Changes in soluble sugar metabolism in loquat fruit during different cold storage

Yingying Wei¹ · Feng Xu¹ · Xingfeng Shao¹

Revised: 11 January 2017 / Accepted: 31 January 2017 / Published online: 13 February 2017
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Abstract To investigate soluble sugar metabolism and its relationship to chilling injury (CI), loquat fruits were stored at 0 and 5 °C for 5 weeks. CI symptoms were monitored during storage, and soluble sugar content and related enzyme activities were measured. Enzymes activities and substrate levels in the ascorbate–glutathione (AsA-GSH) cycle were also determined. Fruit at 0 °C had lower sucrose levels and higher levels of glucose and fructose than fruit maintained at 5 °C, and also manifested less severe CI symptoms. Fruit at 0 °C also exhibited increased activities of acid invertase, neutral invertase, sucrose phosphatesynthase and sucrose synthase. AsA-GSH cycle activity at 0 °C was much higher than that at 5 °C. Glucose levels correlated positively with levels of AsA and GSH and ascorbate peroxidase (APX) activity. The increased glucose levels are sufficient to account for the chilling tolerance of loquat fruit.

Keywords *Eriobotrya japonica* · Chilling injury · Sucrose · Fructose · Glucose

Introduction

Loquat (*Eriobotrya japonica* Lindl.) fruit is highly favored throughout the world for its mild acidity, sweet taste and nutritional value. Because it is harvested when conditions are hot and rainy, it has a short postharvest life

and is subject to large postharvest losses (Cai et al. 2006). Low temperature storage is commonly used to delay senescence and decay. However, the fruit of red-fleshed loquat cultivars is sensitive to low temperatures and is easily injured after 2–3 weeks storage at 1–5 °C (Rui et al. 2010). Chilling injury (CI) is characterized by peel adherence, unusual firmness, juicelessness, and internal browning, possibly the result of tissue lignification (Cao et al. 2009a, b).

Soluble sugars such as sucrose, glucose and fructose are not only the most important factors affecting fruit taste and quality (Itai and Tanahashi 2008), but they also play a crucial role in counter acting oxidative challenge during abiotic stress in plants (Keunen et al. 2013). These sugars serve multiple roles as osmoregulators, cryoprotectants, signaling molecules (Ruelland et al. 2009) and scavengers of reactive oxygen species (den Ende and Valluru 2009) to protect plants against chilling stress. Sugar metabolism may help protect fruit against cold stress, although the data are conflicting. The increase in sucrose content resulting from heat treatment may contribute to the enhanced chilling tolerance of mandarin fruit (Holland et al. 2002). Higher levels of sucrose may also contribute to chilling tolerance in peaches (Wang et al. 2013). However, Abidi et al. (2015) suggested that high sugar content, including glucose as well as sucrose, alleviates CI in peach fruit. Higher chilling tolerance is associated with a higher content of reducing sugars (glucose and fructose) in zucchini (Palma et al. 2014) and apricots (Wang et al. 2016). High hexose content (glucose and fructose) in chilling-resistant loquats probably contributes to chilling tolerance in this cultivar (Cao et al. 2013). Our previous study found that increased levels of reducing sugars maybe related to the heat-induced

✉ Xingfeng Shao
shaoxingfeng@nbu.edu.cn

¹ Department of Food Science and Engineering, Ningbo University, 818 Fenghua Road, Ningbo 315211, People's Republic of China

chilling tolerance of loquat (Shao et al. 2013). Thus, the relationship between sugar metabolism and chilling tolerance is unclear, or perhaps differs between fruit species.

In order to determine whether changes in soluble sugar content play a role in chilling tolerance, and to identify which soluble sugars are positively related to chilling tolerance in loquat fruit, we evaluated the effects of cold temperature storage at 0 °C or 5 °C on CI symptoms, soluble sugar content, and the activities of related enzymes. The relationship between changes in the AsA-GSH (ascorbate-glutathione) cycle and in sugar level was also examined.

Materials and methods

Fruit material and treatment

Loquat (*Eriobotrya japonica* Lindl. cv. “Jiefangzhong”) fruit is an important cultivar in the Chinese market, and is sensitive to chilling stress (Jin et al. 2015). Fresh ripe fruits were harvested at commercial maturity from an orchard in Fujian, China (Fig. 1) and immediately put into a cold chamber (HWS-0450, Ningbo Jiangnan Instrument Factory, Ningbo, People’s Republic of China) at 0 °C or 5 °C and $90 \pm 2\%$ relative humidity. 360 fruits were selected for uniform size, color, and the absence of visual defects, then distributed randomly into two groups of 180, each group consisted of three replicate subgroups of 60 fruits each. Data are presented as replica means $\pm$ standard errors (SE).

Fig. 1 Images of intact “Jiefangzhong” loquat fruit (a) and pulp (b)

Measurements of CI parameters

Fruit firmness (g) was measured on two paired sides (with skin removed) of 5 fruits from each replicate with a TA-XTplus texture analyzer (Stable Micro System Ltd., Haslemere, UK) equipped with a 5 mm diameter probe at a speed of 1 mm/s. Illustration of texture analyzer curves was shown in Fig. 2a, and the peak value of force was recorded as firmness. Two readings were taken per fruit and a mean value was calculated.

Hydrogen peroxide (H_2O_2) was analyzed according to the method of Shao et al. (2013), and expressed as $\mu\text{mol/g}$ fresh weight (FW). Malondialdehyde (MDA) content was determined according to the method of Shao et al. (2012) and expressed as nmol/g .

Determination of fructose, sucrose and glucose levels

Soluble sugar extraction and analysis was performed according to Shao et al. (2013). Measurements were obtained using 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). HPLC analysis for these sugars were shown in Fig. 4, and results were expressed as mg/g .

Determination of sugar metabolism enzymes

Flesh tissue samples (1 g) were ground in 5 mL of 100 mM sodium phosphate buffer (pH 7.5), containing 5 mM MgCl_2 , 1 mM EDTA, 0.1% (v/v) β -

Fig. 2 Illustration of texture analyzer curves (a) and changes in firmness (b) of loquat fruit during cold storage over 5 weeks. Values are the mean $\pm$ SE of triplicate assays. Vertical bars represent the SE of the means

mercaptoethanol and 0.1% (v/v) Triton X-100. Extracts were centrifuged at $10,000\times g$ for 20 min at 4 °C. The resulting supernatant was used for enzyme assays.

Neutral invertase (NI) activity was measured in a mixture containing 100 mM sodium phosphate (pH 7.5), 1% (g/v) sucrose, and crude extract. The mixture was incubated at 34 °C for 1 h and the reactions were stopped by immersion in a boiling water bath for 5 min. The measurement of soluble acid invertase (SAI) activity was similar to that for NI, except that the reaction was conducted at pH 4.5. The concentration of liberated glucose was determined by the DNS method (Miller 1959).

Sucrose synthase (SS) cleavage activity was assayed in a mixture of 80 mM MES [2-(4-Morpholino) ethanesulfonic acid] (pH 5.5), 5 mM NaF, 100 mM sucrose, 5 mM UDP (uridine diphosphate) and crude extract. The mixture was incubated 30 min at 30 °C and terminated by immersion in a boiling water bath for 1 min. Glucose produced by this reaction was assayed using the DNS method (Miller 1959). The activities of SAI, NI and SS-cleavage were expressed as $\text{mg Glu h}^{-1} \text{g}^{-1} \text{FW}$.

To determine SS-synthesis activity, crude extract was incubated 1 h at 34 °C with 0.06 M fructose and 4 mM UDPG (uridine diphosphate glucose) in 100 mM sodium phosphate (pH 8.0) containing 15 mM MgCl_2 . The reaction was stopped by the addition of 0.2 mL 30% (v/v) KOH and immersion in boiling water for 5 min. Sucrose produced by this reaction was assayed using the anthrone assay (van Handel 1963).

Sucrose-phosphate synthase (SPS) was assayed in a reaction containing 100 mM boric acid buffer (pH 8.0), 10 mM UDPG, 5 mM fructose-6-p, 15 mM MgCl_2 , 1 mM EDTA and crude extract. Mixtures were incubated for 30 min at 34 °C and then the reactions were stopped by

immersion boiling water for 3 min. The concentration of sucrose liberated was determined using the anthrone assay (van Handel 1963). SS-synthesis and SPS activities were expressed as $\text{mg Suc h}^{-1} \text{g}^{-1} \text{FW}$.

Determination of AsA-GSH cycle content levels and enzyme activities

To measure ascorbate (AsA), flesh tissue (2 g) was homogenized in 5 ml ice-cold oxalic acid-EDTA solution (oxalic acid 50 mM; EDTA 0.2 mM), and centrifuged at $10,000\times g$ for 20 min at 4 °C. The supernatant was used for the AsA assay according to the method of Wang et al. (2013). The results were expressed as $\text{mg}/100 \text{g FW}$.

Reduced glutathione (GSH) was extracted by grinding 1.0 g of fresh tissue in 5 ml 50 mM sodium phosphate buffer (pH 7.0), and centrifuging at $10,000\times g$ for 20 min at 4 °C. The supernatant was used for the GSH assay using assay kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, China), following manufacturer instructions.

To assay ascorbate peroxidase (APX) and glutathione reductase (GR), 1 g of fresh sample was ground with 5 mL of 100 mM sodium phosphate buffer (pH 7.0), containing 0.1 mM EDTA, 1 mM sodium ascorbate and 1% polyvinyl-pyrrolidone (PVP). The extracts were homogenized, centrifuged at $10,000\times g$ for 20 min at 4 °C, and the supernatant was used for the enzyme assays. The APX assay was conducted as described by Cao et al. (2010). One unit was defined as the amount of enzyme that oxidizes $1 \mu\text{mol ascorbate min}^{-1}$. GR was assayed using assay kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, China), following the manufacturer instructions.

Statistical analysis

One-way analysis of variance (ANOVA) and Pearson correlation coefficients were calculated using SAS (Version 8.2; SAS Institute, Cary, NC, USA). Comparison of means was performed by Duncan's multiple range tests. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

CI symptoms in loquat fruit during cold storage at different temperatures

Images of intact “Jiefangzhong” loquat fruit and pulp were shown in Fig. 1. For firmness measurement, texture analyzer curves was recorded, and the peak value of curve is the firmness of loquat (Fig. 2a). During cold storage, fruit firmness increased with storage time (Fig. 2b). However, firmness at 0 °C was significantly ($P < 0.05$) lower than at 5 °C after 2 weeks storage. H_2O_2 (Fig. 3a) and MDA (Fig. 3b) content in both groups generally increased with storage time. Fruits at 5 °C showed significantly ($P < 0.05$) higher H_2O_2 levels throughout storage, and higher MDA levels after the 1st week of storage.

It is generally accepted that CI develops when oxidative stress generates more reactive oxygen species (ROS) than can be handled by the scavenging capacity of fresh tissue (Hodges et al. 2004). H_2O_2 , the most stable and diffusive ROS, can induce peroxidation and breakdown of unsaturated fatty acids in membrane lipids. MDA is one of the intermediate products of lipid peroxidation and affects membrane structure, disturbing normal physiological metabolism (Shao et al. 2012). In loquat fruit, a major CI symptom is a firm and juiceless texture (Cao et al. 2009a, b, 2010). During cold storage, the chilling-sensitive cultivar has lower levels of H_2O_2 and higher membrane lipid unsaturation than does the chilling-resistant cultivar (Cao et al. 2011). In our tests, loquat fruit stored at 5 °C exhibited more firmness and higher H_2O_2 and MDA levels, and was therefore more sensitive to CI than fruit stored at 0 °C. This result is similar to that reported for peach fruits stored at 0 °C and 5 °C (Zhang et al. 2011; Wang et al. 2013). In contrast, Cai et al. (2006) reported that a storage temperature of 5 °C maintains higher fruit quality than lower (0 °C) temperatures in “Luoyangqing” loquats. The discrepancy between these maybe due to differences between the cultivars selected for study.

Changes in soluble sugar content

In order of abundance, the major soluble sugars in loquat fruit (cv. Jiefangzhong) are fructose, glucose and sucrose

(Fig. 4). At harvest, the levels of those sugars were 29.5, 9.1, and 3.57 mg/g FW, respectively. From HPLC chromatogram (Fig. 4), sorbitol was not detected in our test, in contrast with results reported by Ding et al. (2002) and Xu et al. (2010). The quantity and types of sugar in loquat fruit may be cultivar dependent. Throughout storage, loquat fruit at 0 °C exhibited significantly ($P < 0.05$) higher levels of glucose (Fig. 4a) and fructose (Fig. 4b), and lower levels of sucrose (Fig. 4c), than fruit maintained at 5 °C.

Glucose and sucrose are now recognised as crucial compounds in coordinating plant developmental responses under oxidative stress (den Ende and Valluru 2009). These sugars have several beneficial effects in protecting plants against chilling and oxidative stresses, including osmoregulation, sugar signalling, sugar-modulated gene expression, support of anti-oxidative metabolism, and direct antioxidant activity against reactive oxygen species (Couée et al. 2006; den Ende and Valluru 2009; Ma et al. 2009; Ruelland et al. 2009). Higher sucrose levels are related to the absence of CI symptoms in heat-treated ‘Fortune’ mandarin fruit (Holland et al. 2002) and in peach fruit stored at 0 °C (Wang et al. 2013). However, higher glucose and fructose levels are associated with higher chilling tolerance in heat-treated loquat (Shao et al. 2013) and in a chilling-resistant loquat cultivar (Cao et al. 2013). In this study, we confirmed that increased levels of reducing sugars (glucose and fructose) are associated with reduced CI in loquat fruit stored at 0 °C.

Changes in activities of sugar metabolism enzymes

Several enzymes (SAI, NI, SS and SPS) control sucrose accumulation and degradation in plants. SPS synthesizes sucrose-6-phosphate, which is dephosphorylated by sucrose-phosphate phosphatase to form sucrose. SS plays a dual role in the hydrolysis and synthesis of sucrose. SS cleavage hydrolyzes sucrose to UDP-glucose (uracil-diphosphate glucose) and fructose, while SS synthesis catalyzes the reverse reaction. Invertases (SAI and NI) catalyze the cleavage of sucrose to glucose and fructose (Itai and Tanahashi 2008; Shao et al. 2013). In our study, SAI, NI and SS-cleavage all cleave sucrose (Fig. 5a, b and c), and their activities at 0 °C are significantly ($P < 0.05$) higher than at 5 °C. SS-synthetic and SPS can synthesize sucrose in loquat during cold storage. Both enzymes exhibit similar temporal patterns (Fig. 5d, e), with activities first increasing and then declining. Fruits at 0 °C exhibit significantly ($P < 0.05$) higher activities for both enzymes than at 5 °C. Shao et al. (2013) reported that heat treatment improves the activities of SAI, NI, SS and SPS in loquat fruit. However, Cao et al. (2013) found that the chilling-resistant loquat fruit had higher activities of SAI, NI and SPS, but lower activity of SS, than chilling-sensitive fruit.

Fig. 3 Changes in H_2O_2 (a) and MDA (b) content in loquat fruit during cold storage over 5 weeks. Values are the mean $\pm$ SE of triplicate assays. Vertical bars represent the standard errors of the means

Fig. 4 HPLC chromatogram and the changes in glucose (a), fructose (b) and sucrose (c) content in loquat fruits during cold storage over 5 weeks. Values are the mean $\pm$ SE of triplicate assays. Vertical bars represent the standard errors of the means

In order to better understanding the overall effect of the changes in individual enzyme activities, net activity was calculated for the sucrose-metabolising enzymes after harvest, using the formula (SAI + NI + SS-cleavage

activity) – (SS-synthetic + SPS activity). The resulting value is positive (Fig. 5f), indicating that net sucrose cleavage activity is higher than net sucrose synthesis activity. At both storage temperatures, net activity first

Fig. 5 Changes in activities of SAI (a), NI (b), SS-cleavage (c), SS-synthetic (d), SPS (e) and net sucrose cleavage activity (f), in loquat fruits during cold storage over 5 weeks. Values are the mean $\pm$ SE of triplicate assays. Vertical bars represent the standard errors of the means

increased, declined at the 2nd week, and then rapidly increased thereafter. Fruits at 0 °C showed significantly ($P < 0.05$) higher net cleavage activity than at 5 °C, consistent with the increased level of reducing sugars and the decreased level of sucrose in fruit at 0 °C (Fig. 4). In Hami melons, fruit at 5 °C has a higher sucrose synthesis flux than fruit at 0 °C (Cai et al. 2015). However, peach fruit at 5 °C exhibits a sharp decrease in sucrose content and an increase in reducing sugars (Wang et al. 2013). These

results indicate that changes in fruit sucrose metabolism during cold storage are species-dependent.

Changes in AsA-GSH cycle activity

No significant ($P > 0.05$) differences in AsA and GSH content were observed over the first two weeks of storage (Fig. 6a, b). After two weeks, fruit at 0 °C exhibited markedly ($P < 0.05$) higher values than fruit stored at

Fig. 6 Changes in contents of AsA (a) and GSH (b), and the activity of APX (c) and GR (d) in loquat fruits during cold storage over 5 weeks. Values are the mean $\pm$ SE of triplicate assays. Vertical bars represent the standard errors of the means. Note: net

activity = (SAI + NI + SS-cleave activity) – (SS-synthetic + SPS activity). The value is positive, indicating that sucrose cleavage is predominant

5 °C. Throughout storage, APX and GR (Fig. 6c, d) activity at 5 °C was profoundly ($P < 0.05$) lower than in fruit maintained at 0 °C.

Reductions in CI may be related to increasing activities of antioxidant enzymes (Cao et al. 2009a). AsA and GSH, the main enzymes in AsA-GSH cycle, can function as direct antioxidants and reductants of APX and GR (Nishikawa et al. 2003; Quan et al. 2008), which scavenge H_2O_2 in response to stress (Nishikawa et al. 2003). Increased AsA-GSH cycle activity enhances the chilling tolerance of loquat fruit (Cai et al. 2011; Cao et al. 2011; Shao et al. 2013). We therefore conclude that the enhanced AsA-GSH cycle at 0 °C reduces the levels of H_2O_2 (Fig. 3a) and MDA (Fig. 3b), resulting in less severe CI, compared to fruit stored at 5 °C.

Relationship between soluble sugar and AsA-GSH cycle

The relationship between soluble sugar and the AsA-GSH cycle is shown in Table 1. Only glucose content exhibits a significant positive correlation with AsA, GSH content, and

APX activity ($r = 0.659$, $P < 0.05$; $r = 0.629$, $P < 0.05$; $r = 0.708$, $P < 0.01$, respectively). A very significant ($P < 0.01$) negative correlation exists between sucrose content and AsA content ($r = -0.712$). These results indicate that the higher AsA content in loquat fruit stored at 0 °C may be related to higher glucose and lower sucrose levels.

Glucose can regulate oxidative stress and chilling tolerance by the AsA-GSH cycle (Couée et al. 2006; Ma et al. 2009). Glucose usage by the pentose-phosphate pathway enhances production of NADPH, a major cofactor of ROS scavenging pathways such as the AsA-GSH cycle (Couée et al. 2006). Glucose is also the main precursor for AsA synthesis (Smirnoff et al. 2001), and thus provides the AsA carbon skeleton which is the building block for GSH (Noctor and Foyer 1998). L-galactono-1,4-lactone-dehydrogenase (GLDH), the last limiting enzyme in the AsA biosynthetic pathway, may have multiple roles when fruit is exposed to abiotic stress (Wang et al. 2014). Sucrose originating from cut stems in harvested broccoli florets strongly enhances reducing sugar content and the expression of GLDH, thereby increasing AsA content (Nishikawa

Table 1 Relationship between soluble sugar and AsA-GSH cycle

	Glucose	Fructose	Sucrose	AsA	GSH	APX	GR
Glucose	1						
Fructose	0.489	1					
Sucrose	0.404	0.628*	1				
AsA	0.659*	0.502	−0.712**	1			
GSH	0.629*	−0.021	−0.324	0.378	1		
APX	0.708**	0.155	0.223	0.798**	0.378	1	
GR	0.113	0.154	0.288	0.107	0.200	0.199	1

** $P < 0.01$; * $P < 0.05$

et al. 2005). Glucose content and GLDH activity increased in lettuce and spinach after potassium restriction, resulting in an increase in AsA content (Ogawa et al. 2014). We therefore hypothesize that the reduction of CI at 0 °C is related almost entirely to the higher glucose content, which increases AsA-GSH cycle activity and the scavenging of H_2O_2 .

Conclusion

It has been concluded loquat fruit stored at 0 °C had lower sensitivity to CI and increased activities in all sugar metabolism enzymes, than fruit stored at 5 °C. Sucrose degradation predominates in loquats during storage and the lower storage temperature accelerates sucrose degradation, resulting in lower levels of sucrose and higher levels of glucose and fructose. We conclude that the increase in glucose levels at 0 °C induces higher AsA-GSH cycle activity, which promotes H_2O_2 scavenging. The removal of this major ROS decreases membrane lipid peroxidation and increases chilling tolerance.

Acknowledgements This study was sponsored by the National Science Foundation of China (No. 31671903), the Nature Science Foundation of Zhejiang Province (No. LR15C200002), and the K. C. Wong Magna Fund in Ningbo University.

Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the authors.

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