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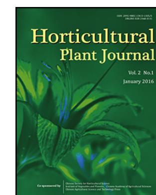

Characterization of Carotenoid Accumulation and Carotenogenic Gene Expression During Fruit Development in Yellow and White Loquat Fruit

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Abstract

Accumulation of carotenoids in peel and pulp of the yellow-fleshed loquat 'Zaozhong 6' (ZZ6) and the white-fleshed loquat 'Baiyu' (BY) were tracked during different fruit development stages, and the expression of 15 carotenogenic genes were analyzed. During loquat fruit ripening the fresh weight content of β-carotene in peel and pulp of ZZ6 increased gradually and peaked at the fully ripe stage, reaching 68.53 $\mu g \cdot g^{-1}$ FW in the peel and 11.92 $\mu g \cdot g^{-1}$ FW in the pulp. In BY, the content of β-carotene in the peel increased and peaked at the fully ripe stage, reaching 38.89 $\mu g \cdot g^{-1}$ FW, while it decreased in the pulp from the original 0.47 $\mu g \cdot g^{-1}$ FW and reduced to 0.29 $\mu g \cdot g^{-1}$ FW. The content of β-cryptoxanthin in the peel and pulp of ZZ6 and BY both increased steadily, and peaked at the fully ripe stage; however, the content of lutein decreased in the peel of ZZ6 and increased in the pulp, but in BY, it dropped and then rose in the peel. There was no significant change of β-cryptoxanthin in the pulp of BY. After the breaker stage, the mRNA levels of phytoene synthase (*PSY*) and chromoplast-specific lycopene β-cyclase (*CYCB*) were higher in the peel, while *CYCB* and β-carotene hydroxylase (*BCH*) mRNAs were higher in the flesh of ZZ6, compared with BY. The results showed that the expression level of *PSY*, *CYCB*, and *BCH* appeared to cooperatively regulate the accumulation of carotenoids.

Keywords: *Eriobotrya japonica*; loquat; carotenoids; gene expression

1. Introduction

Gene cloning and function research of carotenoids biosynthesis pathway have been the focus of recent research. The separation and functional verification of the phytoene synthase (*PSY*) gene in tomato began in 1987. The main chain pathways of carotenoids biosynthesis were elucidated in 1990. In 2000, 1-deoxy-D-xylulose-5-phosphate-synthase (DXS), the first synthase gene of the plant carotenoids biosynthesis, was considered to be a key enzyme for the synthesis of plant carotenoids and other terpenoid substances (Lois et al., 2000). The coding gene was first obtained from *Arabidopsis thaliana* (Mandel et al., 1996) and then isolated and functionally identified in pepper (Bouvier et al., 1998), mint (Lange and Croteau, 1999), and tomato (Lois et al., 2000). Lycopene is a branch point of the further anabolism of carotenoids. Lycopene can be cyclized by lycopene β-cyclase (LCYb) and lycopene ε-cyclase (LCYe) to synthesize α-carotene or cyclized by a lycopene β-cyclase (LCYb)/chromoplast-specific lycopene β-cyclase (CYCB) alone to synthesize β-carotene. Alphacarotene and β-carotene are hydroxylated to produce lutein and zeaxanthin, respectively, catalyzed by β-ring hydroxylase (BCH) and ε-ring hydroxylase (ECH). The other carotenogenic genes, such as 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXP) reductoisomerase (*DXR*), isopentenyl pyrophosphate synthase (*IDS*), lycopene cyclase (*LCY*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), have been cloned early and their functions were studied. A positive correlation was found between enhanced isoprenoid biosynthesis and accumulation of transcripts encoding DXR, which means that it plays a very important role in the carotenoid synthesis (Rodríguez-Concepción and Boronat, 2002). Research on tomato showed that *IDS* is limiting for

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carotenoids biosynthesis during tomato fruit ripening, and its expression might be coordinated with *DXS* and *PSY*. Transferring *IDS* into *A. thaliana* increased carotenoids by 50% (Botella-Pavía et al., 2004). Similarly, studies on the DXPS, PSY1, and PDS expression of tomato demonstrated that the expressions of these genes were significantly enhanced during maturation. For example the expression of *PSY1* increased more than 20 times, which resulted in a 10-fold increase in total carotenoid content (Giuliano et al., 1993; Lois et al., 2000). During fruit ripening, the expression of carotenoid biosynthetic genes in *citrus* fruit shows the same trend as in tomato: a simultaneous increase in the expression of genes (*PSY*, *PDS* and *ZDS*) leading to a massive carotenoids accumulation in the flavedo of 'Valencia' orange (Kato et al., 2004). However, recent researches showed that the carotenoids accumulation may be irrelevant to carotenogenic gene expression. Li et al. (2006) found that the cauliflower containing the *Or* gene confers a high level of β-carotene accumulation in flower buds, turning them orange, but β-carotene accumulation did not result from an increased capacity of carotenoid biosynthesis. Fu et al. (2012) studied plastids and plastid lipid–associated protein (PAP) expression at different ripening stages in 'Luoyangqing' and 'Baisha' loquat, and discovered differences in the quantity and structure of chromoplasts in flesh and peel, as well as PAP expression. Their results indicated that the inability to form chromoplasts in 'Baisha' flesh is the mostly likely explanation for the low carotenoids accumulation. Fu et al. (2014) reported that the failure of carotenoids accumulation in white-fleshed loquat can be due to the non-functional mutant *EjPSY2A*.

In the present study we used yellow-fleshed and white-fleshed loquat cultivars to study the carotenoid dynamics of their peel and flesh; as well as the expression of carotenegenic genes during fruit developmental stages. This study should help to clarify the molecular mechanism of the carotenoids accumulation of loquat and provide a theoretic foundation for scientific regulation of loquat carotenoid biosynthesis.

2. Materials and methods

2.1. Materials

'Zaozhong 6' (ZZ6, yellow-fleshed) and 'Baiyu' (BY, white-fleshed) loquat (*Eriobotrya japonica* Lindl.) were sampled from the Loquat Germplasm Resources Garden in College of Horticulture, South China Agricultural University (SCAU). The fruits were divided into 5 developmental stages: stage I, immature green, 92–98 d after full bloom (DAFB); stage II, breaker, 112–114 DAFB; stage III, degreening, 115–120 DAFB; stage IV, yellow mature, 118–122 DAFB; stage V, full mature, 124–128 DAFB (Zhang et al., 2013a). After the sample was collected, the top and the base of the fruit were quickly removed, the peel and pulp were separated and immediately frozen in liquid nitrogen and then stored at −70 °C for further research.

2.2. Carotenoid extraction and HPLC analysis

Carotenoids were extracted from fruits and analyzed by HPLC, according to a method previously described by Xiong et al. (2007). Components identification and quantitative analysis were done using Agilent 1200 HPLC-DAD analysis system, 5 μm C_{18} m reverse phase column (250 mm × 4.6 mm) and 20 mm × 4.6 mm C_{18} pre column, using external standard method. The chromatographic conditions refer to Hui's (2005). The compounds β-carotenoid, β-cryptoxanthin and lutein at different stages were detected. The standard samples of β-carotenoid, β-cryptoxanthin and lutein were purchased from Sigma Company.

2.3. Expression analysis of carotenogenic genes

Total RNA of peel and pulp from ZZ6 and BY at five stages was extracted from frozen samples following our previously published protocol (Zhang et al., 2013b). cDNA was synthesized according to the manual of M-MLV reverse transcriptase (TaKaRa).

Based on the primer design principle of quantitative real-time PCR (qRT-PCR), *DXS*, *DXR*, *IDI*, *PSY*, *LCYb*, *LCYe*, *BCH* and *ECH* were cloned and sequenced (GenBank number: JX097047, JX089590, JX097049, JX097048, JX089591, JX097050, JX097051 and JX097052, respectively). Primers for these genes (Table 1) were designed by Primer premier 6.0. And the amplification efficiency (E) was detected by qRT-PCR, and the standard curve was produced to check whether the amplification efficiency is in the range of 90%–110%. The primers *PDS*, *ZDS*, *CRTISO*, *CYCB*, *ZEP*, *VDE*, *CCD* stems were from Fu et al. (2012).

The qRT-PCR reactions were performed in a total volume of 20 μL, including 0.5 μL of each primer (10 $\mu mol \cdot L^{-1}$), 1.0 μL cDNA, 8 μL ddH_2O, and 10 μL of SsoAdvanced SYBR Green Supermix (BIO-RAD) on an iQ5 BIO-RAD fluorescence qRT-PCR. The PCR programme was initiated with a preliminary step of 30 s at 94 °C, followed by 40 cycles at 94 °C for 15 s, 50 °C for 15 s, and 72 °C for 25 s. Three replicates were performed with ddH_2O as the negative control. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. The data were analyzed by

Table 1 Primers for quantitative real-time PCR

Gene	Upstream primers (5′–3′)	Downstream primers (5′–3′)	Amply sequence length/bp
DXS	CATAGATGACCGACCAAGT	AGGAGGCAGCCAAACAG	179
DXR	ATCCGCAGTCCATTATACATTC	CAGCAGCATAGGCAAGGT	231
IDI	TCGCCTTGGTTCAGACTTG	AAGCCGCAATCAAAGTTTCC	147
PSY	ACATTCAGCCCTTCAGA	ATCCAACAGTTCCAGCA	117
LCYb	CGGTATGGCTATTCTGCTTA	AGTCATCCTAATCTCTATCCTG	251
LCYe	TTCCTCGGCTCTACATTGT	GGCAGTGACCTCATCTCTT	209
BCH	GCCTTGTTCCTGGTCTTTGTT	ATGAGCCGCAGCAACCTT	152
ECH	AGTTTGCTCTGCTGGAAG	CGAAGACGAGGATGATGT	185

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