

Comparison of Carotenoid Accumulation and Absciscic Acid Content in Fruit-color Altered Mutant and Original Types in Citrus

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Abstract

Carotenoid mutants are useful for elucidating carotenoid metabolism. ‘Kusaie’ is a yellow-fruited form of ‘Rangpur’ (*Citrus limonia* Osbeck). Hyuganatsu (*C. tamurana* hort. ex Tanaka) is a medium-sized, light yellow fruit. Its bud mutation, wherein the fruit rind color becomes orange, is named ‘Orange-hyuga.’ To elucidate the mechanism underlying this alteration, their carotenoid composition and abscisic acid content were analyzed. In the flavedo of ‘Kusaie,’ significant phytoene accumulation, decreased xanthophyll, and ABA contents were observed, suggesting that phytoene desaturase was suppressed. Moreover, the total carotenoids in the juice sac of ‘Kusaie’ was significantly lesser than those in that of ‘Rangpur,’ suggesting that the production of carotenoids is suppressed in ‘Kusaie.’ In ‘Orange-hyuga,’ the total carotenoids was approximately 6-fold that of Hyuganatsu, and a significant xanthophyll accumulation, especially violaxanthin, was observed. Furthermore, ABA content was significantly lower. Accordingly, those might be associated with an altered gene expression balance upstream and downstream of biosynthesis. Difference in biosynthesis regulation in these color-altered cultivars might cause a change in the carotenoid contents and composition in fruits, which seemed to result in color changes from reddish-orange in ‘Rangpur’ to yellow in ‘Kusaie’ and from a light yellow color in Hyuganatsu to an orange color in ‘Orange-hyuga.’ In this study, these color-changing citrus mutants were observed to accumulate carotenoids differently from the original type, and we speculated that different changes occurred in carotenoid biosynthesis regulation.

Discipline: Horticulture

Additional key words: color-changing mutants, Hyuganatsu, ‘Rangpur’, ‘Orange-hyuga’, ‘Kusaie’

Introduction

The amount and composition of carotenoids determine the yellow, red, or orange coloration of many flowers and fruits. Carotenoids represent a diverse and widely distributed class of pigments. They are involved in essential functions in plants as photoprotectors in the chloroplasts of photosynthetic tissues (Deming-Adams & Adams 1996). Moreover, they are nutritional components that are precursors for vitamin A, which is essential to the diet of animals, and antioxidants, which help reduce the

risk of certain forms of cancer (Olson 1989). Carotenoids are synthesized and accumulated in plastids (Bramley 1997). Phytoene, the first carotenoid biosynthesis molecule, is formed from two molecules of geranylgeranyl pyrophosphate by phytoene synthase (PSY) (Fig. 1). The subsequent sequential phytoene desaturation of introducing conjugated double bonds is catalyzed by two enzymes—phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS)—in plants and cyanobacteria (Fig. 1). Carotenoid biosynthesis in plants is related to that of abscisic acid (ABA), produced through C₁₅ intermediates

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Received 14 October 2020; accepted 25 December 2020.

after oxidative cleavage of specific xanthophylls (Fig. 1) (Milborew 2001).

Citrus cultivars have many mutants that produce color-altered fruits. The carotenoid content in the fruits may differ from that in the original type because the content and composition of carotenoids almost determines the color of the citrus fruits. Such changes in fruit rind colors suggest alterations in the enzymes or regulation of carotenoid biosynthesis. Mutant analysis has provided information on plant biology, and carotenoid mutants are helpful in determining the carotenoid metabolism (Li et al. 2001, Isaacson et al. 2002, Park et al. 2002, Tian et al. 2004, Li et al. 2006). Therefore, these mutants are useful materials for acquiring information about carotenoid biosynthesis.

One of the citrus cultivars, 'Rangpur,' is of horticultural importance, primarily as a rootstock in both the Orient and South America and as an ornament (Hodgson 1967). The rind color is reddish-orange, and

the fruit is highly acidic. In the United States, 'Rangpur' is widely used as a dooryard fruit and an ornamental as well as a potted or tubbed plant (Hodgson 1967). Outside the United States, its principal use seems to be a rootstock because of its tolerance of the Citrus tristeza virus and its resistance to soil-borne diseases (Hodgson 1967). 'Kusaie' lime should be considered a yellow-fruited form of the 'Rangpur,' and thus should be called the Kusaie Rangpur. The tree is indistinguishable from other Rangpurs, and the fruit only differs significantly in color (Hodgson 1967). Therefore, 'Kusaie' is considered a spontaneous 'Rangpur' mutation, and the fruit color changes from reddish-orange in 'Rangpur' to distinct yellow (Hodgson 1967). In contrast, the other citrus Hyuganatsu, is a medium-sized, light yellow, globose to oblong, juicy, sweet-flavored, moderately seedy, late-ripening fruit of limited commercial importance in Japan (Iwahori & Kadoya 1999). Its bud mutation, with the orange fruit rind, is named 'Orange-hyuga.' It is indistinguishable from Hyuganatsu, except that the fruit color differs (Iwahori & Kadoya 1999), similar to 'Rangpur' and 'Kusaie.' These mutants ('Kusaie' and 'Orange-hyuga') are considered alterations from each original type ('Rangpur' and Hyuganatsu, respectively) regarding the enzymes or regulation of carotenoid biosynthesis. They may provide novel perspectives on how these processes are regulated in citrus fruits. Therefore, in the present study, the carotenoid contents and compositions in the flavedo and juice sac of 'Kusaie' and 'Rangpur' and the flavedo of Hyuganatsu and 'Orange-hyuga' were analyzed to clarify this alteration's mechanism and to characterize the mutation. Moreover, their ABA contents were measured. Then, the reason for the different carotenoid contents was considered.

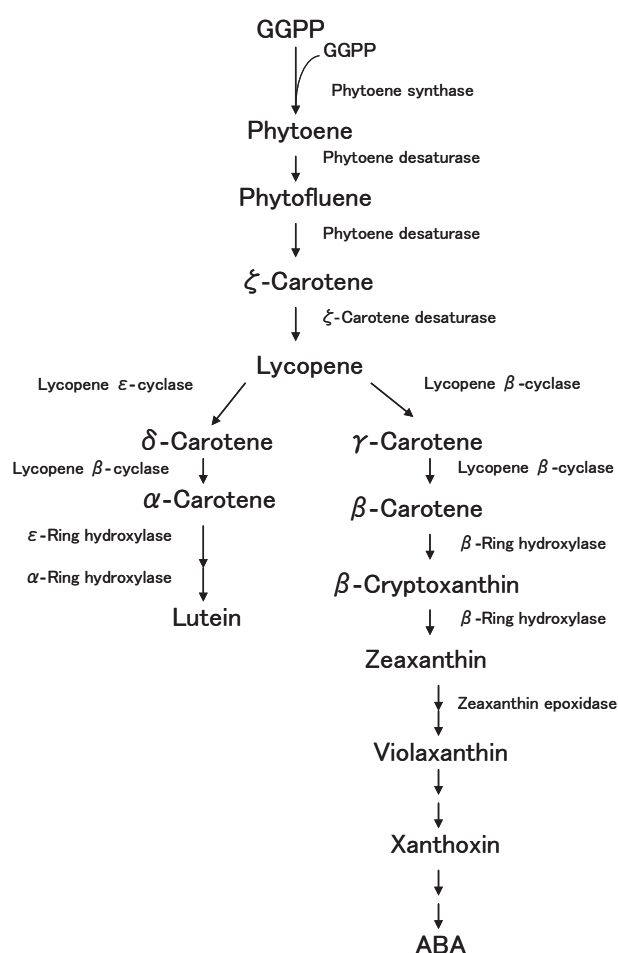


Fig. 1. Pathway of carotenoid biosynthesis and ABA biosynthesis from 9-cis-violaxanthin in plants
GGPP: geranylgeranyl pyrophosphate

Materials and methods

1. Plant material

Fruits of 'Rangpur,' 'Kusaie,' Hyuganatsu, and 'Orange-hyuga' were harvested at their full-colored stage from mature trees in an open field at the Institute of Fruit Tree and Tea Science, National Agricultural Research Organization (Shimizu, Shizuoka, Japan). 'Rangpur' and 'Kusaie' were harvested in January 2005, and Hyuganatsu and 'Orange-hyuga' were harvested in February 2005. After the color of the peel and flesh of the fruits was measured, the flavedos and juice sacs were separated from the sample fruits, immediately frozen in liquid nitrogen, and then stored at -20°C until analysis.

2. Fruit color measurements

Fruit color was measured using a color measuring

system (ΣZ - $\Sigma 90$; Nippon Denshoku Industries Co., Ltd., Tokyo, Japan) at two locations around the equatorial plane of the peel and flesh of the fruit. The CIE (Commission Internationale de l'Eclairage) parameters of L^* , a^* , and b^* were used. The CIE parameter a^* was negative to positive, corresponding to green to red; b^* was negative to positive, corresponding to blue to yellow; and L^* was 0-100, corresponding to white to black. The color of the fruits was expressed as an a^*/b^* ratio, which was negative for green fruits and positive for orange fruits.

3. Extraction and high-performance liquid chromatography (HPLC) analysis of carotenoids

For the HPLC analysis, the carotenoids in the citrus fruits were extracted and refined according to the method described by Kato et al. (2004). The refined samples were analyzed using a reverse-phase HPLC system (Jasco International Co. Ltd., Tokyo, Japan) fitted with a YMC Carotenoid S-5 column of 250 mm \times 4.6 mm i.d. (YMC Co. Ltd., Kyoto, Japan) at a flow rate of 1 mL min⁻¹. The eluent was monitored using a photodiode array detector (MD-910; Jasco International Co. Ltd.). The sample was analyzed using three different gradient elution schedules. Kato et al. (2004) described these schedules in detail.

4. Analysis of endogenous ABA levels

Samples for ABA extraction were lyophilized and ground to a fine powder with a mortar and pestle. Endogenous ABA fractions of the fruits were extracted and refined as reported previously (Okuda 2000, Kato et al. 2006). The sample (dry weight: 0.5 g) was extracted

in 30 mL of acetone overnight at 4°C in the dark. The extract was homogenized in a Polytron homogenizer (Kinematica AG, Luzern, Switzerland). The sample was filtered and evaporated to dryness. The residue was dissolved in 3 mL of ethyl acetone:*n*-hexane (1:9, v/v) solution. After adding 3 mL of water, the sample was evaporated to the aqueous phase using a rotary evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The aqueous phase was subsequently filtered through a nylon membrane (pore size, 0.45 μ m). The step after dissolution with ethyl acetone:*n*-hexane (1:9, v/v) was repeated thrice, and the aqueous phase was pooled. After pH adjustment to 2.5 with 1 N HCl, the aqueous phase was partitioned thrice with a 1/3 (v/v) volume of water-saturated ethyl acetate. The organic phase was collected, evaporated to dryness, and then dissolved in methanol. Endogenous ABA levels were quantified by gas chromatography–mass spectrometry with selected ion monitoring using the method previously described by Okuda (2000).

Results

1. Fruit color

The fruit of 'Kusaie' only differed remarkably from that of 'Rangpur' in color. The rind color of 'Rangpur' was reddish-orange, whereas that of the 'Kusaie' lime was bright yellow (Figs. 2A, B). Furthermore, the flesh of 'Rangpur' was orange, whereas that of 'Kusaie' was faint yellow (Fig. 2C). Fruit color was measured using a color measuring system, and the a^*/b^* ratios of the 'Rangpur' and 'Kusaie' fruits were calculated. The average value of the a^*/b^* ratio of the 'Rangpur' peel was higher than that

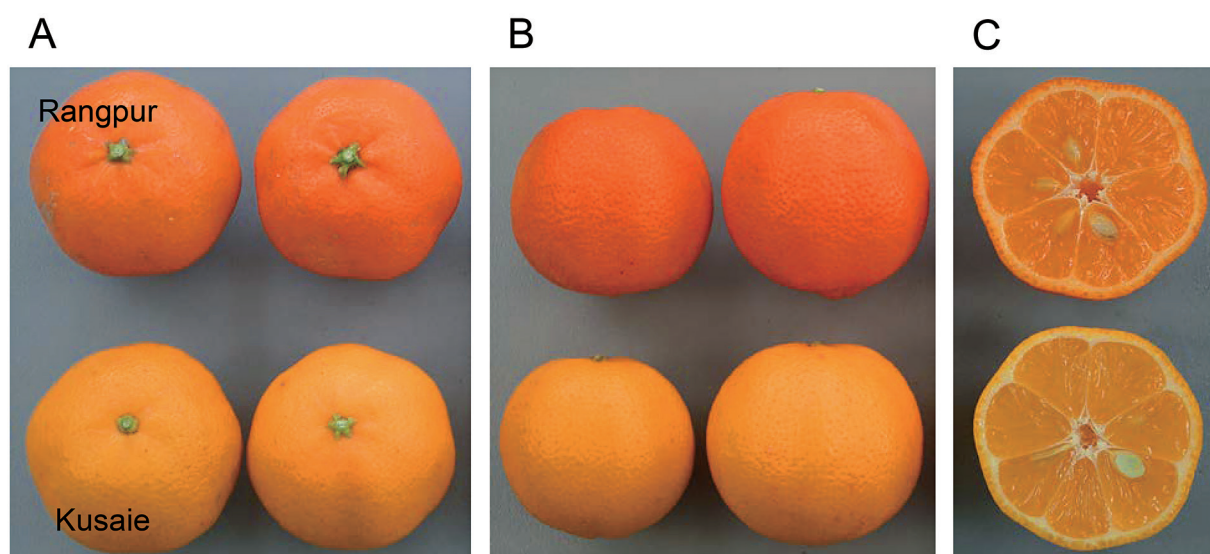


Fig. 2. Mature fruits from 'Rangpur' (orange fruits) and 'Kusaie' (yellow fruits)
Top (A), side (B), and cross-sectional (C) views

Table 1. Differences in peel and flesh color between ‘Rangpur’ and ‘Kusaie’ fruits

Tissue	Cultivar	CIE value measurements			
		L*	a*	b*	a*/b* ratio
Peel	‘Rangpur’	56.5	33.5	58.4	0.57
	‘Kusaie’	69.4	13.7	70.7	0.19
Flesh	‘Rangpur’	38.7	1.9	21.5	0.09
	‘Kusaie’	43.1	−1.3	31.2	−0.04

The CIE parameters of L*, a*, and b* were used.

Table 2. Differences in peel and flesh color between Hyuganatsu and ‘Orange-hyuga’ fruits

Tissue	Cultivar and Species	CIE value measurements			
		L*	a*	b*	a*/b* ratio
Peel	Hyuganatsu	75.8	−0.63	69.1	−0.01
	‘Orange-hyuga’	68.9	16.60	77.9	0.21
Flesh	Hyuganatsu	40.3	−0.60	16.8	−0.04
	‘Orange-hyuga’	37.6	1.60	16.3	0.10

The CIE parameters of L*, a*, and b* were used.

of ‘Kusaie,’ and the CIE parameter of L* of the ‘Rangpur’ peel was slightly lower than that of ‘Kusaie’ (Table 1). In contrast, the average a*/b* ratio of the ‘Rangpur’ flesh was 0.09, whereas that of the ‘Kusaie’ flesh was −0.04, and the parameter L* of the ‘Rangpur’ flesh was not different from that of ‘Kusaie’ (Table 1).

Regarding Hyuganatsu and ‘Orange-hyuga,’ similar to ‘Rangpur’ and ‘Kusaie,’ their fruits only differed in color. The ring color of Hyuganatsu was light yellow, whereas ‘Orange-hyuga’ was orange (Fig. 3). The flesh color of ‘Orange-hyuga’ was slightly orangish compared with Hyuganatsu (Fig. 3). The a*/b* ratio of the Hyuganatsu peel was lower than that of ‘Orange-hyuga,’ and the CIE parameter L* of the Hyuganatsu peel was 75.8, whereas that of the ‘Orange-hyuga’ peel was 68.9 (Table 2). Moreover, the a*/b* ratio of the Hyuganatsu flesh was −0.04 and that of the ‘Orange-hyuga’ flesh was 0.10, and the parameter L* of the Hyuganatsu and ‘Orange-hyuga’ flesh did not differ significantly (Table 2).

2. Concentrations of carotenoids in the flavedo and juice sac of the fruits

The difference in fruit color between ‘Rangpur’ and ‘Kusaie’ was more significant in the peel compared with in the flesh. This tendency was similar in Hyuganatsu and ‘Orange-hyuga.’ To investigate the cause of the fruit-color variation, we performed HPLC carotenoid analysis of the flavedo and juice sac from these fruits and detected eight carotenoid pigments. The identified carotenoids were all-trans-violaxanthin (t-vio), cis-violaxanthin

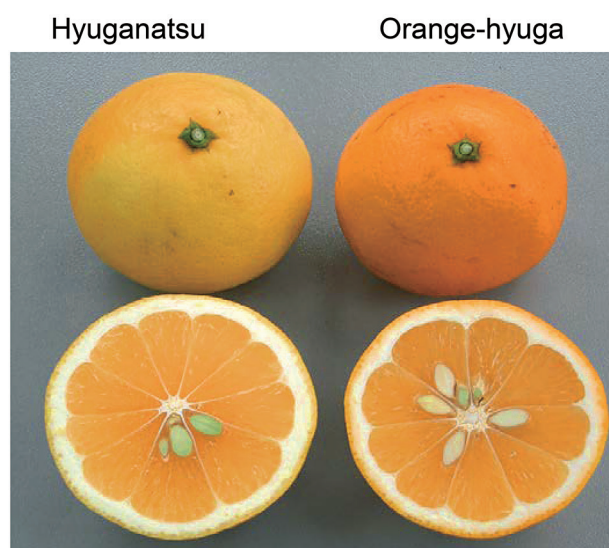


Fig. 3. Mature fruits from Hyuganatsu (yellow fruits) and ‘Orange-hyuga’ (orange fruits)
Top and cross-sectional views

(c-vio), lutein (lut), phytoene (phy), β-cryptoxanthin (β-cry), α-carotene (α-car), β-carotene (β-car), and ζ-carotene (ζ-car). The total carotenoid value (total car) was the sum of the identified carotenoids.

Comparing the concentrations of the carotenoids found in the flavedos from ‘Rangpur’ and ‘Kusaie,’ revealed many qualitative and quantitative differences. The primary carotenoids of the flavedos from ‘Rangpur’ were phy, β-cry, t-vio, and c-vio, whereas in the flavedos from ‘Kusaie,’ the primary carotenoid was phy, which accumulated to ~70% of the total car (Fig. 4). The contents of β-cry, t-vio, c-vio, and lut in the ‘Kusaie’ flavedos

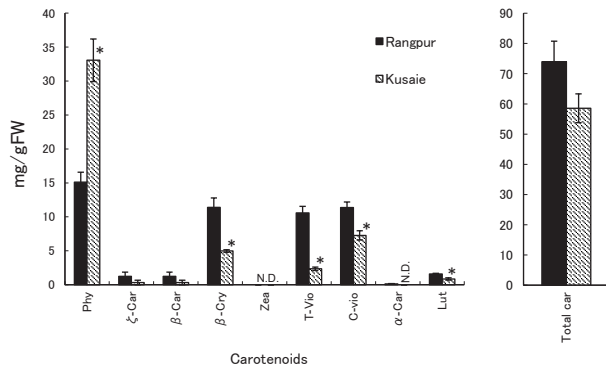


Fig. 4. Carotenoid contents of 'Rangpur' and 'Kusaie' flavedos

Values are means \pm standard error of three 'Kusaie' or four ('Rangpur') replicates.

N.D.: not detected

* Significant differences in individual and total carotenoids between 'Rangpur' and 'Kusaie' samples at the 5% level according to Student's *t*-test

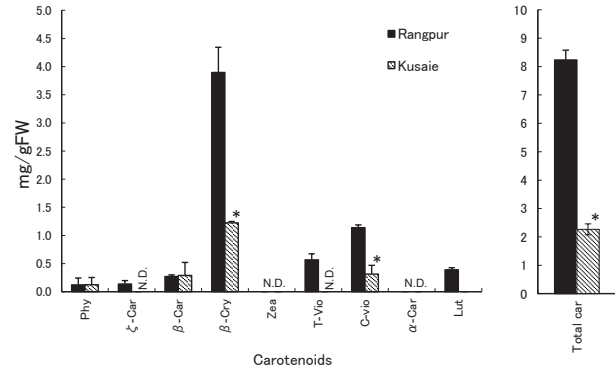


Fig. 5. Carotenoid contents of 'Rangpur' and 'Kusaie' juice sacs

Values are means \pm standard error of three ('Kusaie') or four 'Rangpur' replicates.

N.D.: not detected

* Significant differences in individual and total carotenoids between 'Rangpur' and 'Kusaie' samples at the 5% level according to Student's *t*-test

were significantly smaller than those of the 'Rangpur' flavedos (Fig. 4). Moreover, the total car in the flavedos from 'Kusaie' was ~80% of that from 'Rangpur' (Fig. 4). The carotenoid concentrations in the juice sacs were lower than those in the flavedos. Phytoene accumulation was not observed in the juice sac of 'Kusaie,' and ~45% of the total car in the juice sac of 'Rangpur' was β -cryptoxanthin (Fig. 5). Furthermore, the violaxanthin content was clearly higher in 'Rangpur' than in 'Kusaie' (Fig. 5). Each carotenoid concentration in 'Kusaie' was lower than that in 'Rangpur,' and the total car in 'Kusaie' was ~30% of that in 'Rangpur' (Fig. 5).

Regarding Hyuganatsu and 'Orange-hyuga,' the concentrations of carotenoids in the flavedos from 'Orange-hyuga' were higher than those in the flavenods from Hyuganatsu. In the flavedos from 'Orange-hyuga,' there was a significant accumulation of β -cry, *t*-vio, *c*-vio, and lut. *T*-vio and *c*-vio levels dramatically increased to 6- or 7-times higher values than those in Hyuganatsu (Fig. 6). The total car in the flavedos from 'Orange-hyuga' was approximately 6-times larger than that in the flavenods from Hyuganatsu (Fig. 6). In contrast, the concentrations of carotenoids in the juice sacs from Hyuganatsu and 'Orange-hyuga' were too low to be detected.

3. ABA contents in the flavedo and juice sac of the fruits

The ABA content in the flavedos of the fruit from 'Kusaie' was ~40% lower than that from 'Rangpur' (Table 3). Moreover, the ABA content in the juice sacs of 'Kusaie' was ~76% smaller than that in those of

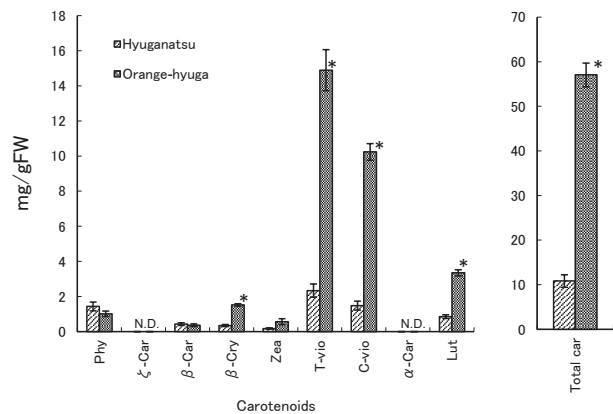


Fig. 6. Carotenoid contents of Hyuganatsu and 'Orange-hyuga' flavedos

Values are means \pm standard error of three replicates.

N.D.: not detected

* Significant differences in individual and total carotenoids between Hyuganatsu and 'Orange-hyuga' samples at the 5% level according to Student's *t*-test

Table 3. Endogenous ABA content in the flavedos and juice sacs of 'Rangpur' and 'Kusaie' fruits

Tissue	ABA (mg g ⁻¹ dry weight)	
	'Rangpur'	'Kusaie'
Flavedos	6.84 \pm 1.57	2.79 \pm 0.64*
Juice sacs	1.94 \pm 0.39	1.48 \pm 0.25

Values are mean \pm standard deviation of three replicates.

* Significant differences in ABA contents by Student's *t*-test ($P \leq 0.05$) between the 'Rangpur' and 'Kusaie' samples

Table 4. Endogenous ABA content in the flavedos of Hyuganatsu and ‘Orange-hyuga’ fruits

Tissue	ABA (mg g ⁻¹ dry weight)	
	Hyuganatsu	‘Orange-hyuga’
Flavedos	2.15 ± 0.50	1.15 ± 0.08*

Values are means ± standard deviation of three replicates.

* Significant differences in ABA contents by Student’s *t*-test ($P \leq 0.05$) between the Hyuganatsu and ‘Orange-hyuga’ flavedo samples

‘Rangpur,’ but the difference was not significant (Table 3). In contrast, the ABA content in the flavedos of the fruit from ‘Orange-hyuga’ was ~50% of that from Hyuganatsu (Table 4). Therefore, the ABA content was also affected.

Discussion

Citrus plants are prone to developing spontaneous mutations in the field. Many of the cultivars currently available in the market have been obtained through the agronomical and nutritional selection of naturally occurring mutants. ‘Kusaie’ and ‘Orange-hyuga’ seemed to be mutants with alterations in fruit color from the wild types ‘Rangpur’ and Hyuganatsu, respectively (Figs. 2 and 3). The *a**/*b** ratio of the peel and flesh was lower in ‘Kusaie’ than in ‘Rangpur’ (Table 1), indicating a change from a reddish-orange to a light yellow color. In contrast, the *a**/*b** ratio of the peel and the flesh of ‘Orange-hyuga’ was higher than that of the peel and of the flesh of Hyuganatsu (Table 2), indicating that the color changed from light yellow to orange. The difference in fruit color between ‘Rangpur’ and ‘Kusaie’ was more significant in the peel compared with in the flesh. This tendency was similar to that in Hyuganatsu and ‘Orange-hyuga.’ Such mutations indicate the occurrence of some alteration in carotenoid biosynthesis, as the color of citrus fruits is almost determined by the amount and composition of carotenoids.

Based on the HPLC analysis of the carotenoid contents in the flavedo, there were no significant differences in total carotenoid contents between the flavedos of ‘Kusaie’ and ‘Rangpur’ (Fig. 4). However, a remarkable accumulation of phy, which was detected in the flavedo tissue of ‘Kusaie,’ was not observed in ‘Rangpur’ (Fig. 4). Furthermore, the contents of β -cry, *t*-vio, and *c*-vio decreased in ‘Kusaie’ compared with those in ‘Rangpur’ (Fig. 4). Therefore, in ‘Kusaie,’ the peel color changed from reddish-orange to yellow due to a significant decrease in orange-colored carotenoids (β -cry, *t*-vio, and *c*-vio) and the accumulation of colorless phy. A previous report (Rodrigo et al. 2003) characterized

the ‘Pinalate’ as a mutant of the ‘Navelate’ (*Citrus sinensis* Osbeck), which has a yellow fruit instead of orange. In the mutant flavedo, linear carotenoids (phytoene, phytofluene, and ζ -carotene) accumulated, and thus ‘Pinalate’ was suggested to be partially blocked during ζ -carotene desaturation. Likewise, in our study, the accumulation of phy in the flavedo of ‘Kusaie’ seemed to indicate a blockage of PDS. Moreover, carotenoids are precursors of the phytohormone ABA. To investigate whether the alteration in carotenoid biosynthesis also affected ABA synthesis, we analyzed the fruits’ ABA contents. The ABA content in the flavedo of ‘Kusaie’ was ~40% lower than that in the flavedo from ‘Rangpur’ (Table 3). Therefore, we speculated that PDS was partially blocked and that ABA biosynthesis downstream of carotenoid biosynthesis was also suppressed. ‘Pinalate,’ a mutant of ABA deficiency in the flavedo, has been used to reveal the effects of ABA on postharvest performance (Alf  rez et al. 2005, Sala et al. 2005) and molecular response in peel ripening (Romero et al. 2019) in the ‘Navelate.’ Like ‘Pinalate,’ ‘Kusaie’ might be a mutant of ABA deficiency in the flavedo and could contribute to informing the action of ABA in the flavedo by comparing its physiology with that of ‘Rangpur.’

In contrast, in the juice sac of ‘Kusaie,’ an accumulation of phytoene as seen in the flavedo was not observed. However, the total carotenoid value in the juice sacs of ‘Kusaie’ was approximately one-fourth of that of ‘Rangpur,’ and β -cry and violaxanthin levels were significantly reduced (Fig. 5). These changes in the carotenoids of the juice sacs were thought to be responsible for the change in flesh color. In the juice sacs of ‘Kusaie,’ the amount of total carotenoids has probably been decreased by reducing the overall production of carotenoids, or the degradation of carotenoids may have been accelerated by promoting downstream enzymes (containing 9-*cis* epoxycarotenoid dioxygenase [NCED]) of carotenoid biosynthesis. In the former case, PSY, which controls flux into the carotenoid pathway, might have been downregulated in the juice sac of ‘Kusaie.’ In the latter case, the ABA content, which is a carotenoid metabolite, may have been increased by upregulated NCED. NCED likely plays a primary role in the cleavage of 9-*cis*-violaxanthin to form C25 epoxy-apocarotenal and xanthoxin to produce ABA in the juice sacs of some citrus species (Kato et al. 2006). However, the ABA content of ‘Kusaie’ was not significantly different from that of ‘Rangpur’ (Table 3), and there was no increase in ABA content due to accelerated carotenoid degradation. Therefore, we speculated that the downregulation of PSY might have suppressed the production of carotenoids in the juice sacs of ‘Kusaie.’ Furthermore, we clarified that

the changes in carotenoid composition in 'Kusaie' and 'Rangpur' differed between the flavedo and juice sac, suggesting that different biosynthetic processes might be involved.

Regarding the flavedo tissue of 'Orange-hyuga,' the contents of phy and β -car were similar to those in the flavedo tissue of Hyuganatsu, but the amount of total carotenoids in 'Orange-hyuga' was approximately 6-fold of that in Hyuganatsu. Furthermore, remarkable accumulation of xanthophylls, especially that of violaxanthin, was observed in 'Orange-hyuga' (Fig. 6). It was thought that the peel color changed from light yellow to orange due to the remarkable accumulation of violaxanthin in 'Orange-hyuga.' Kato et al. (2004) reported that the juice sacs of the satsuma mandarin predominantly accumulated β -cry, whereas the juice sacs of the Valencia orange predominantly accumulated violaxanthins. In these varieties, the expression balance between the upstream genes (PSY, PDS, ZDS, and lycopene β -cyclase) and the downstream genes (β -ring hydroxylase and zeaxanthin epoxidase) in carotenoid synthesis was different. They assumed the expression balance between the upstream and downstream synthesis genes was important in determining the ratio of β -cry/violaxanthin in citrus fruits (Kato et al. 2004). For 'Orange-hyuga,' we speculated that the biosynthesis activity upstream (PSY, ZDS, lycopene β -cyclase, and β -ring hydroxylase) was enhanced relative to that of Hyuganatsu, but that the biosynthesis activity downstream (zeaxanthin epoxidase and 9-*cis*-epoxycarotenoid dioxygenase) was suppressed. This might be due to the accumulation of xanthophylls in the flavedo tissue of 'Orange-hyuga.' The ABA content in the flavedo of 'Orange-hyuga' was ~50% of that from Hyuganatsu. This might have been a result of downstream suppression of carotenoid biosynthesis. It was thought that the oxidative cleavage of 9-*cis*-violaxanthin catalyzed by NCED affects the 9-*cis*-violaxanthin content and the carotenoid profiles in the juice sacs of some citrus species during fruit maturation (Kato et al. 2006).

In the present study, carotenoid metabolism was investigated in color-altered citrus cultivars. The expression levels of synthetic genes may regulate the carotenoid content and composition. In the flavedo of 'Kusaie,' significant accumulation of phy and reduced content of xanthophyll were observed, suggesting that PDS was suppressed. Moreover, the total carotenoid level in the juice sacs of 'Kusaie' was reduced compared with that of 'Rangpur,' and carotenoid production might be suppressed in the juice sacs of 'Kusaie.' In 'Orange-hyuga,' violaxanthin was remarkably accumulated, which might be associated with an altered balance of

gene expression upstream and downstream of biosynthesis. Therefore, the differences in biosynthesis regulation in those color-altered cultivars seemed to be the cause of the opposite results from reddish-orange in 'Rangpur' to yellow in 'Kusaie,' and from a light yellow color in Hyuganatsu to an orange color in 'Orange-hyuga.'

Studies have recently been conducted to elucidate the carotenoid accumulation mechanism in sweet orange and grapefruit by analyzing carotenoid accumulation and biosynthetic gene expression compared with color-changing mutants and original types (Alquezar et al. 2008, 2013, Wei et al. 2014). We speculated that different carotenoid (phy or violaxanthin) accumulation levels from the original type occurred in color-changing citrus mutants whose carotenoid composition and ABA content were revealed in the present study, resulting in different changes in carotenoid biosynthesis regulation. Moreover, they could be used in the future to elucidate the mechanism of specific carotenoid accumulation by analyzing the expression of carotenoid biosynthetic genes. These mutants are potential sources of useful information for genetically and physiologically regulating carotenoid content.

Acknowledgements

We thank the laboratory leader at that time of the research, Toshio Yoshida, for providing information about 'Kusaie' and 'Orange-hyuga.' We also thank Editage (www.editage.com) for English language editing.

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