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Effects of granulation on organic acid metabolism and its relation to mineral elements in *Citrus grandis* juice sacs

Xian-You Wang [a,b], Ping Wang [b,*], Yi-Ping Qi [c], Chen-Ping Zhou [a,b], Lin-Tong Yang [a,d], Xin-Yan Liao [a,d], Liu-Qing Wang [a,d], Dong-Huang Zhu [e], Li-Song Chen [a,b,d,*]

[a] *Institute of Horticultural Plant Physiology, Biochemistry and Molecular Biology, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[b] *College of Horticulture, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[c] *Institute of Materia Medica, Fujian Academy of Medical Sciences, Fuzhou 350001, China*
[d] *College of Resources and Environmental Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[e] *Agricultural Bureau of Pinghe County, Zhangzhou 363700, China*

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ABSTRACT

We investigated the effects of granulation on organic acid metabolism and its relation to mineral elements in 'Guanximiyou' pummelo (*Citrus grandis*) juice sacs. Granulated juice sacs had decreased concentrations of citrate and isocitrate, thus lowering juice sac acidity. By contrast, malate concentration was higher in granulated juice sacs than in normal ones. The reduction in citrate concentration might be caused by increased degradation, as indicated by enhanced aconitase activity, whilst the increase in malate concentration might be caused by increased biosynthesis, as indicated by enhanced phospho*enol*pyruvate carboxylase (PEPC). Real time quantitative reverse transcription PCR (qRT-PCR) analysis showed that the activities of most acid-metabolizing enzymes were regulated at the transcriptional level, whilst post-translational modifications might influence the PEPC activity. Granulation led to increased accumulation of mineral elements (especially phosphorus, magnesium, sulphur, zinc and copper) in juice sacs, which might be involved in the incidence of granulation in pummelo fruits.

1. Introduction

Citrus belongs to evergreen subtropical fruit trees and is the world's leading tree-fruit crop. It is commercially grown in many countries, including China, Brazil, United States, Spain, India, Mexico, Argentina, Iran, Italy, Indonesia, Egypt, Turkey, Pakistan, South Africa, etc. Citrus fruits often suffer from various physiological disorders, including granulation (also called crystallization, sclerification, dryness, *kaosan*, or section drying). Granulation is a physiological disorder of juice sacs in citrus fruits, wherein juice sacs become hard, dry and enlarged, assume a grayish colour and have little extractable juice (Ritenour, Albrigo, Burns, & Miller, 2004; Shomer, Chalutz, Vasiliver, Lomaniec, & Berman, 1989; Singh, 2001). Granulation was first reported by Bartholomew, Sinclair, and Raby (1934) in California, and later by many researchers from different citrus growing countries, like Australia, Brazil and China (Singh, 2001; Xie, Zhuang, Wang, Xu, & Huang, 1998). It has been suggested that sweet orange cultivars (i.e. Pineapple, Washington navel, Hamlin, Blood red, Valencia late) and mandarin cultivars (i.e. Kaula, Nagpur and Dancy) suffer from granulation, and that the disorder is less serious in other citrus species and cultivars, such as grapefruit, pummelo, lemons and limes (Singh, 2001). However, granulation is very serious in 'Guanximiyou' pummelo. Because granulation results in reduced commercial rate and poor quality of fruits, growers often suffer heavy losses (Xie et al., 1998; Zheng, 2006). Fruit acidity, as measured by titratable acidity (TA) and/or pH, is an important factor affecting fruit flavour (Chen, Liu, & Chen, 2009, 2010; Etienne, Génard, Lobit, Mbeguié-A-Mbéguié, & Bugaud, 2013). Increasing evidence has shown that granulation results in a decrease in TA and an increase in pH of the juice sacs in different citrus species and cultivars (Awasthi & Nauriyal, 1972; Sharma & Saxena, 2004; Sharma, Singh, & Saxena, 2006; Singh, 2001). Fruit acidity is due to the presence of organic acids (OAs). In most fruits, the two major OAs are malate and citrate (Chen, Liu, et al., 2009; Chen et al., 2010; Etienne et al.,

* Corresponding authors. Tel.: +86 591 83789169; fax: +86 591 83735681 (P. Wang). Address: College of Resources and Environmental Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China. Tel.: +86 591 83854966; fax: +86 591 83776849 (L.-S. Chen).
E-mail addresses: wangp0518@yahoo.com.cn (P. Wang), lisongchen2002@hotmail.com (L.-S. Chen).

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2013). In citrus juice sacs, citrate is the major OA except for acidless citrus fruits, followed by malate (Yamaki, 1989). However, there is hardly any information available on the effects of granulation on OA metabolism in citrus juice sacs.

The accumulation of OAs in fruit cells is determined by the balance of OA biosynthesis, degradation and vacuolar storage (Chen, Liu, et al., 2009; Diakou, Svanella, Raymond, Gaudillère, & Moing, 2000; Etienne et al., 2013). The major enzymes potentially involved in the biosynthesis and degradation of citrate and malate include phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31), NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37), NADP-malic enzyme (NADP-ME, EC 1.1.1.40), citrate synthase (CS, EC 4.1.3.7), NAD-isocitrate dehydrogenase (NAD-IDH, EC 1.1.1.41), and aconitase (ACO, EC 4.2.1.3) (Chen, Liu, et al., 2009; Etienne et al., 2013). The biosynthesis and breakdown of citrate are mediated by mitochondrial CS, NAD-IDH and ACO (Etienne et al., 2013; Kubo, Kihara, & Hirabayashi, 2002). Sadka, Dahan, Cohen, and Marsh (2000) showed that a decrease in the mitochondrial ACO activity played a role in citrate accumulation during the early stage of citrus fruit development, whilst an increase in the cytosolic ACO activity played a role in citrate decline towards fruit maturation. The biosynthesis of malate mainly occurs in the cytosol and catalyzed by PEPC and NAD-MDH, and cytosolic NADP-ME has been suggested to be involved in the degradation of malate during the ripening of several fruit species (Chen, Liu, et al., 2009; Etienne et al., 2013; Hirai, 1982).

Increasing evidence has shown that citrus fruit granulation is associated with the nutritional status of plants. However, there are some conflicting reports on mineral elements of plants in relation to granulation in citrus. Sinclair and Jolliffe (1961) observed that several mineral elements [i.e. calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and phosphorus (P)] accumulated in granulated juice sacs of 'Valencia' orange. Xie et al. (1998) reported that the concentrations of mineral elements [especially nitrogen (N), P and K] remarkably increased in granulated juice sacs of 'Guanximiyou' pummelo fruits, that both increased concentrations of N, K, boron (B) and zinc (Zn) and decreased concentration of Ca in leaves might contribute to the incidence of granulation, and that granulation increased as the concentration of available copper (Cu) in soils increased. In sweet orange, the lower concentrations of Ca, Zn and B in leaves and pulp were involved in the higher incidence of granulation, but the leaf concentrations of N, K, Mg, sulphur (S), Cu, and manganese (Mn) were not related with the incidence of granulation (Awasthi & Nauriyal, 1972). Munshi, Singh, Vij, and Jawanda (1978) found that N, P, K, Mg, Zn and Cu concentrations in leaves increased, Ca, iron (Fe) and B concentrations in leaves decreased, and Mn concentration in leaves did not change as the granulation progressed in two sweet orange cultivars. Later, they reported that increased levels of K and Mg in the fruit rind and of N, P, K and Ca in the pulp were associated with the higher incidence of granulation in 'Dancy' tangerine (Munshi, Jawanda, Singh, & Vij, 1980). Singh and Singh (1980) demonstrated that granulation had no definite relationship with N, K, Mg, Zn, Cu and Fe concentrations of the Kinnow plants. Similar results have been obtained by Chanana and Nijjar (1984) in sweet orange. To conclude, the relationship between the citrus fruit granulation and the nutrient status of plants has not been fully understood so far.

In this study, we compared granulated juice sacs with normal ones in terms of TA, concentrations of total soluble solids (TSS), malate, citrate, isocitrate and mineral elements, and gene expression and activities of acid-metabolizing enzymes in 'Guanximiyou' pummelo fruits. The objectives of this study were to understand the mechanism of TA reduction in granulated juice sacs and the relationship between the granulation and the nutrient status of juice sacs in pummelo fruits.

2. Materials and methods

2.1. Plant materials

One pummelo (*Citrus grandis*) cultivar, 'Guanximiyou', was used in this study. All the samples were collected from 25-year-old trees on 'Sour pummelo' rootstocks grown at Yanban village pummelo orchard, Xiaoxi town, Pinghe county, Fujian province, China on single-tree replicates for all measurements on 1 October 2012, when the fruit was ripe and the granulation is visible. The trees were grown at a spacing of 4 × 5 m and received standard horticultural practices, and disease and insect control. The cropload of each tree was approximately 150 kg. At the sampling time, the average fruit fresh weight, diameter and length were 2138 ± 111 g FW fruit^{-1}, 18.5 ± 0.3 cm and 19.5 ± 0.9 cm, respectively. Five to ten fruit per tree were chosen from the outer of the mid-upper canopy. After collecting the fruits at noon, each fruit was divided into two halves. One-half of fruit was then used to collect normal and granulated juice sacs. The collected samples were immediately frozen in lipid nitrogen and stored at −80 °C until analysis except for TSS, TA and mineral elements. The second half of the fruit was immediately used to collect normal and granulated juice sacs after being brought to laboratory. The fresh juice sacs were then used to assay TSS and TA. The remaining juice sacs were used for mineral element assay after being dried at 55 °C for 80 h.

2.2. Determination of juice sac TA and TSS

TA was titrated with 0.1 N NaOH to the end point pH 8.1 using a Microprocessor-based Bench pH/mV/°C Meter (pH 211, Henna Instruments, Italy) and the total acidity calculated as malic acid (Chen et al., 2007). TSS was determined with WYT-4 refractometer (Quanzhou Zhongyou Optical Instrument Co., Ltd., Quanzhou, China).

2.3. Extraction and determination of juice sac citrate, isocitrate and malate

Juice sac citrate, isocitrate and malate were extracted and determined according to Chen, Lin, and Nose (2002).

2.4. Measurement of acid metabolizing enzyme activities in juice sacs

CS, ACO, PEPC, phospho*enol*pyruvate phosphatase (PEPP, EC 3.1.3.60), pyruvate kinase (PK, EC 2.7.1.40), NAD-MDH, NADP-MDH, NAD-ME, NADP-ME, NAD-IDH and NADP-IDH were extracted according to Chen, Liu, et al. (2009).

CS and NAD-IDH were measured according to Chen, Liu, et al. (2009). CS activity was determined in 1 mL mixture containing 50 mM Tris–HCl (pH 7.8), 0.1 mM 5,5′-dithiobis-(2-nitrobenzic acid), 0.2 mM acetyl-CoA and 80 μL enzyme extract. The reaction was started by adding 0.5 mM oxaloacetate (OAA), and the increase in A_{412} at 25 °C was monitored. NAD-IDH was measured in 1 mL mixture containing 100 mM Tris–HCl (pH 8.5), 0.1 mM EDTA, 0.8 mM NAD, 0.4 mM $MnSO_4$, 10 mM isocitrate and 160 μL enzyme extract, starting the reaction with isocitrate.

ACO, PEPC, NAD-MDH, NADP-ME, NADP-IDH, PK and PEPP were assayed according to Chen, Tang, et al. (2009). ACO was determined in 1 mL mixture containing 85 mM Hepes–KOH (pH 7.5), 10 mM $MgSO_4$, 5 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM NADP, 2 unit NADP-IDH and 80 μL enzyme extract. The reaction was started by adding 10 mM *cis*-aconitate. PEPC was measured in 1 mL mixture containing 50 mM Tris–HCl (pH 8.0), 5 mM $MnCl_2$, 2 mM DTT, 10 mM $NaHCO_3$, 0.2 mM NADH, 5 unit MDH and 80 μL enzyme extract, starting the reaction with