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Regulation of carotenoid metabolism in response to different temperatures in citrus juice sacs *in vitro*

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ABSTRACT

The present study investigated the regulation of carotenoid accumulation in response to temperatures (10, 20, and 30 °C) in citrus juice sacs *in vitro*. Carotenoid accumulation was induced at 10 °C, but was not affected at 30 °C in the Satsuma mandarin, Valencia orange, and Lisbon lemon. In the Satsuma mandarin and Valencia orange, carotenoid accumulation at 10 °C was transcriptionally regulated by carotenoid biosynthetic genes. The up-regulated expression of nine carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYe*, *CitHYb*, *CitHYe*, *CitLCYb2*, *CitZEP* and *CitVDE*) in the Satsuma mandarin and seven carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitZEP* and *CitVDE*) in the Valencia orange was responsible for enhancing the content of carotenoid at 10 °C. In contrast to 10 °C, no marked changes were observed in carotenoid metabolic gene expression, and carotenoid accumulation was not significantly affected at 30 °C in the Satsuma mandarin and Valencia orange. In the Lisbon lemon, the expression of carotenoid biosynthetic and catabolic genes was involved in the regulation of carotenoid accumulation in response to temperature. The up-regulated expression of biosynthetic genes (*CitPSY* and *CitVDE*) and down-regulated expression of catabolic genes (*CitNCED2* and *CitNCED3*) increased the content of carotenoid at 10 °C in the Lisbon lemon. In contrast to 10 °C, the expression of most carotenoid biosynthetic and catabolic genes was simultaneously up-regulated, whereas carotenoid levels remained unchanged and were constantly low at 30 °C in the Lisbon lemon.

1. Introduction

Carotenoids are natural isoprenoid substances that play an important role in many physiological processes in plants (Peñuelas and Munné-Bosch, 2005; Bartley and Scolnik, 1995; and Bouvier et al., 1998). In plant tissues, carotenoids are major agronomic quality for a number of fruits and vegetables, such as the provision of a diverse range of pigments, aromas, and scent compounds (Howitt and Pogson, 2006; and Yuan et al., 2015). In addition, the important roles of carotenoids in human health cannot be neglected. The antioxidant activities of carotenoids have attracted attention for a long time. Carotenoids have been suggested to reduce the damaging effects of oxidative stress in various chronic diseases, such as eye-related disorders, cardiovascular diseases, and cancers (Männistö et al., 2004; Krinsky and Johnson, 2005; and Rao and Rao, 2007).

Carotenoid metabolism has been extensively studied in citrus fruits, and an almost complete metabolic pathway has been elucidated. Previous studies reported that the accumulation of carotenoid was transcriptionally regulated by genes involved in carotenoid metabolic pathway (Kato et al., 2004; Rodrigo et al., 2004; Kato et al., 2006; Zhang et al., 2012; Ma et al., 2013; and Ma et al., 2016). As shown in Fig. 1, the first step in the biosynthesis pathway is the conversion of two geranylgernyl pyrophosphate (GGPP) molecules derived from the MEP (2-*C*-methyl-*D*-erythritol 4-phosphate) pathway to form phytoene, catalyzed by phytoene synthase (PSY). A series of desaturation by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) converts phytoene into lycopene. The cyclization of lycopene by lycopene-β-cyclase (LCYb) and lycopene-ε-cyclase (LCYe) produces a diverse range of carotenoids. Lycopene is cyclized with one ε-ring and one β-ring by LCYe and LCYb to produce α-carotene. α-Carotene is then converted into lutein catalyzed by β-ring hydroxylase (HYb) and ε-ring hydroxylase (HYe). In addition, lycopene may be cyclized with two β-rings by

Abbreviations: ABA, abscisic acid; HYb, β ring-Hydroxylase; HYe, ε ring-Hydroxylase; LCYb, lycopene-β-cyclase; LCYe, lycopene-ε-cyclase; NCED2, nine-*cis*-epoxycarotenoid dioxygenase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase

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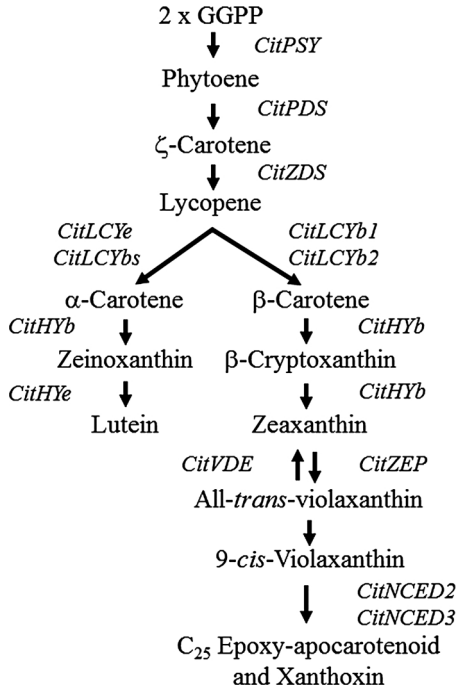

Fig. 1. Carotenoid metabolism pathway in citrus fruits.

LCYb to produce β-carotene. β-Carotene is then hydroxylated by HYb to form β-cryptoxanthin and zeaxanthin. A reversible reaction, called xanthophyll cycle, subsequently occurred. Zeaxanthin is converted into all-*trans*-violaxanthin and 9-*cis*-violaxanthin catalyzed by zeaxanthin epoxidase (ZEP). At this step, the reversal of all-*trans*-violaxanthin into zeaxanthin may be activated by violaxanthin de-epoxidase (VDE). Futhermore, 9-*cis*-violaxanthin may be enzymatically catabolized by 9-cis-epoxycarotenoid dioxygenases (NCED) to produce C_{25} epoxy-apocarotenoid and xanthoxin, which is modified for the synthesis of the phytohormone, abscisic acid (ABA).

Temperature is known to be one of the most important environmental factors affecting the accumulation of bioactive compounds in higher plants (Lee et al., 2005; Krumbein et al., 2012; Manera et al., 2012; and Zhang et al., 2012). In citrus fruits, the biosynthesis of carotenoid during fruit development was found to be strongly influenced by temperature both in the peel and pulp (Iglesias et al., 2007; Alquézar et al., 2008; and Porras et al., 2014). Manera et al. (2012 and 2013) reported that the color change in the peel of lemon fruits was induced by low field temperature. In contrast to low temperature, citrus fruits did not normally accumulate carotenoid and produced greenish-pale color fruits under high field temperature (Joseph Ahrens and Barmore, 1986; Goldschmidt, 1988; Iglesias et al., 2007; and Tadeo et al., 2008). In addition, variations in the content of carotenoids in citrus pulp were also observed among different cultivated regions. The relatively low day/night temperature in Mediterranean was found to be optimum for carotenoid synthesis, whereas the higher temperature in tropical regions inhibited carotenoid synthesis in citrus fruits (Mouly et al., 1999a; and 1999b; and Dhuique-Mayer et al., 2009). These finding suggested that the mechanism underlying the biosynthesis and accumulation of carotenoid in citrus fruits are sensitive to temperature.

The effects of temperature on carotenoid accumulation in citrus fruits have attracted attention for a long time. However, the underlying regulatory mechanisms have not yet been clearly understood (Meredith and Young, 1969; Stewart and Wheaton, 1971; and Lee and castle, 2001). Recent studies that investigated the effects of temperatures on carotenoid accumulation during the ripening process in citrus fruits have been limited by the difficulties associated with controlling temperature in the open field. In our previous study, we successfully established an *in vitro* culture system using citrus juice sacs, which is an efficient technique for controlling undesirable variations during the experimental period. By using an *in vitro* culture system, the effects of temperatures on carotenoid accumulation and the expression of carotenoid metabolic genes were investigated in citrus juice sacs at three different temperatures (10, 20, and 30 °C) in the present study. A better understanding of carotenoid regulation in response to different temperatures during the fruit maturation process will led to novel approaches in the molecular breeding of carotenoid biosynthetic pathway in citrus fruits in future research.

2. Materials and methods

2.1. Plant materials

In the present study, three citrus species with different carotenoid contents and compositions were used as a plant materials: Satsuma mandarin (*Citrus unshiu* Marc), Valencia orange (*C. sinensis* Osbeck), and Lisbon lemon (*C. limon* Burm.f.). The fruits with diameters of approximately 4–5 cm at the immature green stage were randomly harvested from citrus trees. The Satsuma mandarin was harvested from Fujieda Farm, Shizuoka, Japan. The Valencia orange and Lisbon lemon were harvested from NARO Institute of Fruit Science, Department of Citrus Research, Okitsu, Shizuoka, Japan.

2.2. In vitro culture system and temperature treatments

The *in vitro* culture system was performed in accordance with a previously described method (Zhang et al., 2012). Murashige and Skoog (MS) medium was used in the culture system. Medium was supplemented with sucrose (10% w/v) and agar (1% w/v), and pH was adjusted to 5.7. Medium was sterilized using an autoclave. Juice sacs were excised from citrus fruits and placed with the endocarp side up on 10 mL of medium in culture tubes (22 × 120 mm). Citrus juice sacs were cultured at 20 °C during the first two weeks. They were then exposed to different temperatures of 10 °C, 20 °C, and 30 °C for another two weeks in the culture system. Juice sacs were sampled twice, at the second and fourth weeks, in culture system. They were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Carotenoid extraction and quantification

Carotenoid content was measured by HPLC in the three replications with the method described by Kato et al. (2004). The content of β-carotene, β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and lutein were investigated in the juice sacs *in vitro* of the three citrus species. Juice sac samples were homogenized with extraction solvent (hexane-acetone-ethanol, 50:25:25, v/v/v) containing magnesium carbonate basic and centrifuged at 4000 rpm for 20 min. The supernatant containing hexane and the pigment was evaporated to dryness. The dry sample was re-suspended in diethyl ether containing 0.1% (w/v) butylated hydroxytoluene (BHT) and sponified overnight using 20% (w/v) methanolic KOH. After sponification, NaCl-saturated water was added to eliminate water soluble compounds. Anhydrous Na_2SO_4 was added to eliminate residual water from the extract. Retained carotenoids were eluted from anhydrous Na_2SO_4 by diethyl ether, and diethyl ether was subsequently evaporated to dryness. Carotenoid residues were then dissolved in TBME : methanol (1:1, v/v) containing 0.5% (w/v) BHT.

An aliquot (20 μL) was injected into HPLC with a reverse-phase HPLC system (Jasco) fit with a YMC Carotenoid S-5 column of 250 × 4.6-mm-i.d. (Waters, Milford, MA) at a flow rate of 1 mL min^{-1}. The eluent was monitored by a photodiode array detector (MD-910, Jasco). In order to assess carotenoids in the samples, three different gradient elution schedules were used according to a previously described method (Kato et al., 2004). Peaks were identified by comparing their retention times and absorption spectra with authentic standards