extending from the seeds to the placenta as well as around the endocarp in SIVPE3p-GUS and SIVPE5p-GUS throughout fruit development (Figs 3C, E, 4A). Interestingly, SIVPE4p-GUS showed weak GUS activity in the vascular bundles around the endocarp at the MG stage, whereas the GUS activity was exclusively restricted to within seeds at the YL and the RED (DAF39–45) stages (Fig. 3D). Next, the pattern of GUS expression in seeds was carefully observed under a stereo microscope. As shown in Fig. 4B, SIVPE1p-GUS and SIVPE2p-GUS showed GUS activity in the seed coat of developing fruits; this finding was consistent with the fact that SIVPE1 and SIVPE2 are seed coat-type VPEs (Fig. 1). Similarly, both SIVPE3p-GUS and SIVPE5p-GUS showed GUS activity in the seed coat (Fig. 4B). The GUS activity of SIVPE4p-GUS was exclusively evident in the seed endosperm and embryo of developing fruits (Fig. 4B); this observation was also consistent with the notion that the SIVPE4 belongs to the seed-type VPEs (Fig. 1). These spatial expression analyses of the SIVPE genes suggested that SIVPE may play a role(s) during fruit development, especially through vascular bundles and seeds.

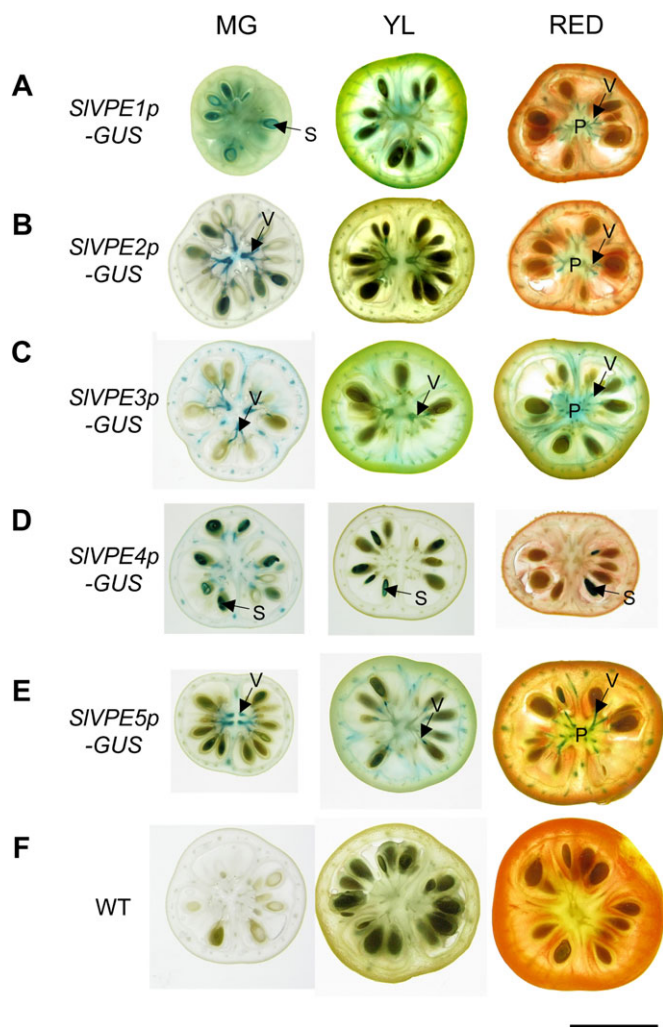
The expression pattern observed using the GUS histochemical assay was not entirely consistent with the results obtained from qRT-PCR. For example, the mRNA expression of SIVPE1 and SIVPE2 was barely detected at the RED stage (Fig. 2B, C), whereas clear GUS activity was evident at the RED stage in SIVPE1p-GUS and SIVPE2p-GUS (Fig. 3A, B). This unexpected GUS staining might be due to the high stability of the GUS protein as reported previously (Kosugi *et al.*, 1990; Debblock and Debrouwer, 1992; Aarts *et al.*, 1997; Wilson *et al.*, 2001; Ariizumi *et al.*, 2002; Ito and Shinozaki, 2002).

#### Measurement of VPE enzyme activity during fruit development

The expression analysis suggested that SIVPE proteins function during fruit development. If this were true, tomato fruits would be expected to exhibit VPE enzymatic activity, because VPE functional activity is most probably correlated with VPE enzyme activity (Gruis *et al.*, 2002, 2004; Shimada *et al.*, 2003; Hara-Nishimura *et al.*, 2005; Yamada *et al.*, 2005; Wang *et al.*, 2009; Kumamaru *et al.*, 2010). A time course experiment was performed to measure the VPE activity of wild-type (WT) Micro-Tom over 45 d during fruit development as depicted in Fig. 2A. VPE activity was barely detected at the early stages (DAF3 and DAF9), but it gradually increased as the fruit developed (i.e. between DAF15 and DAF39) (Fig. 5). The highest VPE activity was recorded at DAF39, but its activity was significantly decreased at DAF45 (~50% compared with DAF39).



**Fig. 2.** Quantitative RT-PCR expression analysis of five *SIVPE* genes. (A) Fruit developmental stages used for this experiment. Bar=15 mm. (B–F) Levels of mRNA expression in fruits, leaves, root, stem, flower, and anther were determined at the indicated time points. Levels of (B) *SIVPE1* mRNA, (C) *SIVPE2* mRNA, (D) *SIVPE3* mRNA, (E) *SIVPE4* mRNA, and (F) *SIVPE5*. The mean values of at least three independent experiments are shown. The error bars represent the SE. DAF, days after flowering.

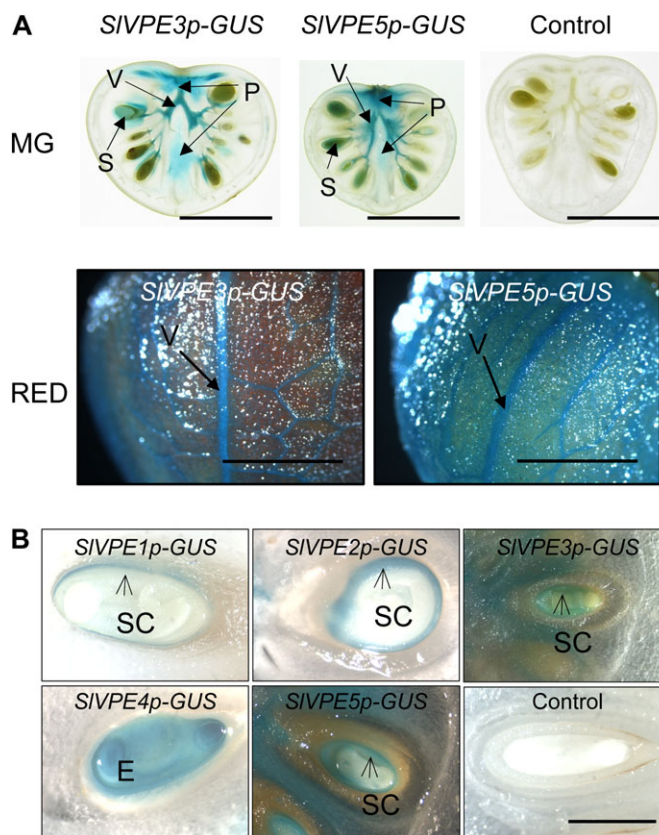


**Fig. 3.** Spatial expression analysis of *SIVPE* genes by histological staining during fruit development. The GUS expression pattern was examined during fruit development in *SIVPE1p-GUS*, *SIVPE2p-GUS*, *SIVPE3p-GUS*, *SIVPE4p-GUS*, and *SIVPE5p-GUS*. WT fruits were also stained as a negative control. MG, mature green stage (DAF27–30). YL, yellow stage (DAF30–33). RED, red stage (DAF39–45). P, placenta; V, vascular bundle; S, seed. Bar=1.0 cm.

This observation was similar to the significant decline observed in *SIVPE3–SIVPE5* mRNA expression at DAF45 (Fig. 2D–F). In addition, VPE activity was detected in vegetative tissues such as root, leaf, and stem, although its activity in these tissues was relatively lower compared with that in developing fruits (Supplementary Fig. S2 at *JXB* online). It seems likely that the pattern of intensity of VPE enzyme activity was similar to that of *SIVPE3–SIVPE5* mRNA expression (Figs 2, 5).

#### Creation of transgenic tomato with decreased *SIVPE* expression

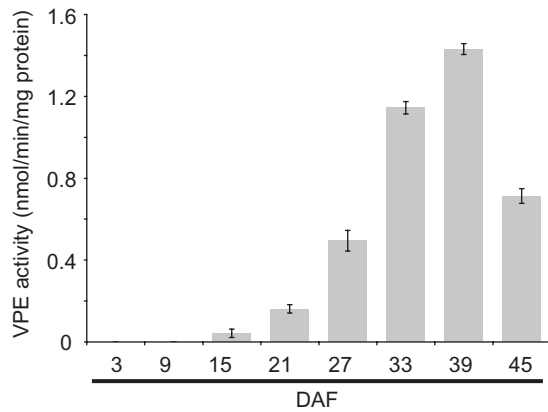
To examine further whether the VPE enzyme activity in tomato was related to the levels of *SIVPE* mRNA expression, and to explore the physiological functions of



**Fig. 4.** Histochemical analysis of *SIVPEp-GUS* lines. (A) Longitudinal sections of *SIVPE3p-GUS* and *SIVPE5p-GUS* at the MG stage (upper panel). Clear GUS activity was evident in the vascular bundles in *SIVPE3p-GUS* and *SIVPE5p-GUS* at the RED stage (lower panel). Bar=1.0 cm. (B) GUS activity pattern in seeds in *SIVPEp-GUS* lines. Bar=0.5 mm. E, embryo; P, placenta; S, seed; SC, seed coat; V, vascular bundle.

*SIVPE* during fruit development, transgenic plants with decreased mRNA levels of each of the *SIVPE* genes were produced using the RNAi strategy. In this experiment, the levels of *SIVPE* mRNA expression in the RNAi lines were compared with those in the control plants (Fig. 6). The mRNA was extracted from fruits, and the mRNA expression of each of the *SIVPE* genes relative to the *UBQ* gene was compared between *RNAi-SIVPE* lines and the control plants by qRT-PCR. The qRT-PCR analysis revealed that the expression levels of *SIVPE1* relative to *UBQ* in the *RNAi-SIVPE1* lines were greatly reduced, reaching only 0.001 (1-a), 0.002 (1-b), and 0.11, (1-c), respectively, when the *SIVPE1* expression level in the control plants was set as 1.0 (Fig. 6A). The same experiment was performed with the *RNAi-SIVPE2* (2-a, 2-b, and 2-c), *RNAi-SIVPE3* (3-a, 3-b, and 3-c), *RNAi-SIVPE4* (4-a), and *RNAi-SIVPE5* (5-a, 5-b, and 5-c) lines. When the mRNA expression level was compared with that of the control plants, the expression of *SIVPE3* in the *RNAi-SIVPE3* line showed a considerable reduction of 0.26 (3-a), 0.11 (3-b), and 0.16 (3-c), respectively (Fig. 6C); on the other hand, the mRNA expression of *SIVPE5* in the *RNAi-SIVPE5* line decreased to 0.12 (5-a), 0.11 (5-b), and 0.07 (5-c), respectively (Fig. 6E). Relative to





**Fig. 5.** Time course analysis of VPE enzymatic activity during fruit development. The VPE enzyme activity was measured at the indicated time points during fruit development. Crude protein was extracted from whole fruits and used for this analysis. The mean values of at least three independent experiments are shown. The error bars represent the SE. DAF, days after flowering.

the expression in the control plants, the mRNA expression of *SIVPE2* in the *RNAi-SIVPE2* line was 0.07 (2-a), 0.03 (2-b), and 0.12 (2-c) (Supplementary Fig. S3A at *JXB* online) and that of *SIVPE4* in the *RNAi-SIVPE4* line was 0.05 (4-a) (Supplementary Fig. S3B at *JXB* online).

Because the vegetative-type *SIVPE* genes showed higher mRNA expression in the leaf (Fig. 2), the relative mRNA levels were examined using leaf tissue in several independent lines of *RNAi-SIVPE3* (3-a, 3-b, 3-c, 3-d, and 3-e) and *RNAi-SIVPE5* (5-a, 5-b, 5-c, and 5-d). The qRT-PCR analysis performed to examine the suppression in *SIVPE3* or *SIVPE5* indicated that both *RNAi-SIVPE3* and *RNAi-SIVPE5* showed a great reduction from 0.05 to 0.66, and from 0.7 to 0.15, respectively, compared with the expression levels in the control plants (the expression level was set as 1.0) (Supplementary Fig. S4A at *JXB* online). These results indicated that a decrease in mRNA expression of all the *SIVPE* genes was effectively achieved by the RNAi strategy.

When the highly conserved region of a gene family is used for RNAi-mediated gene silencing, mRNA expression of other members of the gene family is effectively suppressed (Miki et al., 2005). Since five members of the *SIVPE* gene family show high sequence similarity, it was expected that the *RNAi-SIVPE* plants would show decreased mRNA expression in other members of the *SIVPE* gene family. To examine this hypothesis, the relative mRNA expression of all members of the *SIVPE* genes was determined by qRT-PCR in the *RNAi-SIVPE1*, *RNAi-SIVPE3*, and *RNAi-SIVPE5* lines. This experiment demonstrated that the mRNA levels of five members of the *SIVPE* genes were also highly suppressed to variable degrees in all transgenic plants (Fig. 6). As expected, there was a large suppression in the genes showing high DNA homology to the RNAi-targeted region (Supplementary Table S3 at *JXB* online). In fact, the highest DNA homology between *SIVPE1* and *SIVPE2* in the RNAi-targeted region (89.2%) resulted in the greatest suppression in both *SIVPE* genes (Fig. 6A, B). The sequence

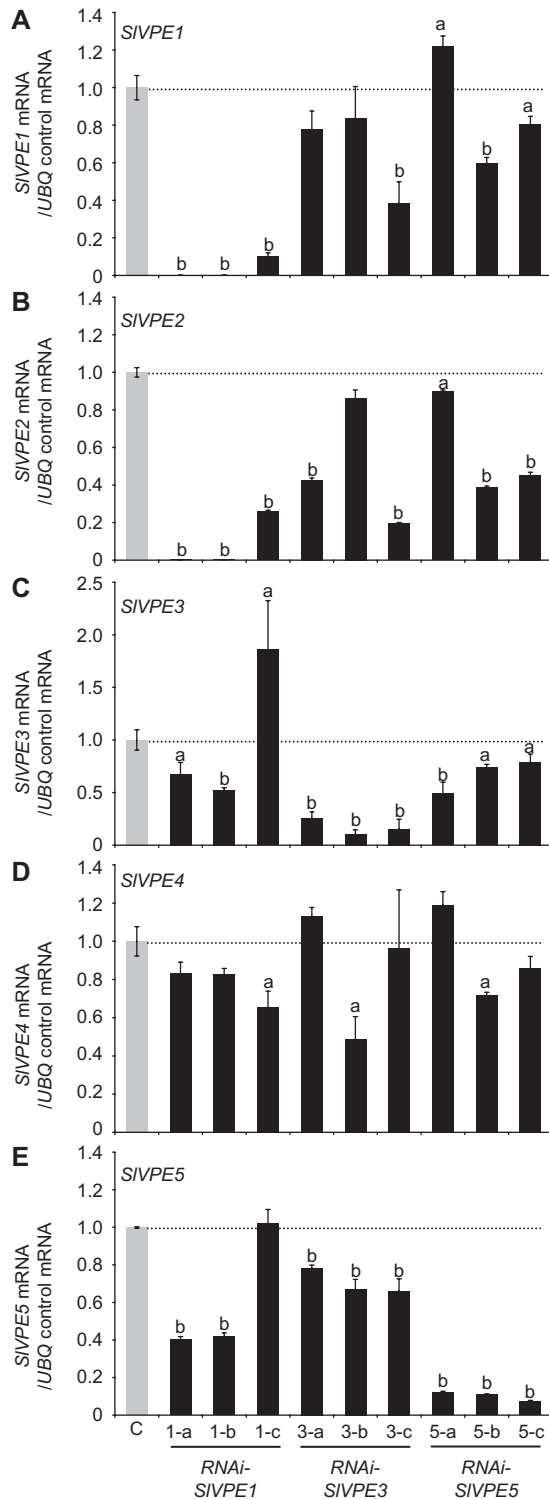
similarity of the RNAi-targeted regions was between 68.7% and 89.2% (Supplementary Table S3 at *JXB* online), suggesting that a sequence homology of ~70% could be effectively targeted by RNAi in tomato; this finding was consistent with a previous report in rice (Miki et al., 2005).

#### *SIVPE* proteins exhibited biochemical VPE activity in tomato

In rice and *Arabidopsis*, loss-of-function mutations in the *VPE* genes result in significantly reduced VPE enzyme activity (Chen et al., 1998; Gruis et al., 2002, 2004; Shimada et al., 2003; Kumamaru et al., 2010). To examine whether a decrease in *SIVPE* mRNA expression was associated with a decrease in VPE activity, the VPE enzyme activity was measured using leaf and fruit tissues in the RNAi lines. Figure 7 shows the VPE enzyme activity in the *RNAi-SIVPE* lines using the same fruit samples used in Fig. 6. The *RNAi-SIVPE1* line exhibited 42.4–70.0% VPE activity, while *RNAi-SIVPE3* exhibited 72.0–75.6% VPE activity, when the VPE activity in the control plants was set as 100%. The *RNAi-SIVPE5* exhibited 71.2–83.5% VPE activity. The *RNAi-SIVPE2* and *RNAi-SIVPE4* lines also showed decreased VPE enzyme activity compared with the control plants (Supplementary Fig. S3C at *JXB* online). Moreover, reduction in *SIVPE* mRNA expression in leaves was also correlated to the levels of VPE enzyme activity (Supplementary Fig. S4B at *JXB* online). These results suggested that the decreased levels of *SIVPE* mRNA were correlated to the decreased levels of VPE activity. Taken together, it is likely that the *SIVPE* genes identified in this study encode proteins exhibiting biochemical VPE activity in tomato.

#### Decrease in VPE activity enhances sugar accumulation in tomato fruit

Since it was previously demonstrated in many plant and animal species that decreased VPE activity results in various developmental arrests (Gruis et al., 2002, 2004; Shimada et al., 2003; Yamada et al., 2005; Wang et al., 2009; Kumamaru et al., 2010), whether the reduction in VPE activity affected tomato growth was next investigated. To examine whether the reduced VPE activity caused defects in inner seed coat formation similar to the *Arabidopsis* *dyve* mutants (Nakaune et al., 2005), transverse sections of developing seeds were observed. However, no difference in the thickness of the inner integument was observed between the *RNAi-SIVPE* lines and the control plants (Supplementary Fig. S5 at *JXB* online). To explore further the roles of the *SIVPE* proteins during fruit development, fruits at the MG stage and the RED stage were harvested, and the contents of fructose, sucrose, and glucose were compared between the control and RNAi lines. As shown in Fig. 8, although no significant difference in content was calculated for any of the sugars at the MG stage between the RNAi lines and the control plants, the suppression of the *SIVPE* genes appeared to cause an increase in the sugar content at the RED stage. For instance, the glucose content in two

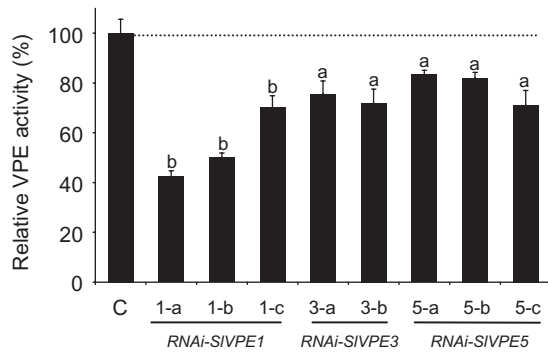


**Fig. 6.** Levels of *SIVPE* mRNA suppression in RNAi lines. The expression levels of (A) *SIVPE1*, (B) *SIVPE2*, (C) *SIVPE3*, (D) *SIVPE4*, and (E) *SIVPE5* in the *RNAi-SIVPE1*, *RNAi-SIVPE3*, and *RNAi-SIVPE5* lines were determined by qRT-PCR. The mRNA was extracted from whole fruits at the MG stage. The mRNA levels in three independent transgenic lines of *RNAi-SIVPE1* (1-a, 1-b, and 1-c), *RNAi-SIVPE3* (3-a, 3-b, 3-c), and *RNAi-SIVPE5* (5-a, 5-b, and 5-c), were determined. The mean values of at least three independent experiments are shown. The error bars represent the SE. a,  $P < 0.05$ ; b,  $P < 0.01$ , as determined by the *t*-test.

lines of *RNAi-SIVPE1* (1-a and 1-b) showed an approximate increase of 2.4- to 2.6-fold compared with the control; interestingly, two lines of *RNAi-SIVPE5* (5-a and 5-c) also showed an increase of ~2.1- to 2.3-fold, respectively (Fig. 8A). In contrast, no significant difference was observed between the *RNAi-SIVPE3* lines and control plants. The level of fructose accumulation showed a small increase from the MG stage to the RED stage, like the control. A larger increase in fructose accumulation was observed in the *RNAi-SIVPE1* and *RNAi-SIVPE5* lines (Fig. 8B). The fructose content in the *RNAi-SIVPE1* (1-a and 1-b) and *RNAi-SIVPE5* (5-c) lines was ~1.4- to 1.7-fold higher than that of the control, respectively, whereas the fructose level in the *RNAi-SIVPE3* lines (3-a and 3-b) was equivalent to that of the control. The level of sucrose accumulation was sharply reduced from the MG stage to the RED stage in the control (Fig. 8C). In contrast to the control, the level of sucrose accumulation in the *RNAi-SIVPE* lines showed a significant increase, except for lines 1-b and 3-a; they showed no significant difference compared with the control plants. The sucrose contents in lines 1-a, 3-b, 5-a, and 5-c were 3.7-, 3.8-, 5.0-, and 8.2-fold higher than that in the control, respectively. The total sugar content in *RNAi-SIVPE1* and *RNAi-SIVPE5* was increased by 1.8- to 2.1-fold, but no significant changes in sugar content were observed in *RNAi-SIVPE3* compared with that in the control (Fig. 9). This result suggests that the suppression of *SIVPE1* and *SIVPE5* had stronger effects on the increased sugar content in fruits. The results also suggest that decreased VPE activity was associated with the increased levels of sugars in the fruits at the RED stage.

#### Measurement of amino acids and related substances

Whether the suppression of the *SIVPE* genes influenced the accumulation of the 22 standard amino acids and 11 other related substances was next explored. In this experiment, fruits at the MG stage and the RED stage were harvested from the control and RNAi lines, and their metabolites were analysed using an amino acid analyser. As shown in Supplementary Table S4 at *JXB* online, no significant difference was calculated in the accumulation of the compounds examined at the MG stage between the control and RNAi lines. At the RED stage, the accumulation of several amino acids was higher in the RNAi lines than that in the control plants. For example, the accumulation of aspartic acid, glutamic acid, valine, histidine, and lysine in *RNAi-SIVPE3* was 1.39-, 1.36-, 1.39-, 1.34-, and 1.30-fold higher than that in the control, respectively. The accumulation of serine and glutamic acid in *RNAi-SIVPE1* was 1.40- and 1.44-fold higher than that in the control, respectively. However, no difference was observed between the control and *RNAi-SIVPE5* at the RED stage. In addition, no difference in the total content of amino acids and related substances between the control and the *RNAi-SIVPE* lines was found. These results suggested that the suppression of



**Fig. 7.** Effect of *SIVPE* suppression on VPE enzyme activity. A decrease in *SIVPE* mRNA expression was associated with decreased levels of VPE enzyme activity in the RNAi lines. VPE enzymatic activity of whole fruit tissue at the MG stage was determined in the control, *RNAi-SIVPE1*, *RNAi-SIVPE3*, and *RNAi-SIVPE5* lines. The VPE activity in the control was set as 100%, and the relative VPE activity is shown. The mean values of at least three independent experiments are shown. The error bars represent the SE. a,  $P < 0.05$ ; b,  $P < 0.01$ , as determined by the *t*-test.

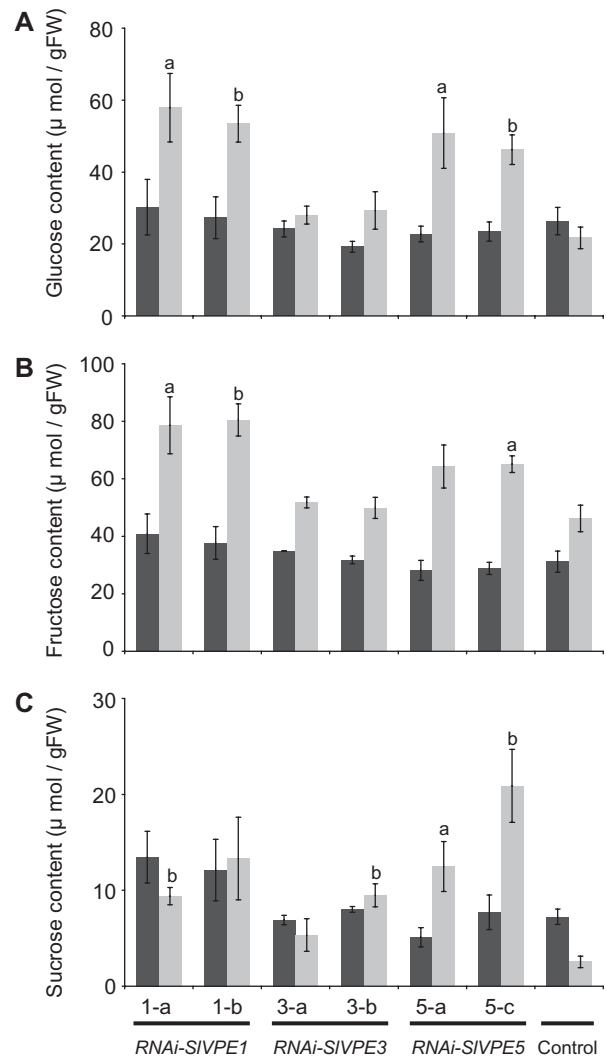
*SIVPE1* and *SIVPE3* may influence the composition of amino acids, whereas the suppression of *SIVPE5* did not have any effect on amino acid accumulation.

## Discussion

### Identification of novel VPE proteins from tomato

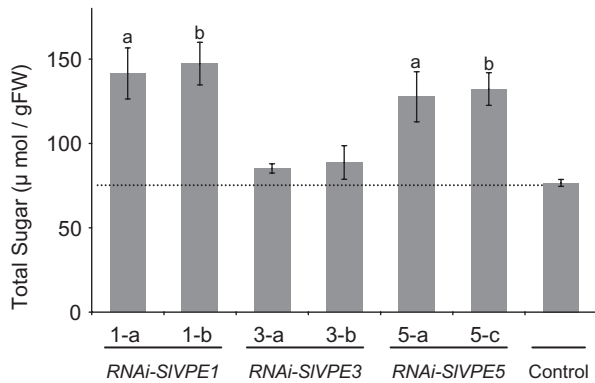
The completion of the tomato genome sequence project has allowed orthologous or homologous genes from the tomato genome to be easily identified using a specific sequence as query. In this study, five *VPE* genes from tomato (*SIVPE*) were identified; the phylogenetic and motif analyses using other VPE proteins (i.e. *Arabidopsis* VPE) revealed that the *SIVPE* proteins showed significant similarity in their protein sequence as well as their protein primary structure (Supplementary Table S2 at *JXB* online). For example, the *SIVPE* proteins are comprised of several conserved amino acid residues that are important for the VPE enzymatic activity (Supplementary Fig. S1 at *JXB* online). Consistent with this, levels of *SIVPE* mRNA expression were correlated with VPE enzymatic activity, suggesting that the tomato *SIVPE* genes encode biochemically active VPE proteins (Figs 6, 7; Supplementary Figs S3, S4 at *JXB* online).

Interestingly, the presence of VPE proteins is well conserved among many organisms including bacteria, animals, and plants (Yamada et al., 2005). Like these proteins, five *SIVPE* proteins were classified into the three categories, seed type (*SIVPE4*), seed coat type (*SIVPE1* and *SIVPE2*), and vegetative type (*SIVPE3* and *SIVPE5*) (Fig. 1). The function of plant VPEs is mainly focused on the processing of storage proteins in developing seeds and on proteins having caspase-1 activity that induce PCD upon



**Fig. 8.** Effect of *SIVPE* suppression on sugar accumulation. A decrease in VPE activity was associated with increased sugar accumulation in mature fruits. The accumulation of (A) glucose, (B) fructose, and (C) sucrose from fruits at the MG stage (black bar) and the RED stage (grey bar) was measured in the control, *RNAi-SIVPE1* (line 1-a and 1-b), *RNAi-SIVPE3* (line 3-a and 3-b), and *RNAi-SIVPE5* (line 5-a and 5-c) lines. The mean values of at least three independent experiments are shown. The error bars represent the SE. A significant difference was calculated between the RNAi lines and the control. a,  $P < 0.05$ ; b,  $P < 0.01$ , as determined by the *t*-test.

the defence response. However, VPEs are also essential for the degradation of invertase, an enzyme that catalyses the hydrolysis of sucrose in senescent leaves (Rojo et al., 2003). Hence, it has been suggested that VPE activity is associated with sugar accumulation in plants. This possibility was explored using one of the fruit crops, the tomato, because it has more advantages in terms of the availability of genetic and molecular tools than other fruit crops (Aoki et al., 2010). In addition, the tomato transformation protocol is well established, and its efficiency is higher than that of other fruit crops (Sun et al., 2006).



**Fig. 9.** Total sugar content in fruits at the RED stage in control plants and *RNAi-SIVPE* lines. Total sugar accumulation of fruits at the RED stage was calculated in the control, *RNAi-SIVPE1* (line 1-a and 1-b), *RNAi-SIVPE3* (line 3-a and 3-b), and *RNAi-SIVPE5* (line 5-a and 5-c) plants. The mean values of at least three independent experiments are shown. The error bars represent the SE. a,  $P < 0.05$ ; b,  $P < 0.01$ , as determined by the *t*-test.

To examine the roles of VPEs during fruit development, the mRNA level of each *SIVPE* gene was determined by qRT-PCR analysis. This analysis indicated that all *SIVPE* genes were expressed in fruit but showed different temporal patterning (Fig. 2). Expression analysis using the *GUS* reporter gene revealed that the *SIVPE* genes were markedly expressed in vascular bundles and in seeds within the fruit (Figs 3, 4). These results suggested that the *SIVPE* proteins function somehow in seeds and/or fruits. Consistent with this, a high level of VPE enzymatic activity was observed in developing fruits compared with other vegetative tissues such as leaf, root, and stem (Fig. 5, Supplementary Fig. S2 at *JXB* online).

To examine whether the *SIVPE* proteins play a role(s) in inducing PCD of the seed inner integument, sections of seeds from developing fruit were made and observed by light microscopy. However, no significant difference in the thickness of the seed coat was observed between the control and the *RNAi-SIVPE* lines (Supplementary Fig. S5 at *JXB* online). Moreover, the distribution of the storage proteins in seeds was similar between the control and the *RNAi-SIVPE* lines when seed storage proteins were examined by Coomassie blue staining (data not shown). These results suggested that *SIVPE* proteins may not play major roles in regulating PCD in the seed inner integument and processing storage proteins in tomato. However, it is possible that the large reduction in VPE enzymatic activity is required to stop PCD and protein processing. The *Arabidopsis*  $\beta$ vpe mutant lacks ~90% of the VPE activity in seeds and shows the accumulation of precursors of globulin and albumin due to a failure in the processing of proteins with a storage function (Gruis *et al.*, 2002, 2004; Shimada *et al.*, 2003). Although  $\beta$ VPE is essential for the processing of storage proteins, mutations in both the  $\alpha$ VPE and  $\gamma$ VPE genes in the  $\beta$ vpe mutant give an additive effect on the decreased VPE activity and on the increased accumulation of precursors; however, single  $\alpha$ vpe and  $\gamma$ vpe mutants do not show

significant defects in processing activity. These findings suggested that the family of *VPE* genes show highly redundant functions. Since the five *SIVPE* genes show very similar spatial expression patterns (Figs 3, 4), it is possible that a reduction in VPE activity in the *RNAi-SIVPE* lines was not sufficient to hinder PCD and protein processing; therefore, the remaining VPE activity in the *RNAi-SIVPE* lines probably compensated the protein function. Further studies are needed to clarify the roles of *SIVPE* proteins in these processes.

#### *SIVPE5* mainly functions in total sugar accumulation

The plant vacuole is the main site for solute accumulation, and the vacuole in fruit accumulates large volumes of sugars, organic acids, and secondary metabolites (Johnson *et al.*, 1988; Shiratake and Martinoia, 2007). To explore whether the suppression of *SIVPE* genes influenced fruit development, sugar accumulation was determined in the *RNAi* lines and control plants. The HPLC analysis to examine the sugar contents indicated that sugar accumulation was unchanged at the MG stage between the *RNAi-SIVPE* lines and the control plants (Fig. 8). However, the effect of *SIVPE* suppression on sugar accumulation was observed at the RED stage, suggesting that the molecular targets of *SIVPE* might function at the RED stage. In contrast, *RNAi-SIVPE1* showed decreased levels of VPE activity associated with increased sugar accumulation in fruit at the RED stage (Figs 7, 8), although the mRNA expression of the *SIVPE1* gene was barely observed at the RED stage (Fig. 2B). To determine whether *RNAi* suppressed expression not only of *SIVPE1* but also of other *SIVPE* genes due to high DNA sequence homology among the *RNAi*-targeted regions (Supplementary Table S3 at *JXB* online), the mRNA expression of the other *SIVPE* genes was determined. As predicted, the mRNA expression of the *SIVPE2*, *SIVPE3*, and *SIVPE5* genes appeared to be effectively suppressed, suggesting that the increased sugar content resulted from the simultaneous reduced expression of *SIVPE2*, *SIVPE3*, and/or *SIVPE5* (Fig. 6). The fact that the mRNA expression of *SIVPE2* was hardly observed at the RED stage (Fig. 2C) and that the total sugar content in the *RNAi-SIVPE3* lines was unchanged suggests that the suppression of *SIVPE5* had the strongest effect among the *SIVPE* genes on the increased total sugar accumulation in fruits (Fig. 9). Consistent with this, the transgenic 5-a line of *RNAi-SIVPE5* showed nearly specific suppression of *SIVPE5*, which was associated with increased total sugar content (Figs 6, 9). A reduction of ~10% in *SIVPE5* mRNA expression in the transgenic 3-a and 3-b lines of *RNAi-SIVPE3* did not result in an increased total sugar content, whereas a reduction of ~40% in *SIVPE5* mRNA in the 1-a and 1-b lines of *RNAi-SIVPE1* caused a large increase in the total sugar levels (Figs 6E, 9). In addition, the reduction in *SIVPE5* expression seemed to have a stronger effect on sugar accumulation than on the reduction of the absolute level of VPE enzymatic activity, since the *RNAi-SIVPE5* lines still exhibited higher levels of VPE activity (line 5-a,

83.5%; line 5-c, 71.2%) than other RNAi lines (i.e. line 1-a, 42.4%; 3-a, 72.0%) (Fig. 7). These results suggested that the greater reduction of the vegetative-type *SIVPE5* is probably important for increasing the total sugar content. However, since none of the *RNAi-SIVPE* lines showed absolute specific suppression of the targeted genes (Fig. 6), the possibility could not be excluded that the simultaneous suppression of several *SIVPE* genes was essential for triggering the sugar accumulation observed.

Interestingly, it is proposed that the vegetative-type VPE is responsible for the maturation and activation of vacuole hydrolytic enzymes (hydrolases), which are involved in the degradation of cellular components sequestered from the cytosol by autophagy during senescence (Yamada *et al.*, 2005). Furthermore,  $\gamma$ VPE targets hydrolases such as the AtFRUCT4 invertase protein,  $\beta$ -glycosidases,  $\alpha$ -mannosidases, and  $\alpha$ -galactosidases for degradation, indicating that  $\gamma$ VPE exhibits proteolytic activity (Rojo *et al.*, 2003). Several lines of evidence have indicated that the sugar content is greatly affected by the activity of hydrolases (Fridman *et al.*, 2004; Baxter *et al.*, 2005). For instance, invertase enzyme activity is most probably correlated with total sugar contents in tomato fruits and carrot taproots (Ohya *et al.*, 1995; Klann *et al.*, 1996; Tang *et al.*, 1999; Husain *et al.*, 2001). Expression of the invertase gene is preferentially observed in the vascular tissues within the placenta that lead to the developing seeds as well as the surrounding pericarp (Fridman *et al.*, 2004). Interestingly, this expression pattern was quite similar to that in vegetative-type *SIVPE3* and *SIVPE5* genes (Figs 3, 4).

The vacuoles in fruit crops accumulate high concentrations of sugars, minerals, and organic acids, resulting in high osmotic pressure (Shiratake and Martinoia, 2007). As a consequence, water is absorbed into the vacuole, and >90% of the cellular volume is occupied by the vacuole at the maturation stage (Johnson *et al.*, 1988). Intriguingly, the vacuoles at this stage are mildly acidic, and this is the optimal environment for activating the VPE protein enzyme by self-catalytic activity (Hara-Nishimura *et al.*, 1993; Kuroyanagi *et al.*, 2002). These findings suggested that the molecular target of the vegetative-type *SIVPE* proteins may be hydrolases in the vacuole of fruits at the RED stage. It is possible that the decreased VPE activity resulting from decreased *SIVPE* expression may slow down the proteolysis of the hydrolases, such as invertases, leading to their accumulation and thus modifying sugar metabolism and sugar accumulation.

Moreover, the movement of substances into the vacuole probably requires an electrochemical proton gradient, which is generated by the vacuolar H<sup>+</sup>-ATPase (V-ATPase) acting as a proton pump in the vacuolar membrane (Shiratake and Martinoia, 2007). The suppression of *V-ATPase* in tomato fruit by the antisense strategy results in an increased sucrose content in fruit, whereas the hexose content was unchanged; consequently, the sugar composition was altered, as for the *RNAi-SIVPE3* lines (line 3-b) (Fig. 8C) (Amemiya *et al.*, 2006). It is possible that the reduced VPE activity influenced the activity of V-ATPase

somewhat. The suppression of *SIVPE5* did not influence other metabolites such as amino acids and related substances (Supplementary Table S4 at *JXB* online), suggesting that the mechanism for *SIVPE5*-dependent regulation specifically targeted sugar accumulation. However, the content of several amino acids was significantly higher in the *RNAi-SIVPE1* and *RNAi-SIVPE3* lines than in the control (Supplementary Table S4). This suggested that *SIVPE1* and *SIVPE3* were somehow involved in amino acid accumulation. However, the fact that *SIVPE* suppression did not cause any change in the total content of amino acids and related substances suggested that the effect could be subtle, if any.

In conclusion, this study has demonstrated that the five *SIVPE* genes isolated encode novel VPE proteins that exhibited biochemical activity in tomato. Tomato fruit quality is largely determined by the content of soluble solids, with soluble sugars being the major components of the soluble solid fraction. Hence the present strategy would be useful for generating high quality tomato fruit by genetic engineering.

#### Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** Key amino acid residues important for VPE self-catalytic activity.

**Figure S2.** Comparison of the intensity of VPE enzyme activity in vegetative tissues and fruits.

**Figure S3.** Suppression of *SIVPE2* and *SIVPE4* mRNA expression was associated with decreased VPE enzyme activity.

**Figure S4.** Decreased mRNA levels in *SIVPE3* or *SIVPE5* caused a reduction in VPE enzyme activity in leaf tissue.

**Figure S5.** Seed coat formation in control and RNAi lines.

**Table S1.** Full-length cDNA and accession numbers of tomato *VPE* genes.

**Table S2.** Predicted primary and secondary features of plant VPE proteins.

**Table S3.** DNA sequence homology in the RNAi-targeted regions among the five *SIVPE* genes.

**Table S4.** Amino acid contents in fruits in control and *RNAi-SIVPE* lines.

**Table S5.** Primers used for cloning and qRT-PCR in this study.

#### Acknowledgements

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