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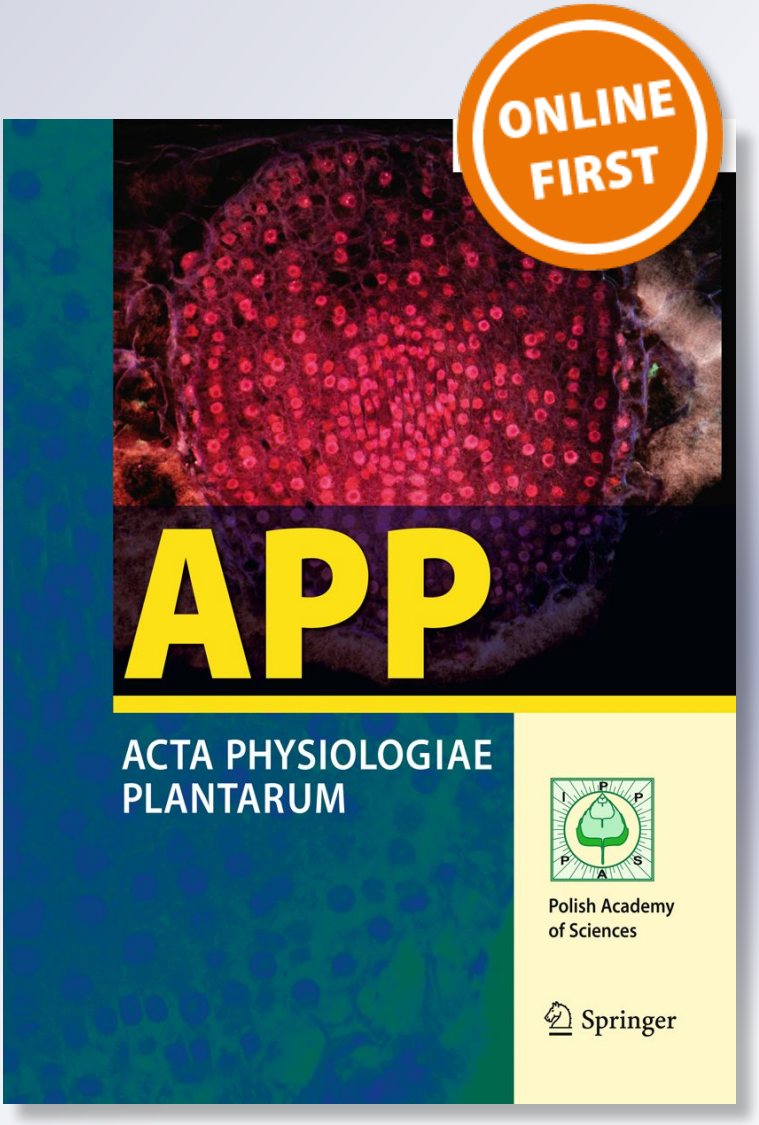

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ORIGINAL PAPER

Cloning and expression of genes related to the sucrose-metabolizing enzymes and carbohydrate changes in peach

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Abstract To shed light on the relationship between sucrose metabolism and expression of genes related to sucrose-metabolizing enzymes, six genes encoding sucrose-metabolizing enzymes were isolated, and the levels of four main carbohydrates and related enzyme activities as well as the expression of these six genes were determined in fruits, leaves and phloem-enriched fraction throughout peach fruit development. Sucrose content in mature fruit ranked first followed by glucose, fructose and sorbitol in that order, while sorbitol was the highest and sucrose lowest in phloem-enriched fraction and leaves. Glucose and fructose had similar change patterns throughout fruit development. Cloning results reveal that the nucleotide sequences of the six genes have high similarity to corresponding genes isolated from other plants. In addition, the expression of these genes and the levels of related enzyme activities varied with tissue and stage of fruit development, suggesting a complexity in relationships between carbohydrates, enzymes activities and related gene expression. Sucrose phosphate synthase maybe a key enzyme involved in sucrose synthesis while sucrose synthase may mainly be responsible for sucrose synthesis in peach fruits at later stages of development. Further studies are needed to genetically and physiologically characterize these genes and enzymes in peach and to gain a better understanding of their functions and relationship with carbohydrate metabolism.

Keywords Peach · Sucrose · Carbohydrate · Sucrose-metabolizing enzyme · Cloning · Gene expression

Introduction

Carbohydrates are synthesized in source leaves and translocated to sink tissues in most species in the form of sucrose to sustain heterotrophic metabolism and growth, or to be stored as sucrose or starch (Roitsch and Gonza'lez 2004). In recent years, it has become evident that sugars, notably sucrose and its breakdown products (glucose, fructose, and sorbitol), are important metabolic substances that affect the expression of different classes of genes (Koch 1996; Rolland et al. 2002) and are involved in regulation of plant development (Wobus and Weber 1999). Due to the importance of sucrose in plant life, considerable research has been done on sucrose metabolism and accumulation, and as a consequence, the major reactions catalyzed and synthesized by an array of enzymes in the sucrose metabolism pathway have been understood.

Enzymes which have a close relationship with sucrose metabolism and accumulation are mainly sucrose synthase (E.C. 2.4.1.13, SS, SUS or SUSy), sucrose phosphate synthase (E.C. 2.4.1.14, SPS), sucrose transporter (SUT), and invertase (E.C. 3.2.1.26, Ivr). SUS catalyzes the reversible conversion of sucrose and UDP or ADP to UDP- or ADP-glucose and fructose (Baroja-Fernandez et al. 2003; Ji et al. 2005). SPS catalyzes the reversible conversion of uridine diphosphate glucose (UDPG) and fructose-6-phosphate (F-6-P) to UDP and sucrose-6-phosphate, then

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sucrose-6-phosphate is further converted to sucrose by phosphatase irreversibly. SUT plays an essential role in loading sucrose into the phloem component of the plant vascular bundles where they are not only key regulators of transport processes but also integral components of signal transduction between sink and source metabolism (Kuhn and Grof 2010). Lastly, Ivr is responsible for irreversible sucrose hydrolysis to glucose and fructose. There are three widely accepted classifications of invertase namely neutral/alkaline (CINV), cell wall (CWINV) and vacuolar (VINV) invertases. The CINVs are found in the cytosol and are considered 'maintenance' enzymes involved in sucrose degradation when the activities of acid invertase and SUS are low. CWINV and VINV are together referred to as the acid invertases (AI). CWINV, or apoplastic invertases, are ionically bound to the cell wall matrix and are thought to play a role in phloem unloading by ensuring a steep concentration gradient from source to sink (Winter and Huber 2000).

In plants, the altered expression of genes involved in sucrose metabolism could affect whole-plant adjustment to changes in carbohydrate supplies at several levels. Although carbohydrate levels may vary widely in plants, maintenance of 'energy homeostasis' has been proposed as one of the functions of carbohydrate-regulated gene expression (Koch 1996). The activity, purification, and expression of many enzymes, such as SUS1 (Moscatelloa et al. 2011; Lingle and Dyer 2001), SPS1 (Heim et al. 1996; Verma et al. 2011), invertase (Ji et al. 2005; Tian et al. 2009), and SUT (ElSayed et al. 2010; Liesche et al. 2011) have been extensively studied in various sink and source tissues of plant species.

Peach [*Prunuspersica* (L.) Batch] is one of the most popular fruits in the world because of its high nutrient level and pleasant flavor (Wan et al. 2009). It is characterized by double-sigmoid growth curve throughout the fruit development period. Among the main carbohydrates, sucrose is predominant with other carbohydrates, such as glucose, fructose, and sorbitol, also being present but at lower concentrations in ripened peach fruit (Robertson et al. 1990; Moing 2000). Great efforts have been made to study sucrose metabolism in leaves (Guo et al. 2004; Moing et al. 1992) and developing fruits (Vizzotto et al. 1996; Moing et al.1998; Lombardo et al. 2011) of peach. However, these studies were primarily focused on sucrose metabolism in either leaves or fruits, and/or did not observe the dynamics of this metabolism during tissue development. Furthermore, information on simultaneous sucrose metabolism as well as related enzyme activity and gene expression in leaves, phloem, and fruits of peach is scanty. To our knowledge, only a *SUS1* had been purified and characterized from peach fruits (Moriguchi and Yamaki 1988) while purification and expression of a *CINV* (partial CDS) in fruits from two peach cultivars was done by Nonis et al. (2007), and *SPS1* and *SPS2* were isolated from fruit by Tong et al. (2007). Only partial CDS of *CWINV1* (Etienne et al. 2002, AF367453), *CINV1* (Nonis et al. 2007, AM409095), *SUS1* (Etienne et al. 2002, AF367450.1), and complete CDS of *SPS1* and *SPS2* (Tong et al. 2007, EF568781.1 and EF568782.1) could be found on the NCBI database.

This study was undertaken primarily to (1) evaluate the carbohydrate content and composition, as well as related enzyme activity in fruits, phloem-enriched fraction, and leaves of peach 'Xiahui6' (XH6) during the period of fruit development, (2) isolate genes encoding *PpCWINV1*, *PpCINV1*, *PpCINV2*, *PpSUS1*, *PpSPS1*, and *PpSUT1*, and (3) analyze the relationships between carbohydrate changes and the activities of related enzymes as well as the expression patterns of the six genes in the three peach tissues during fruit development.

Materials and methods

Plant material

Peach 'XH6' growing at the National Peach Germplasm Repository in Nanjing, China was used as source of tissue samples. All the peach trees used in this experiment were 7-years old growing under the same common field conditions. The peach orchard was divided into three plots from which three trees per plot were selected for tissue collection. Fruits, phloem-enriched fraction and leaves from the same node were sampled from the outer southern canopies of trees at 20-day intervals starting from 45 days after anthesis (DAA) through to fruit ripening. The fifth leaf from the apex of 1-year-old fruiting shoots was picked at each sampling time. Phloem-enriched fraction above and below the node of this leaf was peeled while fruits from the node of the sampled leaves were picked and chopped into pieces after stone removal. For carbohydrate analysis as well as enzyme and RNA extraction, each tissue from individual sampling points and dates was mixed and put into 50-ml tubes and immediately frozen in liquid nitrogen and then stored at −70 °C until use.

A second set of 10-g fresh sample from each tissue was made and dried to constant weight at 70 °C at each sampling date. The ratio of dry weight (DW)/fresh weight (FW) at sampling date was obtained for tissue and used to calculate the DW of the samples for further calculation of carbohydrate and enzyme assays data.

Carbohydrate analysis

Soluble carbohydrate content of fruits, phloem-enriched fraction and leaves were determined according to the

method used by Niu et al. (2006) with slight modifications. Triplicate samples were prepared for fruits, phloem-enriched fraction, and leaves at each stage of development. Frozen samples (about 2-g fresh weight) were ground in liquid nitrogen and extracted in 80 % (v/v) ethanol to which 100-mg mannitol was added as an internal standard. Extracts were centrifuged, supernatant decanted and the pellet re-extracted twice. The combined supernatant was partitioned against chloroform, and the aqueous fraction was dried under vacuum, re-suspended in water, and passed through Dowex-1 and Dowex-50 ion resins. The resulting fraction was further dried under vacuum, re-suspended in water, and passed through a 0.45-μm filter. Aliquots (15 μl) were injected into a high-performance liquid chromatography system (HPLC, Agilent 1100) equipped with a CARBOSep CHO-620 CA carbohydrate column (6 mm × 250 mm, Shoko Co., Ltd., Tokyo) and a refractive index detector (Shen et al. 2007). Column temperature was 80 °C and flow rate was 0.6 ml/min with water as the eluant. The sugars were eluted (with water at 1.0 ml/min) and then identified and quantified by comparison of retention times and peak areas with standards of known sugars.

Enzyme extraction and activity assay

All enzymes were extracted at 4 °C using the method described by Cheng et al. (2009) with some modifications. 1.0 g of fruits and 0.25 g each of leaves and phloem-enriched fraction respectively were homogenized in 10 ml of ice-cold extraction buffer containing 50 mmol/l *N*-2-hydroxyethylpiperazine-*N*-ethane-sulphonic acid (Hepes-NaOH, pH 7.5), 0.5 mmol/l Na-ethylenediaminetetraacetic acid (EDTA), 2.5 mmol/l dithiothreitol (DTT), 0.1 % (w/v) bovine serum albumin (BSA), 10.0 mmol/l magnesium chloride ($MgCl_2$) and 0.05 % (v/v) *t*-octyl-phenoxypolyethoxyethanol (Triton X-100). The homogenate was filtered through four layers of gauze followed by centrifugation at 12,000×*g* for 20 min at 4 °C. The supernatant was dialyzed immediately by a 10-fold volume of diluted extraction buffer (minus Triton X-100) for 20 h at 4 °C where the dialyzate was changed once. These crude enzyme extracts were used for all the downstream enzyme assays below.

The AI activity was assayed as described by Zhang et al. (2011) with slight modification. Each reaction mixture contained 0.2 ml of the crude enzyme extracts from specific tissues and 0.8 ml of reaction solution (containing 0.1 mol/l sodium acetate-acetic acid (pH 4.8) and 0.1 mol/l sucrose. This was incubated at 37 °C for 40 min and then the reaction was stopped by adding 1.0 ml 3,5-dinitrosalicylic acid. Thereafter, the tubes containing the mixtures were kept in boiling water for exactly 5 min and left to cool. The supernatant was diluted to a final volume of 10 ml after centrifugation at 12,000×*g* for 25 min at 4 °C.

The CINV activity was measured in the same manner as described above apart from the use of sodium citrate-citric acid (pH7.2) used in the reaction solution instead of sodium acetate-acetic acid. The activity of the enzyme was measured spectrophotometrically at 20 °C and 520 nm using a UNICAM Helios spectrophotometer (UNIC AM Instruments, Cambridge, UK). The amount of reducing glucose released from sucrose was calculated under the utilization of the standard curve made using 1.0 mg/ml glucose and absorbance value at A_{520}.

Activity of SPS was assayed using the methods applied by Cheng et al. (2009) and Zhang et al. (2011) involving 60 μl of reaction solution and 80 μl crude enzyme extract sample. The reaction solutions were composed of 50 mmol/l Hepes-NaOH (pH 7.5), 10 mmol/l $MgCl_2$, 5 mmol/l sodium fluoride (NaF), 10 mmol/l F-6-P, and 25 mmol/l UDPG. The mixture was incubated for 40 min at 37 °C and then the reaction was stopped by adding 140 μl of 1.0 mol/l NaOH. Non-reacted F-6-P was destroyed by placing the tubes in boiling water for 10 min. After cooling, 0.5 ml of 0.1 % resorcinol solution (w/v, dissolved in 95 % ethanol) and 1.5 ml of 30 % HCl (v/v) were added into the mixture and the tubes were incubated at 80 °C water bath for 8 min. The procedure for the SUS assay was identical to that of SPS except the replacement of F-6-P with 10 mmol/l fructose in the reaction mixtures. The amount of sucrose produced from fructose-6-phosphate was calculated finally under the utilization of the standard curve made using sucrose and absorbance value at A_{520}. All the enzyme activity assays were done for each tissue and stage with each assay being repeated three times. Each replicate represented the tissues picked from trees in each of the three plots in the orchard. Enzyme activity was expressed as micromoles of sucrose or glucose produced per hour per gram dry weight of samples.

RNA extraction, DNase treatment, and cDNA synthesis

Extraction of total RNA was done by modified CTAB method for fruit (Chen et al. 2009) and modified SDS method (Zhang et al. 2010) for the phloem-enriched fraction and leaf tissues. The RNA was then treated with DNase I according to the manufacturer's instructions (Takara) so as to remove contamination by genomic DNA. An aliquot of RNA was quantified spectrophotometrically, and then electrophoretically separated on a 0.8 % agarose gel to check integrity. Each treated total RNA sample (1.0 μg) from different tissues was reverse-transcribed using the Fermentas RevertAid^{TM} First Strand cDNA Synthesis Kit with 1.0 μl of oligonucleotide dT primer and 1.0 μl reverse transcriptase as per instructions from the manufacturer. All cDNA samples were diluted 1:10 with RNase-free water and stored at −20 °C before being used

as template in cloning and quantitative real-time PCR (qRT-PCR).

Cloning of genes related to the sucrose-metabolizing enzymes

Only cDNA reverse transcribed from the fruit was used for the initial cloning of *PpCWINV1*, *PpCINV1*, *PpCINV2*, *PpSUS1*, *PpSPS1*, and *PpSUT1* genes, as well as the 3′ and 5′ RACE (Rapid Amplification of cDNA Ends) of *SPS1*. Using the corresponding full-length cDNA sequences of *CWINV1*, *CINV1*, *CINV2*, *SUS1*, *SPS1*, and *SUT* in *Arabidopsis thaliana* (TAIR: http://www.arabidopsis.org/index.jsp, GenBank accession numbers AT1G55120, AT1G22650, AT3G06500, AT5G20830, AT5G20280 and AT1G22710) as querying probes, a blast search on the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/blast/) database and further search against the *Prunus persica* genomic database in the GDR (The Genome Database for Rosaceae) websites were done. The results showed that the predicted CDS of these genes in peach (Ppa003412m, Ppa003670m, Ppa002847m, Ppa001537m, Ppa000639m, Ppa004033m) were highly homologous to their genes in *Arabidopsis thaliana*. Based on the predicted CDS sequences of these genes in peach, specific primers (Table 1) were designed using Primer Premier 5.0 software, and synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

PCRs were performed in 25 μl reaction volumes containing 2.5 μl 10 × PCR buffer, 2.5 μl dNTP mixture (2.5 mM), 1.5 μl $MgCl_2$, 0.2 μl Ex Taq polymerase (5 U/μl) (TaKaRa), 2.0 μl cDNA, 1.0 μl of each gene-specific primer, and 14.3 μl RNase-free water. PCR was performed in an Eppendorf Authorized Thermal Cycler by running the following program: 94 °C pre-denaturing for 5 min, followed by 38 cycles of 94 °C for 1 min, T_m for 1 min (T_m is as shown in Table 1), 72 °C for 1 min, and 72 °C for 10 min. The PCR products were separated by electrophoresis in 1.0–2.0 % agarose gel and visualized by staining with ethidium bromide. The target DNA fragment was purified with Axyprep^{TM} DNA Gel Extraction Kit (Aygen Biosciences, USA), then ligated into pMD18-T vector and finally transformed into *E.coli* JM 109. The positive clones identified by PCR analysis were sequenced by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). For cloning of *SUS1* and *SPS1*, 0.2 μl of high fidelity PrimeSTAR® HS DNA Polymerase (TaKaRa) was also used.

The similarity between sucrose metabolism genes in peach and those in other plants was determined using NCBI BLAST. The obtained cDNA sequences were further blasted to verify the open reading frame (ORF) of the predicted cDNA sequences and BioXM (Version 2.6) was used to deduce the amino acid sequences of the six genes. Amino acid sequence of each gene was imported to ExPASy server (http://expasy.org/tools/protparam.html) and the molecular weight and theoretical pI (Isoelectric point) recorded.

Primer design for qRT-PCR

The gene-specific primers for qRT-PCR amplification were designed based on the cloned sequences above (five ORFs and one partial cDNA) using Primer Premier 5.0 Software. Amplicon lengths were optimized to 100–200 bp to ensure optimal polymerization efficiency and minimize the impact of RNA integrity on relative quantification of gene expression. The primers were further used to query the predicted peach genome database with Blastn to confirm the identity of these genes. Before qRT-PCR, each primer pair was tested via standard RT-PCR and electrophoresis in ethidium bromide-stained 2.0 % agarose gels so as to verify size specificity of the amplicons. The amplified bands were cloned and sequenced to confirm that they were indeed fragments of the targeted genes. The *Ubiquitin 10* (*UBQ10*, TC2782) and *Translation elongation factor 2* (*TEF2*, TC3544) were used as reference genes based on study by Tong et al. (2009) and followed the guidelines published by Bustin et al. (2009). The primer sequences, amplicon sizes, and melting temperatures for each gene from all PCR products were as listed in Table 2.

qRT-PCR with SYBR green

qRT-PCR was performed using a My-IQ 2 (Bio-Rad, USA) and the SYBR *Premix Ex Taq*^{TM} (TaKaRa, Japan). The qRT-PCR volume was 20.0 μl containing 1.5 μl of diluted cDNA, 0.4 μl each primer, 10.0 μl Master Mix, and 7.7 μl RNase-free water. Thermo cycling conditions were set with an initial polymerase activation step for 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C for template denaturation, 15 s at 60 °C for annealing and 20 s at 72 °C for extension and fluorescence measurement. Each assay was replicated three times with a tissue replicate corresponding to a tissue sample from one of the three plots in the orchard. Raw fluorescence data taken from the My-IQ 2 detection system were exported to Microsoft Excel and relative quantification expression levels of the six genes were finally calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

Statistical analyses were performed using SAS software (Version 9.0) where differences in carbohydrate levels, enzyme activity, and gene expression between the different tissues at different fruit development stages were tested by analysis of variance (ANOVA). Mean separation was

Table 1 Primer sequences and amplicon characteristics designed for cloning of six genes in peach

Gene name	Start and end of sequence	Sequence (5′–3′)	Size of amplified product (bp)	T_m (°C)
PpCWINV1	1–176	ATGGCCATCTCCTCTCCCTTG	176	55.1
		GGCTGGAAATGATAACCAGTTCTGT		
	134–860	ACCCAGTTAATGAACCTTACAG	727	58.6
		GTGTAGTACTCTCGCCTAGTGTT		
	733–1,232	TGGGAATGCCCTGATTTTTT	500	52.3
		TCTGCCTGTGCTGCTGTGAC		
	1,044–1,734	CAAGAAGGGATGGTCTGGACT	691	58.6
		TCAATTGATTTTTGCTGTTTT		
PpCINV1	1–892	ATGGAAGGGGCCAAAGAGT	892	54.7
		TCAGCAACGCCAAAGCACA		
	868–1,674	TTGAGATGTGCTTTGGCGTTG	807	55.1
		TCAGCAAGTCCAAGACGATGA		
PpCINV2	1–471	ATGAGTAGTAGCAATTGTATTGG	471	58.6
		ATAACTAACAACTGAATCCCGAAGC		
	142–955	TTACAATCGAGACGACAAAAAC	814	59.0
		TGACTAACAGGGTAGGAAACAT		
	643–1,650	TGGGAGAAGACGGTTGACT	1,008	55.1
		CCACTGATCCGCAGAAAGC		
	1,544–1,950	CAACACTCCTATGGCAGTTCACCT	407	58.6
		GGTTTTATTAGTTACCCCACCACT		
PpSPS1	~798	CGAGAGAGACAGATAGGAGGGATT	824	58.6
		ATCTTTGGGACCGAATGGTATA		
	779–1,593	TACCATTCGGTCCCAAAGATAA	815	58.6
		AAGAACAGAAGAACTTGTGCCT		
	1,406–1,944	GCTTCTTTACCAATCCTCG	539	52.3
		GTTCTTCAACCCATTCTGC		
	1,919–2,898	GATGTCGGCAGAATGGGTTGA	980	58.6
		ATACCTGAGAGGGCTTGGGAACG		
	2,750–3,277	TGCAGAAGCCCGGAAAGGT	528	59.0
		CCGAATCCCGCAAACAAAC		
	3′	AACTACCCTCTATCAGATGTCCTGGCTCT	417	69.0
	5′	CGCCTCCAGGTAACTGTTTATCCAATCG	189	69.6
PpSUS1	1–570	ATGGCGGAACGTGCCTTGAC	570	59.3
		CTTGCCCTTGTAGCAGTGAA		
	457–1,020	ATTGGAAATGGCGTTGAGT	564	58.6
		AGGAAGAAGCCTTGTGAGA		
	988–1,250	CCCCGCATTATTATTCTCAC	263	55.1
		TTCCCATCACTGTAGTTTCC		
	1,230–1,636	CGGAAACTACAGTGATGGGAA	407	55.1
		GGTGGAATGATGTCAGCCTCT		
	1,586–2,265	GCATTTACTTCCCTTACTCTGA	680	58.6
		GATTTGCCATGTATACTTCTCG		
	2,190–2,421	GGCAGATCCTAGCCACTGGG	232	62.1
		TCACTCCTCCTCGGCACGAG		
PpSUT1	541–1,151	GGTGACGACCCGAAGAGAATG	611	58.6
		GCCAGCAGGAAGTTGACAATG		

Table 2 Primer sequences and amplicon characteristics designed for qRT-PCR of 6 genes in peach 'XH6'

Name of gene	Sequence (5′–3′)	Size of amplified product (bp)	T_m (°C)
PpCWINV1	GTCACAGCAGCACAGGCAG	116	55.1
	CCAATACGAGTAACCCGAAT		
PpCINV1	TGAATGGTGAGCCTGAGA	138	55.1
	GGATAGGGTCGTGAAGAA		
PpCINV2	TATGATTGATAGACGGATGG	144	55.1
	CTAAGTCGGTTATTGATTGC		
PpSPS1	CATACCCCAAACACCACAA	174	55.1
	TATCAACAGGACCCCC		
PpSUS1	ATTGGAAATGGCGTTGAGT	113	53.3
	TTGCCCTTGTAGCAGTGAA		
PpSUT1	GGTGACGACCCGAAGAGAATG	185	58.6
	GTGATGGAAAGGAAGAAGCAG		
UBQ10	AAGGCTAAGATCCAAGACAAAGAG	146	61.0
	CCACGAAGACGAAGCACTAAG		

performed using the Duncan's multiple range test. All tests were done at $p \leq 0.05$ or 0.01.

Results

Carbohydrate analysis

Generally the carbohydrate content in fruits, leaves, and the phloem-enriched fraction varied throughout fruit development (Fig. 1a–c). The sucrose concentration in fruits remained at low levels (54.72 mg/g DW) during the early stages of development, before a spike in accumulation from 65 DAA to reach the highest concentration (490.78 mg/g DW) at around 85 DAA before gradually decreasing until harvest day. In addition, sucrose was still the predominant sugar in the ripened peach fruits, where it was about 3.6 and 10 times higher than that of glucose/fructose, and sorbitol, respectively. Although glucose and fructose were the predominant soluble carbohydrates in fruits during the early stages, their concentrations including that of sorbitol decreased significantly with the rise in sucrose concentration and fruit development until harvest. Interestingly, fruit glucose and fructose not only exhibited the same change patterns but also had comparable concentrations throughout fruit development. The sorbitol content in fruit was higher than sucrose content in the early stages of development but this dropped and remained at the lowest level among four kinds of sugar until fruit harvest (Fig. 1a). In contrast to sucrose whose concentration in fruit fluctuated, the concentration of sorbitol in both phloem-enriched fraction and leaves (Fig. 1b, c) was the highest among the four kinds sugars studied throughout the period of fruit development. However, like in fruits, the concentration and changes in glucose and fructose in the phloem-enriched fraction or leaves were similar.

Enzyme activity analysis

SPS remained relatively stable in activity as the fruit developed with an exception of a significant increase recorded between 65 and 85 DAA (Fig. 1d). SUS, AI, and CINV activity in fruits followed a similar pattern where they all decreased significantly during early stages of fruit development then remained at a relative constant rate until harvest day.

The activity of SUS in the phloem-enriched fraction revealed no major changes except for a significant drop from 65 to 85 DAA (Fig. 1e). The activity of SPS in the phloem-enriched fraction increased significantly as the fruit developed from 45 to 85 DAA and then declined afterwards. In the phloem-enriched fraction, a significant increase of AI activity was present from 45 to 65 DAA followed by a similarly significant decrease which held until harvest day. CINV activity in this tissue declined from 45 to 65 DAA and 85 to 105 DAA which was in contrast to the behavior observed between 65 and 85 DAA.

In leaves, the activities of SUS and CINV enzymes generally declined significantly with fruit development. Both AI and SPS activities presented similar change patterns. Activity of AI was the highest followed by CINV, SUS, and SPS in that order (Fig. 1f).

Cloning of genes related to sucrose-metabolizing enzymes

The fragments of each gene cloned from fruit-derived cDNA were finally linked together using BioXM software

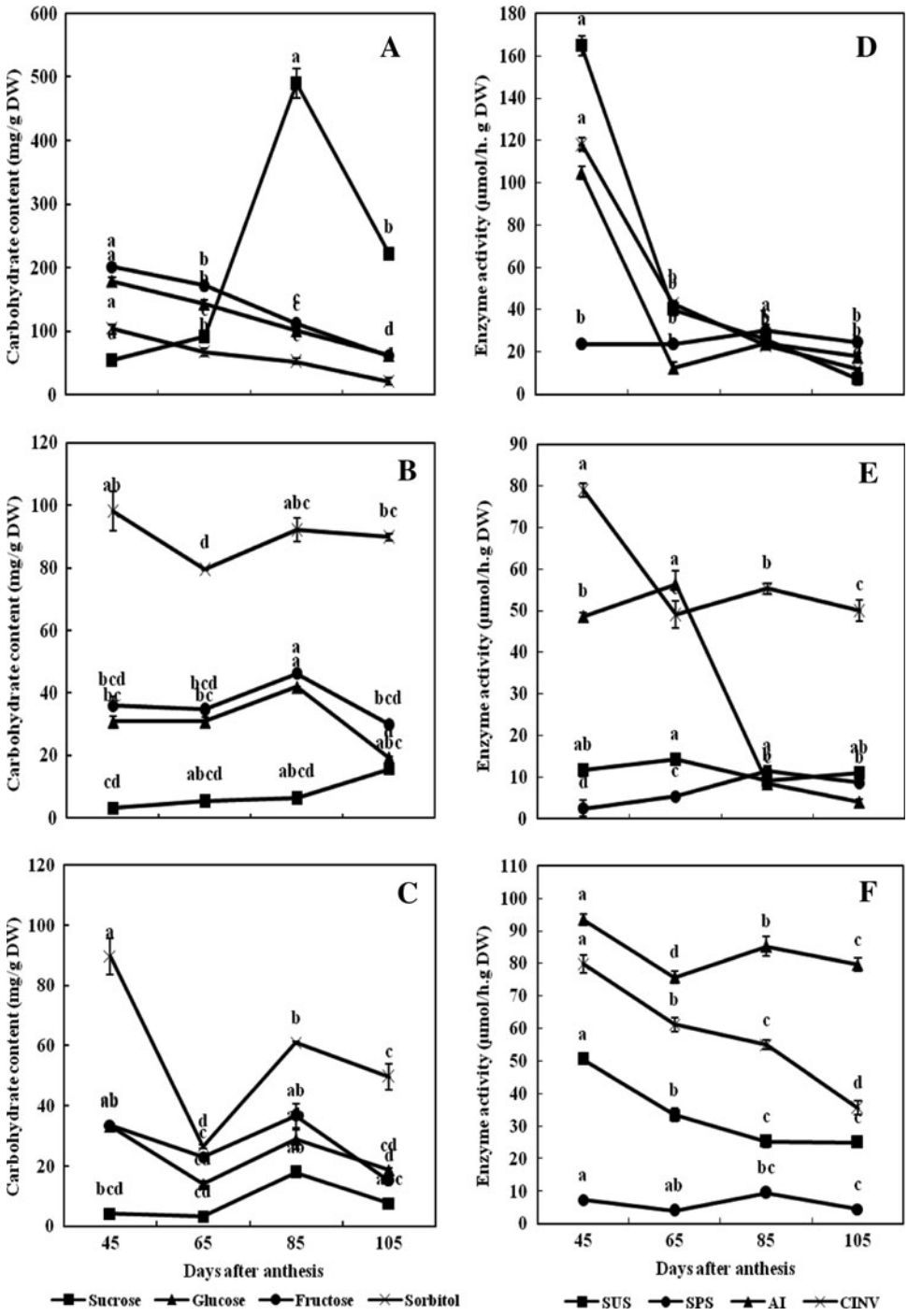

Fig. 1 Changes in content of the main carbohydrates and enzyme activity in three tissues of peach 'XH6' throughout fruit development. Changes in contents of the main carbohydrates (sucrose, glucose, fructose, and sorbitol) in peach fruit (**a**), phloem-enriched fraction (**b**), and leaf (**c**) at 45, 65, 85, 105 DAA. Changes in the enzyme activity (SUS, SPS, AI, CINV) in peach fruit (**d**), phloem-enriched fraction (**e**), and leaf (**f**) at 45, 65, 85, 105 DAA. The *vertical bar* represents ±SE of each mean ($n = 9$). *Bars with the same letters* are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test. *Error bars* smaller than symbol size are not visible

(Version 2.6). This approach resulted in isolation of 1,734, 1,674, 1,950, and 2,421 nucleotide cDNAs with full-length ORF, 1,734, 1,674, 1,887, and 2,421 nucleotides encoding 577, 557, 628, and 806 amino acids for *PpCWINV1*, *PpCINV1*, *PpCINV2*, and *PpSUS1* genes in peach. For *PpSPS1*, a 3,174 nucleotide cDNA fragment was amplified by standard RT-PCR. cDNA fragments with 189 and 417 nucleotides long were produced by 5′ RACE and 3′ RACE, respectively. After sequencing confirmation, a 3,587 nucleotide cDNA clone of *PpSPS1* whose ORF encodes 1,057 amino acids was isolated from 'XH6'. Only 611 nucleotides encoding 204 amino acids were obtained for *PpSUT1*. Their GenBank accession numbers, predicted molecular weight and theoretical pI are as shown in Table 3.

BLAST searching for *PpCWINV1* against the NCBI database revealed the highest identity (81 %) with a *CWINV* precursor of *Fragaria* × *ananassa* (Accession no.

Table 3 Basic information on genes cloned from fruits of peach 'XH6'

Name of genes	Size of amplified product (bp)	GenBank accession no.	ORF length	No. of amino acid	Molecular weight (Da)	Theoretical pI
PpCWINV1	1,734	JQ412748	1,734 (1–1,734)	577	64,768.4	8.77
PpCINV1	1,674	JQ412749	1,674 (1–1,674)	557	63,419.8	5.91
PpCINV2	1,950	JQ412750	1,887 (1–1,887)	628	70,968.9	5.56
PpSPS1	3,587	JQ412751	3,174 (190–3,363)	1,057	118,171.1	6.18
PpSUS1	2,421	JQ412752	2,421 (1–2,421)	806	92,605.0	5.95
PpSUT1	611	JQ412753	591 (19–609)	204	22,463.8	8.97

AF000521) and a 77 % identity with a cell wall apoplastic invertase in *Vitis vinifera* (Accession no. AY538262). *PpCINV1* shared 84 % homology with both neutral invertase (nINV1) of *Manihot esculenta* (Accession no. DQ138370.1) and neutral invertase 2 (NIN2) of *Hevea brasiliensis* (Accession no. GU573727.1). *PpCINV2* shared 99 and 81 % nucleotide sequence identity with putative neutral invertase of *Prunus persica* (Accession no. AM409095.1) and *Vitis vinifera*'s neutral invertase (NIN1) (Accession no. EU016365.1), respectively. *PpSPS1* had a similarity of 83 % with *SPS* in *Citrus unshiu* (Accession no. AB005023.1) while *PpSUS1* showed 82 % max identity with *SUS* in both *Alnus glutinosa* (Accession no. X92378.1) and in *Citrus unshiu* (Accession no. AB02 2092.1). Last, *PpSUT1* presented slightly low homologies of 74 and 72 % with *SUT* in a *Rosa hybrida* cultivar (Accession no. HQ403679.1) and in *Juglans regia* (Accession no. AY504969.1), respectively.

Expression analysis of genes related to sucrose-metabolizing enzymes

qRT-PCR results for gene expression show lack of apparent differences in the expression levels of these genes in the three tissues studied over different stages when both *UBQ10* and *TEF2* were used as reference genes during analysis. Only expression data of these six genes where *UBQ10* was used as the reference gene are therefore presented (Fig. 2).

The expression of *PpCWINV1* in fruit dropped significantly to about one-eighteenth of the initial level, being almost undetectable at 85 DAA while in leaves the expression remained constant up to 65 DAA followed by a significant increase, reaching the highest level (22.74-folds) at harvest. In the phloem-enriched fraction, *PpCWINV1* had no measurable increase from 45 to 65 DAA but a significant decrease in expression of this gene was subsequently observed followed by a relatively low but constant expression until harvest day.

PpCINV1 expression in fruits underwent a nearly 3.5-fold decline (from 45 to 65 DAA), followed by continuous and significant increase until harvest. Apart from a 15.21-fold increase in the transcript level of *PpCINV1* in the leaves between 65 and 85 DAA, no significant change was observed at the other fruit development stages. In the phloem-enriched fraction, a significant decrease in the transcript level of *PpCINV1* from 45 to 65 DAA was detected after which it kept this low level during the other fruit development stages. Generally, expression levels of *PpCINV2* in three tissues closely followed the pattern exhibited by *PpCINV1* throughout the fruit development with slight exception (Fig. 2).

Relative expression of *PpSUT1* in the leaves was characterized by continuously significant increases from 45 to 85 DAA followed by a significant decrease during the last 20 days before harvest. The levels of *PpSUT1* in young fruit were low but thereafter, this expression increased significantly throughout the remaining fruit development stages. Apart from a sharp increase at 65 DAA, no significant changes in the expression of *PpSUT1* were observed in all the other stages in the phloem-enriched fraction.

PpSPS1 was found highly expressed in maturing stages of fruit, while in leaves and the phloem-enriched fraction expression did not follow any particular rule during fruit development. The expression of *PpSUS1* gene in fruit was dramatically higher than that in leaves and the phloem-enriched fraction at the final fruit development stage (Fig. 2) which indicates that the *PpSUS1* gene is predominantly expressed in mature as compared to young fruit, as well as in leaf and phloem-enriched fraction.

Relationships between relative gene expression, enzyme activities, and carbohydrate concentration

In this study, the sucrose concentration in fruits displayed positive correlation to both SPS activity and to the relative expression levels of *PpSPS1* but at the same time had negative correlation to CINV activity in this tissue (Table 5). However, no correlation was observed between SPS activity and the expression of *PpSPS1* in fruit. Fructose, glucose, and sorbitol contents in fruit all have positive correlation with activities of SUS, AI, and CINV, but almost negatively correlated to all genes except

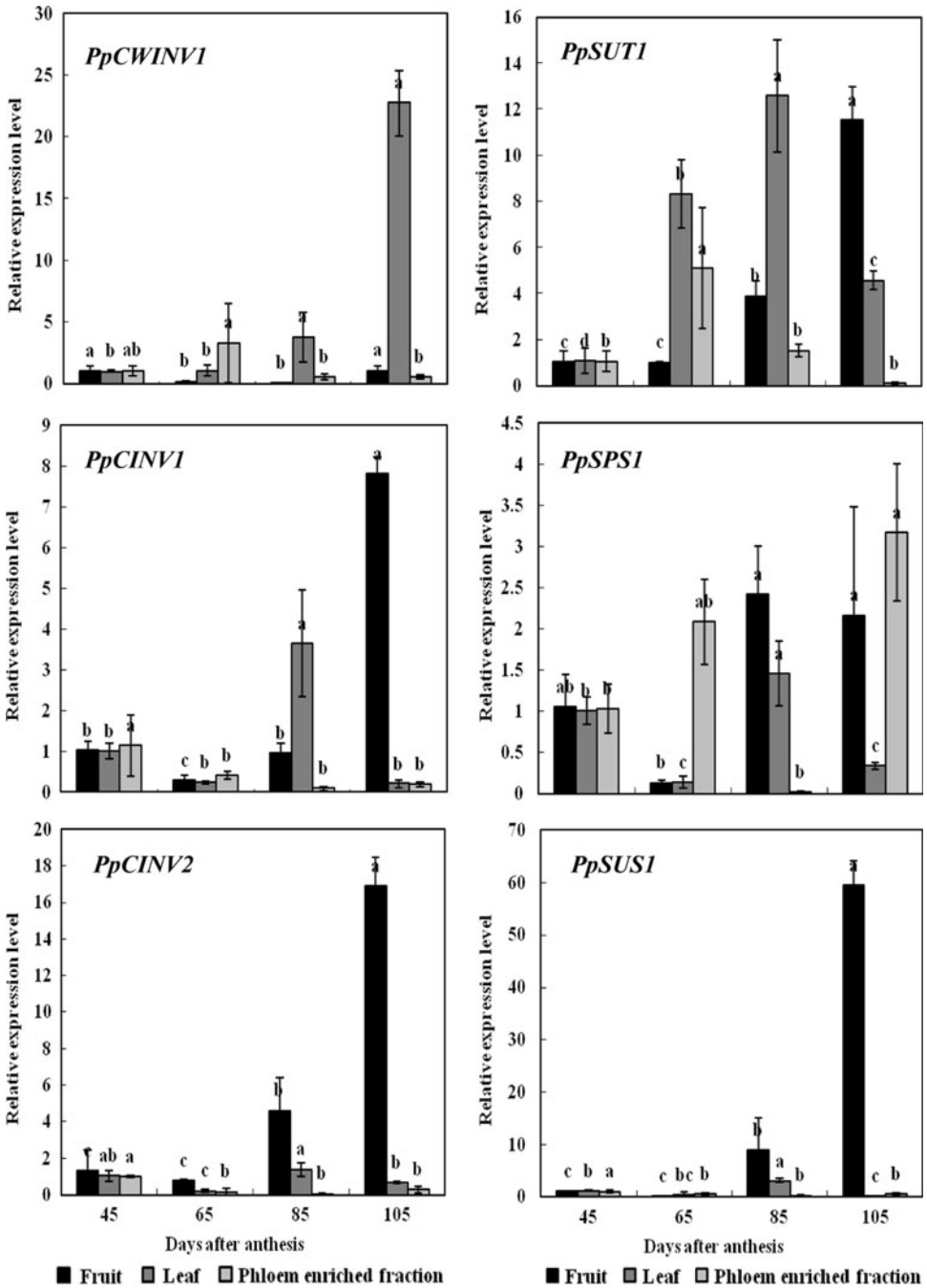

Fig. 2 Expression analysis of genes related to sucrose-metabolizing enzymes in three tissues of peach 'XH6' throughout the fruit development. The mean values of the results obtained, using three independent RNAs as a template, are shown. *UBQ10* was chosen as reference gene. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta CT}$ methods. The *y-axes* show the fold difference in each gene expression level relative to the amount found in each tissue sampled at 45 DAA, respectively. The *vertical bar* represents ±SE of each mean ($n = 9$). *Bars with the same letters* are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test. *Error bars* smaller than symbol size are not visible

PpCWINV1. In leaves, sucrose accumulation had positive correlation with the expression of all the genes except *PpCWINV1* and SUS activity (Table 5). In phloem-enriched fraction, carbohydrate concentrations have no correlation with enzyme activity except for AI, *PpSPS1* was the only gene which has significant positive correlation with the sucrose concentration in all three tissues. In the phloem-enriched fraction, *PpCINV* has negative impacts on the activity of SPS and positive correlation to CINV activity, respectively.

Discussion

The cultivar 'XH6' used in this study is medium-ripening and belongs to the group of juicy peaches. Carbohydrate analysis by HPLC indicates that this is a typical G/F ≈ 1 cultivar as classified by Wang et al. (2008), since the glucose and fructose contents in fruit were similar throughout fruit development (Fig. 1a). On the other hand, the slight decreasing in the sucrose concentration at the final fruit development stage is different from that observed

Table 4 Coefficients of correlation between expression level of genes related to sucrose metabolizing enzymes, enzyme activities (μmol. $h^{-1}g^{-1}$ DW) and carbohydrate content (mg/g DW) in the three tissues of 'XH6' peach at four development stages

Tissue	Carbohydrate content	Correlation coefficients									
		Enzyme activity				Relative expression level					
		SUS	SPS	AI	CINV	*PpCWINV1*	*PpCINV1*	*PpCINV2*	*PpSPS*	*PpSUT*	*PpSUS*
Fruit	Sucrose	-0.571	0.821**	-0.448	-0.626*	-0.466	0.058	0.400	0.655*	0.282	0.178
	Glucose	0.865**	-0.308	0.717**	0.901**	-0.028	-0.769**	-0.902**	-0.627*	-0.890**	-0.842**
	Fructose	0.811**	-0.298	0.644*	0.855**	-0.096	-0.804**	-0.926**	-0.673*	-0.912**	-0.869**
	Sorbitol	0.903**	-0.198	0.793**	0.926**	-0.027	-0.744**	-0.845**	-0.522	-0.856**	-0.811**
Leaf	Sucrose	-0.582*	-0.530	0.114	-0.321	0.087	0.834**	0.694 *	0.720**	0.727**	0.827**
	Glucose	0.487	0.210	0.880**	0.552	-0.340	0.564	0.763**	0.773**	-0.238	0.529
	Fructose	0.356	0.433	0.634*	0.652*	-0.738**	0.760**	0.665*	0.782**	0.219	0.770**
	Sorbitol	0.621*	0.177	0.961**	0.521	-0.175	0.282	0.627*	0.626*	-0.472	0.302
Phloem enriched fraction	Sucrose	-0.158	0.457	-0.710*	-0.495	-0.256	-0.532	-0.345	0.677*	-0.401	-0.362
	Glucose	-0.233	0.274	0.077	-0.035	0.027	-0.045	-0.270	-0.803**	0.246	-0.121
	Fructose	-0.505	0.449	-0.177	-0.106	-0.141	-0.268	-0.309	-0.757**	0.073	-0.329
	Sorbitol	-0.382	-0.097	-0.243	0.395	-0.566	0.460	0.524	-0.348	-0.652*	0.457

*,** Significant at $p \leq 0.05$ or 0.01, respectively

in earlier studies on other peach cultivars where it increased continuously until harvest date (Moing et al. 1998; Nonis et al. 2007; Lombardo et al. 2011). We deduce that this maybe a key characteristic of juicy peach cultivars, since this pattern of sucrose concentration was observed in three juicy peach cultivars tested (the data of juicy peach 'Hujingmilu' and 'Xiaohujing' cultivars are not presented).

In the present research, the change pattern and final concentration of sucrose, fructose, and glucose in the fruit over the fruit development period agree with the findings of a numbers of studies on peach (Wrolstad and Shallenberger 1981; Moing 2000; Wang et al. 2008). In young fruit, high hexose levels are needed to maintain the capacity of cells to divide (Morandi et al. 2008). As tissues develop, a certain level of sucrose is necessary so as to induce storage-associated cell differentiation (Wobus and Weber 1999). Concentration of sorbitol was relatively low and constant in fruits, a trend which agrees with earlier reports (Vizzotto et al. 1996; Lo Bianco and Rieger 2002; Shen et al. 2007). Once unloaded into sink tissues, sorbitol seems to be readily converted into fructose and glucose by sorbitol dehydrogenase and sorbitol oxidase, respectively (Morandi et al. 2008).

The pronounced similarity of glucose and fructose, not only in concentration at each sampling point but also in the changes in concentration throughout the fruit development in each tissue studied (Fig. 1a–c), indicates that as a part of one big system, different tissues are highly related in the aspect of carbohydrate metabolism. Photosynthesis and carbohydrate metabolism in source leaves are known to respond to sink activity (Azcon-Bieto 1983; Paul and Foyer 2001). As strong sinks during some stages of development, fruits have been also shown to alter the carbohydrate concentrations of source leaves (Nii 1997; Wibbe and Blanke 1995). Zhou and Quebedeaux (2003) also concluded that sink strength may regulate carbohydrate metabolism in the source leaves. In general, when sink demand increases, photosynthesis in leaves is stimulated and more carbon is partitioned to sucrose (Dunford 1998). This can be explained from the molecular viewpoint, as shown in Table 4, where both the glucose and fructose contents had significant correlation with the activities of the same enzymes and expression of the same genes in fruits, leaves, and phloem-enriched fraction, respectively. It therefore implies that almost equal amounts of glucose and fructose in each tissue maybe modulated by the same kinds of genes and enzymes in each tissue. On the other hand, fruits have completely contrasting trends in sucrose and sorbitol concentrations in comparison to the trends in both leaves and the phloem-enriched fraction. This further indicates that the metabolic mechanism or pathway maybe slightly different in each tissue since the function and

Table 5 Coefficients of correlation between expression level of genes related to sucrose metabolizing enzymes and enzyme activities (μmol. $h^{-1}g^{-1}$ DW) in the three tissues of 'XH6' peach at four development stages

Tissue	Correlation coefficients				
	Relative expression level	Enzyme activity			
		SUS	SPS	AI	CINV
Fruit	*PpCWINV1*	0.367	-0.452	0.481	0.329
	PpCINV1	-0.447	-0.164	-0.278	-0.484
	PpCINV2	-0.614*	0.180	-0.428	-0.666*
	PpSPS1	-0.357	0.531	-0.148	-0.422
	PpSUT1	-0.620*	0.038	-0.446	-0.660*
	PpSUS1	-0.547	-0.032	-0.377	-0.586*
Leaf	*PpCWINV1*	-0.551	0.774 **	-0.316	-0.844**
	PpCINV1	-0.257	-0.097	0.287	0.068
	PpCINV2	-0.008	-0.183	0.629*	0.121
	PpSPS1	0.094	-0.116	0.681*	0.304
	PpSUT1	-0.665*	-0.301	-0.356	-0.311
	PpSUS1	-0.152	-0.070	0.411	0.177
Phloem enriched fraction	*PpCWINV1*	0.685*	-0.298	0.543	-0.314
	PpCINV1	0.432	-0.876**	0.664*	0.743**
	PpCINV2	0.020	-0.738**	0.442	0.689*
	PpSPS1	0.284	-0.080	-0.101	-0.196
	PpSUT1	0.680*	-0.247	0.588*	-0.219
	PpSUS1	0.446	-0.700*	0.507	0.802**

*,** Significant at $p \leq 0.05$ or 0.01, respectively

nature of fruit tissues (reproductive growth) are different from those of leaves and phloem tissues (vegetative growth). Furthermore, fruit is predominantly a non-photosynthetic organ but leaves and to some extent the phloem are the most important photosynthetic tissues in green plants. Despite these deductions, detailed biochemical and molecular mechanisms behind this aspect are still unknown and need to be studied further.

It has been identified that sink regulation of carbohydrate metabolism involves changes in metabolite levels, the activities of key enzymes involved in sucrose biosynthesis, and the transcription of some specific photosynthetic and carbohydrate metabolism genes (Krapp and Stitt 1995). In this study the sucrose concentration in fruits displayed significantly positive correlation to both SPS activity and the relative expression levels of *PpSPS1* (Table 4). Such responses by SPS activity to the presence/absence of its substrate suggest that sucrose concentration in peach fruits maybe mainly be modulated by SPS activity. This is consistent with previous findings where SPS activity has been suggested to determine the rate of sucrose synthesis and the level of sucrose accumulation in the later stages of fruit development in various plant species such as tomato (Dali et al. 1992; Miron and Schaffer 1991), muskmelon (Hubbard et al. 1989; Lingle and Dunlap 1987), and banana [*Musa acuminate* L.] (Hubbard et al. 1990). A model has been proposed with respect to this aspect of sink regulation whereby higher sink demand removes sucrose in the source and favors sucrose-phosphate synthesis catalyzed by SPS activity and the hydrolysis of sucrose-phosphate as catalyzed by sucrose phosphatase. The resulting increase in phosphate release in the source cytoplasm leads to increased efflux of triose phosphate from the chloroplast via the triose-phosphate transporter. Glucose-6-phosphate, which is formed from triose phosphate, stimulates the activity of SPS, thus providing sucrose to meet the increased sink demand (Dunford 1998). On the other hand, it is observed that the sucrose accumulation in the fruit had negative correlation with the activity of CINV enzyme in this study (Table 4). The CINV are found in the cytosol and are considered 'maintenance' enzymes involved in sucrose degradation when the activities of acid invertase and SUS are low. Since invertase, in the presence of uridine 5'-diphosphate (UDP), can convert sucrose into glucose and fructose in an irreversible manner, it therefore implies that the higher sucrose content the lower CINV activity in fruit.

Noticeably, *PpSPS1* is the only gene which was positively correlated with sucrose contents in all three tissues in this study (Table 4). On one hand, this indicates that *PpSPS1* is likely to be a key gene related to sucrose synthesis or metabolism; while on the other hand, the sucrose content in each tissue may also have affected the expression of this gene since gene expression is widely known to respond to sugar content. As deduced by Huber and Huber (1996), hexose sugars, or other related metabolites, might

be involved in the control of expression of *SPS* as well as other genes.

Although sucrose had significant correlation with both SPS activity and *PpSPS1* expression at same time, no significant correlation was observed between SPS activity and the expression of *PpSPS1* in fruit (Table 5), thus indicating that the activity of an enzyme may not have a strong and direct correlation with the expression of its related gene, or expression of *PpSPS1* was affected by many factors.

Despite the significant positive correlation observed between expression of *PpSPS1* and sucrose content in the leaves, there was no correlation between the sucrose content and SPS activity unlike that observed in fruits (Table 4). This can be attributed to the possibility that sucrose accumulation in the leaves may mainly be due to photosynthetic activity rather than the conversion from the fructose as happens in fruits. Photosynthetic activity in the young peach leaves is not very intense (Zhou and Quebedeaux 2003), hence the content of sucrose is not very high. In addition, the demand for sucrose in young fruit also is not very strong. Due to these aspects, limited amounts of sucrose are exported to the fruit at this stage with only small amounts being needed for vegetative growth of the leaves. As the three tissues develop, the leaves gradually become more photosynthetically active and start producing far more carbohydrates than they require and thus export these assimilates as sucrose to photosynthetically less active or inactive tissues, such as young leaves, roots, or fruits through phloem. We postulate that this may to some degree lead to the low levels of sucrose recorded in the leaves during the active period of fruit development which is in agreement with the observation that after flowering, carbohydrates are mainly directed into the developing fruits or tubers (Dantas et al. 2005). This further confirms the statement that the variation in the sugar level in leaves is a function of a balance of sugar synthesis, translocation, and utilization (Zhou and Quebedeaux 2003).

Multiple sequence alignment indicates that *PpCWINV1*, *PpCINV1*, *PpCINV2*, *PpSUS1*, *PpSPS1*, and *PpSUT1* share a high degree of nucleotide identity with their homologs in species in the Rosaceae family or other species, like strawberry, grape, and soybean, thus confirming that these genes do exist in peach. The results also reveal that using corresponding genes in the TAIR database as a querying probe to search against peach genome database of GDR was an efficient technique to clone new genes. The isolation of the six genes makes a significant contribution for a better understanding of their expression in different tissues.

In conclusion, we find that even though a strict correlation between sucrose metabolism and the six genes studied could not be established, these genes could be seen to be involved in regulating crucial steps in peach development or at least play a supportive role to other sucrose metabolism enzymes by providing substrates to the cells. The exact explanation for the mechanisms underlying diverse expression of some genes in different tissues still needs further studies and elucidation.

Authors' contributions Z-J. Shen and M-L.Yu conceived and designed the study; C-H. Zhang performed the experiments; Y-P. Zhang, N-K. Korir and J. Han did data analysis; C-H. Zhang wrote the paper; R-J. Ma, N-K. Korir, and M-L.Yu revised the paper. All authors read and approved the final manuscript.

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