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Review

Mechanism of Carotenoid Accumulation in Citrus Fruit

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Citrus is a complex source of carotenoids with the largest number of carotenoids found in any fruit. Carotenoid concentration and composition vary greatly among citrus varieties. Satsuma mandarin (*Citrus unshiu* Marc.) predominantly accumulates β-cryptoxanthin in the juice sacs. Valencia orange (*Citrus sinensis* Osbeck) predominantly accumulates violaxanthin isomers in the juice sacs. Lisbon lemon (*Citrus limon* Burm.f.) accumulates low level of carotenoids in the juice sacs. To elucidate the carotenoid accumulation in citrus fruit maturation, the expression of genes related to carotenoid biosynthesis and catabolism was investigated in the three citrus varieties exhibited different carotenoid profile. The results showed that the carotenoid accumulation during citrus fruit maturation is highly regulated by the coordination of the expression for the genes related to carotenoid biosynthesis and catabolism in both flavedo and juice sacs. 'Tamami' is a hybrid between 'Kiyomi' tangor (*Citrus unshiu* Marc. × *Citrus sinensis* Osbeck) and 'Wilking' mandarin (*Citrus nobilis* Lour. × *Citrus deliciosa* Ten.). To elucidate the mechanism of the accumulation of β-cryptoxanthin in 'Tamami', a variety accumulating higher β-cryptoxanthin than Satsuma mandarin, the expression of genes related to carotenoid biosynthesis and catabolism was investigated in the juice sacs of 'Tamami'. The results showed that the mechanism of β-cryptoxanthin accumulation in 'Tamami' was similar to that in Satsuma mandarin. Furthermore, in the recent studies, possible factors, which regulate carotenoid concentration and composition in citrus juice sacs were investigated *in vitro*.

Key Words: β-cryptoxanthin, carotenoid, citrus, fruit maturation, Satsuma mandarin.

Introduction

In nature, there are more than 700 identified carotenoids, which are divided into two groups, carotenes that consist of hydrocarbon structure, and xanthophylls that contain oxygen atoms in the structure. In our dietary, β-carotene and lycopene clarified in the carotenes are responsible for orange color in carrot and red color in tomato, respectively. In xanthophylls, lutein in spinach and broccoli and β-cryptoxanthin in Satsuma mandarin are well-known.

Carotenoids are essential components of the photosynthetic apparatus in plants, algae, and cyanobacteria, in which they protect against photooxidative damage and contribute to light harvesting for photosynthesis (Goodwin, 1980). In higher plants, the bright yellow, orange, and red colors provided by carotenoids accumulate in chromoplasts of flowers and fruits. In these tissues, plants exploit carotenoids as colorants to attract pollinators and agents of seed dispersal. In addition, epoxy-carotenoids, 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, can be metabolized to a plant hormone, abscisic acid (ABA) (Rock and Zeevaart, 1991). Some carotenoids serve as precursors for vitamin A, which play an essential role in human and animal diets and as antioxidants, which play a role in reducing the risk of certain forms of cancer (Olson, 1989). The function of β-cryptoxanthin for human health has been studied (Männistö et al., 2004; Yuan et al., 2003). These studies suggested that high levels of dietary β-cryptoxanthin were associated with a reduced risk of lung cancer. Nishino et al. (2009) reviewed prevention of cancer by carotenoids, especially β-cryptoxanthin. In addition, the study showed that β-cryptoxanthin stimulated bone formation and inhibited bone resorption in tissue culture *in vitro* (Yamaguchi and Uchiyama, 2004). Recently, Sugiura et al. (2011) reported that the combination of vitamin C and β-cryptoxanthin intakes might provide benefit to bone health in post-menopausal Japanese female subjects. Thus, we concluded that β-cryptoxanthin is beneficial to human health. We think that it is important for the promotion of health and the fruit industry in Japan to breed citrus varieties which contain higher β-

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cryptoxanthin and to develop techniques that improve β-cryptoxanthin concentration in citrus fruit after harvest.

1. Carotenoid biosynthesis in plants

The pathway of carotenoid biosynthesis in plants is illustrated in Figure 1 (Cunningham and Gantt, 1998; Ronen et al., 1999). The first committed step in carotenoid biosynthesis is the head-to-head condensation of two molecules of geranylgeranyl pyrophosphate (C_{20}; GGPP) to form colorless phytoene (C_{40}) catalyzed by phytoene synthase (PSY). Phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) introduce four double bonds into phytoene to yield lycopene via phytofluene, ζ-carotene, and neurosporene. Cyclization of lycopene is a crucial branching point in this pathway, yielding α-carotene with one ε-ring and one β-ring, and β-carotene with two β-rings, in which two cyclases, namely, lycopene β-cyclase (LCYb) and lycopene ε-cyclase (LCYe), are responsible for these reactions (Cunningham et al., 1996). α-Carotene is converted to lutein by sequential hydroxylation, which is catalyzed by ε-ring hydroxylase and β-ring hydroxylase (HYb), respectively. β-Carotene is converted to zeaxanthin via β-cryptoxanthin by two-step hydroxylation, which is catalyzed by HYb. Furthermore, zeaxanthin is converted to violaxanthin via antheraxanthin by zeaxanthin epoxidase (ZEP).

Recently, genes encoding enzymes for the main steps of carotenoid metabolism have been isolated and their expression has been characterized in plants (Alquézar et al., 2009; Kato et al., 2004, 2006; Kita et al., 2007). During fruit ripening, transcriptional regulation of carotenoid genes appears to be the major mechanism by which the biosynthesis and accumulation of specific carotenoids are regulated.

Carotenoid biosynthesis and its regulation have been studied in various plant species, such as *Arabidopsis* (Park et al., 2002; Pogson et al., 1996) and tomato (Fraser et al., 1994; Giuliano et al., 1993; Isaacson et al., 2002; Ronen et al., 1999). Bramley (2002) reviewed carotenoid biosynthesis and regulation during ripening and development in tomato fruit. During tomato fruit ripening, the expression of PSY and PDS increased (Fraser et al., 1994; Giuliano et al., 1993; Isaacson et al., 2002; Ronen et al., 1999), whereas the expressions of both LCYb and

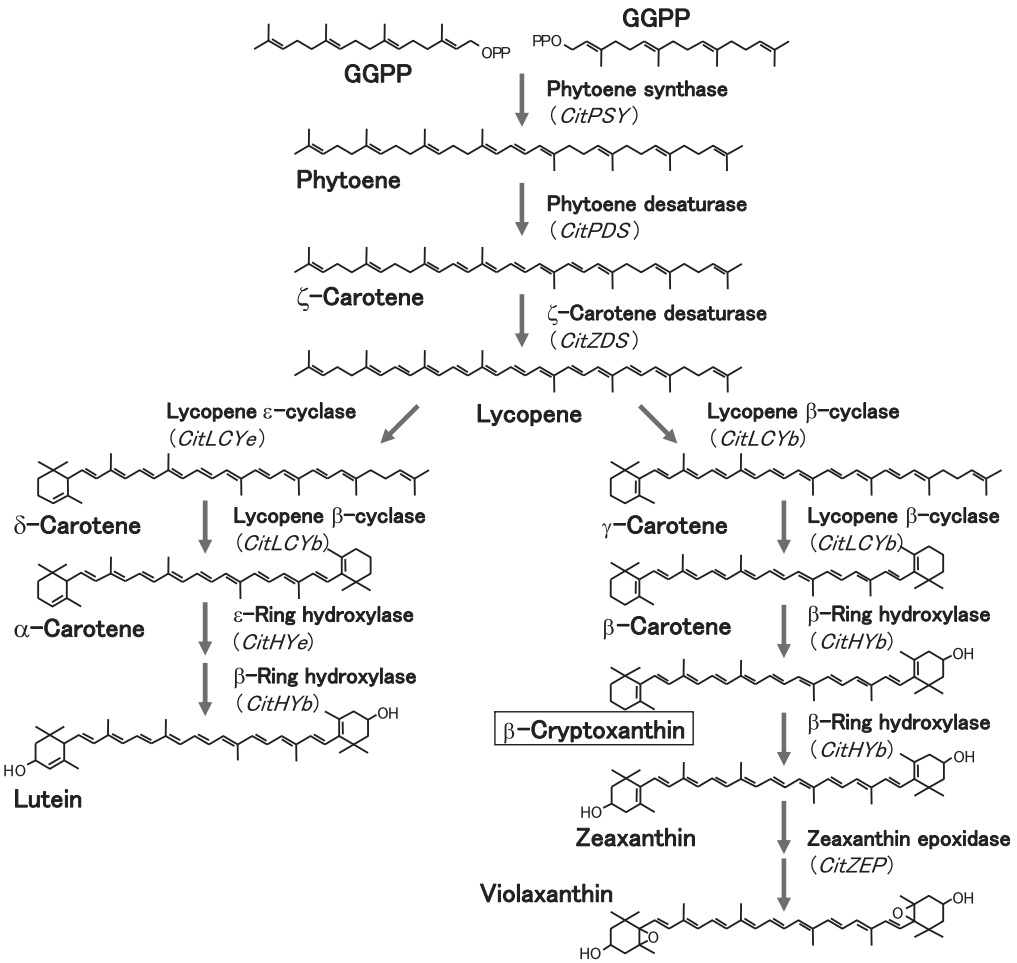

Fig. 1. Carotenoid biosynthetic pathway in citrus. *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, *CitZEP*, and *CitLCYe* were cDNAs for carotenoid biosynthesis genes from Satsuma mandarin, Valencia orange, and Lisbon lemon. β-Cryptoxanthin is catalyzed from β-carotene by β-ring hydroxylase. GGPP, geranylgeranyl pyrophosphate.

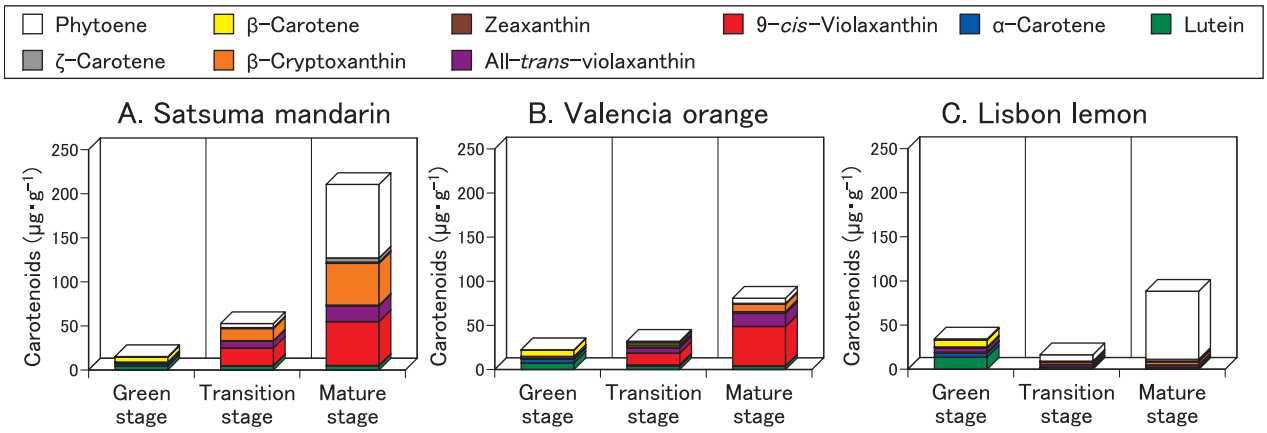

Fig. 2. Changes in carotenoid concentration in flavedos of Satsuma mandarin, Valencia orange, and Lisbon lemon during fruit maturation. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon.

LCYe disappeared (Pecker et al., 1996; Ronen et al., 1999), leading to marked accumulation of lycopene.

Citrus is a complex source of carotenoids, with the largest number of carotenoids found in any fruit (Gross, 1987). During citrus fruit development, marked accumulation of xanthophylls occurred concomitantly with the degradation of chlorophyll. These materials are especially useful for understanding the mechanism of xanthophyll accumulation in fruit, since marked xanthophyll accumulation does not occur in common experimental materials, such as tomato and *Arabidopsis*.

Carotenoid concentration and composition are influenced by growing conditions and fruit maturity. They also differ among geographical origins. Thus, the carotenoid concentration and composition vary greatly among citrus varieties. Mandarin varieties, such as Satsuma mandarin (*Citrus unshiu* Marc.), accumulated β-cryptoxanthin predominantly in the flavedo as well as juice sacs in mature fruit (Goodner et al., 2001; Ikoma et al., 2001). In contrast, mature sweet orange (*Citrus sinensis* Osbeck) accumulated violaxanthin isomers predominantly in fruit (Lee and Castle, 2001; Molnár and Szabolcs, 1980), in which 9-*cis*-violaxanthin was found to be the principal carotenoid (Molnár and Szabolcs, 1980). Mature lemon (*Citrus limon* Burm.f.) showed a light yellow color in the flavedo as well as juice sacs. This light coloration was primarily due to the low concentration of total carotenoids, which was much lower than in sweet orange, such as Navel orange (Yokoyama and Vandercook, 1967). Thus, Satsuma mandarin (variety accumulating β-cryptoxanthin), Valencia orange (variety accumulating violaxanthin),

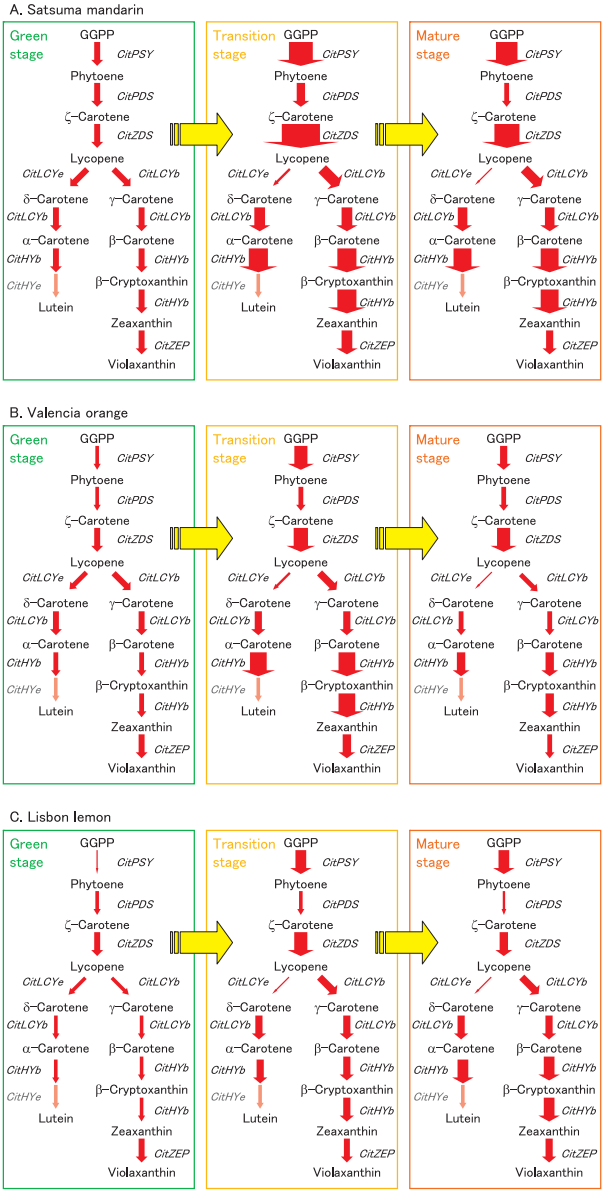

Fig. 3. Expression patterns of carotenoid biosynthetic genes in flavedos of Satsuma mandarin, Valencia orange, and Lisbon lemon during fruit maturation. The width of arrows indicates relative expression levels of carotenoid biosynthesis genes except for *CitHYe* among stages (green, transition, and mature stages) and among the three citrus varieties. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon.

and Lisbon lemon (variety accumulating small amounts of carotenoids) are useful experimental materials to investigate the molecular mechanism regulating carotenoid concentration and composition in fruit because carotenoid profiles in mature fruit were much more diverse among these three citrus varieties.

2. Carotenoid accumulation and expression of carotenoid biosynthetic genes in flavedo

The color of the flavedo changed from green to orange during fruit maturation in Satsuma mandarin and Valencia orange. In Lisbon lemon, it changed from green to yellow during fruit maturation. The green stages in Satsuma mandarin, Valencia orange, and Lisbon lemon were August and September (approximately 90 and 120 days after flowering (DAF)), August, September, and October (approximately 90, 120, and 150 DAF), and August and October (approximately 90 and 150 DAF), respectively.

During the green stage, β-carotene, all-*trans*-violaxanthin, α-carotene, and lutein were predominant, although the amounts of these carotenoids were low in these three varieties (Fig. 2) (Kato et al., 2004). During this stage, high gene expression of *CitLCYe* and low gene expression of *CitPSY*, *CitZDS*, and *CitHYb* were observed (Fig. 3). The high expression of the *CitLCYe* gene suggested that cyclization to the ε-ring in this stage was more active than that in subsequent stages, resulting in the predominant accumulation of β,ε-carotenoid (α-carotene and lutein) in the green stage. In tomato fruit, the LCYe gene was expressed exclusively in chloroplast-containing photosynthetic tissues (Ronen et al., 1999). In addition, it was thought that these low gene expressions of *CitPSY*, *CitPDS*, and *CitZDS*, which produce linear carotenes, were responsible for the low concentrations of carotenoids in this stage.

Transition stages in Satsuma mandarin, Valencia orange, and Lisbon lemon were October and November (approximately 150 and 180 DAF), November and December (approximately 180 and 210 DAF), and December (approximately 210 DAF), respectively. In the transition stage, the gene expression of *CitLCYe* decreased, whereas the gene expression of *CitLCYb* increased in the three varieties (Fig. 3). These results suggested that the pathway changing from β,ε-carotenoid (α-carotene and lutein) synthesis to β,β-carotenoid (β-carotene, β-cryptoxanthin, zeaxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin) synthesis occurred in the flavedo with the transition from the green stage to mature stage. During the transition stage, simultaneous increases in the expression of genes participating in β,β-xanthophyll synthesis (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, and *CitZEP*) and marked accumulation of β,β-xanthophylls (β-cryptoxanthin, zeaxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin) were observed in Satsuma mandarin and Valencia orange (Figs. 2A, B and 3A, B). These results suggested that the simultaneous increases in the gene expressions were responsible for the marked accumulation of β,β-xanthophylls in the flavedos of Satsuma mandarin and Valencia orange. In Lisbon lemon, the concentrations of β,β-xanthophylls remained low, although the expression of a gene set to produce β,β-xanthophylls increased slightly during the transition stage (Figs. 2C and 3C). The increased levels of the gene expression in Satsuma mandarin were higher than those in Valencia orange and Lisbon lemon.

In the mature stage, marked accumulation of phytoene was observed in Satsuma mandarin after the accumulation of β,β-xanthophyll (Fig. 2A). In Lisbon lemon, instead of the marked accumulation of β,β-xanthophylls, phytoene accumulated (Fig. 2C). During the mature stage, the gene expression of *CitPSY* remained high in Satsuma mandarin, whereas the gene expression of *CitPDS* clearly decreased to a low level in Satsuma mandarin (Fig. 3A). These results suggested that, in the case of the flavedo of Satsuma mandarin, the accumulation of phytoene could be primarily explained by the levels of the gene expressions of *CitPSY* and *CitPDS* (high expression of *CitPSY* gene and low expression of *CitPDS* gene) (Fig. 3A). In Lisbon lemon, the gene expression of *CitPDS* was low, which was similar to the decreased level in Satsuma mandarin, suggesting that lack of action in carotenoid desaturases led to the marked accumulation of phytoene and limited accumulation of β,β-xanthophylls (Fig. 3C).

3. Carotenoid accumulation and expression of carotenoid biosynthetic genes in juice sacs

During the green stage of the flavedo (August and September in Satsuma mandarin, August, September, and October in Valencia orange, and August and October in Lisbon lemon), the concentration of carotenoids was low in the juice sacs in the three varieties (Fig. 4). In Satsuma mandarin, β-cryptoxanthin accumulated in the juice sacs, which was about 9.7-fold higher than that in the flavedo. The color of juice sacs in Satsuma mandarin was yellow because of β-cryptoxanthin accumulation; however, in Valencia orange and Lisbon lemon, the color of juice sacs was pale yellow in the green stage because no noticeable accumulation of β-cryptoxanthin was observed (Fig. 4) (Kato et al., 2004). During the transition stage, marked accumulation of carotenoids, especially β,β-xanthophylls, occurred in Satsuma mandarin and Valencia orange (Fig. 4A, B). In the mature stage, Satsuma mandarin accumulated predominantly β-cryptoxanthin, which accounted for 62.1% of the total identified carotenoids (Fig. 4A), whereas Valencia orange accumulated predominantly violaxanthin isomers (all-*trans*-violaxanthin and 9-*cis*-violaxanthin), which accounted for 67.9% of the total identified carotenoids (Fig. 4B). In Lisbon lemon, the concentration of carotenoids was extremely low, although β-cryptoxanthin accumulated slightly (Fig. 4C). Clearly, the gene expres-

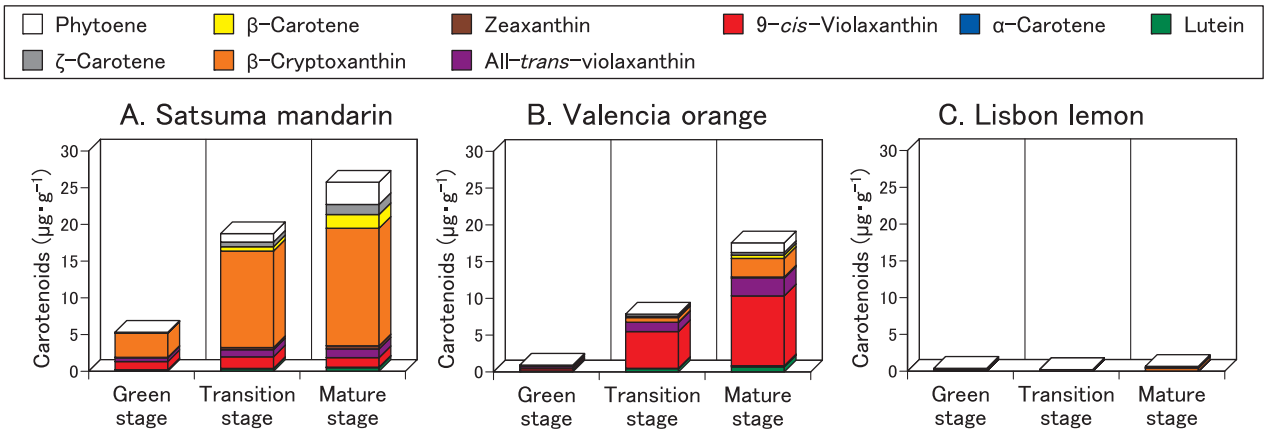

Fig. 4. Changes in carotenoid concentration in juice sacs of Satsuma mandarin, Valencia orange, and Lisbon lemon during fruit maturation. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon.

sion of a set of genes to produce β,β-xanthophylls (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, and *CitZEP*) increased after the green stage, reaching a maximum in Satsuma mandarin and Valencia orange. In Lisbon lemon, the gene expression of a set of genes to produce β,β-xanthophylls also increased; however, the gene expressions were much lower than those in Satsuma mandarin and Valencia orange.

4. Xanthophyll composition and gene expression related to carotene and β,β-xanthophyll syntheses in Satsuma mandarin and Valencia orange fruits

In the transition stage (October and November for Satsuma mandarin, and November and December for Valencia orange), β,β-xanthophylls increased markedly in both the flavedo and juice sacs (Figs. 2A, B and 4A, B); however, a clear difference in the β,β-xanthophyll composition between Satsuma mandarin and Valencia orange fruits was observed. Satsuma mandarin accumulated β-cryptoxanthin as a major carotenoid in mature fruit, whereas Valencia orange mainly accumulated violaxanthin isomers (Fig. 4A, B) (Goodner et al., 2001; Ikoma et al., 2001; Lee and Castle, 2001; Molnár and Szabolcs, 1980). The ratios of β-cryptoxanthin/violaxanthin in juice sacs were 5.17 in Satsuma mandarin and 0.10 in Valencia orange. The difference between these ratios was larger in juice sacs than in flavedo (0.43 and 0.11 in Satsuma mandarin and Valencia orange, respectively).

To compare gene expressions related to the syntheses of carotene and β,β-xanthophyll between Satsuma mandarin and Valencia orange during the period of marked β,β-xanthophyll increase, the transcript levels of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, and *CitZEP* during these two months were examined. In the flavedo, the levels of transcripts involved in carotene synthesis (*CitPSY*, *CitPDS*, *CitZDS*, and *CitLCYb*) and β,β-xanthophyll synthesis (*CitHYb* and *CitZEP*) were higher in Satsuma mandarin than in Valencia orange. In juice sacs, the levels of *CitPSY*, *CitPDS*, *CitZDS*, and *CitLCYb* transcripts involved in biosynthesis from phytoene to β-carotene were higher in Satsuma mandarin (Fig. 5). In contrast, the levels of *CitHYb* and *CitZEP* transcripts involved in the biosynthesis from β-cryptoxanthin to violaxanthin in Valencia orange were much higher than in Satsuma mandarin (Fig. 5).

We isolated cDNAs encoding complete coding regions for HYb from Satsuma mandarin and Valencia orange. The deduced amino acid sequences between these cDNAs were identical except for one amino acid residue, which was located in the transit peptide. This result suggested that the amino acid sequences between these cDNAs were identical in the regions related to enzyme activity. Thus, we thought that the difference in the amino acid sequences between these cDNAs was not responsible for the difference in the β,β-xanthophyll composition between Satsuma mandarin and Valencia orange.

A previous study demonstrated that β-cryptoxanthin instead of zeaxanhtin was mainly accumulated in *Escherichia coli* cells carrying the truncated *Arabidopsis* HYb gene (Sun et al., 1996). The report speculated that the HYb hydroxylated β-rings of β-carotene with greater efficiency than the not-yet-hydroxylated β-ring of β-cryptoxanthin. Because of the high substrate specificity of HYb to β-carotene, HYb would prefer the first-step conversion from β-carotene to β-cryptoxanthin rather than the second-step conversion from β-cryptoxanthin to zeaxanthin under excessive β-carotene supply and/or low HYb activity.

In citrus fruits, the substrate specificity of HYb and expression balance between upstream synthesis genes (*CitPSY*, *CitPDS*, *CitZDS*, and *CitLCYb*) and downstream synthesis genes (*CitHYb* and *CitZEP*) seem important to determine the ratios of β-cryptoxanthin/violaxanthin. As shown in Figure 5, the levels of transcripts for upstream synthesis genes (*CitPSY*, *CitPDS*, *CitZDS*, and *CitLCYb*) in juice sacs were higher in Satsuma mandarin than in Valencia orange. In contrast, the transcript level of the CitHYb gene was lower in

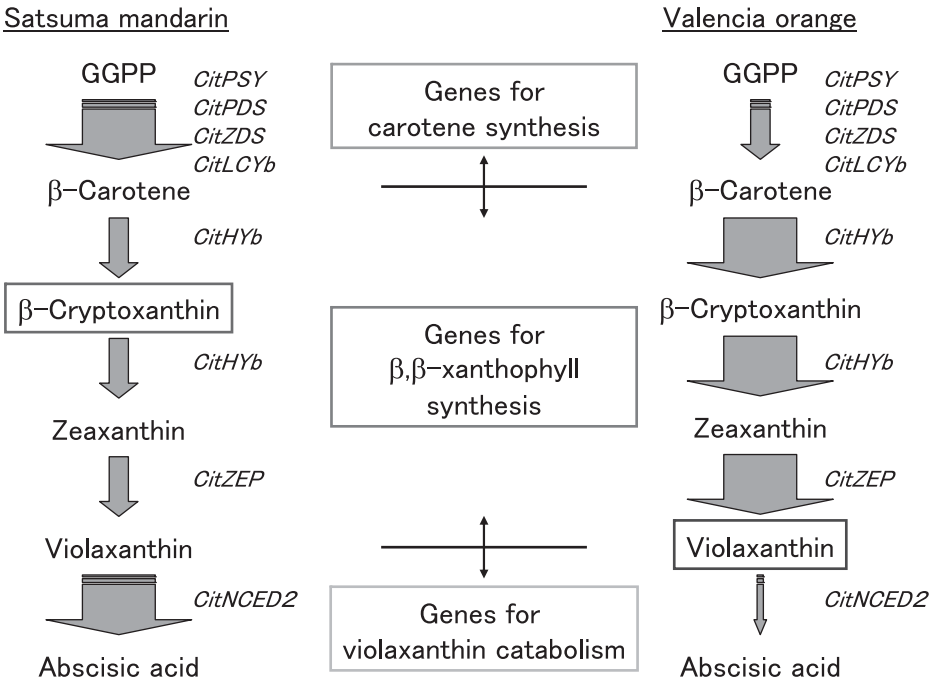

Fig. 5. Comparisons of gene expression for carotenoid biosynthesis and catabolism between Satsuma mandarin and Valencia orange in juice sacs during massive β,β-xanthophyll accumulations (transition stage).

Satsuma mandarin than in Valencia orange. The higher expression of upstream synthesis genes and lower expression of the HYb gene suggested a higher supply of β-carotene and lower activity of CitHYb in juice sacs of Satsuma mandarin than in those of Valencia orange. Indeed, β-carotene increased more during maturation in juice sacs of Satsuma mandarin than in those of Valencia orange (Fig. 4A, B) (Kato et al., 2004). The increased level of β-carotene in the juice sacs of Satsuma mandarin also suggested a higher supply of β-carotene and lower activity of CitHYb than in those of Valencia orange. Therefore, it is thought that, under the balance of high gene expression of upstream synthesis and low gene expression of *CitHYb* (high supply of β-carotene and low activity of CitHYb), CitHYb catalyzes predominantly the first-step conversion by the high substrate specificity of HYb to β-carotene, leading to marked accumulation of β-cryptoxanthin. Moreover, since β-carotene was rarely converted to zeaxanthin via β-cryptoxanthin and the gene expression of *CitZEP* was low in juice sacs of Satsuma mandarin, the accumulation of violaxanthin may be restricted in this tissue.

In contrast, in juice sacs of Valencia orange, CitHYb is likely to sufficiently catalyze the reaction to zeaxanthin via β-cryptoxanthin because of the low gene expression of upstream synthesis and high gene expression of *CitHYb* (low supply of β-carotene and high activity of CitHYb). Moreover, the intensity and duration of the gene expression related to epoxidation, *CitZEP*, were much higher and longer in juice sacs of Valencia orange than in those of Satsuma mandarin. Thus, zeaxanthin would be rapidly converted to violaxanthin in juice sacs of Valencia orange.

In the flavedo, the gene expression involved in carotenoid biosynthesis (both upstream and downstream syntheses) was much higher in Satsuma mandarin than in Valencia orange. The varietal difference in the expression balance between upstream synthesis genes and downstream synthesis genes was much smaller in the flavedo than in juice sacs. Thus, a small difference between Satsuma mandarin and Valencia orange in the expression balance of the genes resulted in a small difference in β,β-xanthophyll composition in the flavedo.

5. Carotenoid catabolism in plants

Carotenoids are metabolized to apocarotenoids through the pathway catalyzed by carotenoid cleavage dioxygenases (CCDs). Apocarotenoids are responsible for the regulation of gene expression in both plants and animals (Moise et al., 2005). Most animals metabolize carotenoids to diterpenoid molecules, such as retinal, a visual chromophore, and retinoic acid, a signal molecule in gene regulation. In higher plants, ABA, which is a well-known apocarotenoid derivative, is necessary for seed development and environmental adaptation (Leung and Giraudat, 1998).

The CCD family consists of different members, which have a different amino acid sequence, subucellular location, double bond cleavage specificity. Among them, CCD1 is one of the best-studied CCDs due to its involvement in C_{13} apocarotenoid-based flower scent as well as fruit and wine aroma biosynthesis. It catalyzes the 9–10 and 9'–10' cleavages of multiple carotenoid substrates to form C_{14} dialdehyde and two C_{13} products,

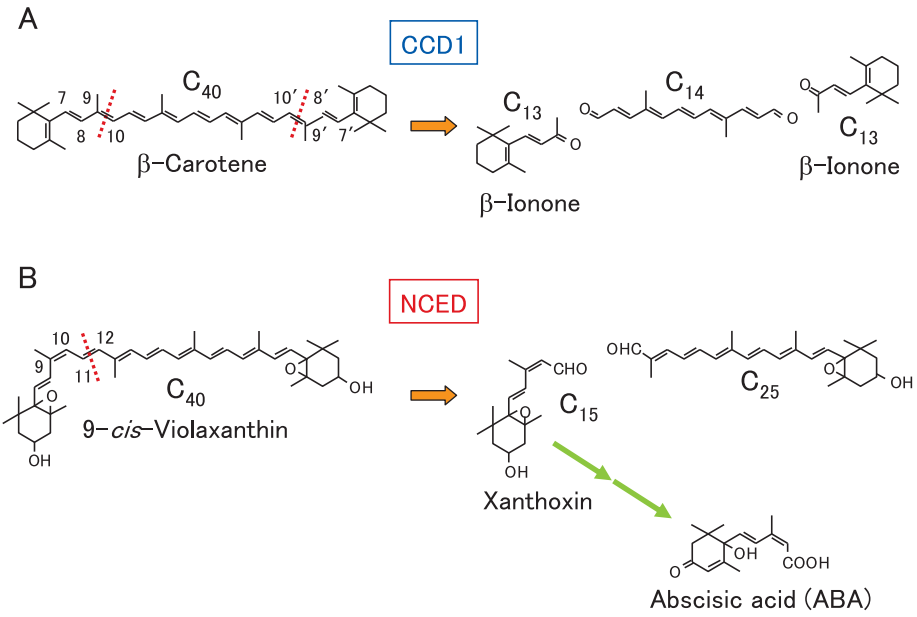

Fig. 6. Enzymatic reactions of CCD1 (A) and NCED (B) in carotenoid catabolism. When CCD1 catalyzes the cleavage of β-carotene, β-ionone is synthesized. NCED catalyzes the cleavage of 9-*cis*-violaxanthin to synthesize xanthoxin, precursor of abscisic acid (ABA).

which vary depending on the carotenoid substrate (Schwartz et al., 2001). When CCD1 cleaved β-carotene as a substrate, the volatile apocarotenoid β-ionone, an important flower fragrance and fruit flavor, was produced (Fig. 6A) (Baldermann et al., 2010; Schwartz et al., 2001; Simkin et al., 2004a, b). The CCD1 gene has been cloned from avocado (*PaCCD1*; Chernys and Zeevaart, 2000), *Arabidopsis* (*AtCCD1*; Schwartz et al., 2001), crocus (*Crocus sativus*; *CsCCD*; Bouvier et al., 2003), tomato (*Solanum Lycopersicon*; *LeCCD1A* and *LeCCD1B*; Simkin et al., 2004a), and petunia (*Petunia hybrida*; *PhCCD1*; Simkin et al., 2004b).

In *Arabidopsis*, there are nine members of the CCD family, five of which are believed to be NCEDs (9-*cis*-epoxycarotenoid dioxygenase) involved in ABA biosynthesis (Tan et al., 2003). NCED catalyzes the cleavage of 9-*cis*-violaxanthin or 9'-*cis*-neoxanthin to form C_{25} epoxy-apocarotenal and xanthoxin (C_{15}), a precursor of ABA (Fig. 6B) (Schwartz et al., 1997, 2003). This reaction is a limiting step in ABA biosynthesis (Chernys and Zeevaart, 2000; Qin and Zeevaart, 1999; Schwartz et al., 2003). The NCED gene has been cloned and characterized in various plant species, such as maize (*Zea mays*; *VP14*; Schwartz et al., 1997), bean (*Phaseolus vulgaris*; *PvNCED1*; Qin and Zeevaart, 1999), cowpea (*Vigna unguiculata*; *VuNCED1*; Iuchi et al., 2000), avocado (*Persea americana*; *PaNCED1* and *PaNCED3*; Chernys and Zeevaart, 2000), and *Arabidopsis* (*AtNCEDs 2*, *3*, *5*, *6*, and *9*; Iuchi et al., 2001; Tan et al., 2003). The expression of NCED is highly activated by stress conditions. Iuchi et al. (2000) found that the induction of VuNCED1 was mainly responsible for ABA biosynthesis under water stress in cowpea. Increases in NCED gene expression in response to drought stress were also observed in *Arabidopsis*, maize, and tomato (Burbidge et al., 1997; Qin and Zeevaart, 1999; Schwartz et al., 1997).

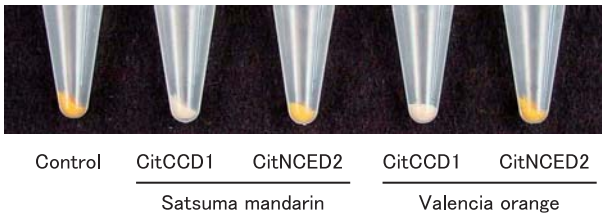

Fig. 7. Functional analyses of CitCCD1 and CitNCED2 from Satsuma mandarin and Valencia orange in zeaxanthin-producing *Escherichia coli*. The zeaxanthin-producing *E. coli* transformed with an empty vector was used as a control.

6. Functional analysis of CCD from citrus

Three CCD cDNAs, *CitCCD1*, *CitNCED2*, and *CitNCED3*, from Satsuma mandarin, Valencia orange, and Lisbon lemon were isolated and their sequences were analyzed. In the putative amino acid sequences of the isolated CCDs from the three citrus varieties, four active center histidines were conserved (Kloer et al., 2005). The recombinant CCD enzymes catalyzed the carotenoid cleavage to yield apocarotenals in the presence of molecular oxygen, ferrous iron, and ascorbate *in vitro*, indicating that CCD enzymes belong to the dioxygenase family (Schwartz et al., 1997).

To confirm the function of CCDs in citrus fruit, plasmids, which produce the recombinant protein for CitCCD1 and CitNCED2 in Satsuma mandarin and Valencia orange, were constructed and transformed into zeaxanthin-producing *E. coli*. The recombinant protein of *CitCCD1* catabolized zeaxanthin and *E. coli* cells

were white (Fig. 7). In contrast, the recombinant protein of *CitNCED2* could not catabolize zeaxanthin, and *E. coli* cells were yellow (Fig. 7).

To investigate the substrate specificity of CitCCDs from Satsuma mandarin, Valencia orange, and Lisbon lemon *in vitro*, we extracted and purified recombinant CCD proteins from *E. coli* cells. As their substrates, β-cryptoxanthin, zeaxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin, which are contained in mature citrus fruit as major β,β-xanthophylls, were tested. The recombinant CitCCD1 protein from the three varieties cleaved β-cryptoxanthin, zeaxanthin, and all-*trans*-violaxanthin to yield C_{14} dialdehyde and C_{13} products (Fig. 8) (Kato et al., 2006). The recombinant CitCCD1 protein from the three varieties also cleaved 9-*cis*-violaxanthin to yield C_{13} product and C_{27} epoxy-apocarotenal (Fig. 8). Previously, recombinant CCD1 proteins were also characterized by their substrate specificity. In *Arabidopsis*, the recombinant AtCCD1 protein cleaved β-carotene, lutein, zeaxanthin, and all-*trans*-violaxanthin of cyclic carotenoids at the symmetrical 9–10 and 9'–10' double bonds to yield two C_{13} products and C_{14} dialdehyde (Schwartz et al., 2001). When 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin were used as a substrate, the recombinant AtCCD1 protein produced a C_{13} product and C_{27} epoxy-apocarotenal (Schwartz et al., 2001). In citrus fruit, it was also found that recombinant CitCCD1 proteins exhibited broad substrate specificity for β,β-xanthophylls (β-cryptoxanthin, zeaxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin).

The recombinant CitNCED2 and CitNCED3 proteins from the three varieties cleaved 9-*cis*-violaxanthin to yield xanthoxin and C_{25} epoxy-apocarotenal, and no products were detected when β-cryptoxanthin, zeaxanthin, and all-*trans*-violaxanthin were tested as substrates (Fig. 6B). These results were consistent with previous results from other plant species, such as maize (Schwartz et al., 1997), bean (Qin and Zeevaart, 1999), and *Arabidopsis* (Iuchi et al., 2001). Therefore, among the three varieties, no difference in substrate specificity was observed for each CCD (CitCCD1, CitNCED2, and CitNCED3).

7. Relationship between ABA accumulation and gene expression of NCEDs in citrus fruit

The localization of NCED proteins has been investigated in various plant species. NCED proteins from other plant species were imported into the chloroplast (Iuchi et al., 2000; Qin and Zeevaart, 1999; Tan et al., 2001, 2003). A previous report suggested that, in the flavedo, the increases in ABA levels were associated with senescence and development of the chromoplast (Harris and Dugger, 1986). Rodrigo et al. (2003) reported that ABA may play a role in the regulation of the rate of fruit coloration in citrus fruit because fruits of the ABA-deficient mutant exhibited a delay in the rate of degreening. The physiological role of ABA accumulation may be involved in chromoplast development and/or the rate of fruit coloration in citrus fruit.

In the flavedo, increases in the ABA level were observed in Satsuma mandarin, Valencia orange, and Lisbon lemon during fruit maturation (Fig. 9). CitNCED genes, which showed different expression patterns, were involved in ABA biosynthesis in the flavedo of each variety (Fig. 9) (Kato et al., 2006). In the flavedo of Satsuma mandarin, the gene expression of *CitNCED2* and *CitNCED3* increased rapidly with ABA accumula-

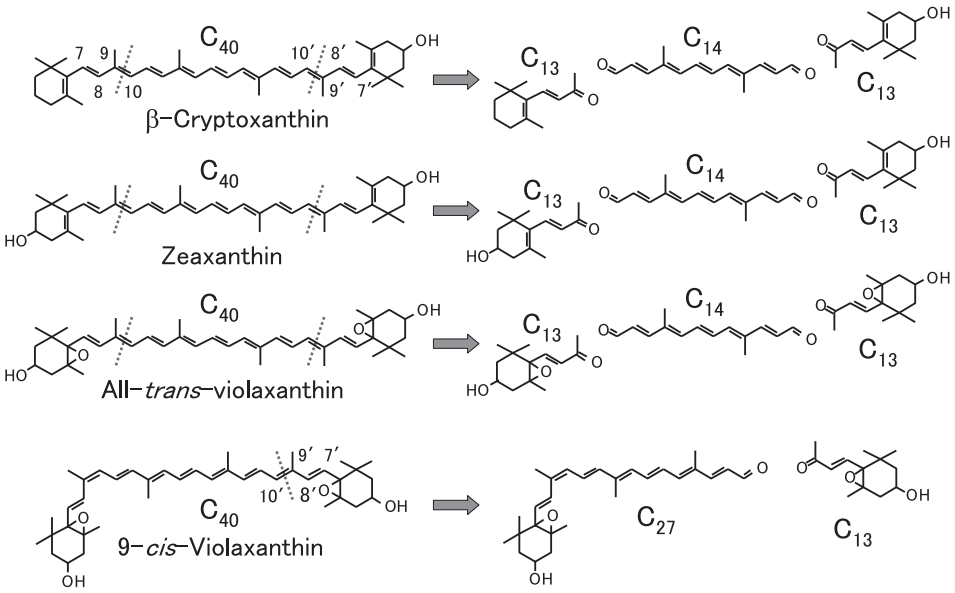

Fig. 8. Function of CitCCD1 from Satsuma mandarin, Valencia orange, and Lisbon lemon. CitCCD1 catalyzes the oxidative cleavage of β,β-xanthophyll (β-cryptoxanthin, zeaxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin). Recombinant CCD enzymes catalyzed β,β-xanthophyll cleavage to yield apocarotenals in the presence of molecular oxygen, ferrous iron, and ascorbate *in vitro*.

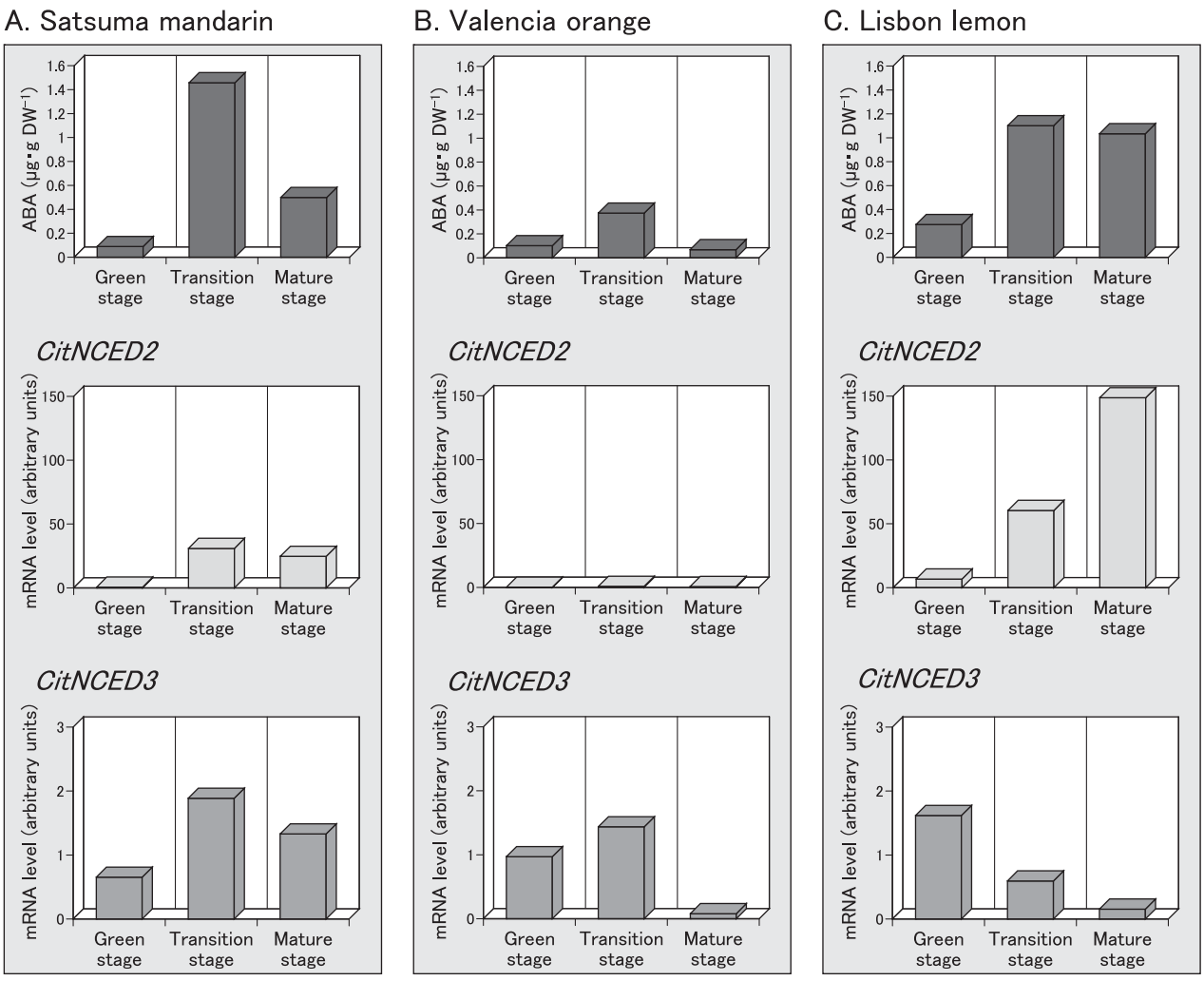

Fig. 9. Changes in ABA content and gene expression of *CitNCED2* and *CitNCED3* in flavedos of Satsuma mandarin, Valencia orange, and Lisbon lemon during fruit maturation. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon.

tion (Fig. 9A). In the flavedo of Valencia orange, no noticeable increase in the gene expression of *CitNCED2* was observed during fruit maturation, whereas the gene expression of *CitNCED3* increased with ABA accumulation (Fig. 9B). It is noteworthy that the level of the gene expression of *CitNCED2* in Lisbon lemon increased marked with ABA accumulation, whereas the gene expression of *CitNCED3* changed irrespective of ABA accumulation (Fig. 9C). These results suggested that the gene expression of *CitNCED2* and *CitNCED3* in Satsuma mandarin, the gene expression of *CitNCED3* in Valencia orange, and the gene expression of *CitNCED2* in Lisbon lemon were primarily responsible for ABA accumulation in their flavedos (Kato et al., 2006).

In the juice sacs, increases in the ABA levels were observed in Satsuma mandarin and Lisbon lemon during fruit maturation (Fig. 10). CitNCED genes, which showed different expression patterns, were involved in ABA biosynthesis in the juice sacs of each variety (Fig. 10) (Kato et al., 2006). In the juice sacs of Satsuma mandarin, the gene expressions of *CitNCED2* and *CitNCED3* increased rapidly with ABA accumulation (Fig. 10A) (Kato et al., 2006). In the juice sacs of Lisbon lemon, the levels of the gene expression of *CitNCED2* increased rapidly with ABA accumulation during the green stage (from August to October), whereas the gene expression of *CitNCED3* changed irrespectively (Kato et al., 2006). These results suggested that the gene expression of *CitNCED2* and *CitNCED3* in Satsuma mandarin and the gene expression of *CitNCED2* in Lisbon lemon were primarily responsible for ABA accumulation in their juice sacs (Kato et al., 2006). In the juice sacs of Valencia orange, the ABA level was much lower than in the two other varieties throughout the experimental period (Fig. 10B). In Valencia orange, no noticeable increase in the gene expression of *CitNCED2* was observed (Fig. 10B). In addition, the gene expression of *CitNCED3* changed irrespective of the ABA level (Kato et al., 2006). These results suggested that, in Valencia orange, the extremely low level of *CitNCED2* was primarily responsible for the

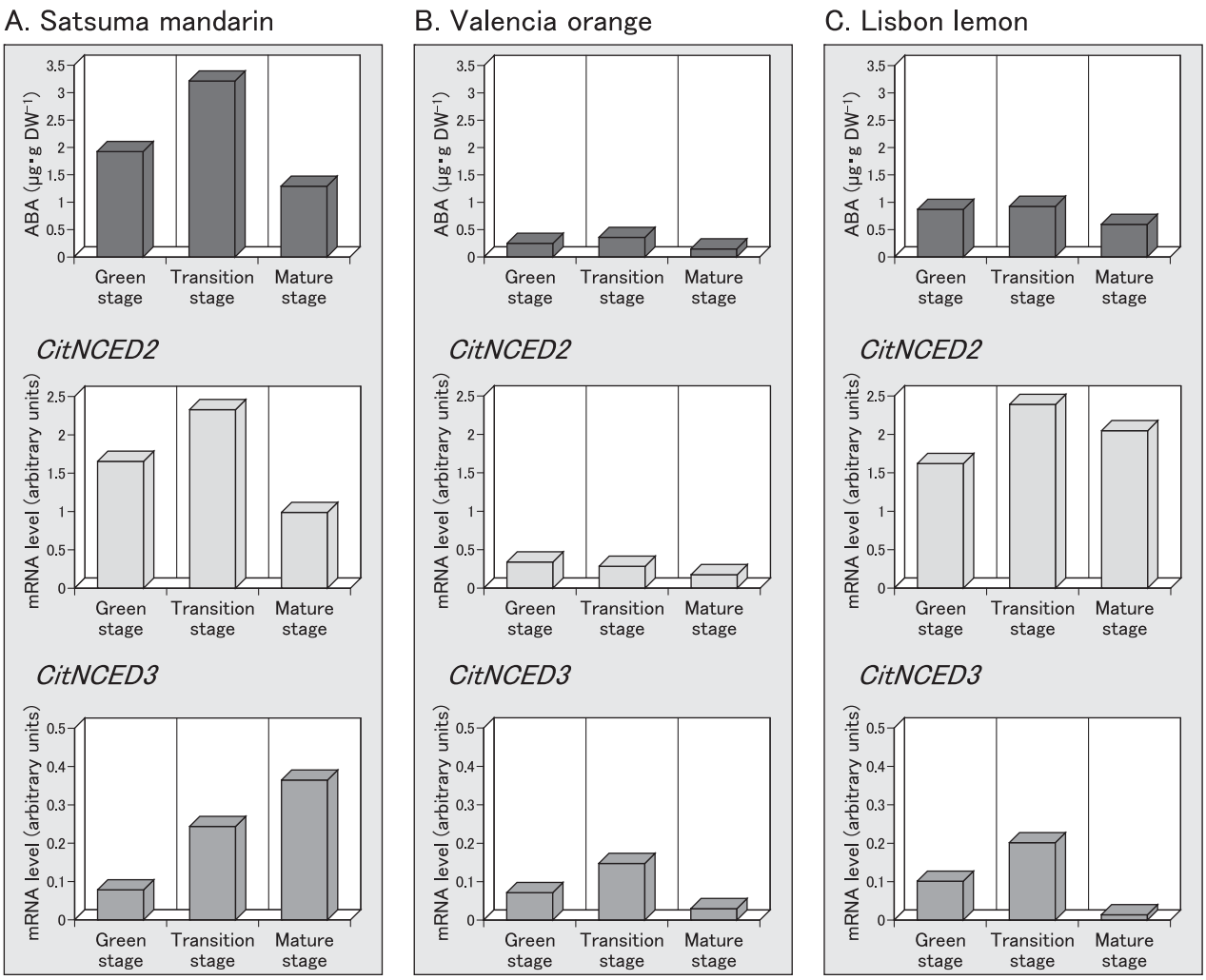

Fig. 10. Changes in ABA content and gene expression of *CitNCED2* and *CitNCED3* in juice sacs of Satsuma mandarin, Valencia orange, and Lisbon lemon during fruit maturation. A, Satsuma mandarin; B, Valencia orange; C, Lisbon lemon.

low level of ABA (Kato et al., 2006). Thus, we thought that the gene expression of *CitNCEDs* was a factor in the regulation of the ABA level during citrus fruit maturation.

8. Relationship between β,β-xanthophyll accumulation and gene expression of CCDs in citrus fruit

In citrus fruit, increases in the gene expression of *CitCCD1* were observed both in the flavedos and juice sacs of the three varieties during maturation (Kato et al., 2006); however, it is likely that the CitCCD1 enzyme did not result in varietal differences in the carotenoid concentration and composition during citrus fruit maturation because, both in the flavedos and juice sacs, no noticeable differences in the levels of the gene expression of *CitCCD1* were observed among Satsuma mandarin, Valencia orange, and Lisbon lemon. In tomato, transgenic plants with reduced expression of *LeCCD1A* and *LeCCD1B* were produced (Simkin et al., 2004a). Their fruits showed significant reductions in the emission rates of β-ionone and geranylacetone but did not show significant changes in the carotenoid concentration. These results are due to the fact that CCD1 protein is a non-plastid targeted enzyme and does not have a significant role in carotenoid turnover (Simkin et al., 2004a). The localization of CCD1 proteins was investigated in various plant species. CCD1 protein from other plant species lacked evident plastid-targeting peptides and was not imported into chloroplasts (Bouvier et al., 2003; Simkin et al., 2004a; Tan et al., 2003). Similarly, CitCCD1 proteins did not contain plastid-targeting peptides, indicating that the proteins probably were not imported into plastids in citrus; therefore, CitCCD1 proteins from the three citrus varieties were not thought to play an important role in carotenoid accumulation.

The level of 9-*cis*-violaxanthin increased more in the flavedos of Satsuma mandarin and Valencia orange than in those of Lisbon lemon (Fig. 2) (Kato et al., 2004). These increases were probably due to not only higher levels of the expression of the gene set to produce β,β-xanthophylls (Kato et al., 2004) but also lower levels of

the gene expression of *CitNCED2* in Satsuma mandarin and Valencia orange than in Lisbon lemon (Kato et al., 2006). In contrast, in the flavedo of Lisbon lemon, the low β,β-xanthophyll concentration, which is responsible for the distinct yellow color of the flavedo in Lisbon lemon, was thought to be caused by the low level of β,β-xanthophyll synthesis by a set of β,β-xanthophyll-synthesizing genes and the high cleavage reaction by NCED.

Clear differences were seen in β,β-xanthophyll concentration and composition among the three varieties in the juice sacs (Fig. 4) (Kato et al., 2004). In mature fruit, the juice sacs of Satsuma mandarin accumulated a low level of 9-*cis*-violaxanthin, whereas those of Valencia orange accumulated a high level of 9-*cis*-violaxanthin (Kato et al., 2004). In Valencia orange, we thought that 9-*cis*-violaxanthin accumulated in the juice sacs did not cleave efficiently by CCDs because the gene expression of *CitNCED2* remained at an extremely low level (Figs. 4 and 5); therefore, in Valencia orange, the accumulation of 9-*cis*-violaxanthin was predominant in the juice sacs. In Satsuma mandarin and Lisbon lemon, we thought that the 9-*cis*-violaxanthin synthesized by carotenoid biosynthesis was cleaved immediately by CCDs because an increase in the gene expression of *CitNCED2* was observed in the juice sacs (Fig. 5). In fact, the levels of ABA in Satsuma mandarin and Lisbon lemon were observed at a higher level than in Valencia orange and the level in Satsuma mandarin noticeably increased (Fig. 10) (Kato et al., 2006); therefore, the accumulation of 9-*cis*-violaxanthin in Satsuma mandarin and Lisbon lemon was much lower than in Valencia orange (Fig. 4) (Kato et al., 2004). In addition, in Lisbon lemon, the accumulation of not only 9-*cis*-violaxanthin but also other β,β-xanthophylls was extremely low because the expression of the gene set to produce β,β-xanthophylls stayed at a much lower level than in Satsuma mandarin during maturation (Fig. 4C) (Kato et al., 2004).

Thus, the oxidative cleavage of 9-*cis*-violaxanthin catalyzed by NCEDs affects the 9-*cis*-violaxanthin concentration and consequently the β,β-xanthophyll composition of the three citrus varieties during fruit maturation; however, it is possible that 9'-*cis*-neoxanthin, another substrate of NCEDs, is also cleaved in citrus fruit and is involved in ABA biosynthesis. The K_m for recombinant PvNCED1 and VP14 is lower with 9'-*cis*-neoxanthin as a substrate relative to 9-*cis*-violaxanthin (Qin and Zeevaart, 1999; Schwartz et al., 2003). In citrus fruit, we did not identify 9'-*cis*-neoxanthin in either the flavedos or juice sacs, probably because of the low concentration. It is unknown whether 9'-*cis*-neoxanthin is used by CitNCEDs to produce ABA during citrus fruit maturation.

9. Mechanism of β-cryptoxanthin accumulation in 'Tamami'

Recent studies have identified that the benefits from carotenoids might be due to β-cryptoxanthin, which is one of the major carotenoids in human blood (Nishino et al., 2009, Sugiura et al., 2011). So far, the dietary sources of β-cryptoxanthin are limited to the fruit of various species, such as Satsuma mandarin, ponkan mandarin, papaya, and loquat (Yano et al., 2005). 'Tamami', a hybrid of 'Kiyomi' tangor (*Citrus unshiu* Marc. × *Citrus sinensis* Osbeck) and 'Wilking' mandarin (*Citrus nobilis* Lour. × *Citrus deliciosa* Ten.), is rich in β-cryptoxanthin, accumulating approximately 20 $\mu g \cdot g^{-1}$

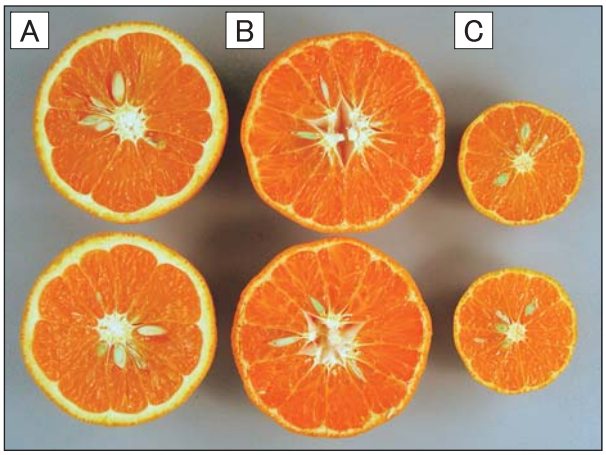

Fig. 11. Sections of citrus fruits. A, 'Kiyomi' tangor (*Citrus unshiu* Marc. × *Citrus sinensis* Osbeck); B, 'Tamami'; C, 'Wilking' mandarin (*Citrus nobilis* Lour. × *Citrus deliciosa* Ten.). 'Tamami' is a hybrid of 'Kiyomi' tangor and 'Wilking' mandarin.

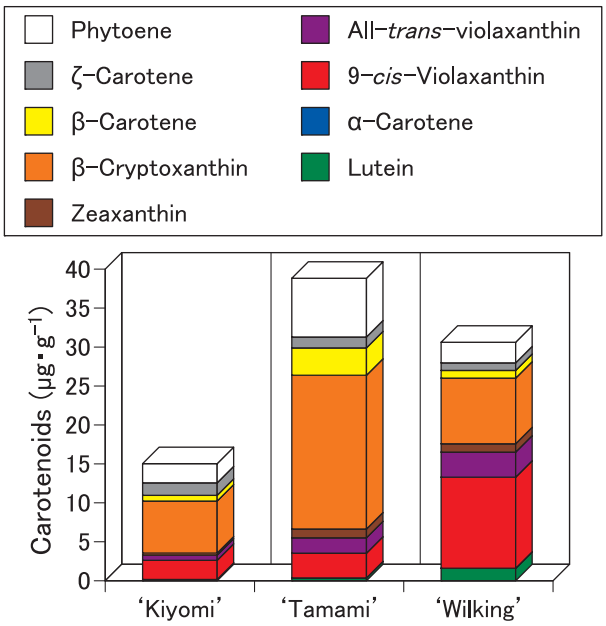

Fig. 12. Carotenoid concentration in juice sacs of 'Kiyomi' tangor, 'Wilking' mandarin, and 'Tamami' of mature fruit.

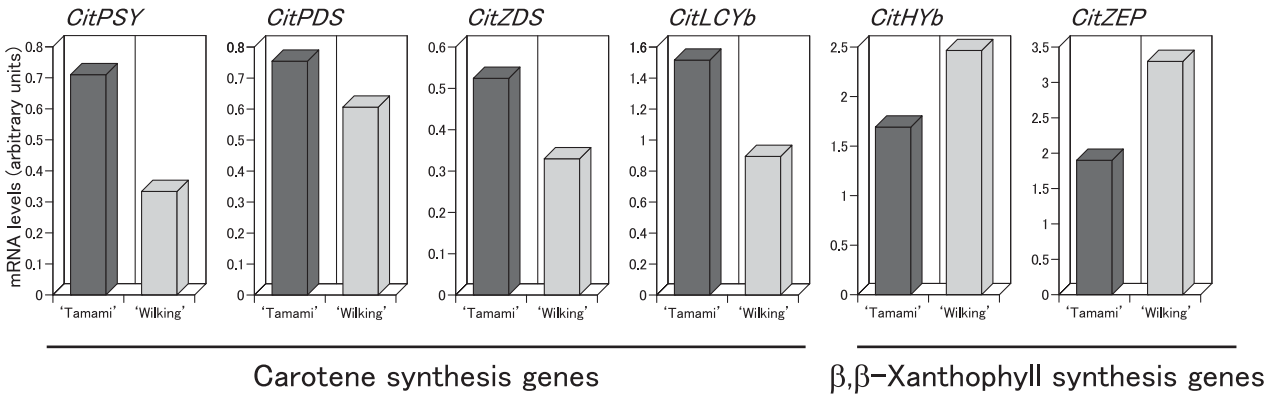

Fig. 13. Comparisons of gene expression for carotenoid biosynthesis between 'Tamami' and 'Wilking' mandarin in juice sacs during marked β,β-xanthophyll accumulation (November–January).

β-cryptoxanthin (Figs. 11 and 12). The level of β-cryptoxanthin in the juice sacs of 'Tamami' was higher than in the juice sacs of Satsuma mandarin, which accumulated 13.5–19.5 μg·g^{-1} β-cryptoxanthin (Yano et al., 2005). To improve the concentration of β-cryptoxanthin in citrus juice sacs, it is important to understand the mechanism of β-cryptoxanthin accumulation in a β-cryptoxanthin-rich variety, such as 'Tamami'.

In the juice sacs of 'Tamami' and 'Wilking' mandarin, β,β-xanthophylls accumulated markedly, accompanying simultaneous increases in the expression of the genes producing β,β-xanthophylls (Fig. 12). Simultaneous increases in the expression of the genes that produce β,β-xanthophylls were also observed in the juice sacs of Satsuma mandarin and Valencia orange, leading to the massive accumulation of β,β-xanthophylls (Kato et al., 2004). Similarly, simultaneous increases in the expression of the genes to produce β,β-xanthophylls were considered to lead to marked β,β-xanthophyll accumulation in the juice sacs of 'Tamami' and 'Wilking' mandarin.

In mature fruit, a clear varietal difference in β,β-xanthophylls was observed in the juice sacs between 'Tamami' and 'Wilking' mandarin (Fig. 12) (Kato et al., 2007). The juice sacs of 'Tamami' accumulated β-cryptoxanthin as a major carotenoid, whereas the juice sacs of 'Wilking' mandarin accumulated violaxanthin isomers (all-*trans*- and 9-*cis*-violaxanthin) as the major carotenoids. As mentioned above, a varietal difference in β,β-xanthophylls was also observed in the juice sacs between Satsuma mandarin and Valencia orange (Fig. 4B, C) (Kato et al., 2004). In these varieties, the expression balance between the upstream synthesis genes (*CitPSY*, *CitPDS*, *CitZDS*, and *CitLCYb*) and the downstream synthesis genes (*CitHYb* and *CitZEP*) was different (Fig. 5) (Kato et al., 2004). A similar expression balance was observed in the juice sacs of 'Tamami' and 'Wilking' mandarin (Fig. 13). During the marked accumulation of β,β-xanthophylls (November–January), the expression of the upstream synthesis genes in

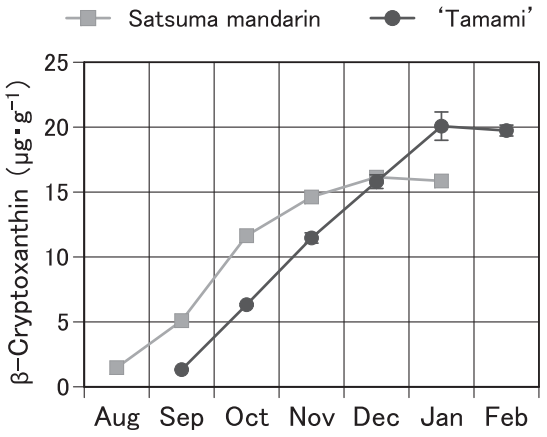

Fig. 14. Changes in β-cryptoxanthin concentration in the juice sacs of Satsuma mandarin and 'Tamami' during fruit maturation. Data are the means ± SE (n = 3).

'Tamami' was higher than that in 'Wilking' mandarin, whereas the expression of the downstream synthesis genes in 'Tamami' was lower than that in 'Wilking' mandarin. These results suggested that the expression balance between the upstream synthesis genes and the downstream synthesis genes was important to determine the ratios of β-cryptoxanthin/violaxanthin in citrus fruit (Kato et al., 2004). Therefore, 'Tamami', whose expression was similar to that of Satsuma mandarin, with a high expression of upstream synthesis genes and a low expression of downstream synthesis genes, accumulated a high level of β-cryptoxanthin (Kato et al., 2007).

It became clear that the juice sacs of 'Tamami' markedly accumulated β-cryptoxanthin and that the level of β-cryptoxanthin in 'Tamami' (20.1 μg·g^{-1} in January) was much higher than that of Satsuma mandarin (15.9 μg·g^{-1} in January) (Fig. 14) (Kato et al., 2004, 2007). The level of β-cryptoxanthin in 'Tamami' increased continuously until January, whereas the level in Satsuma mandarin increased until December. In 'Tamami', the expression of genes producing β,β-xanthophyll remained high during November–January (Kato et al., 2007). In contrast, in Satsuma mandarin,

the expression of genes producing β,β-xanthophylls remained high for less than three months (Kato et al., 2004). The period in which a high level of gene expression was observed in 'Tamami' was longer than in Satsuma mandarin. It was thought that these differences in the respective changing patterns of the expression of the genes producing β,β-xanthophylls between 'Tamami' and Satsuma mandarin affected the level of β-cryptoxanthin during fruit maturation. In addition, in the juice sacs of 'Tamami', the gene expression of *CitZDS* increased noticeably from September, accompanying the rapid increase in the β-cryptoxanthin level (Kato et al., 2007). In 'Tamami', the gene expression of *CitZDS* may play an important role in the high level of β-cryptoxanthin accumulation during fruit maturation.

10. Regulation of carotenoid concentration and composition in citrus cultured juice sacs

In recent years, extensive efforts have been devoted to improving the carotenoid contents in the juice sacs of citrus fruits (Matsumoto et al., 2009; Zhang et al., 2012). In our present study, we first developed an *in vitro* system, in which undefined variables were minimized and medium compositions and environmental factors were carefully controlled. In this system, the juice sacs enlarged gradually with carotenoid accumulation and no callus formed throughout the experimental period in Satsuma mandarin, Valencia orange, and Lisbon lemon (Fig. 14). The changing patterns of carotenoid content and the expression of genes for carotenoid biosynthesis and catabolism in juice sacs *in vitro* were similar to those ripening on trees in the three varieties. Using this system, carotenoid metabolism was investigated in response to different environmental conditions (blue and red LED lights, sucrose and mannitol) and plant hormones (ABA and GA) in the three varieties *in vitro* to understand the regulatory mechanism of carotenoid accumulation in citrus. The results showed that carotenoid accumulation was induced by blue light, sucrose and mannitol treatments, while it was suppressed by ABA and GA treatments in the three citrus varieties. Carotenoid metabolism in the three citrus varieties was not sensitive to red light treatment, by which the total carotenoid content was not significantly affected. In addition, gene expression results showed that carotenoid metabolism in response to these treatments was highly regulated at the transcriptional level in Satsuma mandarin, Valencia orange, and Lisbon lemon (Zhang et al., 2012). Thus, this study provides information on how carotenoid accumulation is regulated, which might produce new strategies to enhance carotenoids, especially β-cryptoxanthin production in citrus.

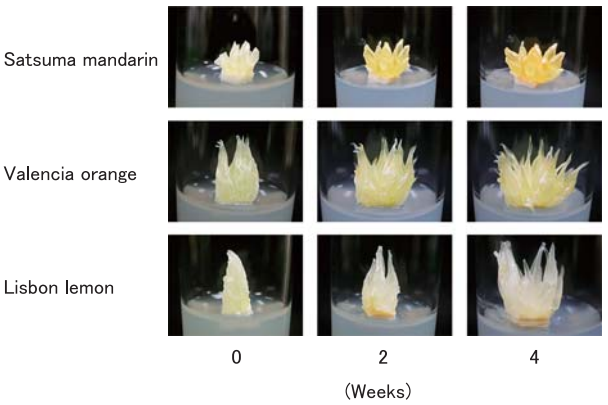

Fig. 15. Changes in the appearance of juice sacs in Satsuma mandarin, Valencia orange, and Lisbon lemon during culture *in vitro*.

11. Future studies

In this review, the mechanism of carotenoid accumulation in citrus fruit was explained by comparing the expression of genes related to carotenoid biosynthesis and catabolism among Satsuma mandarin, Valencia orange, and Lisbon lemon. In addition, the mechanism of β-cryptoxanthin accumulation in 'Tamami', which accumulates higher β-cryptoxanthin, was introduced. As described above, β-cryptoxanthin is a functional component for the prevention of certain lifestyle-related illnesses, especially cancers. Better understanding of the regulation of β-cryptoxanthin may provide new strategies for improving its content in citrus fruit. In the future, new technologies are expected to be introduced to enhance β-cryptoxanthin in citrus fruit. In addition, further research on post-transcriptional factors and other genes in the methyl erythritol phosphate pathway (MEP), which are related to carotenoid metabolism, is needed to elucidate carotenoid accumulation in citrus fruit.

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