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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 |