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Involvement of vacuolar processing enzyme SlVPE5 in post-transcriptional process of invertase in sucrose accumulation in tomato

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Abstract

Enhancing the flavor of fruits plays a fundamental role in improving fruit quality, and volatile compositions as well as acid and sugar accumulation are significant factors that have an impact on the acceptability of sensory responses by human beings. Vacuoles in plants not only function as cell compartments that store amino acids, sugars and other metabolites but also act as lytic organelles where vacuolar proteins are post-translationally processed into mature forms or degraded by the action of vacuolar processing enzyme (VPE). We have previously characterized *VPE* genes (*SlVPE1-5*) during fruit development in tomato and discovered that the VPE enzyme activity negatively interfered with sugar accumulation in mature fruits. Comparative proteomic analysis demonstrated that acid invertase was one of the molecular targets of *SlVPE5*, which is involved in the hydrolysis of sucrose. This study also showed that decreased VPE enzyme activity due to suppression of *SlVPE5* by RNAi strategy (*RNAi-SlVPE5*) accompanied with decreased enzyme activity of acid invertase. Further, we identified the enzyme activity of acid invertase was not well correlated with mRNA levels in the *RNAi-SlVPE5* line. These results suggest that *SlVPE5* regulates post-transcriptional processing through *de novo* synthesis of the acid invertase protein to suppress enzyme activity, thereby eventually ensuring sucrose hydrolysis.

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

1. INTRODUCTION

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

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

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

VPEγ (Rojo et al. 2003), suggested to be involved in degradation of the vacuolar invertase (AtFruct4) in aging tissues. AtFruct4 is degraded in the senescing leaves of the wild type but not in the *vpeγ* mutant. They proposed that senescence induced by aging activates VPE and that its protease activity is required for degradation of the vacuolar invertase AtFruct4 in aging tissues. Tandem MS analysis also found that four glycosidases, including a putative glucosidase (At1g52400), two mannosidases (At3g26720 and At5g13980), and a galactosidase (At3g56310), were not degraded in the *vpeγ* mutant. In tobacco, silencing of the vegetative type VPE (*NtVPE1*) results in the suppression of TMV-induced hypersensitive cell death (Hatsugai et al., 2004). An experiment in Arabidopsis shows that the VPE is involved in two cell death systems: fumonisin-induced cell death (Kuroyanagi et al., 2005) and developmental cell death in seed integuments (Nakaune et al., 2005). The *SlVPE5* gene appeared to be identical to *S. lycopersicum* CYSTEINE PROTEINASE (*SlCp*), which was previously identified to have a dual function as a protease and as an ACS (1-aminocyclopropane-1-carboxylic acid synthase) gene regulator, which is involved in wounding response, ripening and floral senescence. The SlCp was reported to bind to the promoter region of the *ACS* gene and induce downstream GUS expression (Matarasso et al., 2005).

Ariizumi et al. (2011) previously identified five novel *VPE* genes from the tomato, named *SlVPE1* to *SlVPE5*. Based on the sequence structure, they suggested that *SlVPE1* and *SlVPE2* were categorized as the seed coat type, *SlVPE4* was categorized as the seed type, and both *SlVPE3* and *SlVPE5* were categorized as the vegetative type. The suppression of *SlVPE1*, *SlVPE3* and *SlVPE5* using RNAi affected sugar accumulation but did not influence the total amino acid content, showing that VPE is involved in the process of sugar accumulation. Among these genes, suppression of the vegetative type *SlVPE5* using RNAi resulted in the most efficient increase in sucrose accumulation. However, the connection between sucrose accumulation and the vegetative type *SlVPE5* gene is unclear. In the present study, we analyzed the contribution of the vegetative type *SlVPE5* gene in the process of sugar accumulation in the tomato fruits. First, VPE activity was measured in the green and red stages of the *RNAi-SlVPE5* line, and invertase activity was measured in the same samples. Next, proteomic analyses using 2D gel electrophoresis and RT-PCR were performed to confirm post-transcriptional processing. The results suggested that the decreased VPE activity influenced *de novo* synthesis of the acid invertase protein and caused an increase in sucrose accumulation in the *RNAi-SlVPE5* line.

2. MATERIALS AND METHODS

2.1 Plant Materials

Tomato plants of control (cv. Micro-Tom) and transgenic *RNAi-SlVPE5* 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% polyvinylpolypyrrolidone (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

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

2.4 Quantitative Expression Analysis

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

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_2 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_2 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_2 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 α-mannosidase, α-galactosidase and α-glucosidase Activities

The glycosidase activities were measured according to Jagadeesh et al. (2004) with modifications. Whole fruits of control plants and the T_2 generation of transgenic RNAi plants at the MG stage (DAF 27-30) and the RED stage (DAF 39-45) were ground with liquid N_2 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 a-D-mannopyranoside, p-nitrophenyl a-D-galactopyranoside or p-nitrophenyl a-D-glycopyranoside, Sigma) was added to 5 µg/50 µl of the extracted protein in 50 mM

citrate phosphate buffer (pH 5.2) containing 0.5 M NaCl. The mixture was incubated at 30°C for 15 min, and 50 µl of 1 M Na_2CO_3 was added to stop the reaction. Blanks were terminated at zero time with the same 1 M Na_2CO_3 solution. α-Mannosidase, α-galactosidase and α-glucosidase activities were measured as the amount of p-nitrophenol released, which was measured as the increase in absorbance at 410 nm.

3. RESULTS

3.1 Proteomic analysis of *RNAi-SlVPE5* Lines

The densities of the isoelectric points differed markedly between wild type and *RNAi-SlVPE5* lines in RED stage fruits (Fig. 1). Two-dimensional gel electrophoresis (2-DE) of total proteins in the tomato fruit detected 1,271 spots, 5 of which reflected higher differential levels (more than three times) and were further analyzed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Table 1). Three of the 5 well-characterized spots were identified as identical acid invertase proteins, the densities of which were 4.1, 3.6 and 3.3-fold higher in the wild type than that in the *RNAi-SlVPE5* lines. The other 2 spots were identified as a glucan endo-1,3-beta-D-glucosidase and an unknown protein that was expressed in the maturing tomato fruits (Yamamoto et al., 2005); the densities of the isoelectric points for these two proteins were 3.1 and 4.4-fold higher in the wild type than that in the *RNAi-SlVPE5* lines, respectively.

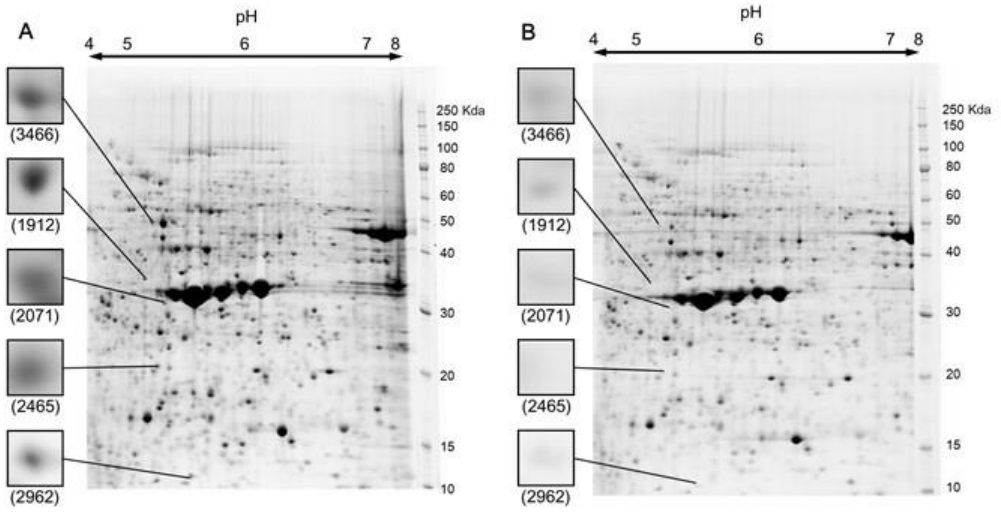

Figure 1. 2D gel electrophoresis of total protein extracts of control (A) and the *RNAi-SlVPE5* line (B). Spots point to 3-fold differences of density in the control compared with the *RNAi-SlVPE5* lines.

Table 1. Identification of differential proteins with more of 3 folds expression in control compared to RNAi *SlVPE5* 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.SNQLDQNFR.A
							561.2745	R.SNQLDQNFR.A
							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.VDSAAELDIEASFEVDK.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.TAYHFQPQK.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.VDSAAELDIEASFEVDK.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.VSTAIETGLTTDTSPPSNGR.F
							2023.0221	K.LEYALFTSPEVVVNDNGR.G
							2245.2542	R.APLLVNLYPYFAVVDNPVIK.L
							2915.4569	K.AGGSSLQIVVSESGWPSAGAGQLTSIDNAR.T

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

significantly more in the mature fruits (DAF33 to 45) compared with earlier time points (DAF0 to 27) (Fig. 2A). Acid invertase mRNA mainly accumulated in the pericarp and placenta and was expressed at a significantly lower level in the seed and jelly (approximately 1%) in comparison with the level in the pericarp (Fig. 2B). The expression levels of acid invertase mRNA in the *RNAi-SlVPE5* transgenic lines were investigated at the MG stages and the RED stage in tissues from whole tomato fruits (Fig. 2C). The mRNA of acid invertase was highly accumulated at the RED stage in comparison to the MG stage. There were no significant differences in the mRNA levels of acid invertase between the wild type and the *RNAi-SlVPE5* transgenic lines.

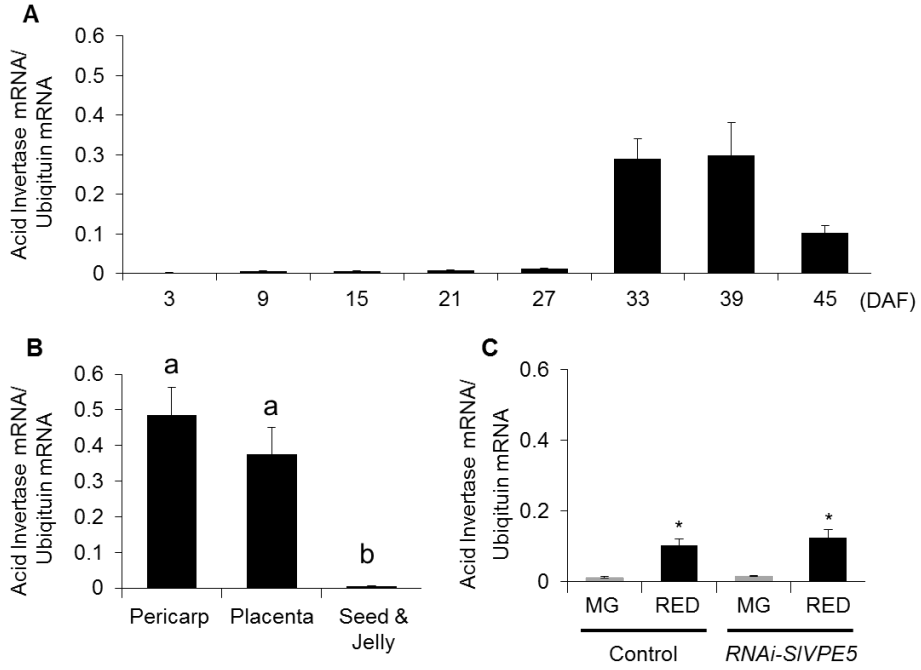

Figure 2. qRT-PCR of acid invertase. A), Time course analysis of acid invertase expression during fruit development. DAF, day after flowering B), Expression analysis of the pericarp, placenta, seed and jelly of wild type tomatoes. Different letters indicate significant differences ($P < 0.05$; Tukey–Kramer test). C), Expression analysis of the *RNAi-SlVPE5* line. Whole fruit tissue at the MG stage (gray) and the RED stage (black) was used. Asterisks indicates significance compared with control ($P < 0.05$; Student's *t*-test).

3.3 **VPE and Invertase Enzyme Activities in Fruit**

We next confirmed that the suppression of *SlVPE5* expression can repress VPE activity at both the MG and RED stages. A previous study revealed that a decrease in *SlVPE* mRNA expression was associated with decreased levels of VPE enzyme activity in the fruits of the *RNAi-SlVPE5* line (Ariizumi et al., 2011). In this study, we also

confirmed that VPE enzyme activity is also repressed in the *RNAi-SlVPE5* lines, which exhibited 80% and 50% of the VPE enzyme activity levels of the control plants at the MG stage and RED stage, respectively (Fig. 3A). VPE enzyme activity in the pericarp, placenta, seed and jelly was also measured at the RED stage in the wild type (Fig. 3B). We observed the highest VPE activity in the seed and jelly, followed by the placenta and pericarp.

The enzyme activity of acid invertase significantly increased 6-fold from the MG stage to the RED stage (Fig. 3C) in the control plant, which perfectly correlated with acid invertase mRNA accumulation (Fig. 2A). The invertase enzyme activity in the fruits was measured in the *RNAi-SlVPE5* lines. Although there was no significant difference in invertase activity at the MG stage, we found that invertase activity in the *RNAi-SlVPE5* lines was repressed to 33.5~50.8% of the activity of wild type at the RED stage (Fig. 3C). A further assay of invertase enzyme activity in the seed and jelly exhibited the lowest activity among the three organs (Fig. 3D).

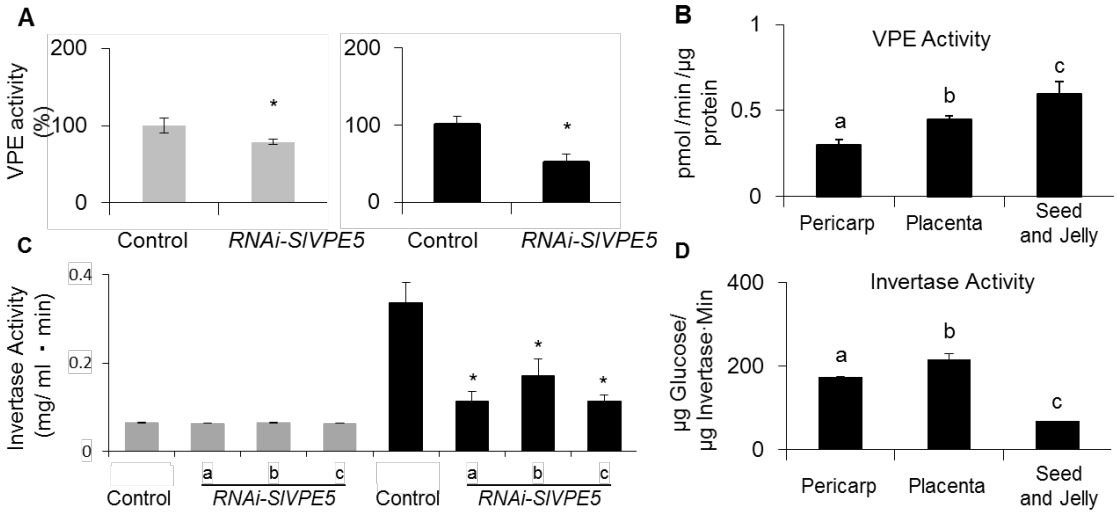

Figure 3. Enzyme activity of VPE (A, B) and invertase (C, D). A, C), Enzymatic activity of whole fruit tissue at the MG stage (gray) and the RED stage (black) of the control and the *RNAi-SlVPE5* line. The activity of the *RNAi-SlVPE5* lines is relative to the control plants. Asterisks indicating significance at <0.05 level as determined by the t-test. B, D), Enzymatic activity in the pericarp, placenta, seed and jelly of wild type tomatoes at the RED stage. a,b,c indicating significance at <0.05 level as determined by Tukey-Kramer test. Different letters indicate significant differences ($P < 0.05$; Tukey–Kramer test).

3.4 Glycosidases Enzyme Activities in Fruit

Rojo et al. (2003) suggested that VPE co-localized with glucosidase, mannosidase and galactosidase and was involved in further processing of these enzymes in the vacuoles. We measured α-mannosidase, α-galactosidase and α-glucosidase activity of the control and the *RNAi-SlVPE5* lines using fruit tissue at the RED stage (Fig. 4). We demonstrated that there was no significant difference in the enzyme activity in the *RNAi-SlVPE5* lines compared with control at the RED stage.

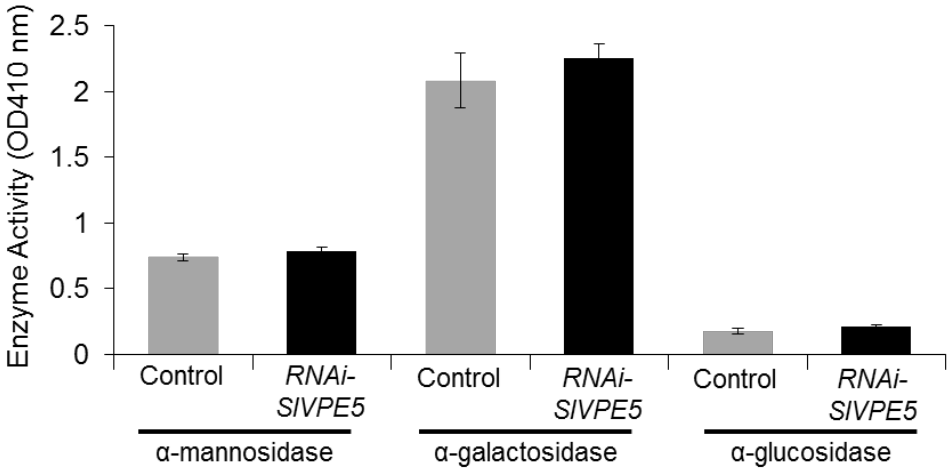

Figure 4. Mannosidase, galactosidase and glucosidase activity in the control (grey) and the *RNAi-SlVPE5* line (black).

4. DISCUSSION

4.1 SlVPE5 Functions in Regulation of Acid Invertase Activity

Although *RNAi-SlVPE5* lines did not show any morphological changes in vegetative and reproductive organ, it has been shown that suppression of *SlVPE5* at the mRNA level results in an increase in sucrose accumulation (Ariizumi et al., 2011). This study demonstrated that suppression of *SlVPE5* expression may suppress synthesis of acid invertase protein resulting in decreasing invertase activity. Acid invertase localizes in the vacuole, and it plays a role as one of the main enzymes that hydrolyses sucrose to glucose and fructose, which is a crucial factor in determining the sugar content of ripened fruits (Ohyama et al., 1995). Ripe tomatoes and grapes contain small amounts of sucrose and accumulate mainly glucose and fructose (Kliewer, 1966; Ohyama et al., 1995). The decrease in sucrose accumulation in red tomato fruits compared to mature green fruits is mainly caused by the high invertase activity in the red tomato (Klann et al., 1993, Endo et al., 1990, Stommel, 1992). Proteomic analysis revealed three fragments corresponding to acid invertase (21, 31 and 49 kDa) proteins that were highly accumulated in the RED stage fruit of the control plant; however, acid invertase decreased to one third of this level in the *RNAi-SlVPE5* lines (Fig. 1, Table 1). The processing of red tomato acid invertase into a smaller sized protein has been reported in previous studies. Although evidence indicating the degradation of acid invertase is typically observed in plants, the biological function of this fragmentation is unclear.

The decreasing molecular size of acid invertase might be required for processing higher enzyme activity. Rojo et al. (2003) reported that senescence induces the VPEγ-dependent breakdown of invertase in aging tissues in Arabidopsis. The levels of invertase decline in senescing leaves of WT plants, but not in *vpeγ* mutants, although the mRNA levels are similar in mutant and WT plants. Tomato would partially differ from Arabidopsis that invertase protein is less apparent in *RNAi-SlVPE5* lines, while the mRNA levels are similar and invertase were degraded in both *RNAi* lines and control. Purification of tomato acid invertase (TIV-1) using its inhibitor (SolyCIF) identified six different size fragments of acid invertase (Reca et al., 2008). Smaller fragments of acid invertase were also found in the western blot analysis of tomato fruits (Endo et al., 1990). Similar results were previously reported by other authors (Greiner et al., 2000; Bucheli and Devaud, 1994). Similar fragmentation of vacuolar invertase was also found in the hypocotyl of the mung bean (Arai et al., 1991) and in carrots (Unger et al., 1994). The increased enzyme activity of invertase was likely due to the *de novo* synthesis of the invertase protein, which occurred because of the relatively high mRNA expression

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

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

4.2 SlVPE5 Post-transcriptionally Processes Acid Invertase

We suggested an increase in invertase activity in ripening stage may not simply due to gene expression but resulting in VPE mediated post-transcriptional processing on *de novo* synthesis and maturation of invertase protein. The expression of acid invertase coordinates spatially and temporally with *SlVPE5*, indicating a high probability of *SlVPE5*-mediated invertase translation or maturation. The mRNA expression of *SlVPE5* is elevated around the IMG stage, and the highest expression is observed from the BR stage to the RED stage, and *SlVPE5* transcript levels are correlated with VPE enzyme activity (Fig. 3A; Ariizumi et al., 2011). The mRNA expression of acid invertase was low at the BR stage and increased as fruit approached maturation, reaching the highest levels at 33 and 39 DAF stages and remained relatively high levels until 45 DAF (Fig. 2A), as similarly shown in previous studies (Klann et al., 1993, Endo et al., 1990, Stommel, 1992). Consistent with the low relative mRNA expression by means of low level of acid invertase protein at the MG stage (Fig. 2A), acid invertase revealed low enzyme activity both in WT and *RNAi- SlVPE5* line and there was no significant

different in its activity between them (Fig. 3C). Although mRNA expression levels were equivalent at RED stage (Fig. 2C), acid invertase activity in *RNAi-SlVPE5* line was repressed to 33.5~50.8% of that of control plant (Fig. 3C). These results suggest that the enzyme activity of acid invertase is not fully correlated with its mRNA abundance, but could be also regulated by post-transcriptional processing of *SlVPE5*.

In the evolutionary process, higher sugar content in the seed and jelly may also play an important role in germination and to attract animals to consume the seeds. Vacuolar invertase increased along with tomato fruit maturation, and its activity accounted for most of the invertase activity in the mature tomato. Most of the acid invertase activity was due to vacuolar invertase and including the activity of cell wall invertase (Jin et al., 2009). We demonstrated that invertase activity and VPE enzyme activity were higher in the placenta, followed by the pericarp. Entry into the seed and jelly was the single exception to the otherwise perfect relationship between VPE and invertase enzyme activity (Fig. 3). Although a considerable amount of enzyme activity of VPE observed (Fig. 3B), acid invertase gene expression was significantly lower in the seeds and jelly. Assuming that lower level of acid invertase protein could be accumulated thus for the comparable low level of enzyme activity in seeds and jelly (Fig. 3D). In tomato, five VPE proteins were classified into the following three categories: seed coat type (SlVPE1 and SlVPE2), seed type (SlVPE4), and vegetative type (SlVPE3 and SlVPE5). Spatial expression analysis of the *SlVPE* genes using a GUS reporter showed that clear GUS activity was observed in vascular bundles extending from the seeds to the placenta as well as around the endocarp in *SlVPE5p-GUS* throughout fruit development (Ariizumi et al., 2011). The SlVPE1 and SlVPE2 lines showed that the majority of GUS activity was evident in the seeds within fruits. Moreover, the GUS activity of SlVPE4p-GUS was exclusively evident in the seed endosperm and the embryo of developing fruits. Co-localization of SlVPE1 SlVPE2 and SlVPE4 ensured the highest VPE activity compared with the pericarp and placenta. Here we further compared VPE activity at RED stage. The *RNAi-SlVPE5* lines showed a decreased activity as 50% level of which in control plants, which is relatively small compared to that in VPE mRNA accumulation (16% of control) (Ariizumi et al., 2011). The other VPE family genes expressing at RED stage, such as *SlVPE3* and *SlVPE4*, could not be suppressed in *RNAi-SlVPE5* lines, although *RNAi-SlVPE5* lines

revealed highly specificity on suppression on *SlVPE5* expression levels. Therefore, total VPE enzymatic activity involving activity of *SlVPE3* and *SlVPE4*.

A possible basis for the increase in sucrose content is that VPE activity was suppressed in the *RNAi-SlVPE5* lines which resulted in a lack of acid invertase protein synthesis and thus for low invertase activity, and given that the sucrose hydrolysis products are slowly decomposed. Although the sucrose content significantly increased, the hexose content decreased due to the lack of sucrose degradation to fructose and glucose due to repression of the enzyme activity of acid invertase (Klann et al., 1993, Ohyama et al., 1995). A pervious study have demonstrated that suppression of *SlVPE5* expression resulting in significant increase in sucrose content (5.0-8.0-fold higher than the control) in tomato fruits at the RED stage (Ariizumi et al., 2011). Fructose and glucose content revealed slightly increased in *RNAi-SlVPE5* lines but which is an extremely low relative level in compare with the increase of sucrose. The mechanism for the increase in the hexose level in the *RNAi-SlVPE5* lines remains elusive.

MS analysis of VPE mutants in *Arabidopsis thaliana* indicated that glucosidase, mannosidase and galactosidase accumulated in the vacuoles and suggested that the processing of this protein was performed by VPE (Rojo et al., 2003). In this study, proteomic analysis revealed glucan endo-1,3-beta-D-glucosidase as a target of *SlVPE5* (Table 1). We suggest that glucan endo-1,3-beta-D-glucosidase is correlated with its caspase-1-like activity on hypersensitive cell death, which was reported to be expressed in tomato plants upon viroid infection (Domingo et al., 1994). Repressed glucan endo-1,3-beta-D-glucosidase translation in the *RNAi-SlVPE5* lines might be the cause of invisible hypersensitive cell death upon induction of the tobacco mosaic virus (TMV) (Hatsugai et al., 2004). The spot with the highest differentiation was an unknown protein (Table 1), a homolog of the legumin type globulin of *Arabidopsis thaliana*. Our results confirmed a previous report that seeds from βVPE knockout mutants are hindered in the maturation of storage proteins in Arabidopsis, such as globulin and albumin (Shimada et al., 2003), given that this is one of the functions of *SlVPE5*, which was expressed in the seed coat (Ariizumi et al., 2011). Since there were no significant differences in the activity levels of α-mannosidase, α-galactosidase and α-glucosidase between the control and the *RNAi-SlVPE5* lines (Fig. 4), future work should clarify how VPE involves in the processing of these three proteins.

In conclusion, our results have demonstrated that decreased VPE enzyme activity due to reduction in *SlVPE5* transcript was associated decreased acid invertase enzyme activity in the fruits at the RED stage, most likely due to a failure in protein synthesis or maturation processed by SlVPE5 (Figs. 1, 2, and 3; Table 1). The decrease in acid inveratse activity could explain the boost of sucrose accumulation in the *RNAi-SlVEP5* lines within fruits at the RED stage compared to those in WT (Ariizumi et al., 2011).

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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.