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Alteration of sugar and organic acid metabolism in postharvest granulation of Ponkan fruit revealed by transcriptome profiling

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ABSTRACT

Granulation is a serious physiological disorder in citrus fruit, accompanied by deterioration of sugars and organic acids, of which the relevant mechanism remains largely unknown. Postharvest granulation was found to begin at the stem region and then gradually extend towards the stylar end of the segment in Ponkan fruit. Hence, stem and stylar juice vesicles within the same fruit with either non-granulation or granulation were analyzed by RNA-Sequencing. Through a comparison of incipient granulated and entire granulated fruit, the results demonstrated that 768 genes were reliably identified to be differentially expressed under granulation. A significant increase in transcript levels was observed in genes encoding enzymes involved in the degradation pathways of sugars and citric acid (*e.g.*, invertase, hexokinase, aconitase, isocitrate dehydrogenase and α-ketoglutarate-dehydrogenase) and in the biosynthesis of cell wall materials including pro-pectin, cellulose and lignin (*e.g.*, UDP-glucuronate 4-epimerase, UDP-glucose pyrophosphorylase and cinnamyl alcohol dehydrogenase) under granulation. Further, there was a distinct decrease in transcript levels of genes encoding enzymes involved in the synthesis pathway of sucrose and citric acid (*e.g.*, sucrose phosphate synthase, sucrose phosphatase, aspartate aminotransferase and malate dehydrogenase) and the degradation pathway of cell wall components (*e.g.*, pectin methylesterase and β-D-xylosidase) under granulation. Together with the decline in content of soluble sugars and acids (sucrose, glucose, fructose, citric acid and malic acid) and the increase in content of cell wall components (lignin, pro-pectin and cellulose) under granulation, the results suggested that sugar and organic acid metabolism adjusted to the synthesis pathway of cell wall components upon granulation at the expense of sugars and acids. This study is the first to unravel the global picture of the network of sugar and organic acid metabolism underlying fruit granulation.

1. Introduction

Citrus is among the most important fruit in the world because of its excellent flavor and high nutritional value. Granulation (also called section-drying, scierification or crystallization) is a serious physiological disorder occurring in either the late-harvest season or postharvest storage in various citrus species, *e.g.*, orange, grapefruit, pummelo, and tangerine (Bartholomew et al., 1941; Shomer et al., 1989; Burns and Albrigo, 1997). The typical symptom of granulation is that the juice vesicle becomes tough, dry, and colorless (Ritenour et al., 2004; Wu et al., 2014). The occurrence of granulation results in the deterioration of fruit quality and the further decline in marketable value. Thus, farmers often suffer heavy losses (Bartholomew et al., 1941; Ritenour et al., 2004). The initiation of granulation in citrus species was associated with various preharvest factors including fruit size, harvest time of fruit, rootstock, tree age and irrigation (Ritenour et al., 2004). Granulation occurred during on-tree ripening in some citrus species and became more severe during postharvest storage; while granulation in some other citrus species, which were free of granulation at harvest, was mainly induced by postharvest storage (Burns and Albrigo, 1998). These causes for the occurrence of granulation were rather complex and varied among citrus types, making the elucidation of the intrinsic mechanism of granulation far more difficult. However, there was a common sign for most granulation of citrus that a dramatic decrease in content of sugars and acids of pulps, regardless of how the juice vesicles get affected by the disorder (Jiang et al., 1991; Wang et al., 2014), which provides important clues for identifying a unifying mechanism for the process of granulation.

Fruit sweetness and acidity, mainly determined by sugars and organic acids, respectively, are vital factors affecting citrus fruit flavor. Sugars are accumulated during fruit development and ripening while remaining relatively stable or decreasing during the postharvest period

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(Chen et al., 2012). The content of sugars in postharvest fruit is determined by the balance of sugar degradation and biosynthesis, which is catalyzed by several critical enzymes such as sucrose synthase (SUS), sucrose phosphate synthase (SPS) and invertase (IN) (Ruan, 2014). The degradation of sucrose begins with the conversion of sucrose into hexose, followed by the glycolysis pathway as a respiratory substrate and/or further synthesis into other carbohydrates (Chen et al., 2012; Gupta et al., 2012). The major organic acid in citrus fruit is citric acid, which increases during fruit development and decreases during the late-ripening and postharvest periods (Ding et al., 2015; Sheng et al., 2017). The decline in citric acid is largely due to use as respiratory substrates or in the synthesis of amino acids (Chen et al., 2012; Sheng et al., 2017). Degradation of citric acid is catalyzed by a series of enzymes such as aconitase (ACO), phosphoenolpyruvate carboxykinase (PEPCK), and isocitrate dehydrogenase (IDH) (Sadka et al., 2000; Chen et al., 2012; Gupta et al., 2012).

Granulation often results in an accelerated decrease in sugars and acids in citrus fruit including pummelo fruit, navel oranges, Valencia oranges and Ponkan mandarins (Sinclair and Jolliffe, 1960; Jiang et al., 1991; Wang et al., 2014). Various plausible reasons for this phenomenon were suggested in previous research. As respiration rate could increase in granulated juice sacs compared to normal fruit in some citrus species including Kaula mandarin, Kinnow mandarin and Ponkan mandarin, one hypothesis was proposed that accelerated respiration rate may be responsible for the depletion of nutrients in granulated citrus fruit (Singh and Singh, 1979; Tan et al., 1985). Citrus granulation was often correlated with secondary wall formation and cell wall thickening in granulated juice vesicles (Shomer et al., 1989; Zhang et al., 1999). The changes in cell wall structure of juice vesicles were suggested to be related with the increasing of content of cell wall components including lignin, hemicellulose and pectin (Burns and Achor, 1989; Shomer et al., 1989; Hwang et al., 1990; Wu et al., 2014; Zhang et al., 2016). Together with the results that respiration was enhanced in the granulated pulp of grapefruit, it was reasonable to suppose that increased respiration in granulated cells aimed to fuel the cell wall changes, and the degradation of sugars and acids generated substrates for biosynthesis of cell wall materials (Shomer et al., 1989; Burns, 1990). Additionally, regrowth of citrus peels was observed during postharvest storage (Bartholomew et al., 1941; Zong et al., 1979), and was further proposed to be a plausible reason for the depletion of nutrients in granulated pulp (Chen et al., 2005).

However, those possible mechanism for the deterioration of nutrients upon granulation were proposed mainly based on physiological data while lack of molecular evidence. Furthermore, there is little information about how these pathways work in the global control of consuming sugars and organic acids in juice vesicles, which would be of importance to elucidate the underlying molecular mechanism of the granulation process. In the present study, RNA-Sequencing (RNA-Seq) transcriptome approach was employed to explore the dynamic changes in gene expression patterns in juice vesicles of Ponkan mandarin (*Citrus reticulata* Blanco cv. Ponkan) during postharvest granulation and to disclose the global picture of sugar and organic acid metabolism alterations in disordered juice sacs.

2. Materials and methods

2.1. Plant material

Ponkan mandarin (*C. reticulata*) fruit were harvested at the commercial ripening stage from a local orchard in Beibei, Chongqing, China. Fruit of uniform color and size were selected and stored at 8–10 °C and 80–85% relative humidity (RH). Stem and stylar juice vesicles of citrus samples were selected from fruit with four months' storage. All the samples were frozen in nitrogen and stored at 10 °C for further analysis.

2.2. Total soluble solids (TSS) and titratable acidity (TA) measurements

One gram of juice sacs from pulp samples were homogenized with 4 mL of ultrapure water and then centrifuged at 6000 × *g* for 5 min at 4 °C. The supernatants were collected for TSS and TA measurements. TSS was measured with a pocket refractometer (PAL-1, Atago, Japan). The supernatants were titrated with 0.01 mol L^{-1} NaOH to the end-point with phenolphthalein as the indicator, and TA was calculated as% of citric acid. Three replicates were performed.

2.3. RNA-seq and data analysis

Total RNA of juice sacs was extracted using the TRIzol reagent (Invitrogen Corp., CA, USA) according to the manufacturer's handbook. Approximately 3 μg of RNA per sample was used for cDNA library construction by NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, MA, USA) following the manufacturer's recommendations. Briefly, mRNA was enriched from total RNA *via* magnetic oligo-dT beads and cleaved using divalent cations. First cDNA strand synthesis was performed *via* random hexamer-primed reverse transcription using M-MuLV reverse transcriptase (RNase H^-), and a subsequent second cDNA strand was synthesized using DNA polymerase I and RNase H. Double-stranded cDNA was processed with end repair and adapter ligation and then amplified by PCR and purified using the AMPure XP system (Beckman Coulter, CA, USA). cDNA libraries were sequenced on an Illumina Hiseq platform. Two biological replicates were performed.

The raw reads were processed through in-house Perl scripts, and clean reads with high quality were obtained by removing reads containing adapter and low-quality sequences. All clean reads were aligned to the reference genome of *Citrus clementina* using TopHat v2.0.12. Gene expression levels were calculated by FPKM (number of fragments per kilobase of transcript sequence per million base pairs sequenced), considering the effect of gene length for the reads and sequencing depth simultaneously (Trapnell et al., 2010). Differential gene expression analysis between normal and granulated juice sacs were performed using the DESeq R package. DESeq employs a negative binomial distribution-based model to determine the differential expression of genes. The Benjamini and Hochberg approach was employed to adjust the resulting *P* values to control the false discovery rate. The criterion of adjusted *P* value < 0.05 was set for a significant difference in gene expression.

2.4. Sugar and organic acid analysis by gas chromatography-mass spectrometry (GC–MS)

The content of soluble sugars and organic acids were measured according to the protocol published previously with little modification (Ding et al., 2015). A 0.3 g pulp sample was homogenized by liquid nitrogen, followed by extraction with 2.7 mL of cold methanol, and then 0.3 mL of 0.2 g L^{-1} ribitol was added as an internal standard for quantification. Samples were subjected to ultrasonic extraction at 4 °C for 15 min, followed by incubation in a 70 °C water bath for 15 min. Then, the sample was centrifuged at 6000 × *g* for 20 min. The supernatant (100 μL) was collected and then concentrated under vacuum. Extracts were subjected to derivatization accompanied by incubation in 50 μL of 20 g L^{-1} methoxyamine hydrochloride in pyridine for 30 min at 50 °C and then treated for 40 min at 60 °C using 50 μL of BSTFA (containing 1% TMCS). A sample of 1 μL was injected into a gas chromatograph through a DB-5 MS capillary column (30 m × 0.25 mm ID, 0.25 μm). The injector temperature was 250 °C, with a carrier helium gas flow rate of 1.0 mL/min and a split ratio of 1:10. The column was maintained at 100 °C for 1 min; increased to 175 °C at a rate of 3 °C/min and held at 175 °C for 1 min; increased to 184 °C at a rate of 2 °C/min, increased to 190 °C at 0.5 °C/min and held at 190 °C for 1 min; and increased to 280 °C at a rate of 7 °C/min and finally held at 280 °C for