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Characterisation of genes encoding key enzymes involved in sugar metabolism of apple fruit in controlled atmosphere storage

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ABSTRACT

Sugars are essential contributors to fruit flavour. Controlled atmosphere (CA) storage has been proved to be beneficial for maintaining harvested fruit quality. To explore regulatory mechanism of sugar metabolism in fruit stored in CA condition, we cloned several genes, encoding key enzymes, involved in sugar metabolism in apple fruit, and analyzed sugar contents, along with gene expression and enzyme activities in fruits stored in air and CA. The results indicated that CA could maintain higher contents of sugars, including sucrose, fructose and glucose. Expression levels of key genes, such as sucrose synthase (*SS*), sucrose phosphate synthase (*SPS*), fructokinase (*FK*) and hexokinase (*HK*), were shown to be correlated with the corresponding enzyme activities. We found that activities of neutral invertase (NI), vacuolar invertase (VI), FK and HK were inhibited, but SPS activity was promoted in apple fruit stored in CA, suggesting that CA storage could enhance sucrose synthesis and delay hydrolysis of sucrose and hexose. These findings provided molecular evidence to explain why higher sugar levels in harvested fruit are maintained under CA storage.

1. Introduction

Carbohydrates not only provide energy for fruit development, but also contribute to the edible quality of fruit (Borsanie et al., 2009). Soluble sugars are derived from photosynthesis in leaves undergoing a series of physiological steps and accumulate in fruit (Ruan, Jin, Yang, Li, & Boyer, 2010). The accumulation of soluble sugars during fruit development largely determines their sweetness at harvest (Li, Feng, & Cheng, 2012). After harvest, sugar catabolism takes place in fruit without a supply from photosynthesis. Changes in composition and contents of soluble sugars lead to easily perceivable alterations in fruit flavour, which is an important, non-visual attribute for customers (Awad & de Jager, 2002; Giovannoni, 2004). Therefore, a study on sugar metabolism in harvested fruit is beneficial for keeping fruit quality in postharvest periods.

Futile cycles of sucrose/hexose interchange were regarded as the system governing fruit sugar contents and composition (Nguyen-Quoc & Foyer, 2001). With regards to harvested fruit, degradation and synthesis of sucrose in the cytosol and in the vacuole are predominant for sugar metabolism and accumulation. Sucrose in the cytosol is converted to fructose and glucose by neutral invertase (NI, EC 3.2.1.26), or to fructose and UDP-glucose (UDPG) by sucrose synthase (SS, EC 2.4.1.13). The fructose and glucose are then phosphorylated to fructose 6-phosphate (F6P) and glucose 6-phosphate (G6P) by fructokinase (FK, EC 2.7.1.4) and hexokinase (HK, EC 2.7.1.1). The F6P enters glycolysis and the TCA cycle to generate energy and intermediates for other processes. Sucrose can also be re-synthesized via either SS or sucrose phosphate synthase (SPS, EC2.4.1.14) (Li et al., 2012). Although SS can either cleave sucrose or catalyze the reverse synthetic reaction, it is widely believed to act in the cleavage direction in postharvest fruit (Mao, Que, & Wang, 2006). Sucrose in the cytosol is transferred to the vacuole, where it is hydrolyzed by vacuolar invertase (VI). Hexoses produced in the vacuole can also be transported to the cytosol for subsequent metabolism or sucrose re-synthesis (Nguyen-Quoc & Foyer, 2001). Sugar metabolism in harvested fruit is affected by various external factors. For instance, temperature can significantly influence sugar contents and enzyme activities of the sugarcane stem (Mao et al., 2006). Some reports indicate that fruit ripening was delayed and sugar composition was changed under anaerobic condition (Lara et al., 2011).

Apples (*Malus domestica* Borkh.) that belongs to the Rosaceae family are an important commercial fruit in the world, but the apple fruit can easily lose internal and external quality due to metabolic degradation, respiration and synthesis processes during inappropriate storage (Veberic, Schmitzer, Petkovsek, & Stampar, 2010). To extend the postharvest life of the apple fruit, CA storage has been successfully applied, because of its positive effect on maintaining fruit quality (Tian, Xu, Jiang, & Gong, 2002; Wang,

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Tian, & Xu, 2005). The main rationale of CA storage is to inhibit respiration and delay ripening of the fruit (Jiang, Tian, & Xu, 2002; Tian, Li, & Xu, 2005). However, there is little information regarding sugar metabolism in harvested fruit during storage periods, particularly the biochemical and molecular basis for regulating sugar metabolism still remains unclear. In this study, we evaluated the changes in contents of sugars in apple fruit stored in air and CA condition, then cloned several genes encoding key enzymes involved in sugar metabolism by combining the methods of RT-PCR, RACE with *in silico* cloning. Moreover, transcript levels of the genes and corresponding enzymes activities were also analyzed during long-term CA storage.

2. Materials and methods

2.1. Fruit and storage condition

Apple (*M. domestica* Borkh. cv. Fuji) fruit was harvested approximately 150 days after bloom, from an orchard in Beijing, China, and were immediately transported to the Institute of Botany, Chinese Academy of Sciences. Fruits were manually sorted for uniformity of colour, size and firmness, then placed in a plastic tray and transferred to a controlled atmosphere (CA) cabinet (Fruit s.r.l. Control, type FC-701, Milano, Italy) with 2% O_2 + 1% CO_2 at 0 °C. The same trays, wrapped in polyethylene film to maintain about 95% relative humidity, were stored in air at 0 °C as the control. About 30 fruits from each treatment were used for firmness, solid soluble content (SSC) measurements. Flesh tissue was also collected from the different sample fruit, immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Determination of firmness and SSC

Flesh firmness was determined on opposite peeled cheeks of the fruit using a hand-held fruit firmness tester (FT-327, Italy), equipped with a cylindrical plunger, 8 mm in diameter. SSC was determined using an Abbe refractometer (10481 S/N, USA).

2.3. Soluble sugars measurement

Soluble sugars were extracted and determined according to Liang et al. (2011). Frozen tissue (3 g) was homogenized with 10 ml of ultrapure water and then centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was passed through a SEP-C18 cartridge (Supelclean ENVI C18 SPE) and filtered over 0.45-μm membrane filters for high-performance liquid chromatography (HPLC) analysis. The separation of the sugars was carried out using a Transgenomic CARB Sep Coregel 87C column (300 × 7.8 mm), with a guard column cartridge (Transgenomic CARB Sep Coregel 87C cartridge), at 85 °C. The mobile phase was double-distilled water, with a flow rate of 0.6 ml min^{-1}, and a refractive index detector was used to monitor the eluted carbohydrates. The Chromeleon chromatography data system was used to integrate peak areas, according to external standard solution calibrations (sucrose, glucose, fructose and sorbitol were purchase from Sigma Chemical Co.).

2.4. Cloning of VI, SS and SPS gene by RT-PCR and RACE

Total RNA was extracted from apple fruit using RNAplant Plus reagent (Tiangen, Beijing). First-strand cDNA was synthesized with the *TransScript* First-Strand cDNA Synthesis kit (TransGen, Beijing). To obtain the partial sequences of *VI*, *SS* and *SPS* gene, degenerate primers were designed based on the conserved amino acid sequences (Table 1). PCR products were cloned into pMD19-T vector (TaKaRa) and sequenced. RACE was performed to amplify the 3′ ends of the partial sequence (3′-Full RACE Core Set Kit, TaKaRa). Reverse transcription was carried out using an adaptor primer provided by the kit. PCR was then performed using the outer primer provided by the kit and a gene-specific primer (GSP1) designed according to the sequence of the cloned conserved domain (Table 1). In order to improve specificity, a further round of PCR was performed with the inner primer provided by the kit and a nested gene-specific primer (GSP2) (Table 1). The resulting PCR products were cloned and sequenced. Full-length coding sequences for *VI*, *SS* and *SPS* genes were obtained by RT-PCR. The degenerate primers were designed based on the conserved amino acid sequences as the forward primers, and the specific primers were designed according to the obtained sequences of the reverse primers (Table 1). The resulting PCR products were cloned and sequenced. The nucleotide sequence of the full-length cDNA was analyzed by using a BLAST search of the GenBank Database at NCBI.

2.5. In silico cloning of NI, FK and HK gene and their experimental verification by RT-PCR

Prunus persica NI coding sequences (GenBank accession No. JQ412750), *Eriobotrya japonica FK* coding sequences (JF414124) and *E. japonica HK* coding sequences (JF414121) were respectively used as queries to perform BLAST searches in *M. domestica* EST database to retrieve homologous sequences. Apple ESTs with high identity were assembled into a contig using CAP3 program, respectively (http://pbil.univ-lyon1.fr/cap3.php). The putative full-length cDNA of apple *NI*, *FK* and *HK* were analyzed by the ORF Finder procedure of NCBI, and the specific primers (Table 2) were designed for RT-PCR experiments to test the presence of these genes in *M. domestica* Borkh. cv. Fuji. The resulting PCR products were cloned and sequenced.

Table 1
Primer sequences used for cloning of *VI*, *SS* and *SPS* gene.

Gene	Primer sequence (5′–3′)
VI partial CDS	GTTT(C/T)ACAAGG(C/G)ATGGTATCACCTGATGCATAGTA(C/T)CT(C/T)CCATAGTC
SS partial CDS	CAAGGAATGGGTTTTGAGC(A/G)AGGCAAAAGCTTC(A/G)TA(A/G)AA(A/T)GCAGGCT
SPS partial CDS	GGTCACTCACTTGGACGAGATAACCCCA(A/G)CG(A/G)TATTCAATGTGTGA
VI GSP1	ACTGATTTCCGTGACCCCACAAC
VI GSP2	GCTATAAACGGGTCCAAGGGTT
SS GSP1	ACCAAGAGATTGCAGGAACGAAG
SS GSP2	TATTCTCAATGGCAAGGCTCGAC
SPS GSP1	CCACAAGCAGTCTGATGTTCCTG
SPS GSP2	GGTGTTTGTGAAAAGGCTGGTTC
VI full-length CDS	ATGGACT(A)CCAACAACACTTCCTACTTAAATCTGGTCTAATGGGAAAGGAT
SS full-length CDS	ATGGC(A)GAATCGCCC(G)TAAGTTCACTTTAATGTGCATCGTCGATGGCTTC
SPS full-length CDS	ATGGCGGGAAAT(C)GACTGGGTGAACCTACCGCTTGAGAAACCCTAGTTT

Table 2
Primer sequences used for experimental verification of *NI*, *FK* and *HK* gene.

Gene	Primer sequence (5′–3′)
NI full-length CDS	ATGAGTACTAGCAATTGTATTGGCTAGACCGCAACTTGAGATTTTG
FK full-length CDS	ATGGCTCTTCATTCTACTGCTTTCTCTAGGCTACAGACTTGAGAAGGAC
HK full-length CDS	ATGGGGAAGAAGGCGGTGATAATCTCAGGATTCATCGATTCCTGGGTA

Table 3
Primer sequences used for real-time PCR.

Gene	Primer sequence (5′–3′)
NI	GAAAAGGAGGCCTGGAGGTTCCGGATCAGTAGCAGCAAGAG
VI	CGGCCAAATCGAGTCCATATGGCAGTTCGTTGCCAAGAAA
SS	AAGTTCACTCGCGCCTTAAGCAGGAGGGCAACGAGTTCGT
SPS	CTCCTGACCCTCCAATTTGGCCTGGCAAGGGCAAGTATCA
FK	ATTGGCTGAAGCACCTGCATTGAGCCACCAAGACGAGCTA
HK	GTTGTCGCTGCCGTGATTTTGCAGACCATGCCATTTTGGT
Actin	CTCCCAGGGCTGTGTTTCCTAGGCATCCTTCTGACCCATACC

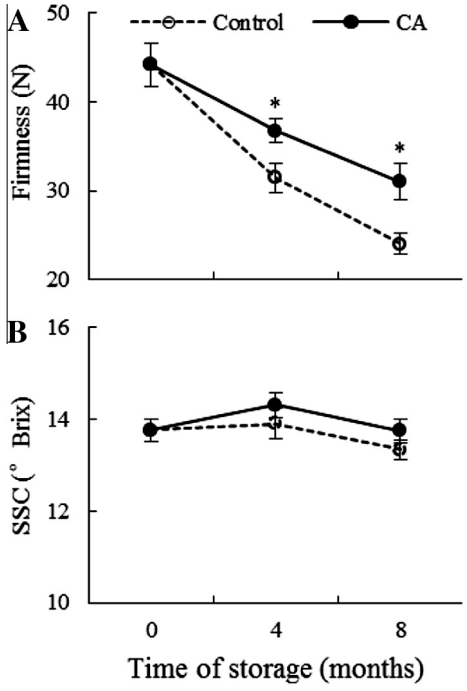

Fig. 1. Change in firmness (A) and soluble solids content (SSC) (B) of apple fruit stored in air and controlled atmosphere at 0 °C. Bars represent standard deviations of the means. Asterisks indicate significant differences between controls and CA-treated fruit according to the Student's *t*-test ($P < 0.05$).

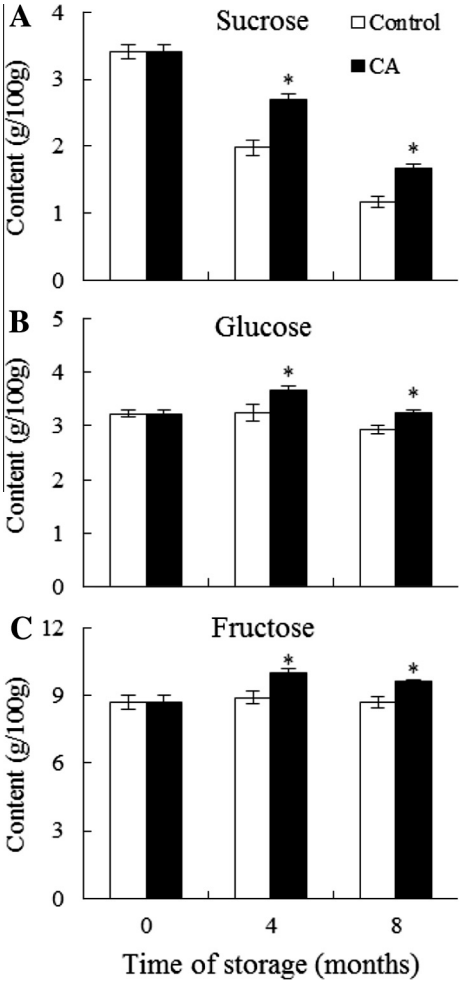

Fig. 2. Change in contents of sucrose (A), glucose (B) and fructose (C) of apple fruit stored in air and controlled atmosphere at 0 °C. Bars represent standard deviations of the means. Asterisks indicate significant differences between controls and CA-treated fruit according to the Student's *t*-test ($P < 0.05$).

2.6. Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) was performed with a Mx3000P system (Stratagene) using the SYBR green detection protocol (TaKaRa). Primers (Table 3) were designed using PrimerExpress 3.0, and primer specificity was determined by melt curve analysis and agarose gel electrophoresis of the products. *Actin* (AB638619) mRNA was used as an internal control, and relative amounts of mRMA were calculated using the comparative threshold cycle method. Each RNA sample was run in triplicate and repeated in three independent sets of treatments for a total of nine replicates per gene per sample.

2.7. Enzyme assays

Approximately 1 g of flesh frozen tissue was homogenized in 3 ml of 50 mM HEPES–NaOH buffer (pH 7.5) containing 5 mM $MgC1_2$, 1 mM EDTA, 2.5 mM DTT, 10 mM ascorbic acid and 5% PVPP. After centrifugation at 12,000 rpm for 30 min, supernatants were dialyzed for about 16 h against 25 mM HEPES–NaOH (pH 7.5) and 0.5 mM EDTA and used as the crude enzyme extract.

Neutral invertase (NI): the reaction mixture was 50 mM HEPES–NaOH (pH 7.5) and 100 mM sucrose. The mixture was incubated for 30 min at 37 °C. The reaction was stopped and the reducing sugars were measured using dinitrosalicylic acid (Zrenner, Salanoubat, Willmitzer, & Sonnewald, 1995).

Vacuolar invertase (VI): the reaction mixture was 100 mM sodium acetate (pH 4.8) and 100 mM sucrose and otherwise was as for neutral invertase (Zrenner et al., 1995).

Sucrose synthase (SS): SS activity was measured in the direction of sucrose degradation. The reaction mixture was 20 mM HEPES–NaOH (pH 7.8), 100 mM sucrose and 5 mM UDP. Incubation was carried out at 25 °C for 30 min, and stopped at 95 °C for 5 min. The determination of UDPG was performed in 200 mM glycine

(pH 8.9), 5 mM $MgC1_2$ and 0.6 mM NAD, the reaction was started with 0.05 U UDPG dehydrogenase (Zrenner et al., 1995).

Sucrose phosphate synthase (SPS): The reaction mixture was 50 mM HEPES–NaOH (pH 7.4), 5 mM $MgC1_2$, 1 mM EDTA, 2 mM F6P, 10 mM G6P and 3 mM UDPG. Incubation was carried out at 25 °C for 30 min, and stopped at 95 °C for 5 min. UDP was measured in 50 mM HEPES–NaOH (pH 7.0), 5 mM $MgC1_2$, 0.3 mM NADH, 0.8 mM phosphoenolpyruvate and 14 U of lactate dehydrogenase, starting the reaction with 4 U of pyruvate kinase (Stitt, Wilke, Feil, & Heldt, 1988).

Hexokinase (HK): The reaction mixture was 100 mM Tris–HCl (pH 8.0), 5 mM $MgC1_2$, 0.5 mM NAD, 10 mM glucose and 2U G6PDH, the reaction was started with 2 mM ATP.

Fructokinase (FK): The reaction mixture was as for hexokinase, with 2 U phosphoglucoisomerase and 10 mM fructose instead of glucose (Renz & Stitt, 1993).

The chemicals including UDP, UDPG, UDPG dehydrogenase, NAD, NADH, F6P, G6P, phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase, G6PDH, ATP, phosphoglucoisomerase were purchased from Sigma–Aldrich (Shanghai) Trading Co., Ltd.

2.8. Statistical analysis

Statistical analysis was performed with SPSS 13.0. Data were compared in a Student's *t*-test. Differences at $P < 0.05$ were considered as significant.

3. Results

3.1. Quality attributes of 'Fuji' apple

During storage, the firmness of the apple fruit decreased rapidly under normal air conditions, while the softening was impaired in CA conditions (Fig. 1A). On the other hand, the soluble solid content (SSC) was not modified by CA treatment and changed slightly during storage time (Fig. 1B). Regarding the sugar contents, the sucrose level that declined under normal condition was also decreased in CA, but to a lesser extent in fruit in comparison with the control (Fig. 2A). Neither the glucose nor fructose level was obviously altered during storage in air. Nevertheless, both contents in CA were higher compared to the control fruit (Fig. 2B and C).

3.2. cDNA cloning

Vacuolar invertase, sucrose synthase and sucrose phosphate synthase genes were cloned from apple fruit using RT-PCR and RACE and designated as *VI*, *SS* and *SPS* (GenBank accession Nos. JX459594, JX459593 and JX459592, respectively). *VI*, *SS* and *SPS* showed 90%, 98% and 98% amino acid sequence identity with those of sequences from *Pyrus pyrifolia* (BAG30919, BAB20799 and BAG30918, respectively).

Moreover, we obtained *NI*, *FK* and *HK* EST contigs by *in silico* cloning, ORF analysis based on ORF finder program of NCBI showed

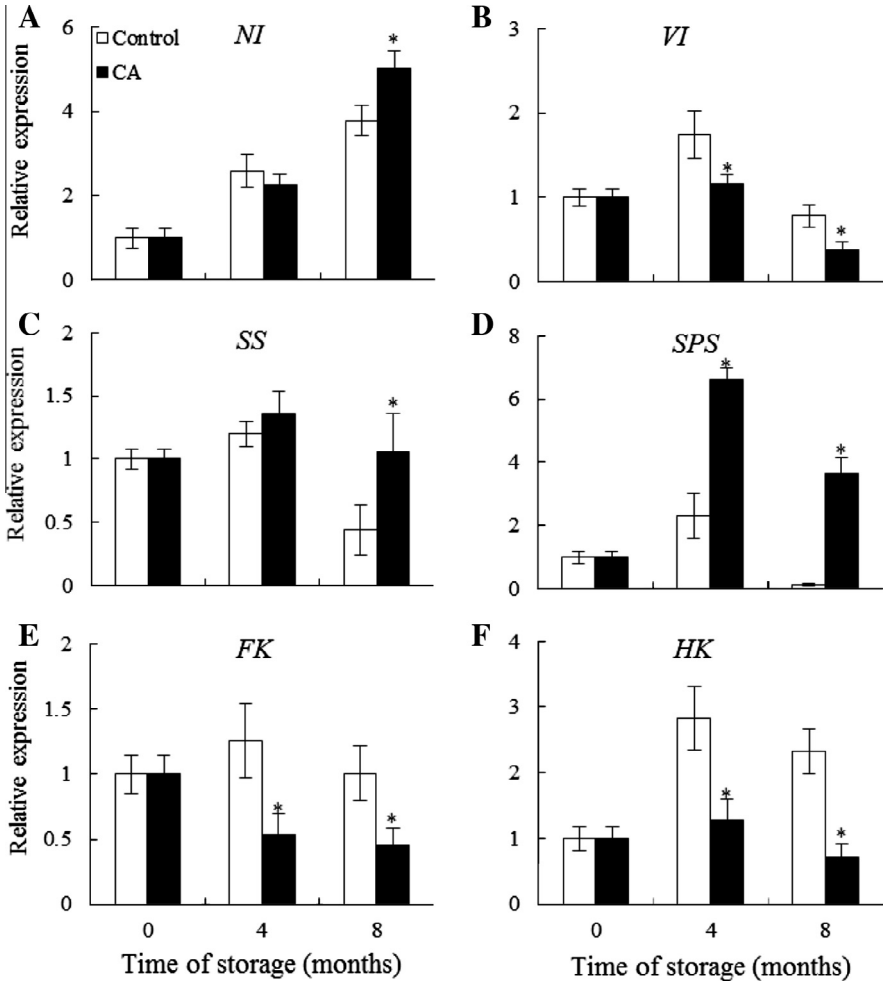

Fig. 3. Relative mRNA expression of genes encoding enzymes involved in apple fruit sugar metabolism stored in air and controlled atmosphere at 0 °C. *NI*, neutral invertase; *VI*, vacuolar invertase; *SS*, sucrose synthase; *SPS*, sucrose phosphate synthase; *FK*, fructokinase; *HK*, hexokinase. Bars represent standard deviations of the means. Asterisks indicate significant differences between controls and CA-treated fruit according to the Student's *t*-test ($P < 0.05$).

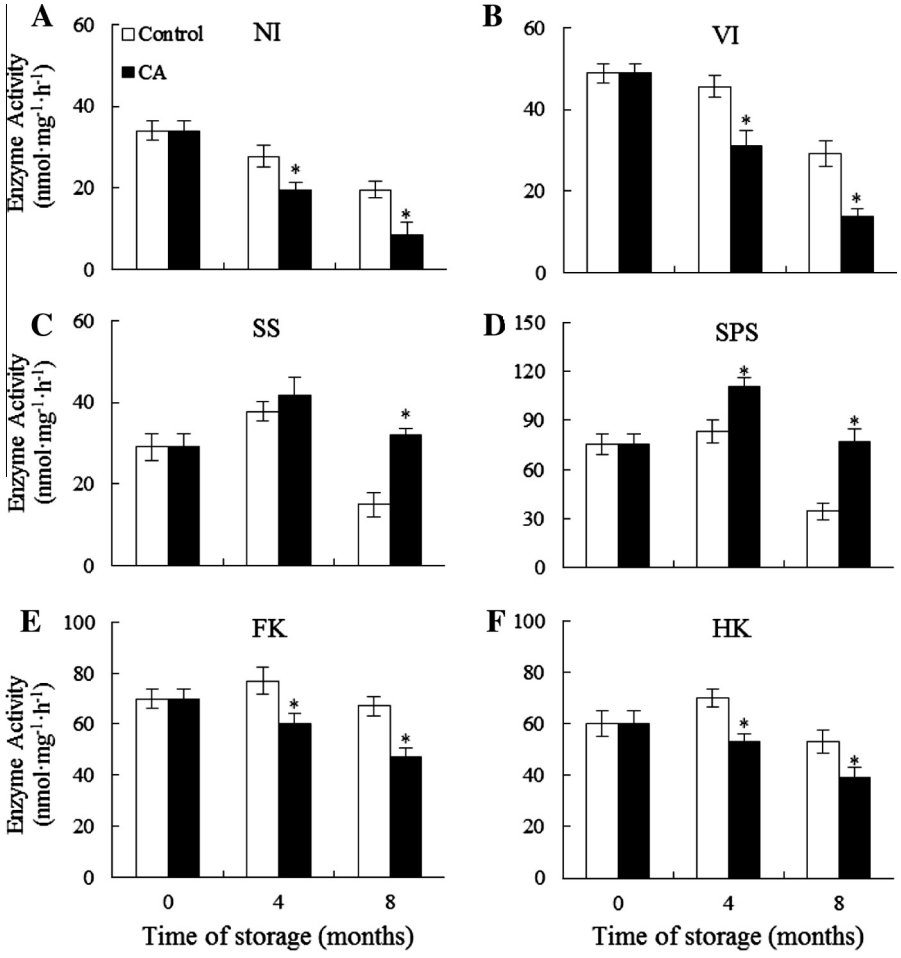

Fig. 4. Activities of key enzymes involved in apple fruit sugar metabolism stored in air and controlled atmosphere at 0 °C. Bars represent standard deviations of the means. Asterisks indicate significant differences between controls and CA-treated fruit according to the Student's *t*-test ($P < 0.05$).

NI, *FK* and *HK* EST contigs contained a 2049, 1051 and 1494 bp ORF, respectively. According to the predicted sequences, three pairs of specific primers were designed and PCR amplifications were performed from apple fruit. The cDNA of the *NI*, *FK* and *HK* genes were sequenced and consistent with the sequences obtained through *in silico* cloning. *NI* had a high amino acid sequence identity, showing 91% with the sequence from *Prunus persica* (AFI57906); *FK* and *HK* showed a 96% and a 95% sequence identity with *E. japonica* (ADZ96381 and ADZ96378, respectively). The sequences of *NI*, *FK* and *HK* have also been submitted to Genbank (accession Nos. JX459591, JX459589 and JX678841, respectively).

3.3. Expression of genes encoding key enzymes during fruit storage

Expression levels of cloned genes in fruits stored in air and CA were determined through QRT-PCR. Except for a continuous increase in the transcript level observed for *NI* during storage under air condition, expression of the other genes showed an increase after 4 months of storage, and then dropped to different extent (Fig. 3). *VI* transcript abundance was much lower in CA-treated fruit (Fig. 3B), whereas both *NI* and *SS* expression in treated fruit had an obvious rise after 8 months of storage compared to the control (Fig. 3A and C). In addition, CA notably affected *SPS* expression, with a 3- and 26-fold increase in treated fruit, respectively, with respect to the fruit of the same postharvest age (Fig. 3D). With regards to *FK* and *HK* transcript levels, a decrease was observed during storage after CA treatment, compared to the untreated fruit (Fig. 3E and F).

3.4. Activities of key enzymes in sugar metabolism during fruit storage

The activity of SS, SPS, FK and HK in control fruit increased initially and then decreased, which correlated with their transcript levels. Activity changes in NI and VI exhibited a similar pattern of decreasing gradually during the storage, whereas both invertase activities in CA-treated fruit decreased to a much lower level as compared to the control (Fig. 4A and B). Activities of SS and SPS were also modified in CA-treated fruit, with the levels of both being 2 times higher than that in control, after 8 months of storage (Fig. 4C and D). Moreover, the activity of FK and HK in fruit stored in CA had a constant decrease, and remained at a relatively lower level throughout the storage time, compared to the control (Fig. 4E and F).

4. Discussion

The CA storage condition is beneficial for maintaining higher fruit firmness and sugar contents in 'Fuji' apple, compared to storage in air (Fig. 1). With regards to the individual sugars in apple fruit, the content of fructose was higher than that of sucrose or glucose after harvest (Fig. 2). Moreover, sucrose declined sharply over the entire storage time (Fig. 2A). It is supposed that sucrose degradation, catalyzed by NI, VI and SS, became predominant to satisfy the requirement for respiration and intermediates formation. Apple fruit kept in CA conditions showed down-regulated activities of NI and VI (Fig. 4A and B), indicating sucrose utilization, via SS or invertase, was modified (Fig. 5). Nevertheless, SS activity match-

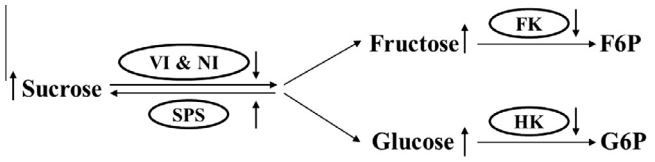

Fig. 5. Simplified scheme of sugar metabolism regulated by controlled atmosphere during postharvest storage of apple fruit. Key enzymes and main sugars that are increased or decreased under CA condition compared to fruit of the same postharvest age under aerobic atmosphere at 0 °C are indicated by ↑ and ↓, respectively. F6P, fructose-6-phosphate; G6P, glucose-6-phosphate.

ing expression level of the relative gene was promoted by CA storage (Figs. 3C and 4C). Previous studies indicated that the transcript level and activity of SS were induced by anoxic treatment in many species (Geigenberger, 2003), while VI gene expression was strongly repressed by low oxygen concentrations (Zeng, Wu, Avigne, & Koch, 1999). It is likely that the sucrose degradation pathway through SS would be favourable for invertase pathway under anoxia (Bologa, Fernie, Leisse, Loureiro, & Geigenberger, 2003), but we found that the expression level of the *NI* gene was not correlated with the change in NI activity during CA storage (Figs. 3A and 4A). Ruan et al. (2010) considered that invertase might be regulated at the post-translational levels. Nascimento, Cordenunsi, Lajolo, and Alcocer (1997) reported that sucrose accumulation was accompanied by increased activity and mRNA level of SPS in banana fruit. In our experiment, activity and expression of SPS were enhanced by CA treatment (Figs. 3D and 4D). A similar result was reported by Lara et al. (2011), who found that the *SPS* gene was up-regulated in peach fruit in the anoxic conditions. SPS expression in banana fruit was also regulated by abiotic factors and phytohormones, such as white light and ethylene (Choudhury, Roy, Das, & Sengupta 2008). However, Ziliotto, Begheldo, Rasori, Bonghi, and Tonutti (2008) thought that the SPS gene did not respond to 1-methylcyclopropene treatment in peach fruit, suggesting it is probably regulated in an ethylene independent manner. Our results indicated that CA treatment promoted the transformation to sucrose by enhancing SPS activity, and delayed hydrolysis of sucrose by inhibiting the activities of NI and AI, leading to the higher sucrose level of apple fruit in storage (Fig. 5).

With regards to the further metabolism of fructose and glucose, high activities of FK and HK were observed in fruit kept in air storage. The fact, that fructose and glucose contents did not change, suggested that these sugars might be synthesized and consumed at the same rates. In contrast, apple fruit stored in CA showed lower activities of FK and HK (Fig. 4E and F), resulting in higher levels of fructose and glucose (Fig. 2B and C). FK and HK have been proved to be important factors for regulating glycolytic flux, especially under low oxygen (Fox, Green, Kennedy, & Rumpho, 1998). Notably, in anoxic conditions, activities of HK and FK were promoted in maize, while they were decreased in wheat and particularly in rice (Mustroph & Albrecht, 2003). This may be attributed to the different requests for energy and substrates in different species.

In conclusion, our results indicated that CA treatment maintained higher contents of sucrose and hexose, by regulating the genes encoding key enzymes involved in sugar metabolism. This work contributed to a better understanding of the molecular mechanism of sugar metabolism in harvested fruit subjected to CA.

Acknowledgements

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