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Transcriptional regulation of lycopene metabolism mediated by rootstock during the ripening of grafted watermelons

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ABSTRACT

Rootstocks have comprehensive effects on lycopene accumulation in grafted watermelon fruits. However, little is known about lycopene metabolic regulation in grafted watermelon. To address this problem, parallel changes in lycopene contents and the expression of its metabolic genes were analyzed during the fruit ripening of nongrafted watermelon and watermelon grafted onto bottle gourd, pumpkin, and wild watermelon. Results showed that rootstocks mediated the transcriptional regulations of lycopene accumulation in different ways. Bottle gourd and wild watermelon promoted lycopene accumulation in grafted watermelon fruits by upregulating the biosynthetic genes *phytoene synthase* (*PSY*) and ζ-carotene desaturase (*ZDS*), and downregulating the catabolic genes *β-carotene hydroxylase* (*CHYB*), *zeaxanthin epoxidase* (*ZEP*), *9-cis-epoxycarotenoid dioxygenase* (*NCED*), and *carotenoid cleavage dioxygenase* (*CCD*). However, pumpkin did not affect lycopene accumulation by upregulating both biosynthetic and catabolic genes. The rootstock-dependent characteristic of lycopene accumulation in grafted watermelon fruits provided an alternative model for investigating lycopene metabolic regulation.

1. Introduction

Watermelon (*Citrullus lanatus*) is an economically important horticultural crop with large fruits for fresh consumption. Aside from its delicious flavours, watermelon is also rich in nutritional compounds, such as sugar, carotenoids, citrulline, and flavonoids. Thus, watermelon is recognized as a functional food that provides important nutritional and bioactive benefits (Perkins-Veazie, Davis, & Collins, 2012). Carotenoids are not only micronutrients in human foods but they are also colouring agents in watermelon. Synthesized and accumulated carotenoids in the chromoplasts of watermelon give the fruits their different flesh colours. For example, white-fleshed watermelon has small amounts of carotenoids, yellow-fleshed watermelon contains mainly violaxanthin and/or neoxanthin, orange-fleshed watermelon consists of more β-carotene, and red- and pink-fleshed watermelons are pigmented by lycopene (Lewinsohn et al., 2005; Liu et al., 2012; Tadmor et al., 2005; Yuan, Zhang, Nageswaran, & Li, 2015).

Lycopene is a non-provitamin A-active carotenoid with valuable health-promoting benefits. It is a powerful antioxidant and free-radical antagonist, which prevents prostate cancer, breast cancer, atherosclerosis and associated coronary artery disease (Sharma & Goswami, 2011). Tomato and watermelon are the main sources of dietary intake of lycopene. Red-fleshed watermelons contain more lycopene per unit fresh weight than do fresh tomatoes (Perkins-Veazie, Collins, Davis, & Roberts, 2006). The high content of lycopene attracts the attention of consumers and farmers. Understanding lycopene metabolism and its regulation is fundamental for enhancing its contents in watermelon fruits.

Carotenoid metabolism pathways have been well established in horticultural crops (Yuan et al., 2015). The metabolic pathway of lycopene and its related genes were also studied in watermelon by comparative genomics and transcriptome analysis (Grassi et al., 2013). The lycopene biosynthetic pathway starts with the condensation of two geranylgeranyl diphosphates by phytoene synthase (PSY) to produce the 15-*cis*-phytoene. Then, through a series of desaturations by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) and isomerizations by ζ-carotene isomerase (Z-ISO) and carotene isomerase (CRTISO), phytoene is converted through the *trans* configuration, yielding red-coloured all-*trans*-lycopene. In the lycopene catabolic pathway, all-*trans*-lycopene is cyclized by lycopene cyclase (LCY) to produce the

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α-carotene and β-carotene branches. These carotenes are further hydroxylated by β-carotene hydroxylase (CHYB) to produce lutein and zeaxanthin. Zeaxanthin epoxidase (ZEP) hydroxylates zeaxanthin to yield antheraxanthin and then violaxanthin. The carotenoids are degraded by carotenoid cleavage dioxygenase (CCD) or 9-*cis*-epoxycarotenoid dioxygenase (NCED) to produce apocarotenoids (Grassi et al., 2013; Nisar, Li, Lu, Khin, & Pogson, 2015; Yuan et al., 2015). Transcriptome analysis showed that *PSY*, *PDS*, *CRTISO*, *ZDS*, *CHYB*, *ZEP*, *NCED*, and *CCD* were differentially expressed during the ripening of red-fleshed watermelon (Grassi et al., 2013). However, recent transcriptome analysis showed that only *PSY*, *CHYB*, *NCED*, and *CCD* were differentially expressed during watermelon fruit ripening (Guo et al., 2015). The expression profiles of these genes were also surveyed during the fruit ripening of red-, pink-, yellow-, and white-fleshed watermelons (Lv, Li, Liu, Gu, & Zhao, 2015). According to the results, transcriptional regulation is a major mechanism that regulates lycopene metabolism during watermelon fruit ripening (Grassi et al., 2013; Kang, Zhao, Hou, & Tian, 2010; Lv et al., 2015).

Grafting is an efficient method for controlling soil-borne diseases and therefore, it has become widely applied in watermelon production. Bottle gourd (*Lagenaria siceraria*), pumpkin (*Cucurbita* spp.), and wild watermelon (*Citrullus lanatus* var. *citroides*) are the commonly used rootstocks in watermelon grafting (King, Davis, Zhang, & Crosby, 2010; Kong et al., 2014). However, recent studies have demonstrated that grafting affected both the quality and the ripening behaviour of watermelons (Soteriou, Kyriacou, Siomos, & Gerasopoulos, 2014). Lycopene content is one of the major criteria used in ranking fruit quality of red-fleshed watermelons. The effects of grafting on lycopene accumulation in watermelon fruits were also widely examined. Higher, lower, and unchanged lycopene contents were observed in the fruits of grafted watermelons compared with nongrafted watermelons in different studies (Petropoulos et al., 2014; Proietti et al., 2008; Soteriou et al., 2014; Turhan, Ozmen, Kuscu, Serbeci, & Seniz, 2012; Wimer, Inglis, & Miles, 2015). These results demonstrate that lycopene content in grafted watermelon fruit is a rootstock-scion combination-dependent trait. However, how lycopene metabolism is regulated in grafted watermelon fruit is still unknown.

The negative effect of grafting on lycopene content impedes the application of the grafting technique in watermelon production and influences the perception of consumers. Thus, measures must be taken to eliminate the negative effects of grafting, and the beneficial effects of grafting must be used to enhance lycopene contents in grafted watermelons. Understanding the regulation of lycopene metabolism in grafted watermelon fruits is a prerequisite for achieving this goal. In this study, parallel changes of lycopene contents and expression of lycopene metabolic genes were investigated during the ripening of watermelons that were grafted onto different rootstocks, with the aim of clarifying the regulating roles of rootstocks in lycopene metabolism in grafted watermelon fruits. The results are not only helpful for understanding the regulatory mechanism of lycopene metabolism, but will also provide guidance for rootstock selection and breeding, aiming to improve watermelon quality by grafting.

2. Materials and methods

2.1. Plant materials and treatments

The diploid seeded watermelon Zaojia (*Citrullus lanatus* var. *lanatus*) was used as the scion. It is a popular red-fleshed cultivar with maturity of 30 days after pollination (DAP). Commercial rootstock cultivars representing the three genera of watermelon rootstocks were used in this study, namely, Jingxinzhen No.1 (*Lagenaria siceraria*), Qingyanzhen No.1 (*Cucurbita maxima* × *C. moschata*), and Yongshi (*Citrullus lanatus* var. *citroides*). The hole-insertion method was used for grafting (Hassell, Memmott, & Liere, 2008). When Zaojia was grafted onto Jingxinzheng No.1, the grafting combination was abbreviated as Z/J. Similarly, Z/Q, and Z/Y indicated that Qingyanzhen No.1 and Yongshi were used as rootstocks, respectively. The nongrafted Zaojia was used as the control and abbreviated as Z. At the third true leaf stage, each seedling was transplanted into a cylinder-shaped pot containing 18 L substrate (peat: vermiculite: perlite = 1:1:1, v/v) and grown in a plastic greenhouse. A randomized complete block design with three replications was adopted for the four treatments. Each plot consisted of a single row of 30 watermelon plants spaced 50 cm apart. The distance between two rows was 150 cm. The plants were irrigated with full strength Hoagland nutrient solution through a drip-irrigation system with two emitters per plant and a flow rate of 2 L/h. The same amount of irrigation solution was applied for each plant, which was 0–3 L per day, depending on plant growth stage and environmental conditions. Disease and pest controls were applied, following commercial production practices. Only the main vine of each plant was allowed to grow and trained vertically. Pollinations were performed on the same day for the grafted and nongrafted plants. The pollinated female flowers were tagged, and only one fruit was allowed to develop on each plant. Two fruits in each plot were randomly sampled at 10, 18, 23, 27, 30, and 35 DAP. Flesh tissues at these sampling times exhibited the similar phenotypic traits of the four critical development stages of immature white (10 DAP), white–pink flesh (18 DAP), red flesh (23 and 27 DAP), fully ripe or overripe (30 and 35 DAP) that were described previously (Guo et al., 2011, 2015). The harvested fruits were cut longitudinally. Flesh tissues were taken from the centres of the two fruits in the same plot and mixed together. All the samples were immediately frozen in liquid nitrogen and then stored at −80 °C for lycopene and RNA extractions.

2.2. Lycopene extraction and measurement

Lycopene was extracted and measured according to the previously described method (Liu et al., 2012). Briefly, the frozen sample was ground into fine powder in liquid nitrogen and 3 g of powder were homogenized with 15 ml of extraction solvent (hexane/acetone/ethanol, 2:1:1, containing 0.01% butylated hydroxytoluene). After centrifugation at 4000×*g* for 10 min, the supernatant was washed with saturated NaCl solution and concentrated to dryness. The residue was then dissolved in 2 ml of methyl *tert*-butyl ether (MTBE). The solution was treated with an additional 2 ml of MTBE with 1 ml of KOH: water: methanol (10 g: 25 ml: 75 ml), and kept overnight in darkness for saponification. The sample was then rinsed with saturated NaCl solution until it became neutral. After evaporation under vacuum, the residue was dissolved in 1 ml of MTBE. The solution was then analyzed by a Waters 1525 reversed phase HPLC with a C_{30} carotenoid column. Lycopene was identified by comparing the retention time and absorption spectra with the authentic standard of all-*trans* lycopene (Sigma–Aldrich, USA) and quantified by the standard curve. Two technical replicates were performed for each biological replication.

2.3. Gene expression analysis

In this study, lycopene metabolic genes identified for differential expression in watermelon fruits through transcriptome analyses, namely, biosynthetic genes *PSY*, *PDS*, *CRTISO*, and *ZDS*, and catabolic genes *CHYB*, *ZEP*, *NCED1*, and *CCD1*, were selected (Grassi et al., 2013; Guo et al., 2015). Gene-specific primers were designed by Primer3Plus with the product size ranging from 70 to 160 bp (Untergasser et al., 2007). The generated primer pairs