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REVIEW
The Characteristics of Carotenoid Biosynthesis in Citrus Fruit

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Abstract

To learn how to regulate carotenoid content in citrus fruit, we studied the mechanism of carotenoid accumulation using physiological and genetic methods. Based on differences in the carotenoid profiles of flavedo and juice sacs, citrus species could be roughly divided into three groups: carotenoid-poor, violaxanthin-abundant, and β-cryptoxanthin-abundant groups. A comparison of the gene expression among several citrus species with different carotenoid profiles showed that the difference in carotenoid profiles among citrus species was highly regulated by coordination of the expression for genes related to carotenoid biosynthesis and catabolism. Quantitative trait loci related to carotenoid content were also identified to select progeny with high carotenoid content. Moreover, we showed that the effect of postharvest ethylene on carotenoid accumulation in flavedo varied with temperature. Under an ethylene atmosphere, in flavedo, carotenoid accumulation was enhanced more dramatically than under an ethylene-free atmosphere at 20°C but repressed at 5°C.

Discipline: Biotechnology
Additional key words: β-cryptoxanthin, DNA marker, ethylene, gene expression, Satsuma mandarin

Introduction

Carotenoids are important components of fruit quality and their presence as pigments dictates peel and juice color in citrus fruit. Citrus carotenoids also boost human health. Some carotenoids are precursors of vitamin A, which is essential to humans, and antioxidants, which reduce the risk of chronic diseases[17,35]. The study of the function of β-cryptoxanthin, a carotenoid rich in mandarin fruit, has been particularly emphasized with human health in mind[13,37]. These studies suggested that high levels of dietary β-cryptoxanthin were associated with a reduced risk of lung cancer. Moreover, recent nutritional epidemiologic studies showed that β-cryptoxanthin intakes may reduce the risk of lifestyle-related diseases, such as liver dysfunction[27], osteoporosis[29,30], and metabolic syndrome[28].

Regulating carotenoid content in citrus fruit is thus important for fruit quality and human health. In this paper, we discuss diversity in the carotenoid profiles of fruit among citrus species. Moreover, the relationship between the accumulation of carotenoids, especially β-cryptoxanthin, and the expression of carotenoid-related genes is explored. Finally, the potential to regulate carotenoid content genetically and physiologically is explained.

Diversity in carotenoid profiles among citrus species

Citrus is a complex source of carotenoids, containing more than any other fruit. Generally, high-performance liquid chromatography (HPLC) with a photodiode array detector and a C_{30} column has been used to study citrus carotenoids. However, several carotenoids could not be distinctly separated in HPLC analysis due to the

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complicated composition of carotenoids in citrus fruit. In addition, some carotenoids could not be quantified with this method due to insufficient detector sensitivity. Therefore, to investigate the diversity in carotenoid profiles among citrus species, we developed a highly sensitive liquid chromatography mass spectrometry (LC-MS) method for the simultaneous quantification of 18 carotenoids[14].

With this method, the carotenoid contents of 39 varieties were determined monthly from October to January. Based on the carotenoid contents, patterns of seasonal changes of 39 varieties were classified into three types, H (high content transition), M (middle content transition), and L (low content transition), in each carotenoid. After the categories of the patterns of the seasonal changes, H, M, and L, had been converted into numerical values (ordinal scale), 39 varieties were classified based on similar numerical values for the patterns of seasonal changes in phytoene, β-cryptoxanthin, and violaxanthin by hierarchical clustering (Table 1). In this study, the concentrations of phytoene, β-cryptoxanthin, and violaxanthin exceeded those of others, and the difference in concentrations among the varieties exceeded that in other carotenoids. Accordingly, three carotenoids, phytoene, β-cryptoxanthin, and violaxanthin, were selected to classify the citrus varieties.

In flavedo, 39 species were classified into 5 clusters, in which the carotenoid profiles were carotenoid-poor, phytoene-abundant, violaxanthin-abundant, violaxanthin- and β-cryptoxanthin-abundant, and phytoene-, violaxanthin-, and β-cryptoxanthin-abundant. In juice sacs, they were classified into 4 clusters, in which the carotenoid profiles were carotenoid-poor, violaxanthin-abundant, violaxanthin- and phytoene-abundant, and violaxanthin-, phytoene-, and β-cryptoxanthin-abundant. Based on a comparison of carotenoid profiles between flavedo and juice sacs, it emerged that the carotenoid profiles of the flavedo and juice sacs were similar in each species, with the exception of a few varieties. Several reports have indicated that differences in the carotenoid composition can be used to classify citrus fruit. Goodner et al. reported that the difference in the concentration of β-cryptoxanthin in juice can be used as a factor to discriminate mandarins, oranges, and their hybrids[7]. Fanciullino et al. reported that *cis*-violaxanthin and β-cryptoxanthin in juice were strong determinants in classification of the 25 citrus genotypes[5]. The present study showed that violaxanthin and β-cryptoxanthin were important determinants in citrus classification; not only in juice sacs but also flavedo[14].

Moreover, the present results showed that most species, including many mandarins, such as Satsuma mandarin, Ponkan, and Mediterranean mandarin, were classified into the β-cryptoxanthin-abundant category in both flavedo and juice sacs. In contrast, oranges such as Trovita and Washington navels were classified into the violaxanthin-abundant category both in flavedo and juice sacs. Many varieties, including lime, lemon, grapefruit, and pummelo, were separated from oranges and mandarins due to the low violaxanthin and β-cryptoxanthin content in these species both in flavedo and juice sacs. These results suggested that citrus species could be roughly divided into three groups; namely carotenoid-poor, violaxanthin-abundant, and β-cryptoxanthin-abundant respectively (Table 1).

Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit

The pathway of carotenoid biosynthesis in plants has been extensively studied[2,3,4,8,18,21]. In citrus fruit, violaxanthin and β-cryptoxanthin, the predominant carotenoids during ripening, are synthesized as follows (Fig. 1). The first committed step of carotenoid biosynthesis is the condensation of two molecules of geranylgeranyl pyrophosphate (C_{20}) to form a colorless phytoene (C_{40}) catalyzed by phytoene synthase (PSY). Phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) introduce four double bonds into phytoene to produce lycopene. Lycopene β-cyclase (LCYb) changes lycopene into β-carotene with two β-rings. β-Carotene is converted to zeaxanthin via β-cryptoxanthin via two-step hydroxylation, which is catalyzed by β-ring hydroxylase (HYb). Furthermore, zeaxanthin is converted to violaxanthin via antheraxanthin by zeaxanthin epoxidase (ZEP).

β-Cryptoxanthin is an intermediate of a two-step hydroxylation by HYb. Sun et al. demonstrated that β-cryptoxanthin, rather than zeaxanthin, was mainly accumulated in *Escherichia coli* cells carrying the truncated HYb gene[32]. Based on this result, they indicated that HYb hydroxylated the β-rings of β-carotene more efficiently than the not-yet-hydroxylated β-ring of β-cryptoxanthin. Li et al. also analyzed the function of two HYb cDNAs (Zmbch1 and Zmbch2), which were isolated from maize, in *E. coli* cells carrying their cDNAs without truncation[11]. ZmBCH1 could convert β-carotene into β-cryptoxanthin and zeaxanthin, but ZmBCH2 could form β-cryptoxanthin alone and had lower overall activity than ZmBCH1. Based on the results in truncated HYb and ZmBCH2, we thought that the accumulation of β-cryptoxanthin predominated in two-step hydroxylation by HYb when the enzyme activity was insufficient. Moreover, it was thought that HYb preferred the first-step conversion from

Table 1. Classification of citrus species based on patterns of seasonal changes in phytoene, β-cryptxanthin and violaxanthin in both flavedo and juice sacs

Carotenoid profiles[a]		Flavedo				
		Carotenoid-poor	PHY-abundant	VIO-abundant	VIO- and BCR-abundant	PHY-, VIO-, and BCR-abundant
Juice sacs	Carotenoid-poor	(Carotenoid-poor) Tahiti lime[b] Sweet lime Eureka lemon Hirado buntan Red blush grapefruit Marsh grapefruit Kinukawa Naruto Kikudaidai Hyuganatsu Kawabata Shunkokan Henkamikan Kabuchi	Yuzu			
	VIO-abundant at a middle level	Yamamikan Hassaku Natsudaidai Attani Rokugatsumikan Ujukitsu Yatsushiro		Iyo	Rangpur lime	
	VIO-abundant at a high level and PHY-abundant			(Violaxanthin-abundant) Trovita orange Washington navel orange Tachibana Shiikuwasha	Tankan	
	VIO-abundant at a medium level and PHY- and BCR-abundant		Tengu		(β-cryptoxanthin-abundant) Limonia Kunenbo Ponkan Mediterranean mandarin Obenimikan Dancy tangerine Cleopatra	(β-cryptoxanthin-abundant) Satsuma mandarin Hirakishu

[a] Abbreviations in the carotenoid profile: PHY, phytoene; BCR, β-cryptoxanthin; VIO, violaxanthin.
[b] Underlined species were carotenoid-poor, violaxanthin-abundant, or β-cryptoxanthin-abundant both in flavedo and juice sacs.

β-carotene to β-cryptoxanthin rather than the second-step conversion from β-cryptoxanthin to zeaxanthin under low HYb activity and/or excessive β-carotene supply.

In citrus fruit, the substrate specificity of HYb seems important to regulate β-cryptoxanthin accumulation. We investigated the mechanism causing the diversity of carotenoid profiles in juice sacs between citrus species, Satsuma mandarin (β-cryptoxanthin-abundant species) and Valencia orange (violaxanthin-abundant species), based on profiles in the gene expression of carotenoid biosynthetic enzymes[9]. This study showed that the gene expression of upstream carotene synthesis (PSY, PDS, ZDS, and LCYb) in the Satsuma mandarin exceeded that in the Valencia orange, whereas the gene expression of downstream xanthophyll synthesis (HYb and ZEP) in the Satsuma mandarin was lower than that in the

latter (Fig. 1). The higher expression of upstream synthesis genes and lower expression of the HYb gene suggested a higher supply of β-carotene and lower HYb activity in juice sacs of the Satsuma mandarin than in those of the Valencia orange. Therefore, it was thought that, amid an equilibrium of a high gene expression of upstream synthesis and low gene expression of HYb (high supply of β-carotene and low HYb activity), HYb predominantly catalyzed the first-step conversion due to its high substrate specificity to β-carotene, leading to a marked accumulation of β-cryptoxanthin in juice sacs of the Satsuma mandarin.

In contrast, in the juice sacs of the Valencia orange, HYb was likely to sufficiently catalyze the reaction to zeaxanthin via β-cryptoxanthin due to the low gene expression of upstream synthesis and high gene expression of HYb (low supply of β-carotene and high HYb activity). Moreover, the intensity in the gene expression of ZEP was much higher in the juice sacs of the Valencia orange than those of the Satsuma mandarin. Accordingly, it was assumed that zeaxanthin was rapidly converted to violaxanthin by ZEP in the juice sacs of the Valencia orange.

Relationship between carotenoid accumulation and gene expression of 9-*cis*-epoxycarotenoid dioxygenase in citrus fruit

Carotenoids are metabolized to apocarotenoids through the pathway catalyzed by carotenoid cleavage dioxygenases. CCD1, a carotenoid dioxygenase, catalyzes the symmetrical 9-10 and 9'-10' cleavages of multiple carotenoid substrates to form a C_{14} dialdehyde and two C_{13} products, which vary depending on the carotenoid substrate[24]. Nine-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the cleavage of 9-*cis*-violaxanthin or 9'-*cis*-neoxanthin to form a C_{25} epoxy-apocarotenal and xanthoxin (C_{15}), a precursor of a plant hormone, abscisic acid[23,25] (Fig. 2).

We investigated the relationship between the carotenoid profile and carotenoid cleavage reaction by NCED in the juice sacs of the Satsuma mandarin, Lisbon lemon, and Valencia orange[10]. In the juice sacs, increased abscisic acid levels were observed in the Satsuma mandarin during fruit maturation. As the abscisic acid accumulated, the gene expressions of NCED2 and NCED3 also increased. In the juice sacs of the Lisbon lemon, the levels of the NCED2 gene expression soared as abscisic acid accumulated during the green stage (from August to Octo-

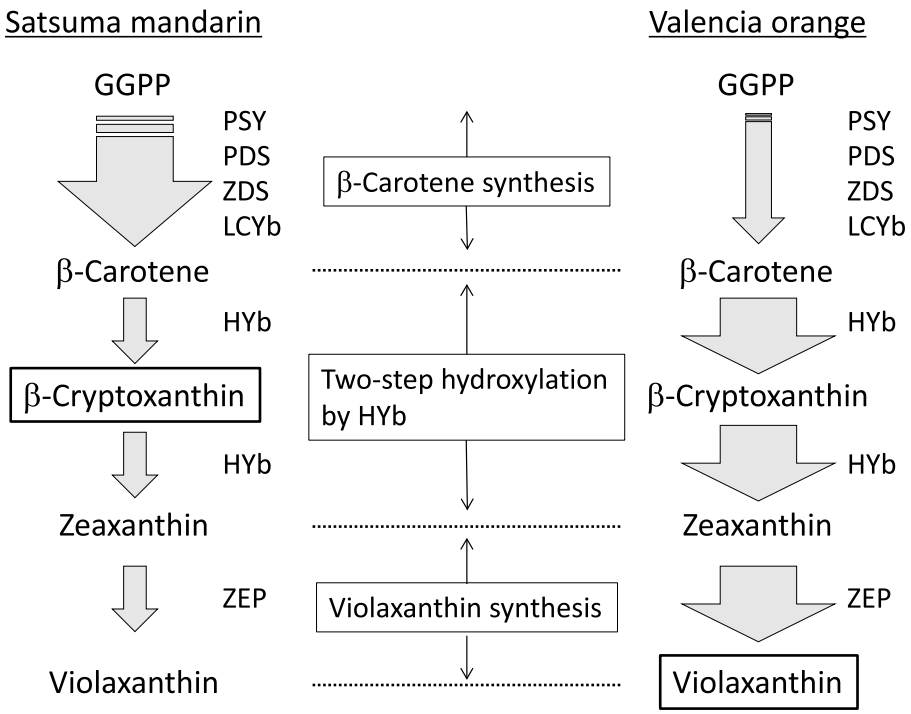

Fig. 1. Comparison of gene expression for carotenoid biosynthesis between the Satsuma mandarin and Valencia orange in juice sacs during massive carotenoid accumulation

The width of the arrows indicates the relative gene expression levels for carotenoid biosynthetic enzymes. Abbreviations of carotenoid biosynthetic enzymes: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ξ-carotene desaturase; LCYb, lycopene β-cyclase; HYb, β-ring hydroxylase; ZEP, zeaxanthin epoxidase.

ber), whereas the NCED3 gene expression changed, regardless of the abscisic acid level. These results suggested that the NCED2 gene expression and NCED3 in the Satsuma mandarin and the NCED2 gene expression in Lisbon lemon were primarily responsible for the abscisic acid accumulation in their juice sacs. In the juice sacs of the Valencia orange, the abscisic acid level was much lower than those of the Satsuma mandarin and Lisbon lemon. In the Valencia orange, no noticeable increase in NCED2 gene expression was observed. In addition, the NCED3 gene expression changed, regardless of the abscisic acid level. This result suggested that, in the Valencia orange, the extremely low level of NCED2 was primarily responsible for the low level of abscisic acid.

In mature fruit, the juice sacs of the Satsuma mandarin accumulated a low level of 9-*cis*-violaxanthin, whereas those of the Valencia orange accumulated a high level of 9-*cis*-violaxanthin. We thought that in the Valencia orange, 9-*cis*-violaxanthin in the juice sacs was not cleaved efficiently by NCED because the NCED2 gene expression remained minimal. Therefore, in the Valencia orange, the content of 9-*cis*-violaxanthin was high in the juice sacs. In contrast, we thought that in the Satsuma mandarin and Lisbon lemon, the 9-*cis*-violaxanthin was cleaved immediately by NCED because an increase in the

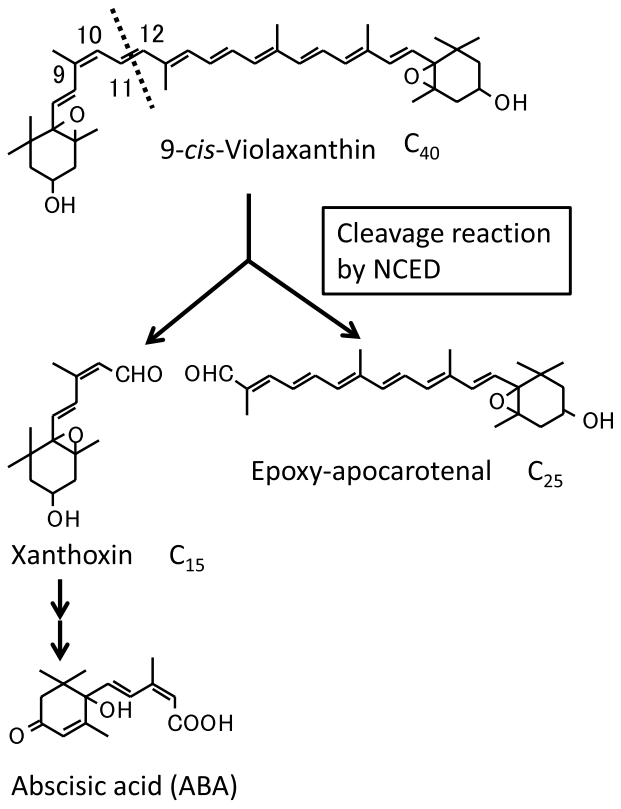

Fig. 2. Cleavage of 9-*cis*-violaxanthin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) and abscisic acid synthesis

NCED2 gene expression was observed in the juice sacs. Therefore, the content of 9-*cis*-violaxanthin in the Satsuma mandarin and Lisbon lemon was much lower than that in the Valencia orange. Thus, the oxidative cleavage of 9-*cis*-violaxanthin catalyzed by NCED affected the 9-*cis*-violaxanthin concentration and, consequently, the carotenoid profiles of the three citrus species during fruit maturation.

Quantitative trait loci (QTL) analysis of carotenoid content in citrus fruit

An increase in carotenoids, especially the β-cryptoxanthin content, is an important breeding objective for citrus in Japan. However, to date, there have been no detailed genetic analyses of carotenoid content in citrus. Analysis of quantitative trait loci (QTL) using a segregating population is an effective means of obtaining genetic information on agronomically important traits. This method allows identification of genetic regions associated with certain quantitative traits on linkage groups, whereupon genetically linked selection markers can be obtained for breeding.

QTL analyses of carotenoid content have been reported for the tomato[1] and carrot[22]. In maize kernels, two QTLs were detected and their locations on the genetic map were associated with the loci of phytoene synthase and ξ-carotene desaturase genes[34]. In the cauliflower, Lu et al. showed that the Or gene, which regulates the synthesis of a chaperone protein, that of the DnaJ cysteine-rich-domain, regulated β-carotene accumulation[12]. Thus, these previous studies detected QTLs related to the accumulation of various carotenoids such as β-carotene and lycopene. However, QTL analysis was not conducted in a plant with high β-cryptoxanthin content. Thus, we performed QTL analysis to identify loci related to carotenoid content in citrus fruit[31].

In our study, QTL in a mapping population derived from a cross between two citrus parents, ‘Okitsu-46’ and ‘Nou-5,’ were investigated. The female parent, Okitsu-46, was derived from hybridization between Ueda unshiu (*C. unshiu* Marc.) × Hassaku (*C. hassaku* Hort. ex Tanaka). The male parent, Nou-5, was derived from hybridization between Lee (Clementine mandarin (*C. clementina* Hort. ex Tanaka) × Orlando tangelo (Duncan grapefruit (*C. paradisi* Macf.) × Dancy tangerine (*C. tangerine* Hort. ex Tanaka))) × Mukaku kishu (*C. kinokuni* Hort. ex Tanaka).

Most of the QTL for each carotenoid were mapped to different locations on the linkage map. QTL for β-cryptoxanthin content was detected on three linkage groups. The strongest QTL for β-cryptoxanthin content was detected on the linkage group 6 of ‘Nou-5.’ The QTL

with the largest effect on β-cryptoxanthin content were broadly located from 4.1 to 7.0 cM on linkage group 6 of 'Nou-5', including the Gn0005 locus. The Gn0005 marker was derived from the CitPAP cDNA sequence (DDBJ Acc. No. AB011797). In the bell pepper (*Capsicum annuum* L.), the PAP gene is thought to encode a carotenoid association protein[19], which is involved in carotenoid accumulation, although the relation between CitPAP and carotenoid accumulation could not be confirmed in the citrus[16].

A distribution of β-cryptoxanthin contents among progenies genotyped using the Gn0005 marker on linkage group 6 of 'Nou-5' is shown in Fig. 3. The mean of the β-cryptoxanthin content was 1.3 mg/100g for homozygous progenies genotyped with the Gn0005 marker, whereas that for heterozygous genotypes was 0.9 mg/100g. There was a significant difference in the means between the marker genotypes of progenies.

This study provides preliminary data because we obtained data in only one season and from a limited number of individuals (n = 51). However, the results that we obtained suggest that information on QTL could be used to generate DNA markers to select progeny with high carotenoid content.

Physiological regulation of carotenoid content by a plant hormone, ethylene

Previous studies showed that an ethylene treatment at temperatures within the range 15-25°C noticeably accelerated carotenoid accumulation in the flavedo of postharvest citrus fruit[20,26,33,36]. However, few studies have focused on the effect of low temperature (5°C) on carotenoid accumulation and the difference in the effect of ethylene on carotenoid accumulation among different temperatures. Postharvest citrus fruit may be exposed to ethylene at different temperatures when the citrus fruit and ethylene-producing fruits and vegetables coexist in a storage room, on a store shelf, or during transportation. We therefore investigated the effect of ethylene on carotenoid accumulation in citrus fruit at different temperatures[15]. In our study, the effect of postharvest ethylene was investigated at ambient (20°C) and low (5°C) temperatures.

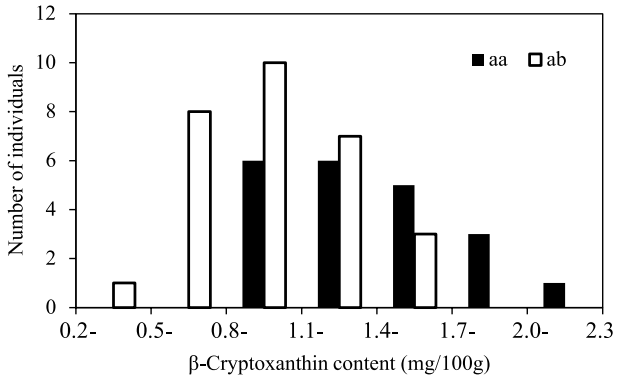

Fig. 3. Distribution of β-cryptoxanthin contents among progeny genotyped using the Gn0005 marker on linkage group 6 of 'Nou-5'

The number of homozygous (aa) and heterozygous (ab) genotype progeny are shown by black and white bars, respectively. The mean β-cryptoxanthin content of homozygous and heterozygous individuals was 1.3 and 0.9 mg/100g, respectively, and a significant difference was shown by the t-test ($P < 0.01$).

In flavedo, at 20°C, ethylene distinctly enhanced the accumulation of phytoene, ζ-carotene, β-carotene, and β-cryptoxanthin with promotion in the gene expression of PSY, PDS, LCYb, HYb, and ZEP (Fig. 4). In contrast, ethylene did not clearly enhance the accumulation of 9-*cis*-violaxanthin and all-*trans*-violaxanthin in flavedo at 20°C, although the gene expression of PSY, PDS, LCYb, HYb, and ZEP was promoted by ethylene. The biosynthesis of violaxanthin was probably stimulated by ethylene as massively as that of phytoene, ζ-carotene, β-carotene, and β-cryptoxanthin. However, a drastic increase in the gene expression of NCED by ethylene seemed to promote the metabolism from 9-*cis*-violaxanthin to abscisic acid. In fact, the content of abscisic acid in flavedo under ethylene exceeded that under air. Thus, we assumed that ethylene enhanced not only the biosynthesis of violaxanthin but also the metabolism from violaxanthin to abscisic acid by NCED at 20°C. Consequently, the enhanced accumulation of 9-*cis*-violaxanthin by ethylene was inconspicuous at 20°C.

In contrast, in flavedo at 5°C, ethylene repressed the accumulation of phytoene and ζ-carotene with repressed PSY and PDS gene expression. The contents of β-carotene, β-cryptoxanthin, and violaxanthin in flavedo under ethylene were lower than those under air, although the gene expression of LCYb and HYb was promoted by ethylene. In addition, under ethylene at 5°C, the content of 9-*cis*-violaxanthin in flavedo clearly decreased. The repressed gene expression of PSY and PDS by ethylene probably led to the low biosynthesis of β-carotene, β-cryptoxanthin, and violaxanthin. Under such conditions, drastic increases in the gene expression of NCED by ethylene appeared to enhance the metabolism of 9-*cis*-violaxanthin to abscisic acid, consequently decreasing the content of 9-*cis*-violaxanthin. Thus, we assumed that, under ethylene at 5°C, the repressed gene expression of PSY and PDS by ethylene was primarily responsible for the repressed accumulation of carotenoids. In addition, the promoted gene expression of NCED by ethylene seemed to encourage the reduction of 9-*cis*-violaxanthin

content in flavedo under ethylene at 5°C.

These results suggested that a difference in the gene expression response of PSY and PDS to ethylene between temperatures was observed in flavedo. However, no such difference was observed in previous studies[6,20], in which the gene expression of PSY, ZDS, HYb, and NCEDs was enhanced by ethylene at around 20°C, because the effect of ethylene on gene expression was not tested at low temperature then. Accordingly, the difference in the gene expression response of PSY and PDS to ethylene between temperatures is novel knowledge. Based on these results, we suggest that storage at around 20°C may dramatically enhance carotenoid accumulation, but storage at 5°C represses the carotenoid accumulation in the flavedo, because the gene expression response of PSY and PDS to ethylene between temperatures differs in the latter. Accordingly, artificial ethylene treatment can improve the orange color of citrus peel at around 20°C rather than at low temperature. Moreover, we think that simultaneous treatment of ethylene and a certain inhibitor to NCEDs is effective in elevating carotenoid content in flavedo because ethylene stimulates not only biosynthesis of carotenoid but also metabolism from carotenoid to abscisic acid by NCEDs. In future, research into the NCED-inhibitor will be required to improve the orange color of citrus peel.

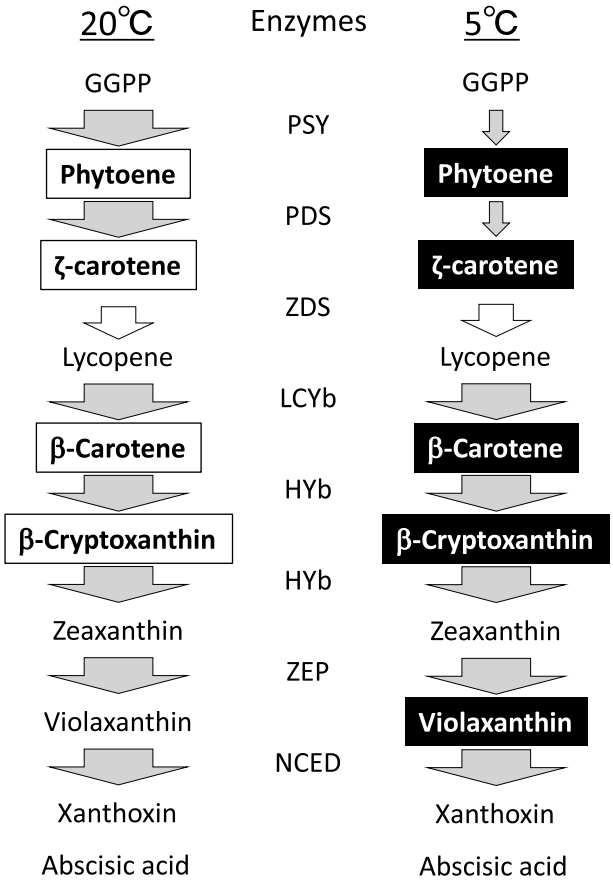

Fig. 4. The effects of ethylene on carotenoid content and the gene expression of carotenoid-related enzymes at different temperatures (20 and 5°C)

Accumulation was promoted and repressed respectively in carotenoids written in bold black and white characters. The gray and white arrows indicate the responsiveness to ethylene in the gene expression for carotenoid-related enzymes. In the wide gray arrows, the gene expression was promoted by ethylene, while in the medium-width white arrows, the gene expression was not influenced by ethylene. In the narrow gray arrows, the gene expression was repressed by ethylene. Abbreviations of carotenoid-related enzymes: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYb, lycopene β-cyclase; HYb, β-ring hydroxylase; ZEP, zeaxanthin epoxidase; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

Conclusion

In this paper, the diversity of carotenoid profiles among citrus species was discussed. The mechanism of carotenoid accumulation in citrus fruit was also explained by comparing the expression of genes related to carotenoid biosynthesis and catabolism among citrus species. Moreover, we showed possible methods to regulate carotenoid content by DNA markers and a plant hormone, ethylene. We hope that in future, the information reported in this paper will aid further technical development toward controlling carotenoid content in the citrus and boosting its quality.

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