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# The metabolism of soluble carbohydrates related to chilling injury in peach fruit exposed to cold stress

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ABSTRACT

In order to elucidate the metabolism of soluble carbohydrates related to chilling injury (CI) in peach fruit (*Prunus persica* L. Batsch cv. Yulu) exposed to cold storage, we systematically investigated the changes in soluble carbohydrate content, transcript levels for genes encoding related enzymes, and the ascorbic acid (AsA) regeneration cycle in fruit stored at 0 °C and 5 °C for 28 days. At 0 °C, sucrose levels gradually increased up to day 21 before declining, but no significant changes of reducing-sugar levels (glucose and fructose) were observed during this time. However, the levels of sucrose decreased sharply and reducing-sugar levels increased during storage at 5 °C. Thus, fruit stored at 0 °C showed significantly higher levels of sucrose and lower levels of glucose and fructose than fruit stored at 5 °C. This difference was primarily due to lower transcription levels for neutral invertase 2 (NI/2), acid invertase (AI) and sucrose synthase (SS) and lower activities of NI and AI, and higher levels for sucrose phosphate synthase 1 (SPS/1). Both groups of fruit displayed an early, dramatic increase in the transcription levels of sorbitol and sorbitol dehydrogenase (SDH). Additionally, stable membrane permeability, a lack of CI symptoms and a regular AsA cycle were observed throughout the test period in fruit stored at 0 °C. On the other hand, membrane permeability increased sharply in fruit stored at 5 °C, which caused severe internal browning and stimulated AsA cycle activity later in storage. These results suggest that the higher levels of sucrose, resulting from the balance between its degradation and biosynthesis, contribute to membrane stability and enhanced chilling tolerance in peach fruit. Additionally, higher levels of glucose may have provided more reducing power and served as a substrate for AsA biosynthesis, which resulted in enhanced AsA cycle activity in chilling-sensitive fruit.



## 1. Introduction

Changes in soluble carbohydrates, including soluble sugars (sucrose, glucose and fructose) and sugar alcohols, have often been reported in plants exposed to low temperatures (Guy et al., 1992; Cao et al., 2013). Although the functions of these different carbohydrates have not been investigated in detail, they may serve multiple roles as osmoregulators, cryoprotectants, signaling molecules (Ruelland et al., 2009) and scavengers of reactive oxygen species (Van den Ende and Valluru, 2009) to protect plants against chilling stress.

In terms of fruit under postharvest conditions, soluble carbohydrates not only affect quality and taste but also are closely related to chilling tolerance during cold storage (Agopian et al., 2011; Cao et al., 2013). In 'Fortune' mandarin fruit, heat-conditioning increased cold tolerance by protecting sucrose levels and favoring the loss of reducing sugars (Holland et al., 2002). In other instances, increased chilling tolerance was related to higher reducing-sugar levels, especially glucose in loquat fruit (Shao et al., 2012; Cao et al., 2013). Although several studies concerning soluble carbohydrates have been conducted in fruit during cold storage, the relationship between individual carbohydrates and chilling tolerance is still unclear.

There are several enzymes that control soluble carbohydrate metabolism in plants exposed to chilling stress. Sucrose phosphate synthase (SPS) is responsible for the synthesis of sucrose from glucose and fructose. Acid invertase (AI) and neutral invertase (NI) catalyze the cleavage of sucrose to glucose and fructose, displaying crucial function(s) in harvest ripening fruit (Borsani et al., 2009; Lombardo et al., 2011). In spite of its dual role in the synthesis and hydrolysis of sucrose, sucrose synthase (SS) acts mainly in the direction of cleavage (Xu et al., 1989; Fallahi et al., 2008). In many

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species of the *Rosaceae*, sorbitol is the main photosynthetic product. As the predominant enzyme of sorbitol metabolism, sorbitol dehydrogenase (SDH) is primarily responsible for the cleavage of sorbitol to fructose in the presence of $NAD^+$ (Bianco and Rieger, 2002). The activities of sucrose phosphate synthase (SPS) and SS are positively correlated with sucrose content, and glucose and sorbitol content also display a positive correlation with sucrose-metabolizing enzyme levels and SDH activity in loquat (Ni et al., 2011).

Peaches are economically important fruit but which deteriorate rapidly when stored at ambient temperatures. The conventional method for prolonging their shelf-life is cold storage, but chilling injury (CI) often occurs during such storage. CI symptoms for peach fruit are often observed within 1 or 2 weeks when stored at 2–5 °C and after 3 or more weeks when stored at 0 °C (Lurie and Crisosto, 2005). These symptoms manifest primarily as flesh browning, resulting from membrane lipid oxidation. It has been reported that peach fruit exhibit differential sensitivity to 0 °C and 5 °C (Lurie and Crisosto, 2005; Zhang et al., 2010). CI in plants such as peach fruit is caused by an accumulation of reactive oxygen species (ROS) as a result of an imbalance between production and depletion. In addition to causing damage, ROS may act as a signal that induces cellular antioxidant systems to protect against chilling-induced damage. Ascorbic acid (AsA) metabolism is a crucial part of the antioxidant system, and it has been argued that chilling tolerance of peach fruit is closely correlated with AsA metabolism (Yang et al., 2011, 2012), a process containing a regeneration cycle, ascorbic acid–glutathione (AsA–GSH), ascorbate peroxidase (APX) and glutathione reductase (GR) as key enzymes.

The objective of this study was to investigate the metabolism of soluble carbohydrates related to CI in peach fruit exposed to cold stress. To do this, we investigated changes in the soluble carbohydrate content, the transcript levels and activities of related enzymes, and the AsA regeneration cycle in peach fruit stored at 0 °C and 5 °C for 28 days.

## 2. Materials and methods

### 2.1. Plant materials and experiment design

Peach fruit (*Prunus persica* L. Batsch cv. Yulu) were handpicked at a commercially ripe stage (138 days after full bloom) from a commercial orchard in Fenghua, Zhejiang Province, China. They were selected for uniformity of color and size, as well as a lack of any visual defects, and then stochastically divided into two groups. They were placed into cold storage at 0 °C or 5 °C with relative humidity between 92% and 95%. Batches of 6 fruit were sampled after periods of 0, 7, 14, 21, or 28 days of cold storage. Slices of mesocarp (approximately 1 cm thick) were combined, frozen in liquid nitrogen, and stored at −80 °C for further use. Three replicates, consisting of 6 fruit per replicate, were used for biochemical as well as molecular analyses, and the experiments were conducted twice.

### 2.2. Measurements of CI index, relative electrolyte leakage (REL) and firmness

CI was assessed every 7 days of cold storage as the percentage of internally browned area visible on the mesocarp surface following a cut parallel to the axial diameter. The severity of internal browning in the peach fruit was evaluated on a scale of 0–4: 0 (no signs of internal browning), 1 (<5.0%), 2 (5.0–25.0%), 3 (25.0–50.0%) and 4 (>50.0%). The results are expressed as the CI index, calculated using the following formula: CI index = [(CI scale) × (number of fruit at that CI)]/(4 × total number of fruit in each treatment).

The REL was measured as described by Saltveit (2002) with some modification. Flesh discs (10 mm diameter, 4 mm thickness) from fruit were washed with ultrapure water, and then 6 discs were transferred to a small breaker containing 20 mL ultrapure water. The conductivity of the solution ($E_0$) was determined with a conductivity meter (DDS-11A, Shanghai Precision Scientific Instrument Co., Ltd., PR China). After incubation for 3 h, the conductivity of the solution ($E_1$) was measured again. Then, the solution and discs were heated in a boiling water bath for 15 min, and the total conductivity ($E_T$) was measured. The REL was calculated according the following formula: $(E_1 - E_0)/E_T \times 100\%$.

Firmness was measured on two pared sides of suture using a TA-XT*plus* texture analyzer (Stable Micro System Ltd., UK) with a 5 mm diameter probe. The test speed was 1 mm/s, the puncture distance was 5 mm.

### 2.3. Analysis of soluble carbohydrates content

Soluble carbohydrates were detected using a method described by Shao et al. (2012). Five grams of frozen tissue was homogenized with 1 mL of solution I [5.48% (w/v) zinc acetate:glacial acetic acid (97:3)] and 1 mL of solution II [potassium ferrocyanide 2.65% (w/v)]. The homogenate was diluted to 25 mL with water and filtered with a 0.2 μm filter unit. A 20 μL aliquot was injected into a high performance liquid chromatography (HPLC) system (Model 2695, Waters, USA) equipped with a refractive index (RI) detector (Model 2414, Waters, USA) and an amino column (Kromasil® 100A, Sweden). Acetonitrile/water (75:25, v/v) was used as the solvent at a flow rate of 1 mL $min^{-1}$ at 35 °C.

### 2.4. RNA isolation and RT-PCR

#### 2.4.1. RNA extraction, DNase treatment, and cDNA synthesis

Total RNA was obtained from peach fruit mesocarp according to the method described by Meisel et al. (2005). RNA samples were treated with RNase-free DNase (TaKaRa, Japan) to remove genomic DNA according to the instruction manual. The final RNA pellet was resuspended in RNase-free water and checked for integrity on a 1% agarose gel. The concentration of DNA-free RNA was determined using a spectrophotometer (Nanodrop 1000, Thermal, USA). RNA samples were stored at −80 °C.

An aliquot (2 μg) of total RNA was reverse-transcribed with the SYBR PrimeScript RT-PCR kit II (TaKaRa, Japan) for first-strand cDNA synthesis, according to the manufacturer's instructions. All cDNA samples were stored at −20 °C and diluted 1:10 with RNase-free water before being used as template in RT-qPCR analysis.

#### 2.4.2. Selection of peach sequences and primer design

PCR primers (Table 1) were designed based on primer sequences published by Borsani et al. (2009) and the *P. persica* expressed sequence tag (EST) database (TIGR Plant Transcript Assemblies; http://plantta.tigr.org) (Childs et al., 2007). To recover the 3′-untranslated region for qPCR primer design, 3′ race of the AI and GalLDH genes was conducted. The PCR products were cloned into the pMD19-T vector (TaKaRa, Japan) and sequenced. The sequenced clones were assembled and then compared with the non-redundant database of the National Center for Biotechnology Information (NCBI) using blastn and blastx.

Primer pairs for RT-qPCR amplification were designed based on selected sequences using AlleleID 6.0 (Premier Biosoft International, Palo Alto, CA, USA). In order to test the gene specificity of these primer sets, the primers were further used to search the peach EST database with blastn. Then, melting curves were monitored during qPCR (Eppendorf/Mastercycler ep realplex, Eppendorf, Germany). Additionally, individual qPCR products were examined for size.