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Spatio-temporal distribution and natural variation of metabolites in citrus fruits

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ABSTRACT

To study the natural variation and spatio-temporal accumulation of citrus metabolites, liquid chromatography tandem mass spectrometry (LC–MS) based metabolome analysis was performed on four fruit tissues (flavedo, albedo, segment membrane and juice sacs) and different *Citrus* species (lemon, pummelo and grapefruit, sweet orange and mandarin). Using a non-targeted metabolomics approach, more than 2000 metabolite signals were detected, from which more than 54 metabolites, including amino acids, flavonoids and limonoids, were identified/annotated. Differential accumulation patterns of both primary metabolites and secondary metabolites in various tissues and species were revealed by our study. Further investigation indicated that flavedo accumulates more flavonoids while juice sacs contain more amino acids. Besides this, cluster analysis based on the levels of metabolites detected in 47 individual *Citrus* accessions clearly grouped them into four distinct clusters: pummelos and grapefruits, lemons, sweet oranges and mandarins, while the cluster of pummelos and grapefruits lay distinctly apart from the other three species.

1. Introduction

Citrus, including kumquats, lemons, pummelos, grapefruits, oranges and mandarins (including mandarin and tangerine), is the largest fruit crop in the world, and cultivated world-wide (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2011b). Constituting the richest dietary sources of flavonoids and vitamins, citrus fruits are becoming more attractive for maintaining human health (Gattuso, Barreca, Gargiulli, Leuzzi, & Caristi, 2007). A number of citrus species have been recorded in the Chinese pharmacopoeia as appropriate for medical use, and the dried tangerine or orange peel of Chinese traditional medicine has been used for relieving stomach upset, skin inflammation, muscle pain, cough and dyspnea, and ringworm infections (Li, Gu, Dou, & Zhou, 2007). Identifying bioactive components present in different parts of citrus fruits has been carried out in an attempt to gain a deeper understanding of the correlation between diet, health benefits and reduced risk of diseases (Abad-Garcia, Garmon-Lobato, Berrueta, Gallo, & Vicente, 2012).

Identification of bioactive compounds, such as flavonoids (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2011a; Li, Lo, & Ho, 2006; Patil, Jayaprakasha, Chidambara Murthy, & Vikram, 2009) and limonoids (Hsu, Berhow, Robertson, & Hasegawa, 1998), in citrus fruits has been highlighted in many studies. Derivatives of

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different flavonoids in citrus species have been detected. These flavonoids can be divided into four groups: flavanones, flavones, flavonols and anthocyanins (Benavente-García, Castillo, Marin, Ortuño, & Del Río, 1997), and are mainly present as their glycosyl derivatives (flavanone *O*-glycosides, FGs) and polymethoxylated flavones (PMFs) (Gonzalez-Molina, Dominguez-Perles, Moreno, & Garcia-Viguera, 2010; Scordino et al., 2011). Of these, flavanones constitute the majority of flavonoids in citrus fruits such as sweet and sour oranges and their near relatives-mandarins (*Citrus reticulata*). Furthermore, FGs, such as naringin, neohesperidin and poncirin, are responsible for the citrus bitterness, which is mainly affected by their sugar moieties (Gonzalez-Molina et al., 2010; Li et al., 2014). Sugar moieties, such as rutinosides, hesperidin and narirutin in lemons, however, are tasteless (Garg, Garg, Zaneveld, & Singla, 2001; Peterson et al., 2006; Tomás-Barberán & Clifford, 2000). Citrus fruits also contain significant amounts of limonoids (Chinapongtitiwat, Jongaroontaprangsee, Chiewchan, & Devahastin, 2013; Hsu et al., 1998), which are usually present in neutral (noncarboxylated/aglycone) and acidic (carboxylated/glucoside) forms. The former are insoluble and bitter, while the latter are soluble and tasteless (Roy & Saraf, 2006). Investigation into both flavonoids and limonoids both revealed their broad spectrum of biological activities. For example, PMFs, which are exclusively found in the citrus genus, particularly in the flavedo part of mandarins and sweet oranges (Liu, Xu, Cheng, Yao, & Pan, 2012; Scordino et al., 2011), exhibited excellent anti-inflammatory, anti-carcinogenic and anti-atherogenic properties (Li et al., 2006).

Accumulation of metabolites, such as flavonoids and limonoids, in citrus has attracted the attention of a number of researchers, who mainly focused on juice in terms of its dietary property. Citrus juice is characterized by the presence of significant amounts of two flavanones, hesperidin and eriocitrin. Flavones with their polymethoxyl and di-*C*-glucosyl derivatives have also been recognized as its main flavonoid components. Based on LC–MS/MS analysis, the qualitative and quantitative flavonoid composition in sour orange (*Citrus aurantium* L.) and crude *Citrus limetta* Risso (limetta, Mediterranean sweet lemon) juice was elucidated (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2011c; Barreca et al., 2011a). Seven flavonoids, including three *C*-glucosides, two *O*-glycosides and two 3-hydroxy-3-methylglutaryl flavanone glycosides, and eight compounds, including *C*-glucosyl flavones, *O*-glycosyl flavone and the *O*-glycosyl flavanone were identified for the first time in the citrus juice. Recently, a number of metabolites were also tentatively identified in the fruits of two isogenic cultivars, an orange bud mutant, 'Hong Anliu', along with its parental wild-type, 'Anliu' (Pan, Li, Deng, & Xiao, 2013). Significant differences in metabolites of orange fruits between two isogenic orange genotypes provided further insights into understanding how the bud mutation in the orange impacts the physiological and biochemical processes of orange fruits. Accumulation of metabolites in different plants differed depending on the tissue and the stage of development (Dong et al., 2014, 2015). An examination of the qualitative and quantitative differences in both primary and secondary metabolites present in various tissues of citrus allow us to study their comprehensive profiling, thereby contributing towards a possible understanding of their functions.

Application of the non-targeted metabolomics method represented a promising method for the direct chemical screening of metabolites in plants. The structure and accumulation of more than 130 metabolites in the orange bud mutant with its parental wild-type species have also been illustrated (Pan et al., 2013). However, studies of the accumulation of various metabolites in citrus have so far covered only a few citrus varieties. Besides this, comprehensive analysis of the draft genome of sweet orange (*Citrus sinensis*) has been presented recently, and the genome represents a valuable resource for citrus functional genomic research (Xu et al., 2013). It has also been demonstrated that a genome-wide association study coupled with metabolomics analysis is a powerful tool for metabolic pathway reconstruction when using a number of diverse accessions (Chen et al., 2014). Germplasm resources based on their genetic variation have been widely used in many studies (Chen et al., 2014; Wen et al., 2014). Understanding the natural variation of non-targeted metabolites in citrus using germplasm resources thus allows an exploration of their genetic basis and the construction of various biosynthetic pathways. So far, naturally occurring variation of metabolic profiles in citrus has received little attention.

In this study, comprehensive metabolic profiling and natural variation analysis of both primary and secondary metabolites were carried out in citrus and more than 54 of them were identified/annotated and quantified using an LC–MS-based non-targeted metabolomics method. Tissue-specific accumulations were observed for various amino acids and flavonoids, together with their differential accumulations in five major *Citrus* species. Population structure of the citrus germplasm collection has also been characterized based on small-molecule profiles.

2. Materials and methods

2.1. Plant materials

To study the citrus metabolome, citrus fruits of different cultivars from different citrus production areas of China were analyzed. Fruits were collected for analyses at the commercial mature stage and the species were as follows: lemon (*Citrus lemon* [L.] Burm f.), pummelo (*Citrus grandis* (L.) Osbeck), grapefruit (*Citrus paradisi* Macf), sweet orange (*C. sinensis* [L.] Osbeck), and mandarin (*C. reticulata* Marcf.) (Supplementary Table S3).

Cultivars were harvested randomly from trees in three positions and pooled for each biological replicate. According to Li et al. (2014), the fruits were washed and the tissues of flavedo, albedo, segment membrane (SM) and juice sacs (JS) were separated with sterilized scalpels, then placed in liquid nitrogen and stored at −80 °C before being lyophilized in Heto-Holten (A/S, Allerød, Denmark).

2.2. Reagents and standards

HPLC-grade acetonitrile, acetic acid and methanol were purchased from Merck (Darmstadt, Germany); water was purified with a MilliQ ULTRA purification system (Millipore, Vimodrone, Italy). The internal standard used in this study is lidocaine which was bought from Shanghai New Asiatic Pharmaceuticals Co., Ltd (http://www.xinyapharm.com/).

Authentic standards were provided by Sigma-Aldrich, USA (http://www.sigmaaldrich.com/united-states.html). Standard stock solutions of flavonoids and limonoids were prepared in methanol, except for 4′,5,7-Trimethyl-3′,6-dimethoxy Apigenin (sinensetin), 4′,5,7-Trimethyl-6,8-dimethoxy Apigenin (tangeretin), methelEriodictyol 7-*O*-rutinoside (hesperidin) and methelEriodictyol 7-*O*-neohesperidoside (neohesperidin) which were dissolved with methanol-dimethyl sulfoxide (50:50, v/v), and the amino acids were prepared using deionized water as a solvent. All stock standard solutions were stored at −20 °C in darkness.

2.3. Sample preparation and extraction

The lyophilized tissues of different accessions were ground using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. A 100 mg powder was weighed and extracted overnight at 4 °C with 1.0 ml 70% aqueous methanol and pure