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Changes in carotenoid profiles and in the expression pattern of the genes in carotenoid metabolisms during fruit development and ripening in four watermelon cultivars

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ABSTRACT

Changes in carotenoid profiles during fruit ripening were investigated in four watermelon cultivars: red-fleshed "CN66", pink-fleshed "CN62", yellow-fleshed "ZXG381" and white-fleshed "ZXG507". The expression pattern of twelve genes (*GGPS*, *PSY*, *PSY-A*, *PDS*, *ZDS*, *CRTISO*, *LCYB*, *CHYB*, *ZEP*, *NCED1*, *NCED2* and *NCED3*) was analysed. In "CN66" and "CN62", lycopene appeared at 12 DAP and became a main carotenoid increased at the later stages. The transcript levels of carotenogenic genes in "CN66" sharply increased during 18–30 DAP, and concomitantly, fruit accumulated the massive amounts of carotenoids. In "ZXG381", violaxanthin and lutein contents were positively correlated, respectively, with *CHYB* and *ZEP* transcript levels during fruit ripening. The trace amounts of carotenoids in "ZXG507" were accompanied with the low transcript levels of most biosynthetic genes. The results suggest that differential transcriptional regulation of carotenoid metabolic genes is very important in determining the amount and type of specific carotenoids accumulated during fruit development and ripening.

1. Introduction

The watermelon (*Citrullus lanatus*), member of cucurbitaceae family, is the third most popular fruit vegetable in the world (Guner & Wehner, 2004). Flesh colour is an agronomically important trait of watermelon. Carotenoids are responsible for the different flesh colours in watermelon fruit. Their biosynthetic and catabolic pathways have been determined in higher plants (Cazzonelli & Pogson, 2010; Hirschberg, 2001; Taylor & Ramsay, 2005). In plastids, geranylgeranyl pyrophosphate (GGPP) is synthesized in a reaction catalysed by GGPP synthase (GGPS) enzymes via methylerythritol phosphate (MEP), isopentenyl pyrophosphate (IPP), and dimethylallyl pyrophosphate (DMAPP). Two molecules of GGPP (C_{20}) are converted to colourless 15-*cis*-phytoene (C_{40}) by phytoene synthase (PSY) in the first committed reaction of the carotenoid pathway. Subsequently, four additional double bonds are introduced by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), producing phytofluene, ζ-carotene, and lycopene. ζ-Carotene isomerase (ZISO) and carotenoid isomerase (CRTISO) are required to catalyse the poly-*cis*-carotenoids to all-*trans*-carotenoids during desaturation. The cyclisation of lycopene is the branching point of this pathway, producing α-carotene by lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) or β-carotene by lycopene β-cyclase (LCYB) alone. Thereafter, lutein and zeaxanthin are produced by ε-carotene hydroxylase (CHYE) and β-carotene hydroxylase (CHYB). Zeaxanthin is mediated by zeaxanthin epoxidase (ZEP) to form violaxanthin via antheraxanthin and then is converted into neoxanthin. Carotenoids are metabolized to apocarotenoids through symmetrical 9–10 and 9′-10′ cleavages catalysed by carotenoid cleavage dioxygenases (CCDs). Nine-*cis*-epoxycarotenoid dioxygenases (NCEDs) catalyses the cleavage of 9-*cis*-violaxathin or 9′-*cis*-neoxanthin to yield epoxy-carotenal (C_{25}) and xanthoxin (C_{15}), a precursor of abscisic acid (ABA) (Auldridge, McCarty, & Klee, 2006; Walter & Strack, 2011).

Red-fleshed watermelons consisted mostly of lycopene (Perkins-Veazie, Collins, Davis, & Roberts, 2006; Tadmor et al., 2005). Small amount of phytoene, phytofluene, ζ-carotene α-carotene, lutein, zeaxanthin, and violaxanthin were also reported (Liu et al., 2012) in red-fleshed cultivar. The major pigments in orange-fleshed cultivar were prolycopene, phytoene and ζ-carotene, however, an orange-fleshed cultivar "NY162003" contained mostly β-carotene and traces of lycopene (Tadmor et al., 2005). In canary yellow-fleshed and pale yellow-fleshed cultivars, the predominant carotenoid was

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neoxanthin, followed by violaxanthin and neochrome (Bang, Davis, Kim, Leskovar, & King, 2010). But another study showed that violaxanthin and luteoxanthin were the dominant carotenoids in the yellow-fleshed cultivar (Liu et al., 2012). It is quite evident that these results reported are not consistent completely in carotenoid composition of yellow-fleshed watermelon. This shows that carotenoid composition in yellow-fleshed watermelon has not been completely understood yet. Moreover, information is lacking about changes in carotenoid profiles during watermelon fruit development and ripening.

The genes involved in carotenoid metabolism have been recently studied in watermelon. The expression of carotenogenic genes (*PSY-A*, *PSY-B*, *PDS*, *ZDS*, *CRTISO*, *LCYB*, *CHYB* and *ZEP*) was measured by semi-quantitative RT-PCR only in different coloured watermelon ripening fruits (Bang, 2005). *PSY-A* was expressed in various coloured fleshes, but *PSY-B* transcript was not found in any coloured fleshes. Three single nucleotide polymorphism (SNPs) in the coding region of *LCYB* were identified between canary yellow and red watermelon, and showed perfect co-segregation with flesh colour phenotypes. The results suggested that the SNPs of *LCYB* may be the genetic determinant for canary yellow or red flesh colour (Bang, Kim, Leskovar, & King, 2007; Bang et al., 2010). In our laboratory the transcript levels of nine genes involved in carotenoid metabolism (*PSY*, *PDS*, *ZDS*, *CRTISO*, *LCYB*, *CHYB*, *NCED1*, *NCED2* and *NCED3*) were detected by dot blot hybridization, respectively, at 10, 20, and 30 day after pollination based on 30 day fruit development and ripening period (Kang, Zhao, Hou, & Tian, 2010). There were significant differences in *LCYB* expression patterns between yellow and red flesh cultivars. Lycopene accumulation in the red cultivar was considered to be related to significantly different *LCYB* transcript levels, which was not in accordance with the Bang's report. Low carotenoid content may be due to high transcript levels of *NCEDs* in the yellow cultivar. Transcriptome analysis during fruit development and ripening was recently performed in red-fleshed cultivar "Dumara" (Grassi et al., 2013) and inbred line 97103 (Guo et al., 2011). The expression of the genes in carotenoid metabolism pathway was analysed at four ripening stages (white, ~10 DAP; white–pink, ~18 DAP; pink, ~28 DAP; and red-ripe, ~34 DAP). Changing levels displayed in the gene expression during fruit ripening generated a metabolic flux towards carotenoid synthesis (Grassi et al., 2013). It has been shown that the expression patterns of *PSY* and *GGPS*, key enzyme genes in carotenogenic pathway, have an important bearing on the balance between isoprenoid pathway precursor supply and utilisation (Fraser, Enfissi, & Bramley, 2009). However, much less is known about the effects of their expression on carotenoid accumulation in other colour-fleshed watermelon fruits except red fruits. Furthermore, the cause on trace amounts of carotenoids in white-fleshed cultivar has yet not been well defined.

In the present study we investigated more detailed changes in carotenoid profiles by HPLC method and examined the expression pattern of twelve genes involved in carotenoid metabolism during fruit development and ripening in four flesh-coloured watermelon cultivars by quantitative real-time PCR and elucidated the possible relationship between carotenoid accumulation and the transcript levels of the specific genes involved.

2. Materials and methods

2.1. Plant materials

Four different coloured watermelon cultivars, red-fleshed "CN66"; pink-fleshed "CN62"; yellow-fleshed "ZXG381" and white-fleshed "ZXG507" (Fig. 1), were obtained from The National Mid-term Gene Bank for Watermelon and Melon (Zhengzhou, Henan, China). Seeds of each watermelon cultivar were sown at the same time in greenhouse. These cultivars showed uniform fruit development stages. Freshly opened female flowers were hand-pollinated and tagged to identify fruit with a known development age. Generally, it takes 36 d for the development of watermelon fruit. Fruits were harvested every 6 d after pollination until the fully ripe stage. At least three fruits at each developmental stage were collected for various measurements. Fruits were quickly delivered to the laboratory and cut longitudinally from the stem-end to the blossom-end. Flesh samples were taken from the heart area of each fruit, deseeded and homogenised. The colour of flesh samples were measured immediately, then the samples were frozen in liquid nitrogen, and stored at −80 °C until carotenoid analysis and RNA extraction.

2.2. Flesh colour measurement

The colour changes during fruit development and ripening of the four watermelon cultivars were quantified through CIELAB parameters (L^*, a^*, and b^*) measured by a spectrocolorimeter (Model SP60, X-rite, USA), equipped with D65 optical sensor and using an observation angle of 10°. ΔE^* represents the total colour difference of the flesh colour in each period compared with that at 6 DAP (day after pollination), and was calculated using the equation: $\Delta E^* = (\Delta a^{*2} + \Delta b^{*2} + \Delta L^{*2})^{1/2}$ (Zepka, Borsarelli, Ma, & Mercadante, 2009).

2.3. Carotenoid extraction and HPLC analysis

Carotenoids were extracted from 5 g of homogenised flesh with petroleum ether and acetone (2:1, v/v), as described before (Kang et al., 2010). The pooled organic phase was washed twice with distilled water. The petroleum ether extract was reduced to dryness at <40 °C and kept under a nitrogen atmosphere at −80 °C until HPLC analysis.

HPLC analysis of carotenoids was carried out using a Waters liquid chromatography system equipped with a 1525 pump and 2998 photodiode array detector. Data were analysed with Empower Software (Waters, USA). Samples, supplied as dried extracts, were redissolved in mobile phase (acetonitrile: methanol: dichloromethane, 7:2:1, v/v) and filtered through 0.45 μm membranes. An aliquot (10 μL) of samples was injected into the HPLC C_{18} column (250 mm × 4.6 mm, 5 μm, Waters Spherisorb) (column temperature 25 °C, flow rate 1.0 mL/min). Compounds were detected at 280, 366 and 450 nm. Carotenoids were identified on the basis of their retention time and absorption spectra compared with authentic standards (lycopene and β-carotene from Sigma, violaxanthin and lutein from CaroteNature) and were quantified according to their standard curves. Other carotenoids without authentic standards were identified in the light of their spectral information published in the handbook (Britton, Liaaen-Jensen, & Pfander, 2004) and were quantified by their absorption coefficients (phytoene, 915 at 286 nm; phytofluene, 1580 at 347 nm; and ζ-carotene, 3550 at 400 nm).

2.4. Total RNA isolation and cDNA synthesis

Total RNA was extracted from the homogenised flesh using the total RNA isolation kit (Tiangen, China) according to manufacturer's instructions. Extracted RNA was treated by RNase-free DNase I (Takara, Japan) at 37 °C for 30 min to eliminate genomic DNA. UV absorption spectrophotometry and gel electrophoresis were performed to test RNA purity and quality. cDNA was synthesized with reverse transcriptase M-MLV (RNase H^-) following the manufacturer's procedures (Takara, Japan). The reaction mixture contained 1.0 μg total RNA, 1 μL oligo$(dT)_{18}$ primer (50 μM), 1 μL dNTP mixture (10 mM), 20 U RNase inhibitor, 200 U RTase M-MLV, 4 μL