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Research article

Accumulation of carotenoids in a novel citrus cultivar 'Seinannohikari' during the fruit maturation

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ABSTRACT

In the present study, carotenoid metabolism was investigated in the fruits of a novel citrus cultivar, 'Seinannohikari' (*Citrus* spp.). During the maturation, β,β-xanthophylls were accumulated rapidly with β-cryptoxanthin being the dominant carotenoid compound in the flavedo and juice sacs of 'Seinannohikari'. In the juice sacs of mature fruits, 'Seinannohikari' accumulated high amount of carotenoids, especially β-cryptoxanthin. The content of β-cryptoxanthin in the juice sacs of 'Seinannohikari' was approximately 2.5 times of that in 'Miyagawa-wase' (*Citrus unshiu*), which is one of its parental cultivars, at the mature stage. Gene expression results showed that the massive accumulation of β-cryptoxanthin might be attributed to the higher expression of carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, *CitHYb*, and *CitZEP*), and lower expression of carotenoid catabolic genes (*CitNCED2* and *CitNCED3*) in the juice sacs of 'Seinannohikari'.

1. Introduction

Carotenoids are a group of colorful pigments widely distributed in nature, providing attractive colors to fruits and flowers. To date, more than 750 carotenoids have been identified; they not only fulfill multiple functions in regulating plant growth and development, but also are important for maintaining human health and preventing the occurrence of diseases (Schwartz et al., 1997; Havaux, 1998; Krinsky et al., 2003; Cazzonelli and Pogson, 2010; Nisar et al., 2015; Yuan et al., 2015; Sun et al., 2017). Recently, a growing number of epidemiological studies suggested that carotenoids, such as β-carotene, β-cryptoxanthin, lutein, and astaxanthin, acted as precursors of vitamin A or antioxidants in human body, and dietary intakes of carotenoids reduced risks of eye diseases, certain cancers, and inflammation (Takayanagi et al., 2011; Yamaguchi, 2012; Iskandar et al., 2013; Pouchieu et al., 2014; Sugiura et al., 2015; Bahonar et al., 2017; Mohammadzadeh Honarvar et al., 2017).

Carotenoids are synthesized in plants and certain bacteria and fungi, however, they cannot be biosynthesized in the human body, and humans need to get them from daily diet. Citrus fruits are a rich and complex source of carotenoids. So far, approximately 115 carotenoids have been identified in citrus fruits, among them β,β-xanthophylls, which account for up to 90% of total carotenoids, are predominantly accumulated in most citrus cultivars (Rodrigo et al., 2013a,b; Endo et al., 2016). Ikoma et al. (2016) reported that according to the difference in β,β-xanthophyll composition, citrus cultivars could be roughly divided into three groups: β-cryptoxanthin abundant cultivars, violaxanthin abundant cultivars, and cultivars with low β-cryptoxanthin and violaxanthin contents. In the recent years, carotenoid metabolism has been investigated in several citrus cultivars with different carotenoid profiles, and the results suggested that carotenoid accumulation was highly regulated at the transcriptional level in citrus fruits during the maturation (Kato et al., 2004; Rodrigo et al., 2004; Rodrigo and Zacarías, 2007; Alquézar et al., 2008; Ríos et al., 2010; Zhang et al., 2012; Ma et al., 2013; Wei et al., 2014; Sugiyama et al., 2017; Zhu et al., 2017). As shown in Fig. 1, β,ε-carotenoids (carotenoids with one β-ring and one ε-ring), such as α-carotene and lutein, are mainly accumulated with a high expression level of *CitLCYe* in the flavedo of immature fruits. During the maturation, the shift from β,ε-carotenoids synthesis to β,β-carotenoids (carotenoids with two β-rings) synthesis occurs along with the decrease in the expression of *CitLCYe*, and the increases in the expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*,

Abbreviations: GGPP, geranylgeranyl diphosphate; CitPSY, phytoene synthase; CitPDS, phytoene desaturase; CitZDS, ζ-carotene desaturase; CitCRTISO, ζ-carotene isomerase; CitLCYb, lycopene β-cyclase; CitLCYe, lycopene ε-cyclase; CitHYb, β-ring hydroxylase; CitHye, ε-ring hydroxylase; CitZEP, zeaxanthin epoxidase; CitCCD, carotenoid cleavage dioxygenase; CitNCED, 9-*cis*-epoxycarotenoid dioxygenase

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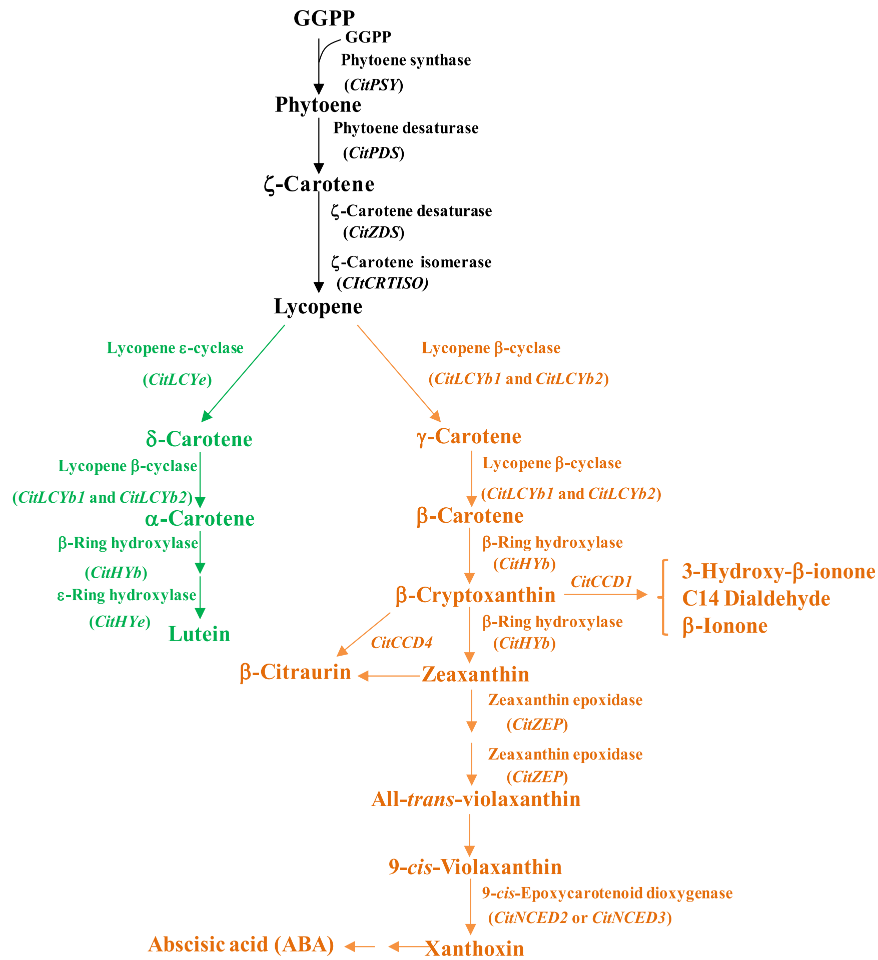

Fig. 1. Carotenoid metabolism in citrus fruits during the maturation. GGPP, geranylgeranyl diphosphate.

CitLCYb2, *CitHYb*, and *CitZEP* (Kato et al., 2004). In addition, the accumulation of carotenoid is affected by the expression levels of carotenoid cleavage dioxygenases (CCDs), including *CitCCD1*, *CitCCD4*, and *CitNCEDs* (9-*cis*-epoxycarotenoid dioxygenases). The cleavage of carotenoids by *CCDs* not only regulates carotenoid accumulation in citrus fruits, but also produces a range of apocarotenoids that are important for the maturation and quality of citrus fruits (Kato et al., 2006; Ma et al., 2013; Rodrigo et al., 2013a,b).

'Seinannohikari' (*Citrus* spp.), a novel citrus cultivar, is originated from a cross between EnOw No.21 ['Encore' (*C. nobilis* × *C. deliciosa*) × 'Okitsu wase' (*C. unshiu*)] and 'Youkou' ['Kiyomi' (*Citrus* sp.) × 'Nakano No.3' (*C. reticulata*)]. 'Seinannohikari' fruits have high market values because they are easily peelable, the flesh is very soft and juicy with distinctive flavor. Moreover, the juice sacs of 'Seinannohikari' exhibit deep orange color, and accumulate high amounts of carotenoids, especially β-cryptoxanthin. Yoshioka et al. (2015) reported that in the juice sacs of 'Seinannohikari', the average amount of β-cryptoxanthin in the three years was around 2.76 mg 100 g^{-1} FW, which was much higher than other citrus cultivars. To date, however, the mechanism of carotenoid accumulation in 'Seinannohikari' was still unknown. In the present study, the changes in carotenoid accumulation and expression of genes related to carotenoid metabolism were investigated in the flavedo and juice sacs of 'Seinannohikari' during the fruit maturation. In addition, the molecular mechanism of carotenoid accumulation in the fruits of 'Seinannohikari' was discussed by comparing the gene expression profiles between 'Seinannohikari' and 'Miyagawa-wase' (*Citrus unshiu*), which is one of the parental cultivars of 'Seinannohikari' (Fig. S1).

2. Materials and methods

2.1. Plant materials

'Seinannohikari' (*Citrus* spp.) and 'Miyagawa-wase' (*Citrus unshiu*) cultivated at the NARO Institute of Fruit Tree Science, Department of Citrus Research, Okitsu (Shizuoka, Japan) were used as materials. Fruit samples were collected periodically from September to January. In this study, approximately 50 juice sacs from 5 citrus fruits were used at each sampling point. The flavedo and juice sacs were separated from sampled fruits, immediately frozen in liquid nitrogen, and kept at −80 °C until used.

2.2. Extraction and determination of carotenoids

The identification and quantification of carotenoids were conducted according to the methods described by Kato et al. (2004). Carotenoids were extracted from the flavedo and juice sacs using a hexane:acetone:ethanol (2:1:1 [v/v]) solution containing 0.1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and 10% (w/v) magnesium carbonate basic. The organic solvents were evaporated, and saponified overnight with 20% (w/v) methanolic KOH. After the water-soluble extracts were removed by adding NaCl-saturated water, the carotenoids were re-extracted with diethyl ether, and evaporated to dryness. Carotenoid extracts were then redissolved in 5 mL of a methyl *tert*-butyl ether: methanol (1:1 [v/v]) solution, and an aliquot (20 μL) was injected to the reverse-phase HPLC system (Jasco, Tokyo, Japan) fitted with a YMC Carotenoid S-5 column of 250- × 4.6-mm-i.d. (Waters, Milford, MA) at