

# Involvement of vacuolar processing enzyme SIVPE5 in post-transcriptional process of invertase in sucrose accumulation in tomato

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1 **Involvement of vacuolar processing enzyme SIVPE5 in**  
2 **post-transcriptional process of invertase in sucrose**  
3 **accumulation in tomato**

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1 **Abstract**

2

3 Enhancing the flavor of fruits plays a fundamental role in improving fruit quality, and  
4 volatile compositions as well as acid and sugar accumulation are significant factors that  
5 have an impact on the acceptability of sensory responses by human beings. Vacuoles in  
6 plants not only function as cell compartments that store amino acids, sugars and other  
7 metabolites but also act as lytic organelles where vacuolar proteins are  
8 post-translationally processed into mature forms or degraded by the action of vacuolar  
9 processing enzyme (VPE). We have previously characterized *VPE* genes (*SIVPE1-5*)  
10 during fruit development in tomato and discovered that the VPE enzyme activity  
11 negatively interfered with sugar accumulation in mature fruits. Comparative proteomic  
12 analysis demonstrated that acid invertase was one of the molecular targets of *SIVPE5*,  
13 which is involved in the hydrolysis of sucrose. This study also showed that decreased  
14 VPE enzyme activity due to suppression of *SIVPE5* by RNAi strategy (*RNAi-SIVPE5*)  
15 accompanied with decreased enzyme activity of acid invertase. Further, we identified the  
16 enzyme activity of acid invertase was not well correlated with mRNA levels in the  
17 *RNAi-SIVPE5* line. **These results suggest that *SIVPE5* regulates post-transcriptional**  
18 **processing through *de novo* synthesis of the acid invertase protein to suppress enzyme**  
19 **activity**, thereby eventually ensuring sucrose hydrolysis.

20

21

22 **Keywords:** fruit, RNA interference, vacuolar processing enzyme, acid invertase,  
23 post-transcriptional regulation

24

1 **1. INTRODUCTION**

2

3 Fruit quality can be improved by producing plants that have added value. An  
4 alternative method can be used for generating high quality fruit using genetic  
5 manipulation to add or change phenotypic characteristics (e.g., fruit flavor and nutrition).  
6 There are concerns regarding the perceived quality of the tomatoes, especially regarding  
7 the fruit flavor. There are many complex factors that determine the flavor and quality of  
8 the tomato fruit. The intensity of flavor of tomato fruit is determined largely by the  
9 composition of sugars (primarily fructose and glucose), the organic acid content  
10 (primarily citric, malic and total acidity), and the presence of volatile compounds  
11 (Mikkelsen, 2005). High sugar and acid content generally provides a favorable effect on  
12 taste. Many researchers have tried to find ways to obtain a high sugar content in the  
13 tomato fruit, such as by using the acid invertase gene as a molecular marker for the trait of  
14 sucrose accumulation, finding factors that control sugar metabolism-associated genes,  
15 carbohydrate accumulation and sugar-regulated starch biosynthetic genes under  
16 conditions of salinity and osmotic stress (Lu et al., 2010; Yin et al., 2010).

17 Most sugar accumulates in the vacuole, the size of which accounts for more than  
18 half of the cell size in fruits (Winter et al., 1993), therefore, manipulation of the vacuole  
19 might be an alternative method to increase the sugar content of fruits. As an approach for  
20 this vacuole manipulation, the inhibition of vacuolar sucrose transport was used to  
21 increase cytoplasmic sucrose concentrations (Endler et al., 2006). A novel sucrose  
22 transporter (*SUT2*) localized to the vacuole of rice (*Oryza sativa*) resulted in significant  
23 increases in sucrose, glucose and fructose in rice leaves (Eom et al., 2011). Suppression  
24 *SUT2* homolog in tomatoes (*Solanaum lycopersicum*) resulted in unexpected decrease in  
25 the concentrations of sucrose, glucose and fructose in tomato fruits, however, significant  
26 changes of soluble sugars was found in the tomato leaves (Hackel et al., 2006).

27 VPE is a cysteine proteinase enzyme that is responsible for the maturation of  
28 various vacuolar proteins (Nakaune et al., 2005) that involves in proteolytic pathways  
29 extending between cellular compartments. This enzyme is known to play an essential role  
30 in plant growth and development, such as in senescence and programmed cell death, the  
31 accumulation of storage proteins, such as in seeds, and storage protein mobilization  
32 (Grudkowska and Zagdanska, 2004). Studies of the vegetative type of VPE proteins have  
33 been conducted mainly under abiotic stress conditions such as wounding, senescence, and  
34 treatment with hormones such as jasmonic acids, ethylene, or salicylic acid (Kinoshita et  
35 al., 1999; Yamada et al., 2004), and in cell death due to aging (Rojo et al. 2003) or disease  
36 (Woltering et al., 2002; Hatsugai et al., 2004). Arabidopsis vegetative type VPE, namely

1 *VPE $\gamma$*  (Rojo et al. 2003), suggested to be involved in degradation of the vacuolar invertase  
2 (AtFruct4) in aging tissues. AtFruct4 is degraded in the senescing leaves of the wild type  
3 but not in the *vpe $\gamma$*  mutant. They proposed that senescence induced by aging activates  
4 VPE and that its protease activity is required for degradation of the vacuolar invertase  
5 AtFruct4 in aging tissues. Tandem MS analysis also found that four glycosidases,  
6 including a putative glucosidase (At1g52400), two mannosidases (At3g26720 and  
7 At5g13980), and a galactosidase (At3g56310), were not degraded in the *vpe $\gamma$*  mutant. In  
8 tobacco, silencing of the vegetative type VPE (*NtVPE1*) results in the suppression of  
9 TMV-induced hypersensitive cell death (Hatsugai et al., 2004). An experiment in  
10 Arabidopsis shows that the VPE is involved in two cell death systems: fumonisin-induced  
11 cell death (Kuroyanagi et al., 2005) and developmental cell death in seed integuments  
12 (Nakaune et al., 2005). The *SIVPE5* gene appeared to be identical to *S. lycopersicum*  
13 CYSTEINE PROTEINASE (*SICp*), which was previously identified to have a dual  
14 function as a protease and as an ACS (1-aminocyclopropane-1-carboxylic acid synthase)  
15 gene regulator, which is involved in wounding response, ripening and floral senescence.  
16 The SICp was reported to bind to the promoter region of the ACS gene and induce  
17 downstream GUS expression (Matarasso et al., 2005).

18 Ariizumi et al. (2011) previously identified five novel *VPE* genes from the  
19 tomato, named *SIVPE1* to *SIVPE5*. Based on the sequence structure, they suggested that  
20 *SIVPE1* and *SIVPE2* were categorized as the seed coat type, *SIVPE4* was categorized as  
21 the seed type, and both *SIVPE3* and *SIVPE5* were categorized as the vegetative type. The  
22 suppression of *SIVPE1*, *SIVPE3* and *SIVPE5* using RNAi affected sugar accumulation  
23 but did not influence the total amino acid content, showing that VPE is involved in the  
24 process of sugar accumulation. Among these genes, suppression of the vegetative type  
25 *SIVPE5* using RNAi resulted in the most efficient increase in sucrose accumulation.  
26 However, the connection between sucrose accumulation and the vegetative type *SIVPE5*  
27 gene is unclear. In the present study, we analyzed the contribution of the vegetative type  
28 *SIVPE5* gene in the process of sugar accumulation in the tomato fruits. First, VPE activity  
29 was measured in the green and red stages of the *RNAi-SIVPE5* line, and invertase activity  
30 was measured in the same samples. Next, proteomic analyses using 2D gel  
31 electrophoresis and RT-PCR were performed to confirm post-transcriptional processing.  
32 The results suggested that the decreased VPE activity influenced *de novo* synthesis of the  
33 acid invertase protein and caused an increase in sucrose accumulation in the  
34 *RNAi-SIVPE5* line.

35

## 2. MATERIALS AND METHODS

### 2.1 Plant Materials

Tomato plants of control (cv. Micro-Tom) and transgenic *RNAi-SIVPE5* lines prepared by previous studies (Ariizumi et al., 2011) were germinated with sterile water at 37°C, approximately 50-60% humidity and 16 hours of light. The seedlings were transferred to rock wool and grown under the same conditions. Fruits were sampled during the developmental stages from 3 days after flowering (DAF) to 45 DAF. The fruits at 15–27 DAF are referred to as the Immature Green (IMG) stage, at 27-30 DAF are referred to as the Mature Green (MG) stage, at 31-34 DAF are referred to as the Breaker stage (BR), and at 39-45 DAF are referred to as the Mature Red (RED) stage.

### 2.2 Protein Extraction

Protein was extracted based on the TCA/acetone extraction and phenol extraction methods (Isaacson et al., 2006). Whole fruits were ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle, and 5 g of the respective powders was resuspended in 5 ml of extraction buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 2% polyvinylpyrrolidone (PVPP)). After centrifugation at 12,000 x g for 20 min, the supernatants were collected. A total of 15 ml of 10% TCA in acetone containing 20 mM was added to the supernatant and incubated at -20°C for 1 hour. The solution was centrifuged at 12,000 x g for 10 min, and the supernatant was discarded. The pellet was washed with 15 ml acetone, centrifuged at 12,000 x g for 10 min, and the supernatant was discarded. The pellet was resuspended in 15 ml of Tris-saturated phenol and washed twice with 5 ml of extraction buffer, followed by centrifugation at 5,000 x g for 30 min. The phenol phase was collected. Then, to precipitate the protein, 5 volumes of cold 0.1 M ammonium acetate in methanol were added and incubated overnight at -20°C. The solution was centrifuged at 12,000 x g for 20 min, and the supernatant was discarded. The pellet was washed once with ice cold methanol and three times with ice cold acetone, then air dried. Proteins were extracted from the pellet with 7 M urea, 2 M thiourea, 20 mM DTT, 2 mM Tris-(2-cyanoethyl) phosphine, 2% CHAPS, and 0.2% (v/v) BioLyte 3-10. Then, the solution was centrifuged at 20,000 x g for 5 min and the supernatant was used as the protein extract. The total protein amount was quantified by the Bradford assay (Bradford, 1976).

### 2.3 Two Dimensional Gel Electrophoresis (2-DE) and the Identification of Proteins

1 A total of 120 µg of protein was loaded onto immobilized pH gradient (IPG)  
2 strips (pH 3–10 nonlinear, 17 cm; IPG ReadyStrip, Bio-Rad) during more than 12 hours  
3 of passive rehydration of the strips. First-dimensional isoelectric focusing was performed  
4 with IPG ReadyStrips (Bio-Rad) according to the manufacturer’s protocol. Next, the IPG  
5 strips were reduced for 15 min with 50 mM Tris-HCl (pH 8.5), 6 M urea, 30% glycerol,  
6 2% SDS, 1% DTT, and 0.005% bromophenol blue. Then, the strips were alkylated for 15  
7 min with 50 mM Tris-HCl (pH 8.5), 6 M urea, 30% glycerol, 2% SDS, 4.5%  
8 iodoacetamide, and 0.005% bromophenol blue. The second dimension of SDS-PAGE  
9 was carried out using a 10/16% gradient gel (19 x 17 cm). After electrophoresis, gels  
10 were stained with Sypro Ruby (Invitrogen) and imaged using the Molecular Imager FX  
11 system (Bio-Rad). Spots in the control with a density more than three times higher than in  
12 the RNAi lines were in-gel digested with trypsin, and the digested proteins were  
13 subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry  
14 (MALDI-TOF MS) and liquid chromatography–tandem mass spectrometry  
15 (LC-MS/MS) (Apro Science, Japan). Proteins were identified by a homology search of  
16 the NCBI nr and dbEST databases using the Mascot Server  
17 ([http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html), Version: 2.0, Matrix Science,  
18 London, UK).

19

## 20 **2.4 Quantitative Expression Analysis**

21 The acid invertase mRNA expression at various stages and in tissues from the  
22 transgenic tomato plants was determined using quantitative RT-PCR. Total RNA was  
23 isolated from frozen tomato tissues using an RNeasy Plant Mini kit (Qiagen, Japan) with  
24 treatment of DNase I to remove genomic DNA contamination. Total RNA (5 µg) was  
25 used to synthesize the first-strand cDNA using the SuperScript III First-Strand Synthesis  
26 System (Invitrogen, Carlsbad, CA). The cDNA was used for quantitative RT-PCR with  
27 SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan), and the PCR thermal cycling  
28 conditions were set according to the manufacturer’s instructions. Relative quantification  
29 of acid invertase gene expression was calculated using the tomato *UBIQUITIN3 (UBI3)*  
30 gene (accession number X58253) as an internal control (Kim et al., 2010). The primer  
31 sequences were as follows: acid invertase gene forward,  
32 5'-TCGCCCTACCTTAGGATTAGATGTC-3', and reverse,  
33 5'-GTCAACATTCCCAAGTTGTTTCTGT-3'; reference gene *UBI3*, forward,  
34 5'-CACCAAGCCAAAGAAGATCA-3', and reverse,  
35 5'-TCAGCATTAGGGCACTCCTT-3'.

36

## 2.5 Measurement of VPE Enzyme Activity

The VPE enzyme activity was measured according to Shimada et al. (2003). Whole fruits of control plants transformed with empty vector and the T<sub>2</sub> generation of transgenic RNAi plants at the mature green (MG) stage (DAF 27-30) and the mature red (RED) stage (DAF 39-45) were ground with liquid N<sub>2</sub> and resuspended in 50 mM sodium acetate buffer (pH 5.5), 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM E-64-d. After centrifugation at 10,000 x g for 30 min, 30 µl of the supernatant was resuspended in 130 µl distilled deionized water, 40 µl of 500 mM sodium acetate (pH 5.5), and 500 mM dithiothreitol (DTT) buffer. The mixture was incubated at 37°C for 10 min, and 4 µl 10 mM fluorescent VPE-specific substrate z-AAN-MCA (Benzyloxycarbonyl-L-Alanyl-L-Alanyl-L-Asparagine 4-Methyl-Coumaryl-7-Amide; Peptide Institute) was added. The mixture was incubated for 2 hours at 37°C, and the fluorescence intensity was determined using a Wallac 1420 ARVO MX/Light microplate reader (Perkin-Elmer). The fluorescence was monitored at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

## 2.6 Measurement of Invertase Activity

The invertase activity was measured according to Ohyama et al. (1995) with modifications. Whole fruits of control plants and transgenic RNAi plants at the MG stage (DAF 27-30) and the RED stage (DAF 39-45) were ground with liquid N<sub>2</sub> and resuspended in 20 mM Tris-HCl (pH 7.2) containing 0.5 M NaCl. After centrifugation at 12,000 x g for 20 min, the supernatants were desalted on a column of Sephadex G-50 (GE Healthcare) and used as the source of soluble enzymes. A total of 0.5 µg in 50 µl distilled deionized water was incubated with 150 µl of 3% (w/v) sucrose and 50 mM sodium acetate buffer (pH 5.2) and then boiled at 95°C for 5 min to stop the reaction. The concentration of glucose was then determined using a D-Glucose assay kit (Megazyme, Ireland).

## 2.7 Measurement of $\alpha$ -mannosidase, $\alpha$ -galactosidase and $\alpha$ -glucosidase Activities

The glycosidase activities were measured according to Jagadeesh et al. (2004) with modifications. Whole fruits of control plants and the T<sub>2</sub> generation of transgenic RNAi plants at the MG stage (DAF 27-30) and the RED stage (DAF 39-45) were ground with liquid N<sub>2</sub> and resuspended in 50 mM citrate phosphate buffer (pH 5.2) containing 0.5 M NaCl. A total of 50 ml of 10 mM enzyme substrate (p-nitrophenyl  $\alpha$ -D-mannopyranoside, p-nitrophenyl  $\alpha$ -D-galactopyranoside or p-nitrophenyl  $\alpha$ -D-glycopyranoside, Sigma) was added to 5 µg/50 µl of the extracted protein in 50 mM



1 citrate phosphate buffer (pH 5.2) containing 0.5 M NaCl. The mixture was incubated at  
2 30°C for 15 min, and 50 µl of 1 M Na<sub>2</sub>CO<sub>3</sub> was added to stop the reaction. Blanks were  
3 terminated at zero time with the same 1 M Na<sub>2</sub>CO<sub>3</sub> solution. α-Mannosidase,  
4 α-galactosidase and α-glucosidase activities were measured as the amount of  
5 p-nitrophenol released, which was measured as the increase in absorbance at 410 nm.  
6

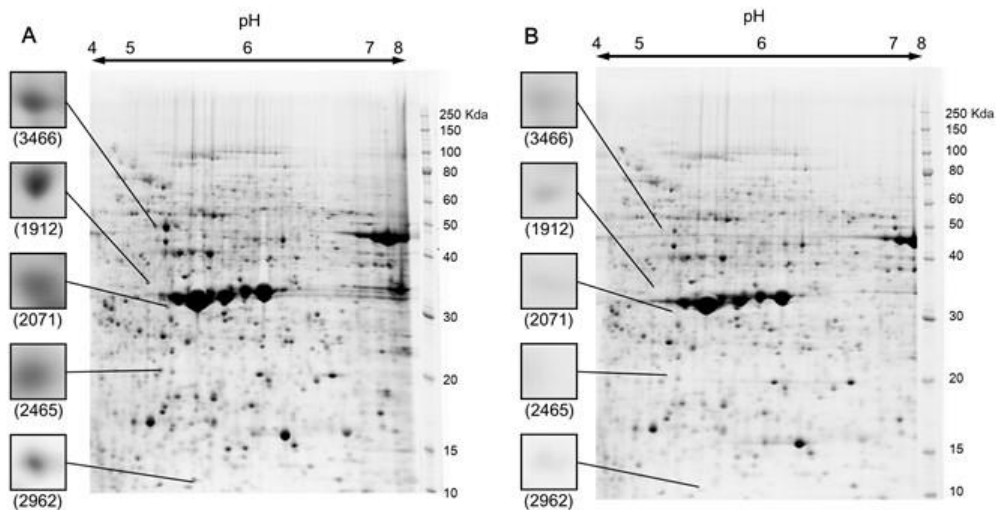
1 **3. RESULTS**

2

3 **3.1 Proteomic analysis of *RNAi-SIVPE5* Lines**

4 The densities of the isoelectric points differed markedly between wild type and  
5 *RNAi-SIVPE5* lines in RED stage fruits (Fig. 1). Two-dimensional gel electrophoresis  
6 (2-DE) of total proteins in the tomato fruit detected 1,271 spots, 5 of which reflected  
7 higher differential levels (more than three times) and were further analyzed using  
8 matrix-assisted laser desorption/ionization-time of flight mass spectrometry  
9 (MALDI-TOF MS) and liquid chromatography–tandem mass spectrometry  
10 (LC-MS/MS) (Table 1). Three of the 5 well-characterized spots were identified as  
11 identical acid invertase proteins, the densities of which were 4.1, 3.6 and 3.3-fold higher  
12 in the wild type than that in the *RNAi-SIVPE5* lines. The other 2 spots were identified as a  
13 glucan endo-1,3-beta-D-glucosidase and an unknown protein that was expressed in the  
14 maturing tomato fruits (Yamamoto et al., 2005); the densities of the isoelectric points for  
15 these two proteins were 3.1 and 4.4-fold higher in the wild type than that in the  
16 *RNAi-SIVPE5* lines, respectively.

17



18

19 **Figure 1.** 2D gel electrophoresis of total protein extracts of control (A) and the *RNAi-SIVPE5* line (B).

20 Spots point to 3-fold differences of density in the control compared with the *RNAi-SIVPE5* lines.

**Table 1.** Identification of differential proteins with more of 3 folds expression in control compared to RNAi *SIVPE5* lines.

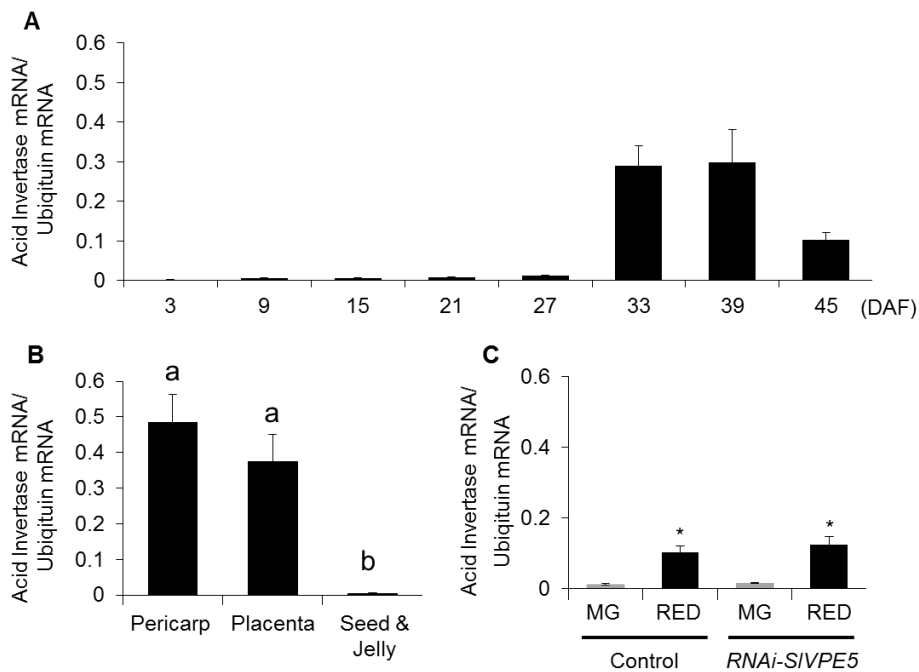
Spot Number	Gi Number	Expression differential	Protein Name	pI	MW (kDa)	Characterization Method	Observed Mass	Peptide
2962	58212712	4.4	Maturing fruit Solanum lycopersicum cDNA clone FA03DH12	5.5	11	LC-MS/MS	561.2698	R.SNQLDQNFRA
							561.2745	R.SNQLDQNFRA
							727.3752	R.AFNTELMAEAFNIPVEIVR.R + Oxidation
							1090.5608	R.AFNTELMAEAFNIPVEIVR.R + Oxidation
							1090.5631	R.AFNTELMAEAFNIPVEIVR.R + Oxidation
2071	19849290	4.1	Acid Invertase	5.3	31	TOF-MS/MS	1100.5864	K.GWASVQSIPR.T
							1467.822	K.QVDLQPGSIELLR.V
							1742.8181	K.WTPDNPELDCGIGLR.L
							1837.8712	R.VDSAAELDIEASFVDDK.V
							2008.0503	K.TGTHLLQWPVEEIESLR.V
							2190.0931	R.RVLWGWIGETDSESADLQK.G
							2224.0554	K.ASLDDNKQDHYAIGTYDLGK.N
2465	19849290	3.6	Acid Invertase	5.2	21	TOF-MS/MS	1119.5474	R.TAYHFQPK.N
							1343.7866	K.GNPVLVPPPGIGVK.D
							1467.7915	K.QVDLQPGSIELLR.V
							1618.9544	K.FKGNPVLVPPPGIGVK.D
3466	19849290	3.3	Acid Invertase	5.2	49	TOF-MS/MS	1100.5961	K.GWASVQSIPR.T
							1467.8332	K.QVDLQPGSIELLR.V
							1742.8326	K.WTPDNPELDCGIGLR.L + Carbamidomethyl
							1837.8648	R.VDSAAELDIEASFVDDK.V
							2008.0594	K.TGTHLLQWPVEEIESLR.V
							2190.1257	R.RVLWGWIGETDSESADLQK.G
							2224.0629	K.ASLDDNKQDHYAIGTYDLGK.N
1912	498924	3.1	Glucan endo-1,3-beta-D-glucosidase	5.1	34	TOF-MS/MS	906.492	R.FKDEVLR.F
							1177.6737	K.YVPVLFNAVR.N
							1408.6561	K.HFGLYSANMQPK.Y + Oxidation
							1559.8455	R.IYEPDQLTLQALR.G
							1575.8992	R.FIEPIINFLVTNR.A
							1585.8382	R.NIQTAISGAGLGDQIK.V
							1767.842	R.YIAVGNEVSPFNENSK.Y
							1783.8958	K.NLFDAILDATYSALEK.A
							2003.9942	K.VSTAIETGLTDTSPPSNGR.F
							2023.0221	K.LEYALFVSPEVVNDNGR.G
							2245.2542	R.APLLNLVYPYFAVVDNPIK.L
			2915.4569	K.AGSSSLQIVVSESGWPSAGAGQLTSIDNAR.T				

1  
2  
3

### 3.2 Quantitative RT-PCR Analysis of Acid Invertase

Quantitative RT-PCR analysis was carried out to determine the temporal expression pattern of the acid invertase gene. In addition to the mRNA isolated at the different developmental stages from the whole tomato fruits, mRNA was also isolated from the pericarp, placenta, seed and jelly, as depicted in Fig. 2A, B. Although quantitative PCR analysis showed that acid invertase exhibited a continuous pattern of gene expression throughout fruit development, transcript abundance accumulated

1 significantly more in the mature fruits (DAF33 to 45) compared with earlier time points  
 2 (DAF0 to 27) (Fig. 2A). Acid invertase mRNA mainly accumulated in the pericarp and  
 3 placenta and was expressed at a significantly lower level in the seed and jelly  
 4 (approximately 1%) in comparison with the level in the pericarp (Fig. 2B). The  
 5 expression levels of acid invertase mRNA in the *RNAi-SIVPE5* transgenic lines were  
 6 investigated at the MG stages and the RED stage in tissues from whole tomato fruits  
 7 (Fig. 2C). The mRNA of acid invertase was highly accumulated at the RED stage in  
 8 comparison to the MG stage. There were no significant differences in the mRNA levels  
 9 of acid invertase between the wild type and the *RNAi-SIVPE5* transgenic lines.  
 10



11 **Figure 2.** qRT-PCR of acid invertase. A), Time course analysis of acid invertase expression during  
 12 fruit development. DAF, day after flowering B), Expression analysis of the pericarp, placenta, seed  
 13 and jelly of wild type tomatoes. Different letters indicate significant differences ( $P < 0.05$ ;  
 14 Tukey–Kramer test). C), Expression analysis of the *RNAi-SIVPE5* line. Whole fruit tissue at the MG  
 15 stage (gray) and the RED stage (black) was used. Asterisks indicates significance compared with  
 16 control ( $P < 0.05$ ; Student’s *t*-test).  
 17

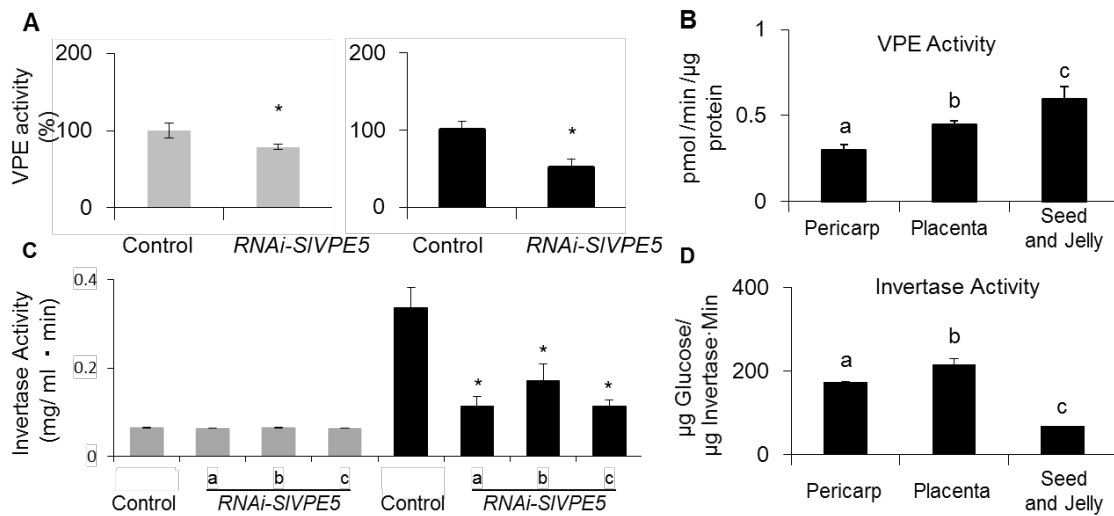
18  
 19

### 20 3.3 VPE and Invertase Enzyme Activities in Fruit

21 We next confirmed that the suppression of *SIVPE5* expression can repress VPE  
 22 activity at both the MG and RED stages. A previous study revealed that a decrease in  
 23 *SIVPE* mRNA expression was associated with decreased levels of VPE enzyme activity  
 24 in the fruits of the *RNAi-SIVPE5* line (Ariizumi et al., 2011). In this study, we also

1 confirmed that VPE enzyme activity is also repressed in the *RNAi-SIVPE5* lines, which  
 2 exhibited 80% and 50% of the VPE enzyme activity levels of the control plants at the  
 3 MG stage and RED stage, respectively (Fig. 3A). VPE enzyme activity in the pericarp,  
 4 placenta, seed and jelly was also measured at the RED stage in the wild type (Fig. 3B).  
 5 We observed the highest VPE activity in the seed and jelly, followed by the placenta  
 6 and pericarp.

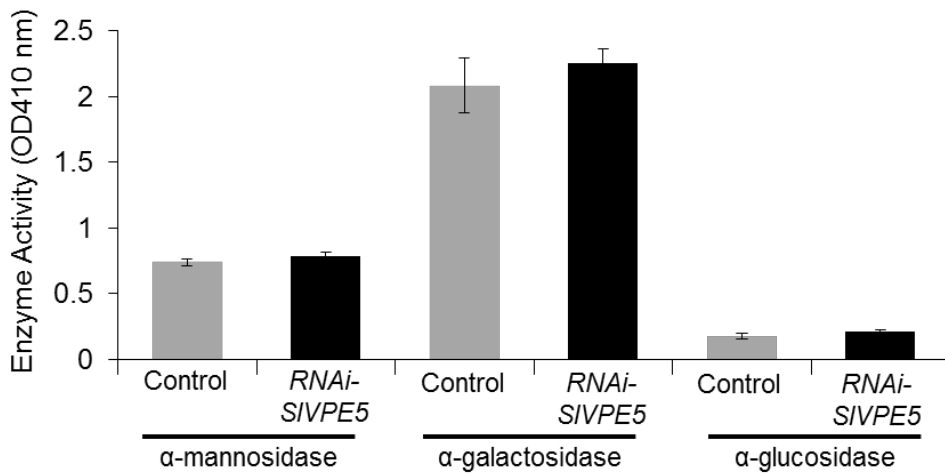
7 The enzyme activity of acid invertase significantly increased 6-fold from the  
 8 MG stage to the RED stage (Fig. 3C) in the control plant, which perfectly correlated  
 9 with acid invertase mRNA accumulation (Fig. 2A). The invertase enzyme activity in the  
 10 fruits was measured in the *RNAi-SIVPE5* lines. Although there was no significant  
 11 difference in invertase activity at the MG stage, we found that invertase activity in the  
 12 *RNAi-SIVPE5* lines was repressed to 33.5~50.8% of the activity of wild type at the RED  
 13 stage (Fig. 3C). A further assay of invertase enzyme activity in the seed and jelly  
 14 exhibited the lowest activity among the three organs (Fig. 3D).  
 15



16  
 17 **Figure 3.** Enzyme activity of VPE (A, B) and invertase (C, D). A, C), Enzymatic activity of whole  
 18 fruit tissue at the MG stage (gray) and the RED stage (black) of the control and the *RNAi-SIVPE5* line.  
 19 The activity of the *RNAi-SIVPE5* lines is relative to the control plants. Asterisks indicating  
 20 significance at <0.05 level as determined by the t-test. B, D), Enzymatic activity in the pericarp,  
 21 placenta, seed and jelly of wild type tomatoes at the RED stage. a,b,c indicating significance at <0.05  
 22 level as determined by Tukey-Kramer test. Different letters indicate significant differences  
 23 ( $P < 0.05$ ; Tukey-Kramer test).  
 24  
 25

1 **3.4 Glycosidases Enzyme Activities in Fruit**

2 Rojo et al. (2003) suggested that VPE co-localized with glucosidase,  
3 mannosidase and galactosidase and was involved in further processing of these enzymes  
4 in the vacuoles. We measured  $\alpha$ -mannosidase,  $\alpha$ -galactosidase and  $\alpha$ -glucosidase  
5 activity of the control and the *RNAi-SIVPE5* lines using fruit tissue at the RED stage  
6 (Fig. 4). We demonstrated that there was no significant difference in the enzyme  
7 activity in the *RNAi-SIVPE5* lines compared with control at the RED stage.



8  
9 **Figure 4.** Mannosidase, galactosidase and glucosidase activity in the control (grey) and the  
10 *RNAi-SIVPE5* line (black).  
11  
12

## 1 4. DISCUSSION

### 3 4.1 SIVPE5 Functions in Regulation of Acid Invertase Activity

4 Although *RNAi-SIVPE5* lines did not show any morphological changes in  
5 vegetative and reproductive organ, it has been shown that suppression of *SIVPE5* at the  
6 mRNA level results in an increase in sucrose accumulation (Ariizumi et al., 2011). This  
7 study demonstrated that suppression of *SIVPE5* expression may suppress synthesis of  
8 acid invertase protein resulting in decreasing invertase activity. Acid invertase localizes  
9 in the vacuole, and it plays a role as one of the main enzymes that hydrolyses sucrose to  
10 glucose and fructose, which is a crucial factor in determining the sugar content of  
11 ripened fruits (Ohyama et al., 1995). Ripe tomatoes and grapes contain small amounts  
12 of sucrose and accumulate mainly glucose and fructose (Kliwer, 1966; Ohyama et al.,  
13 1995). The decrease in sucrose accumulation in red tomato fruits compared to mature  
14 green fruits is mainly caused by the high invertase activity in the red tomato (Klann et  
15 al., 1993, Endo et al., 1990, Stommel, 1992). Proteomic analysis revealed three  
16 fragments corresponding to acid invertase (21, 31 and 49 kDa) proteins that were highly  
17 accumulated in the RED stage fruit of the control plant; however, acid invertase  
18 decreased to one third of this level in the *RNAi-SIVPE5* lines (Fig. 1, Table 1). The  
19 processing of red tomato acid invertase into a smaller sized protein has been reported in  
20 previous studies. Although evidence indicating the degradation of acid invertase is  
21 typically observed in plants, the biological function of this fragmentation is unclear.

22 The decreasing molecular size of acid invertase might be required for  
23 processing higher enzyme activity. Rojo et al. (2003) reported that senescence induces  
24 the VPE $\gamma$ -dependent breakdown of invertase in aging tissues in Arabidopsis. The levels  
25 of invertase decline in senescing leaves of WT plants, but not in *vpe $\gamma$*  mutants, although  
26 the mRNA levels are similar in mutant and WT plants. Tomato would partially differ  
27 from Arabidopsis that invertase protein is less apparent in *RNAi-SIVPE5* lines, while the  
28 mRNA levels are similar and invertase were degraded in both *RNAi* lines and control.  
29 Purification of tomato acid invertase (TIV-1) using its inhibitor (SolyCIF) identified six  
30 different size fragments of acid invertase (Reca et al., 2008). Smaller fragments of acid  
31 invertase were also found in the western blot analysis of tomato fruits (Endo et al.,  
32 1990). Similar results were previously reported by other authors (Greiner et al., 2000;  
33 Bucheli and Devaud, 1994). Similar fragmentation of vacuolar invertase was also found  
34 in the hypocotyl of the mung bean (Arai et al., 1991) and in carrots (Unger et al., 1994).  
35 The increased enzyme activity of invertase was likely due to the *de novo* synthesis of  
36 the invertase protein, which occurred because of the relatively high mRNA expression

1 at the RED stage (Fig. 2A). The comparison of two tomatoes species, *Solanum*  
2 *lycopersicum* and *Solanum pimpinellifolium*, revealed that invertase activities were  
3 much lower in the MG stage than in the RED stage from all cultivars (Husain et al.,  
4 2001). The purification of invertase from the mature red tomato with partitioning also  
5 shows that the most active phase with activity of more than 90% contains mainly the 22  
6 kDa protein.

7 Processing of acid invertase with VPE might be a self-defense mechanism in  
8 the tomato as a reaction to impulse by pathogens or stresses. Vegetative VPE was  
9 reported to be up-regulated in the rosette leaves of *Arabidopsis thaliana* by wound  
10 treatment, ethylene, salicylic acid or jasmonate (Kinoshita et al., 1999). The acid  
11 invertase gene has also been shown to be regulated by wounding, jasmonate, bacterial  
12 infection and stresses (Sturm and Chrispeels, 1990; Kim et al., 2000; Li et al., 2011).  
13 Higher invertase activity was suggested to contribute to heat tolerance in the young  
14 tomato fruit, which possibly occurs through increasing glucose signaling activities and  
15 repressing the programmed cell death pathway (Li et al., 2011). Pressman et al. (2006)  
16 suggested that sucrose-cleaving enzymes in tomato anthers respond, at both the mRNA  
17 and enzyme activity levels, to high-temperature conditions. Heat stress was found to  
18 cause a reduction in acid invertase activity in the anthers 5 days before anthesis and an  
19 induction in acid invertase activity in the maturing anthers. The response is dependent  
20 upon the stage of flower development and it may involve post-transcriptional control.

21

#### 22 **4.2 SIVPE5 Post-transcriptionally Processes Acid Invertase**

23 We suggested an increase in invertase activity in ripening stage may not simply  
24 due to gene expression but resulting in VPE mediated post-transcriptional processing on  
25 *de novo* synthesis and maturation of invertase protein. The expression of acid invertase  
26 coordinates spatially and temporally with *SIVPE5*, indicating a high probability of  
27 *SIVPE5*-mediated invertase translation or maturation. The mRNA expression of *SIVPE5*  
28 is elevated around the IMG stage, and the highest expression is observed from the BR  
29 stage to the RED stage, and *SIVPE5* transcript levels are correlated with VPE enzyme  
30 activity (Fig. 3A; Ariizumi et al., 2011). The mRNA expression of acid invertase was  
31 low at the BR stage and increased as fruit approached maturation, reaching the highest  
32 levels at 33 and 39 DAF stages and remained relatively high levels until 45 DAF (Fig.  
33 2A), as similarly shown in previous studies (Klann et al., 1993, Endo et al., 1990,  
34 Stommel, 1992). Consistent with the low relative mRNA expression by means of low  
35 level of acid invertase protein at the MG stage (Fig. 2A), acid invertase revealed low  
36 enzyme activity both in WT and *RNAi-SIVPE5* line and there was no significant



1 different in its activity between them (Fig. 3C). Although mRNA expression levels were  
2 equivalent at RED stage (Fig. 2C), acid invertase activity in *RNAi-SIVPE5* line was  
3 repressed to 33.5~50.8% of that of control plant (Fig. 3C). These results suggest that the  
4 enzyme activity of acid invertase is not fully correlated with its mRNA abundance, but  
5 could be also regulated by post-transcriptional processing of *SIVPE5*.

6 In the evolutionary process, higher sugar content in the seed and jelly may also  
7 play an important role in germination and to attract animals to consume the seeds.  
8 Vacuolar invertase increased along with tomato fruit maturation, and its activity  
9 accounted for most of the invertase activity in the mature tomato. Most of the acid  
10 invertase activity was due to vacuolar invertase and including the activity of cell wall  
11 invertase (Jin et al., 2009). We demonstrated that invertase activity and VPE enzyme  
12 activity were higher in the placenta, followed by the pericarp. Entry into the seed and  
13 jelly was the single exception to the otherwise perfect relationship between VPE and  
14 invertase enzyme activity (Fig. 3). Although a considerable amount of enzyme activity  
15 of VPE observed (Fig. 3B), acid invertase gene expression was significantly lower in  
16 the seeds and jelly. Assuming that lower level of acid invertase protein could be  
17 accumulated thus for the comparable low level of enzyme activity in seeds and jelly  
18 (Fig. 3D). In tomato, five VPE proteins were classified into the following three  
19 categories: seed coat type (*SIVPE1* and *SIVPE2*), seed type (*SIVPE4*), and vegetative  
20 type (*SIVPE3* and *SIVPE5*). Spatial expression analysis of the *SIVPE* genes using a  
21 GUS reporter showed that clear GUS activity was observed in vascular bundles  
22 extending from the seeds to the placenta as well as around the endocarp in  
23 *SIVPE5p-GUS* throughout fruit development (Ariizumi et al., 2011). The *SIVPE1* and  
24 *SIVPE2* lines showed that the majority of GUS activity was evident in the seeds within  
25 fruits. Moreover, the GUS activity of *SIVPE4p-GUS* was exclusively evident in the  
26 seed endosperm and the embryo of developing fruits. Co-localization of *SIVPE1*  
27 *SIVPE2* and *SIVPE4* ensured the highest VPE activity compared with the pericarp and  
28 placenta. Here we further compared VPE activity at RED stage. The *RNAi-SIVPE5* lines  
29 showed a decreased activity as 50% level of which in control plants, which is relatively  
30 small compared to that in VPE mRNA accumulation (16% of control) (Ariizumi et al.,  
31 2011). The other VPE family genes expressing at RED stage, such as *SIVPE3* and  
32 *SIVPE4*, could not be suppressed in *RNAi-SIVPE5* lines, although *RNAi-SIVPE5* lines

1 revealed highly specificity on suppression on *SIVPE5* expression levels. Therefore, total  
2 VPE enzymatic activity involving activity of *SIVPE3* and *SIVPE4*.

3 A possible basis for the increase in sucrose content is that VPE activity was  
4 suppressed in the *RNAi-SIVPE5* lines which resulted in a lack of acid invertase protein  
5 synthesis and thus for low invertase activity, and given that the sucrose hydrolysis  
6 products are slowly decomposed. Although the sucrose content significantly increased,  
7 the hexose content decreased due to the lack of sucrose degradation to fructose and  
8 glucose due to repression of the enzyme activity of acid invertase (Klann et al., 1993,  
9 Ohyama et al., 1995). A pervious study have demonstrated that suppression of *SIVPE5*  
10 expression resulting in significant increase in sucrose content (5.0-8.0-fold higher than  
11 the control) in tomato fruits at the RED stage (Ariizumi et al., 2011). Fructose and  
12 glucose content revealed slightly increased in *RNAi-SIVPE5* lines but which is an  
13 extremely low relative level in compare with the increase of sucrose. The mechanism  
14 for the increase in the hexose level in the *RNAi-SIVPE5* lines remains elusive.

15 MS analysis of VPE mutants in *Arabidopsis thaliana* indicated that glucosidase,  
16 mannosidase and galactosidase accumulated in the vacuoles and suggested that the  
17 processing of this protein was performed by VPE (Rojo et al., 2003). In this study,  
18 proteomic analysis revealed glucan endo-1,3-beta-D-glucosidase as a target of *SIVPE5*  
19 (Table 1). We suggest that glucan endo-1,3-beta-D-glucosidase is correlated with its  
20 caspase-1-like activity on hypersensitive cell death, which was reported to be expressed  
21 in tomato plants upon viroid infection (Domingo et al., 1994). Repressed glucan  
22 endo-1,3-beta-D-glucosidase translation in the *RNAi-SIVPE5* lines might be the cause of  
23 invisible hypersensitive cell death upon induction of the tobacco mosaic virus (TMV)  
24 (Hatsugai et al., 2004). The spot with the highest differentiation was an unknown  
25 protein (Table 1), a homolog of the legumin type globulin of *Arabidopsis thaliana*. Our  
26 results confirmed a previous report that seeds from  $\beta$ VPE knockout mutants are  
27 hindered in the maturation of storage proteins in *Arabidopsis*, such as globulin and  
28 albumin (Shimada et al., 2003), given that this is one of the functions of *SIVPE5*, which  
29 was expressed in the seed coat (Ariizumi et al., 2011). Since there were no significant  
30 differences in the activity levels of  $\alpha$ -mannosidase,  $\alpha$ -galactosidase and  $\alpha$ -glucosidase  
31 between the control and the *RNAi-SIVPE5* lines (Fig. 4), future work should clarify how  
32 VPE involves in the processing of these three proteins.

1            In conclusion, our results have demonstrated that decreased VPE enzyme  
2 activity due to reduction in *SIVPE5* transcript was associated decreased acid invertase  
3 enzyme activity in the fruits at the RED stage, most likely due to a failure in protein  
4 **synthesis or maturation** processed by SIVPE5 (Figs. 1, 2, and 3; Table 1). The decrease  
5 in acid invertase activity could explain the boost of sucrose accumulation in the  
6 *RNAi-SIVPE5* lines within fruits at the RED stage compared to those in WT (Ariizumi  
7 et al., 2011).

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1   **REFERENCES**

2

3   Arai, M., Mori, H., Imaseki, H., 1991. Roles of sucrose-metabolizing enzymes in  
4   growth of seedlings: purification of acid invertase from growing. *Plant Cell Physiol.* **32**,  
5   1291-1298.

6

7   Ariizumi T., Higuchi K., Arakaki S., Sano T., Asamizu E., Ezura H., 2011. Genetic  
8   suppression analysis in novel vacuolar processing enzymes reveals their roles in  
9   controlling sugar accumulation in tomato fruits. *J. Exp. Bot.* **62**, 2773-2786.

10

11   Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram  
12   quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**,  
13   248-254.

14

15   Bucheli P., Devaud S., 1994. Sugar accumulation in tomato and partial-purification of  
16   buffer-insoluble invertase. *Phytochem.* **364**, 837–841.

17

18   Domingo C., Conejero V., Vera P., 1994. Genes encoding acidic and basic class III  
19   beta-1,3-glucanases are expressed in tomato plants upon viroid infection. *Plant Mol.*  
20   *Biol.* **24**, 725-732.

21

22   Endler A., Meyer S., Schelbert S., Schneider T., Weschke W., Peters S.W., Keller F.,  
23   Baginsky S., Martinoia E., Schmidt U.G., 2006. Identification of a vacuolar sucrose  
24   transporter in barley and Arabidopsis mesophyll cells by a tonoplast proteomic  
25   approach. *Plant Physiol.* **141**, 196–207.

26

27   Endo M., Nakagawa H., Ogura N., Sato T., 1990. Size and levels of mRNA for acid  
28   invertase in ripe tomato fruit. *Plant Cell Physiol.* **31**, 655-659.

29

30   Eom J.S., Cho J.I., Reinders A., Lee S.W., Yoo Y., Tuan P.Q., Choi S.B., Bang G., Park  
31   Y.I., Cho M.H., Bhoo S.H., An G., Hahn T.R., Ward J.M., Jeon J.S., 2011. Impaired  
32   function of the tonoplast-localized sucrose transporter in rice, OsSUT2, limits the  
33   transport of vacuolar reserve sucrose and affects plant growth. *Plant Physiol.* **157**,  
34   109-119.

35

36   Greiner S., Koster U., Lauer K., Rosenkranz H., Vogel R., Rausch T., 2000. Plant

1 invertase inhibitors: expression in cell culture and during plant development. *Aust. J.*  
2 *Plant Physiol.* **27**, 807–814.

3

4 Grudkowska M., Barbara Z., 2004. Multifunctional role of plant cysteine proteinases.  
5 *Acta Biochim. Pol.* **51**, 609-624.

6

7 Hackel A., Schauer N., Carrari F., Fernie A.R., Grimm B., Kühn C., 2006. Sucrose  
8 transporter LeSUT1 and LeSUT2 inhibition affects tomato fruit development in  
9 different ways. *Plant J.* **45**, 180-192.

10

11 Hatsugai N., Kuroyanagi M., Yamada K., Meshi T., Tsuda S., Kondo M., Nishimura M.,  
12 Hara-Nishimura I., 2004. A plant vacuolar protease, VPE, mediates virus-induced  
13 hypersensitive cell death. *Science* **305**, 855–858.

14

15 Husain S.E., Thomas B.J., Kingston-Smith A.H., Foyer C.H., 2001. Invertase protein,  
16 but not activity, is present throughout development of *Lycopersicon esculentum* and *L.*  
17 *pimpinellifolium* fruit. *New Phytol.* **150**, 73–81.

18

19 Isaacson T., Damasceno C.M.B., Saravanan R.S., He Y., Catalá C., Saladié M., Rose  
20 J.K.C., 2006. Sample extraction techniques for enhanced proteomic analysis of plant  
21 tissues. *Nat. Protoc.* 1, 769-774.

22

23 Jagadeesh B. H., Prabha T, N., Srinivasan K., 2004. Activities of glycosidases during  
24 fruit development and ripening of tomato (*Lycopersicum esculantum* L.) implication in  
25 fruit ripening. *Plant Sci.* **166**, 1451-1459.

26

27 Jin Y., Ni D.A., Ruan Y.L., 2009. Posttranslational elevation of cell wall invertase  
28 activity by silencing its inhibitor in tomato delays leaf senescence and increases seed  
29 weight and fruit hexose level. *Plant Cell* **21**, 2072-2089.

30

31 Kim J.Y., Mahé A., Brangeon J., Prioul J.L., 2000. A maize vacuolar invertase, IVR2, is  
32 induced by water stress. Organ/tissue specificity and diurnal modulation of expression.  
33 *Plant Physiol.* **124**, 71–84.

34

35 Kim Y.W., Kato K., Hirai T., Hiwasa-Tanase K., Ezura H., 2010. Spatial and  
36 developmental profiling of miraculin accumulation in transgenic tomato fruits

1 expressing the miraculin gene constitutively. *J. Agric. Food Chem.* **58**, 282–286.

2

3 Kinoshita T., Yamada K., Hiraiwa N., Kondo M., Nishimura M., Hara-Nishimura I.,  
4 1999. Vacuolar processing enzyme is upregulated in the lytic vacuoles of vegetative  
5 tissues during senescence and under various stressed conditions. *Plant J.* **19**, 43–53.

6

7 Klann E.M., Chetelat R.T., Bennett A.B., 1993. Expression of acid invertase gene  
8 controls sugar composition in tomato (*Lycopersicon*) Fruit'. *Plant Physiol.* **103**, 863-870.

9

10 Kliewer W.M., 1966. Sugars and organic acids of *Vitis vinifera*. *Plant Physiol.* **41**,  
11 923-931.

12

13 Kuroyanagi M., Yamada K., Hatsugai N., Kondo M., Nishimura M., Hara-Nishimura I.,  
14 2005. VPE is essential for mycotoxin-induced cell death in *Arabidopsis thaliana*. *J. Biol.*  
15 *Chem.* **280**, 32914–32920.

16

17 Li Z., Palmer W.M., Martin A.P., Wang R., Rainsford F., Jin Y., Patrick J.W., Yang Y.,  
18 Ruan Y.L., 2011. High invertase activity in tomato reproductive organs correlates with  
19 enhanced sucrose import into, and heat tolerance of, young fruit. *J. Exp. Bot.* **63**,  
20 1155-1166.

21

22 Lu S., Li T., Jiang J., 2010. Effects of salinity on sucrose metabolism during tomato  
23 fruit development. *Afr. J. Biotechnol.* **9**, 842-849.

24

25 Matarasso N., Schuster S., Avni A., 2005. A novel plant cysteine protease has a dual  
26 function as a regulator of 1-aminocyclopropane-1-carboxylic acid synthase gene  
27 expression. *Plant Cell* **17**, 1205-1216.

28

29 Mikkelsen R.L., 2005. Tomato Flavor and Plant Nutrition: A Brief Review. *Better*  
30 *Crops* **89**.

31

32 Nakaune S., Yamada K., Kondo M., Kato T., Tabata S., Nishimura M., Hara-Nishimura  
33 I., 2005. A vacuolar processing enzyme,  $\Delta$ VPE, is involved in seed coat formation at the  
34 early stage of seed development. *Plant Cell* **17**, 876-887.

35

36 Ohyama A., Ito H., Sato T., Nishimura S., Imai T., Hiria M., 1995. Suppression of acid

1 invertase by antisense RNA modified the sugar composition of tomato fruit. *Plant Cell*  
2 *Physiol.* **36**, 369-376.

3

4 Pressman E., Harel D., Zamski E., Shaked R., Altahan L., Rosenfeld K., Firon N., 2006.  
5 The effect of high temperatures on the expression of sucrose cleaving enzymes during  
6 tomato (*Lycopersicon esculentum*) anther development. *J. Hortic. Sci. Biotech.* **81**,  
7 341-348.

8

9 Reca I.B., Brutus A., D'Avino R., Villard C., Bellincampi D., Giardina T., 2008.  
10 Molecular cloning, expression and characterization of a novel apoplastic invertase  
11 inhibitor from tomato (*Solanum lycopersicum*) and its use to purify a vacuolar invertase.  
12 *Biochimie* **90**, 1611-1623.

13

14 Rojo E., Zouhar J., Carter C., Kovaleva V., Raikhel N.V., 2003. A unique mechanism  
15 for protein processing and degradation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci.*  
16 *USA* **100**, 7389-7394.

17

18 Shimada T., Yamada K., Kataoka M., Nakaune S., Koumoto Y., Kuroyanagi M., Tabata  
19 S., Kato T., Shinozaki K., Seki M., Kobayashi M., Kondo M., Nishimura M.,  
20 Hara-Nishimura I., 2003. Vacuolar processing enzymes are essential for proper  
21 processing of seed storage proteins in *Arabidopsis thaliana*. *J. Biol. Chem.* **278**,  
22 32292–32299.

23

24 Stommel J.R., 1992. Enzymic components of sucrose accumulation in the wild tomato  
25 species *Lycopersicon peruvianum*. *Plant Physiol.* **99**, 324-328.

26

27 Sturm A., Chrispeels M., 1990. cDNA cloning of carrot extracellular  $\beta$ -fructosidase and  
28 its expression in response to wounding and bacterial infection. *Plant Cell* **2**, 1107-1119.

29

30 Unger C., Hardegger M., Lienhard S., Sturm A., 1994. Soluble acid  
31  $\beta$ -fructofuranosidase and comparison with the cell wall isoenzyme. *Plant Physiol.* **104**,  
32 1351-1357.

33

34 Winter H., Robinson D.G., Heldt H.W., 1993. Subcellular volumes and metabolite  
35 concentrations in barley leaves. *Planta* **191**, 180–190.

36

- 1 Woltering E.J., van der Bent A., Hoerberichts F.A., 2002. Do plant caspases exist? *Plant*  
2 *Physiol.* **130**, 1764–1769.
- 3
- 4 Yamada K., Nishimura M., Hara-Nishimura I., 2004. The slow wound-response of c  
5 VPE is regulated by endogenous salicylic acid in Arabidopsis. *Planta* **218**, 599–605.
- 6
- 7 Yamamoto N., Tsugane T., Watanabe M., Yano K., Maeda F., Kuwata C., Torki M., Ban  
8 Y., Nishimura S., Shibata D., 2005. Expressed sequence tags from the laboratory-grown  
9 miniature tomato (*Lycopersicon esculentum*) cultivar Micro-Tom and mining for single  
10 nucleotide polymorphisms and insertions/deletions in tomato cultivars. *Gene* **356**,  
11 127-134.
- 12
- 13 Yin Y.G., Kobayashi Y., Sanuki A., Kondo S., Fukuda N., Ezura H., Sugaya S.,  
14 Matsukura C., 2010. Salinity induces carbohydrate accumulation and sugar regulated  
15 starch biosynthetic genes in tomato (*Solanum lycopersicum* L. cv. ‘Micro-Tom’) fruits  
16 in an ABA- and osmotic stress-independent manner. *J. Exp. Bot.* **61**, 563–574.
- 17
- 18



**Contributions**

Ning Wang and Narendra Duhita performed and analyzed the experiments, and Ning Wang, Narendra Duhita, Tohru Ariizumi and Hiroshi Ezura wrote the manuscript. Tohru Ariizumi and Hiroshi Ezura designed and supervised the research work and provided funding for this work.