Protocols of Largely different carotenogenesis in two pummelo fruits with different flesh colors

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Abstract

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Protocol

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Step 1.

Materials and reagents

'CH' and 'FC' pummelo (*Citrus maxima*) fruits exhibiting typical characters at each corresponding fruit development stage were collected from the scientific research orchard of the Forestry Science Academy of Lishui, Zhejiang Province, in 2011. The fruits were collected at 45, 75, 100, 125 and 150 days postanthesis (DPA) (Fig 2). The fruits of 'CH' and 'FC' were commercially mature between 125 DPA and 150 DPA. The flavedo, albedo, SMs and JS of the fruits were carefully dissected [22]. The JS of Huanong Red (HR, red-fleshed), Chandler (QDC, red-fleshed), Hirado Buntan (HB, pink-fleshed), Wubu Red-fleshed (WBH, red-fleshed), Thai (T, pale yellow-fleshed), Kao Pan (KP, pale green-fleshed), Fenghuang (FH, pale green-fleshed) and Acidless (WS, pale green-fleshed) pummelo fruits at full maturity were collected from the National Citrus Breeding Center at Huazhong Agricultural University. Three biological replicates for each sample were prepared, with each biological replicate consisting of 6-8 fruits.

All the samples were treated with liquid nitrogen and then stored at –80°C. Some of them were lyophilized in a Heto Lyolab 3000 (Heto-Holten A/S, Allerød, Denmark) and homogenized to a powder in liquid nitrogen for the extraction of metabolites.

Extraction and determination of carotenoids

Carotenoids were extracted from 1.0 g of lyophilized samples and then measured in accordance with the methods of Liu *et al*. [22] and Lee [34]. The carotenoids were identified by comparing the retention times and the absorption spectra with those of authentic standards and were quantified by peak areas. The peak areas were converted to concentrations by comparisons with the authentic standards of known concentrations measured by high-performance liquid chromatography (HPLC). Authentic carotenoid standards of antheraxanthin, α-carotene, β-cryptoxanthin, all-*trans*-lutein, all-*trans*-violaxanthin and phytoene were purchased from CaroteNature (Lupsingen, Switzerland), while those of β-carotene and all-*trans*-lycopene together with standards of limonin and nomilin were purchased from Sigma Co. Ltd. (St. Louis, MO, USA).

Extraction and determination of limonin and nomilin in limonoid aglycones

In accordance with the methods of Li *et al*. [35], 3.0 g of lyophilized powder was extracted with 50 mL of dichloromethane via a FexIKAvarioControl system (IKA-Werke GmbH & Co. KG, Staufen, Germany). The extraction solution was collected and then dried under vacuum in a 5301 concentrator (Eppendorf,

Hamburg, Germany) after 15 cycles of Soxhlet extraction (approximately 3 h), after which 1 mL of acetonitrile was ultimately added. Prior to the HPLC analysis, 1 mL of sample tissue was filtered through a micropore film filter (0.22 μm).

With respect to HPLC analyses, the same instrument was used to analyze the carotenoids, while a C_{18} HPLC column (4.6×150 mm, 5 μm, Agilent, USA) was used for limonin and nomilin separation. For quantification, an isocratic elution model was applied with acetonitrile:10% methanol (40:60, v/v) at an elution speed of 1 mL/min and an injection volume of 20 μL (Manners, 2007). Both limonoids were detected at a wavelength of 210 nm under ambient temperature.

Extraction and determination of phytohormones

In accordance with the methods of Pan *et al*. [36] and Ding *et al*. [37], phytohormone extractions were carried out with 50 mg of lyophilized powder in 500 μL of chilled buffer (isopropanol:water:concentrated hydrochloric ac>

In accordance with the methods of Ma *et al*. [38], the phytohormone extracts were separated with HPLC (Agilent 1100, Agilent Technologies, Palo Alto, CA, USA) and measured via HPLC-ESI-MS/MS (API 3000 mass spectrometer, Applied Biosystems, Foster City, CA, USA), and the MS/MS conditions of each analysis were set in accordance with the methods of Pan *et al*. [36].

Authentic standards of IAA, ABA, SA and JA were purchased from OlChemIm (OlChemIm, Olomouc, Czech Republic).

RNA extractions and qRT-PCR analysis

In accordance with the methods of Gao *et al*. [39], total RNA was extracted and purified from the tissues of pummelo fruits. To avoid genomic DNA contamination, the total RNA was then treated with DNase I at 37°C for 1 h. The concentration and purity of the total RNA were then determined via a spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE, USA). The transcription levels of *CmDXS* (encoding 1-deoxy-D-xylulose-5-phosphate synthase), *CmDXR* (encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase), *CmPSY* (encoding phytoene synthase), *CmPDS* (encoding phytoene desaturase), *CmZISO* (encoding ζ-carotene isomerase), *CmZDS* (encoding ζ-carotene desaturase), *CmCRTISO* (encoding carotenoids isomerase), *CmLCYb1* (encoding lycopene β-cyclase gene1), *CmLCYB2* (encoding lycopene β-cyclase gene2), *CmLCYe* (encoding lycopene ε-cyclase gene), *CmBCH* (encoding β-carotene hydroxylase), *CmZEP* (encoding zeaxanthin epoxidase) and *CmNSY* (encoding neoxanthin synthase) were examined via quantitative real-time PCR (qRT-PCR) analysis. The primer sequences of the 8 genes above and of an endogenous control gene (*Actin*) were designed in accordance with the methods of Liu *et al.* [22], while those of *CmDXS*, *CmDXR*, *CmPSY* and *CmZISO*, which are listed in S1 Table, were designed with Primer Premier 5 (Premier, Canada).

Reverse transcription was performed as described by Liu *et al.* [22], while qRT-PCR was performed using an ABI 7500 Real Time System (PE Applied Biosystems, Foster City, CA, USA) in accordance with the methods of Liu *et al.* [22]. For each sample, the expression level of each biological replicate was determined by the mean value of at least three technical replicates. The mean threshold cycle (*Ct*) and the standard deviation (SD) for each sample were obtained in accordance with the methods of Gao *et al.* [39].

Amplification and sequencing of gene alleles

To amplify the whole coding sequences of the eleven genes identified mainly from the JS of both pummelo fruits, primers were designed in accordance with the methods of Liu *et al.* [22] and Gao *et al.* [39] or via Primer Premier 5; the primers are listed in S2 Table. The whole coding sequences of the *CmDXS* alleles could not be amplified, possibly due to their low expression levels in all fruit tissues. The whole coding sequences of at least 10 clones for each carotenogenesis gene were sequenced from the cDNA mainly from the JS in each pummelo fruit. In particular, with respect to *CmPSY*, *CmLCYb1*, *CmLCYb2*,

CmLYCe and *CmNSY*, nearly 50 clones of each gene in each pummelo fruit were obtained. However, due to the difficulties in obtaining new clones, only 15 clones of *CmZEP* were obtained for 'FC'.

A Phanta® Max Super-Fidelity DNA Polymerase Kit (Vazyme Biotech Co., Ltd) was used for allele amplification. The PCR products were electrophoresed and then screened on 1.5% agarose gels, after which they were recovered using an EZNA® Gel Extraction Kit (Omega, USA) in accordance with the manufacturer's instructions.

A vector was connected to the PCR amplification products using a Zero Background pTOPO-Blunt Simple Cloning Kit (Aidlab Biotechnologies Co., Ltd) in accordance with the manufacturer's instructions. For the selection of positive clones, 100 ng/μL ampicillin and universal M13 primers were used, and the positive clones were sent to Tshingke Biological Technology Co. (Wuhan, China) for sequencing.

The nucleotide sequences were aligned using MultAlin (http://multalin.toulouse.inra.fr/multalin/multalin.html). The amino acid sequences were deduced using Primer Premier 5, after which sequence alignment was carried out by ClustalW online (http://www.genome.jp/tools-bin/clustalw) and GeneDoc software. Phylogenetic analysis

Homologous gene sequences of each carotenogenesis gene were obtained from the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Their phylogenetic relationships were analyzed using MEGA 7.0.14. The maximum likelihood method was used to construct phylogenetic trees with 1000 replicate bootstrap supports, and values greater than 50% are shown at nodes.

Data analysis

The developmental stages of 'CH' and 'FC' (Fig 2) were processed via Photoshop software. The metabolite and gene expression profiles were processed by OriginPro 8. Average significant difference analyses were examined at a threshold of $P<0.05$ and were conducted with ANOVA in SAS (SAS Institute, Inc., USA).

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Step 2.