Running head: The pathway of β-citraurin biosynthesis

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Enzymatic formation of β-Citraurin from β-Cryptoxanthin and Zeaxanthin by Carotenoid Cleavage Dioxygenase4 in the Flavedo of Citrus Fruit

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One-sentence summary:
The biosynthesis of β-citraurin from β-cryptoxanthin and zeaxanthin contributed to the attractively reddish peel color of citrus fruits.
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The online version of this article contains Web-only data.
ABSTRACT

In the present study, to investigate the pathway of β-citraurin biosynthesis, carotenoids contents and the expression of genes related to carotenoid metabolism were investigated in two citrus varieties of Satsuma mandarin (Citrus unshiu Marc.), ‘Yamashitabeni-wase’, which accumulates β-citraurin predominantly, and ‘Miyagawa-wase’, which does not accumulate β-citraurin. The results suggested that CitCCD4 was a key gene contributing to the biosynthesis of β-citraurin. In the flavedo of ‘Yamashitabeni-wase’, the expression of CitCCD4 increased rapidly from September, which was consistent with the accumulation of β-citraurin. In the flavedo of ‘Miyagawa-wase’, the expression of CitCCD4 remained at an extremely low level during the ripening process, which was consistent with the absence of β-citraurin. Functional analysis showed that the CitCCD4 enzyme exhibited substrate specificity. It cleaved β-cryptoxanthin and zeaxanthin at the 7,8 or 7',8' position. But other carotenoids tested in the present study (lycopene, α-carotene, β-carotene, all-trans-violaxanthin and 9-cis-violaxanthin) were not cleaved by CitCCD4 enzyme. The cleavage of β-cryptoxanthin and zeaxanthin by CitCCD4 enzyme led to the formation of β-citraurin. Additionally, with ethylene and red LED light treatments, the gene expression of CitCCD4 was up-regulated in the flavedo of ‘Yamashitabeni-wase’. These increases in the expression of CitCCD4 were consistent with the accumulation of β-citraurin in the two treatments. These results might provide new strategies to improve the carotenoids contents and compositions of citrus fruits.
INTRODUCTION

Carotenoids, a diverse group of pigments widely distributed in nature, fulfill a variety of important functions in plants and play a critical role in human nutrition and health (Schwartz et al., 1997; Cunningham and Gantt, 1998; Havaux, 1998; Krinsky et al., 2003; Ledford and Niyogi, 2005). The pathway of carotenoid biosynthesis has been well documented in various plant species, including Arabidopsis (Park et al., 2002), tomato (Isaacson et al., 2002), pepper (Bouvier et al., 1998), citrus (Kato et al., 2004, 2006, 2012; Rodrigo et al., 2004; Rodrigo and Zacarías, 2007; Zhang et al., 2012a) and apricot (Kita et al., 2007). Genes encoding the enzymes in the carotenoid biosynthetic pathway have been cloned and their expression profiles have also been characterized (Fig. 1). As carotenoids contain a series of conjugated double bonds in the central chain, they can be oxidatively cleaved in a site-specific manner (Mein et al., 2011). The oxidative cleavage of carotenoids not only regulates their accumulation, but also produces a range of apocarotenoids (Walter et al., 2010). In higher plants, many different apocarotenoids derive from the cleavage of carotenoids, and have important metabolic functions, such as plant hormones, pigments, aroma and scent compounds, as well as signaling compounds (Fig. 1). A well-known example is abscisic acid (ABA), which is a C₁₅ compound derived from the cleavage of the 11,12 double bond of 9-\textit{cis}-violaxanthin and 9'-\textit{cis}-neoxanthin (Schwartz et al., 1997; Tan et al., 1997; Cutler and Krochko, 1999; Chernys and Zeevaart, 2000; Giuliani et al., 2003).

Carotenoid cleavage dioxygenases (CCDs) are a group of enzymes that catalyze the oxidative cleavage of carotenoids (Ryle and Hausinger, 2002). CCDs are nonheme iron enzymes present in plants, bacteria and animals. In plants, CCDs belong to an ancient and highly heterogeneous family (CCD1, CCD4, CCD7, CCD8, and NCEDs). The similarity among the different members is very low apart from four strictly conserved histidine residues and a few glutamate residues (Kloer and Schulz, 2006; Walter et al., 2010). In Arabidopsis, the CCD family contains nine members (CCD1, NCED2, NCED3, CCD4, NCED5, NCED6, CCD7, CCD8, and NCED9), and orthologues in other plant species are typically named according to their homology with an Arabidopsis CCD (Huang et al., 2009). In our previous
study, the functions of CitCCD1, CitNCED2, and CitNCED3 were investigated in citrus fruits (Kato et al., 2006). The recombinant CitCCD1 protein cleaved β-cryptoxanthin, zeaxanthin, and all-trans-violaxanthin at the 9,10 and 9’,10’ positions and 9-cis-violaxanthin at the 9’,10’ position. The recombinant CitNCED2 and CitNCED3 proteins cleaved 9-cis-violaxanthin at the 11,12 position to form xanthoxin, a precursor of ABA (Kato et al., 2006). To date, information on the functions of other CCDs in citrus fruits remains limited, while the functions CCD7 and CCD8, as well as NCED5, NCED6, and NCED9 in Arabidopsis have been characterized (Kloer and Schulz 2006; Walter et al., 2010). In Arabidopsis, CCD7 cleaves all-trans-β-carotene at the 9’,10’ position to form all-trans-β-apo-10’-carotenal (C27). All-trans-β-apo-10’-carotenal is further shortened by AtCCD8 at the 13,14 position to produce β-apo-13-carotenone (Alder et al., 2012). NCED5, NCED6 and NCED9 cleaved 9-cis-violaxanthin at the 11,12 position to form xanthoxin (Tan et al., 2003). Compared with other CCDs, the function of CCD4 is poorly understood. In Chrysanthemum morifolium, CmCCD4a contributed to the white color formation by cleaving carotenoids into colorless compounds (Ohmiya et al., 2006). Recently, it has been reported that CsCCD4, CmCCD4a and MdCCD4 could cleave β-carotene to yield β-ionone (Rubio et al., 2008; Huang et al., 2009).

β-Citraurin, a C30 apocarotenoid, is a color-imparting pigment responsible for the reddish color of citrus fruits (Farin et al., 1983). In 1936, it was first discovered in Sicilian oranges (Cual, 1965). In citrus fruits, the accumulation of β-citraurin is not a common event, it is only observed in the flavedos of some varieties during the fruit ripening. The citrus varieties accumulating β-citraurin are considered more attractive because of their red-orange color (Ríos et al., 2010). Although more than seventy years have passed since β-citraurin was first identified, the pathway of its biosynthesis is still unknown. As its structure is similar to that of β-cryptoxanthin and zeaxanthin, β-citraurin was presumed to be a degradation product of β-cryptoxanthin or zeaxanthin (Oberholster et al., 2001; Rodrigo et al., 2004; Ríos et al., 2010; Fig. 1). To date, however, the specific cleavage reaction producing β-citraurin has not been elucidated. In the present study, we found that CitCCD4 gene was involved in the synthesis of β-citraurin, using two citrus varieties of Satsuma mandarin,
‘Yamashitabeni-wase’, which accumulates β-citraurin predominantly, and ‘Miyagawa-wase’, which does not accumulate β-citraurin. To confirm the role of the CitCCD4 gene further, functional analyses of the CitCCD4 enzyme were performed in vivo and in vitro. Additionally, the regulation of β-citraurin content and CitCCD4 gene expression in response to ethylene and red light-emitting diode (LED) light treatments was also examined. The present study is the first to investigate the biosynthesis of β-citraurin in citrus fruits. The results might provide new strategies to enhance the nutritional and commercial qualities of citrus fruits.

RESULTS

Isolation and Identification of β-Citraurin

The crude extract of carotenoids from the flavedo of ‘Yamashitabeni-wase’ was loaded on a column of silica gel. The red pigment was collected and analyzed by HPLC. As shown in Fig. 2, the peak eluted at 24 min was isolated. The mass spectrum of the eluent showed a molecular ion at m/z 433 ([M+H]+). The absorption maximum of the eluent was 456 nm in ethanol. The mass spectrum and absorption maximum of the eluent were consistent with those of β-citraurin (Farin et al., 1983; Agócs et al., 2007). Therefore, the peak eluted at 24 min was identified as β-citraurin.

Changes in the Contents and Compositions of Carotenoids in the Flavedos

According to changes in the color of the flavedo, the ripening process of citrus fruits can be divided into three stages, a green stage (September), a transition stage (from October to November) and a mature stage (from December to January; Kato, 2012). As shown in Fig. 3A, two varieties of Satsuma mandarin, ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’, showed a color break gradually from the transition stage. In the mature stage, ‘Yamashitabeni-wase’ displayed a reddish color in peel, while ‘Miyagawa-wase’ displayed a yellowish color in peel.

The changes in the contents and compositions of carotenoids were examined in the flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the fruit ripening. As shown
in Fig. 3B, a significant difference in the \( \beta \)-citraurin content was observed between the two varieties. In ‘Yamashitabeni-wase’, \( \beta \)-citraurin content increased rapidly from October. In contrast, \( \beta \)-citraurin was undetectable in ‘Miyagawa-wase’ throughout the ripening process. During the fruit ripening, the contents of \( \beta,\varepsilon \)-carotenoids, lutein and \( \alpha \)-carotene, decreased rapidly to a low level from September, while massive accumulation of \( \beta,\beta \)-xanthophylls (\( \beta \)-cryptoxanthin, zeaxanthin, all-\( trans \)-violaxanthin and 9-\( cis \)-violaxanthin) occurred in the two varieties during the ripening process (Fig. 3B). The contents of \( \beta \)-cryptoxanthin and zeaxanthin increased rapidly from October in ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’. The content of \( \beta \)-cryptoxanthin was similar between the two varieties throughout the ripening process (Fig. 3B). The content of zeaxanthin was higher in ‘Miyagawa-wase’ than that in ‘Yamashitabeni-wase’ in November. The contents of all-\( trans \)-violaxanthin and 9-\( cis \)-violaxanthin increased gradually from September in ‘Yamashitabeni-wase’. In ‘Miyagawa-wase’, the contents of all-\( trans \)-violaxanthin and 9-\( cis \)-violaxanthin increased significantly from October, and their contents were much higher than those in ‘Yamashitabeni-wase’ from November to January (Fig. 3B).

Changes in the Expression of Genes Related to Carotenoid Metabolism

The expression of a set of genes related to carotenoid metabolism (\textit{CitPSY}, \textit{CitPDS}, \textit{CitZDS}, \textit{CitLCYb1}, \textit{CitLCYb2}, \textit{CitHYb}, \textit{CitZEP}, \textit{CitVDE}, \textit{CitCCD1}, and \textit{CitNCED2}) increased from September to December in the flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’. Interestingly, we found that the expression of \textit{CitCCD4} increased significantly with a peak in December in the flavedo of ‘Yamashitabeni-wase’, however, it remained at a low level in the flavedo of ‘Miyagawa-wase’ during the ripening process (Fig. 4). Moreover, in the juice sacs, where \( \beta \)-citraurin was not accumulated, the expression level of \textit{CitCCD4} was extremely low in the two varieties during the ripening process (Fig. 5 and Supplemental Fig. S1). These results indicated that \textit{CitCCD4} might be involved in the biosynthesis of \( \beta \)-citraurin in the flavedo of ‘Yamashitabeni-wase’.

Isolation of Full-length cDNA of \textit{CitCCD4}
In the present study, full-length cDNAs of *CitCCD4* gene was isolated from ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’, and their sequences were analyzed. The sequences of CitCCD4 in ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ were identical at the nucleic acid level and amino acid level (Supplemental Fig. S2). The nucleotide sequence of *CitCCD4* contained 1,692 bp, and encoded a putative protein of 563 amino acids with an estimated molecular mass of 63 kD. CitCCD4 protein was predicted to localize in cytoplasm (utility:8.0) or chloroplast (utility:5.0) by WoLF PSORT (Horton et al., 2007, http://wolfpsort.org/). Using the online prediction server TMpre, the protein of CitCCD4 was predicted to have two transmembrane helices, which indicated that it was an integral membrane protein (Supplemental Fig. S2). In N-terminal region of the protein encode by *CitCCD4*, no characteristic transit peptide was detected by TargetP.

A BLAST search in Citrus Genome Database (http://www.citrusgenomedb.org) revealed that the cDNA sequence of *CitCCD4* was identical to scaffold_128:294016:295707 of the citrus gene clementine0.9_006329m.g. The gene structure of clementine0.9_006329m.g included a 5’ UTR, a CDS and a 3’ UTR. To further identify the genome organization of *CitCCD4*, the genomic DNAs of *CitCCD4* were amplified by PCR from the start codon to the stop codon and the sequences were analyzed in ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’. The sequence analysis revealed that the *CitCCD4* gene did not contain introns in either ‘Yamashitabeni-wase’ or ‘Miyagawa-wase’ (Supplemental Fig. S3).

**Subcellular localization of CitCCD4-GFP fusion protein expressed in tobacco leaves**

To determine the subcellular localization of CitCCD4, the cDNA of *CitCCD4* without the stop codon was fused with GFP reporter gene under the control of Cauliflower mosaic virus 35S promoter and bombarded in tobacco leaves. After 16-18h, the localization of GFP fusion proteins was observed by confocal laser-scanning microscopy. Confocal imaging of GFP fluorescence showed that the CitCCD4 fusion protein was located in the chloroplast (Fig. 6).
Functional Analyses of Recombinant CitCCD4 Protein

In the present study, the functions of CitCCD4 were investigated in vivo and in vitro. Lycopene, α-carotene, β-carotene and zeaxanthin were used as substrates to assay the enzyme activity of the CitCCD4 in vivo. β-Cryptoxanthin, zeaxanthin, all-trans-violaxanthin and 9-cis-violaxanthin were used as substrates to assay the enzyme activity of CitCCD4 in vitro.

To examine the enzyme activity of CitCCD4 in vivo, the cDNA of CitCCD4 isolated from the flavedo of ‘Yamashitabeni-wase’ was cloned into the pRSF-2 Ek/LIC vector. The recombinant plasmid was transformed to the lycopene-accumulating E. coli BL21 (DE3) cells, α-carotene and β-carotene-accumulating E. coli BL21 (DE3) cells, as well as zeaxanthin-accumulating E. coli BL21 (DE3) cells. Carotenoids were extracted from bacteria and their contents and compositions were analyzed by HPLC. No cleaved products were observed when CitCCD4 was expressed in E. coli BL21 (DE3) cells accumulating lycopene, α-carotene and β-carotene (Fig. 7, A and B). Thus, the CitCCD4 enzyme cannot cleave lycopene, α-carotene and β-carotene. When CitCCD4 was expressed in E. coli BL21 (DE3) cells accumulating zeaxanthin, the peak for β-citraurin, which eluted at 24 min, was observed (Fig. 7C).

To examine the enzyme activity of CitCCD4 in vitro, the cDNA of CitCCD4 isolated from the flavedo of ‘Yamashitabeni-wase’ was cloned into the pCold I vector. The recombinant protein expressed in E. coli cell was extracted and confirmed by SDS-PAGE. When all-trans-violaxanthin and 9-cis-violaxanthin were used as substrates for the cleavage reaction of the recombinant CitCCD4 enzyme, no cleavage product was detected (Fig. 8, C and D). When β-cryptoxanthin and zeaxanthin were used as substrates, however, the peak for β-citraurin, which eluted at 24 min, was observed (Fig. 8, A and B). In the case of β-cryptoxanthin, the other cleaved product, which eluted at 43 min, was also detected (Fig. 8A). The absorption maximum of the peak at 43 min was 460 nm. Both the elution time and absorption maximum of this eluent were identical to that of standard trans-β-apo-8’-carotenal (Fig. 8A). Therefore, the eluent at 43 min was identified as trans-β-apo-8’-carotenal. These results suggested that CitCCD4 enzyme could cleave β-cryptoxanthin and zeaxanthin at the 7, 8 or 7’, 8’ position, and the cleavage of β-cryptoxanthin and zeaxanthin contributed to the
biosynthesis of \(\beta\)-citaurin.

**Effects of Ethylene and Red LED Light on \(\beta\)-Citaurin Content and CitCCD4 Expression**

To enhance the content of \(\beta\)-citaurin, the effects of ethylene and red LED light (660 nm) on \(\beta\)-citaurin content and the expression of *CitCCD4* were investigated using the fruits of ‘Yamashitabeni-wase’ harvested in October. During the experimental periods, the accumulation of \(\beta\)-citaurin was observed in the control, as well as the ethylene and red light-treated groups (Fig. 9). Compared with the control, the content of \(\beta\)-citaurin was significantly increased by ethylene treatment. The expression of *CitCCD4* was up-regulated by ethylene and red LED light treatments (Fig. 9). These increases in the expression of *CitCCD4* were well consistent with the accumulation of \(\beta\)-citaurin in the two treatments.

**DISCUSSION**

**Accumulation of \(\beta\)-Citaurin in the Flavedos of Citrus Fruits**

The accumulation of \(\beta\)-citaurin, a red pigment, has been detected in only a few citrus varieties (Xu et al., 2011). Ríos et al. (2010) reported that \(\beta\)-citaurin was observed in the flavedo of a citrus clementine mutant 39B3, while it was absent in the flavedo of the other citrus clementine mutant 39E7. The defective synthesis of \(\beta\)-citaurin was proposed to cause the yellowish color of fully ripe 39E7 flavedo. Because \(\beta\)-citaurin is a characteristic pigment, it can be isolated in pure form among other carotenoids from the flavedos of citrus fruits (Agócs et al., 2007). In the present study, we isolated \(\beta\)-citaurin from the flavedo of ‘Yamashitabeni-wase’ using a silica gel column and HPLC. The mass spectrum and absorption maximum of the \(\beta\)-citaurin were consistent with those reported previously (Farin et al., 1983; Agócs et al., 2007; Fig. 2). In ‘Yamashitabeni-wase’, the content of \(\beta\)-citaurin increased significantly from October. To the best of our knowledge, this is the first report to show that \(\beta\)-citaurin is a main carotenoid that accumulates in Satsuma mandarin.
‘Yamashitabeni-wase’. In the other Satsuma mandarin variety, ‘Miyagawa-wase’, β-citraurin was undetectable throughout the ripening process (Fig. 3B). This difference led to different peel colors between the two varieties. The accumulation of β-citraurin contributed to the reddish peel in ‘Yamashitabeni-wase’, while the complete absence of β-citraurin led to the yellowish peel in ‘Miyagawa-wase’ (Fig. 3A). Although more than seventy years have passed since β-citraurin was first identified in citrus fruits, the pathway of β-citraurin biosynthesis has yet to be elucidated (Cual, 1965). In the present study, the contents of β-cryptoxanthin, zeaxanthin, and β-citraurin rapidly increased in ‘Yamashitabeni-wase’ from October. The concomitant increases in β-cryptoxanthin, zeaxanthin, and β-citraurin indicated that β-citraurin may be a breakdown product of β-cryptoxanthin and zeaxanthin. Additionally, β-citraurin did not accumulate in ‘Miyagawa-wase’, zeaxanthin was further converted into all-trans-violaxanthin and 9-cis-violaxanthin, and as a result, the contents of all-trans-violaxanthin and 9-cis-violaxanthin were higher than those in ‘Yamashitabeni-wase’.

Isolation and Sequence Analysis of CitCCD4

To find the enzyme responsible for β-citraurin biosynthesis, we compared the expression of genes related to carotenoid metabolism between the two varieties of Satsuma mandarin, ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’. In the flavedo, a significant difference in the expression of CitCCD4 was observed between the two varieties. In ‘Yamashitabeni-wase’, the expression of CitCCD4 increased rapidly with a peak in December, which was consistent with the accumulation of β-citraurin (Fig. 4). In ‘Miyagawa-wase’, the expression of CitCCD4 remained at a low level during the ripening process, which was consistent with the absence of β-citraurin in the flavedo (Fig. 4). Moreover, in the juice sacs, where β-citraurin was not accumulated, the expression of CitCCD4 was extremely low in both ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ (Fig. 5 and Supplemental Fig. S1). These results suggested CitCCD4 to be a key factor regulating β-citraurin biosynthesis.

In the present study, full-length cDNAs of the CitCCD4 gene were isolated from ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’, and their sequences were analyzed. The
homology of CitCCD4 between ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ was 100% identity at the nucleic acid level and amino acid level (Supplemental Fig. S2). In Clementine, two types of CCD4, CcCCD4a and CcCCD4b, were isolated (Agustí et al., 2007). The identities of CitCCD4 investigated herein between CcCCD4a and CcCCD4b at the amino acid level were 52% and 99%, respectively (Supplemental Fig. S4). The nucleotide sequence of CitCCD4 contained 1,692 bp, and encoded a putative protein of 563 amino acids with an estimated molecular mass of 63 kD. The nucleotide sequence of CcCCD4b contained 1,683 bp, and encoded a putative protein of 560 amino acids. The nucleotide sequence of CitCCD4 was not identical to CcCCD4a or CcCCD4b. In addition, we isolated the 2000-bp genomic DNA sequences of CitCCD4 promoters from ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’, and no significant difference was observed between the two varieties (Data not shown). Ríos et al. (2010) reported that CcGCC1, a transcriptional regulator, played an important role in regulating the color break in citrus fruits. Thus, it is possible that some transcriptional regulators are involved in controlling the expression of CitCCD4 in the flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’. Further research is needed to confirm this.

In contrast to the CCD1, CCD7 and CCD8 where the introns were conserved presence, in CCD4 the intron-exon structure was highly dynamic (Mein et al., 2011). In the present study, no intron was observed in CitCCD4 in ‘Yamashitabeni-wase’ or ‘Miyagawa-wase’ (Supplemental Fig. S3). Huang et al. (2009) reported that RdCCD4 and AtCCD4 contained no intron, while MdCCD4, OfCCD4 and CmCCD4 contained introns. The absence or presence of introns was related to the different expression patterns and biochemical functions of these CCD4 genes (Huang et al., 2009).

Unlike CCD1 enzymes, which were located in the cytoplasm, most CCD4 enzymes were located in plastids (Ytterberg et al., 2006; Rubio et al., 2008). In the N-terminal region of the protein encoded by CitCCD4, no characteristic transit peptide was predicted. However, the subcellular localization of CitCCD4-GFP fusion protein expressed in tobacco leaves suggested that CitCCD4 fusion protein was imported into plastids (Fig. 6). This similar phenomenon was also observed in CsZCD enzyme. Bouvier et al. (2003) found that CsZCD enzyme was compartmentalized to the plastid, although it did not contain a typical cleavable transit peptide. In plants, carotenoids are synthesized and stored in plastids (Kloer and Schulz
The location of CitCCD4 enzyme within plastids allowed it to access its carotenoids substrates, such as β-cryptoxanthin and zeaxanthin.

**Functional Analyses of Recombinant CitCCD4 Enzyme**

Compared with other CCDs, the information about the functions of CCD4 is limited. In potato, down-regulation of CCD4 gene expression using RNAi resulted in increase violaxanthin content (Campbell et al., 2010). In Chrysanthemum morifolium, CmCCD4a contributed to the white color formation by cleaving carotenoids into colorless compounds (Ohmiya et al., 2006). A common feature of CCD4 identified in several recent studies is a 9,10 or 9’,10’ cleavage activity to yield β-ionone (Rubio et al., 2008; Huang et al., 2009). However, the 7,8 or 7’,8’ cleavage activity of CCD4 remains to be confirmed. Bouvier et al. (2003) reported that CsZCD specifically catalyzed the cleavage of zeaxanthin at the 7,8 and 7’,8’ positions of the chromophore and initiated the formation of saffron secondary metabolites. In the present study, we found that CitCCD4 enzyme exhibited substrate specificity. It cleaved β-cryptoxanthin and zeaxanthin at the 7,8 or 7’,8’ position (Figs. 7 and 8). But other carotenoids tested in the present study (lycopene, α-carotene, β-carotene, all-trans-violaxanthin and 9-cis-violaxanthin) were not cleaved by CitCCD4 enzyme (Figs. 7 and 8). Moreover, the cleavage of β-cryptoxanthin and zeaxanthin by CitCCD4 enzyme led to the formation of β-citraurin. In addition, when β-cryptoxanthin and zeaxanthin were added together in the same reaction solution, the content of β-cryptoxanthin and zeaxanthin decreased simultaneously along with the biosynthesis of β-citraurin (Data not shown). The specific cleavage reaction of CitCCD4 enzyme presented herein further confirmed the previous speculations that β-citraurin was a breakdown product of β-cryptoxanthin and zeaxanthin (Fig. 10). In addition, when β-cryptoxanthin was used as the substrate for the cleavage reaction of the recombinant CitCCD4 enzyme, trans-β-apo-8’-carotenal, which eluted at 43 min, was also detected except for β-citraurin (Fig. 10). In Clementine, extremely low content of trans-β-apo-8’-carotenal was detected in the fruits harvested in November, while in the fully ripened fruits it was absence (Ríos et al., 2010). In the present study, we were unable to detect trans-β-apo-8’-carotenal in the flavedo or juice sacs of...
‘Yamashitabeni-wase’. It is possible that trans-β-apo-8’-carotenal may be further cleaved by other CCDs in ‘Yamashitabeni-wase’.

**Effects of Ethylene and Red LED Light on β-Citraurin Content and CitCCD4 Expression**

It has been reported that ethylene treatment increased the contents of carotenoids; as a result, the degreening process of citrus fruits was accelerated (Rodrigo and Zacarías, 2007). We previously found that red LED light was effective to enhance carotenoids contents, especially the content of β-cryptoxanthin, while blue LED light had no significant effect on the carotenoid content in the flavedo of Satsuma mandarin (Ma et al., 2012). In the present study, to investigate the regulatory effects of ethylene and red LED light on β-citraurin accumulation, fruits harvested in October were used as materials, as the changes in β-citraurin content were most significant at this stage. As shown in Fig. 9, the content of β-citraurin was increased by the ethylene and red LED light treatments. Additionally, with ethylene and red LED light treatments, the gene expression of CitCCD4 was up-regulated in the flavedo of ‘Yamashitabeni-wase’. These increases in the expression of CitCCD4 were well consistent with the accumulation of β-citraurin in the two treatments. The results presented herein provide more insights into the regulatory mechanism of β-citraurin metabolism in citrus fruits.

**CONCLUSION**

In the present study, the biosynthetic pathway of β-citraurin was investigated using two citrus varieties of Satsuma mandarin, ‘Yamashitabeni-wase’, which accumulates β-citraurin predominantly, and ‘Miyagawa-wase’, which does not accumulate β-citraurin. The results suggested that CitCCD4 was a key gene regulating the biosynthesis of β-citraurin. The cleavage of β-cryptoxanthin and zeaxanthin by CitCCD4 enzyme led to the formation of β-citraurin. In addition, the ethylene and red light treatments were effective to enhance the content of β-citraurin by up-regulating the expression of CitCCD4 in the flavedo of
‘Yamashitabeni-wase’. These results presented herein might contribute to elucidate the mechanism of β-citraurin accumulation in citrus fruits, which might facilitate the improvement in the citrus nutritional and commercial qualities.

MATERIALS AND METHODS

Plant Material

Two varieties of Satsuma mandarin (Citrus unshiu Marc.), ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’, cultivated at the Fujieda Farm of Shizuoka University (Shizuoka, Japan) were used as materials. Fruit samples were collected periodically from September to January. The flavedos and juice sacs were separated from sampled fruits, immediately frozen in liquid nitrogen, and kept at -80 °C until used.

Extraction and Determination of Carotenoids

The identification and quantification of carotenoids were conducted according to the methods described by Kato et al. (2004). Pigments were extracted from the samples using an hexone:acetone:ethanol (2:1:1 [v/v]) solution containing 0.1% (w/v) 2,6-di-tert-butyl-4-methylphenol and 10% (w/v) magnesium carbonate basic. After the organic solvents had been completely evaporated, the extracts containing carotenoids esterified to fatty acids were saponified with 20% (w/v) methanolic KOH. Water-soluble extracts were then removed by adding NaCl-saturated water. The pigments repartitioned into the diethylether phase were recovered and evaporated to dryness. Subsequently, the residue was redissolved in a TBME: methanol (1:1 [v/v]) solution. An aliquot (20 μL) was separated by a reverse-phase HPLC system (Jasco, Tokyo, Japan) fitted with a YMC Carotenoid S-5 column of 250×4.6-mm-i.d. (Waters, Milford, MA) at a flow rate of 1 mL min⁻¹. The eluent was monitored by a photodiode array detector (MD-2015, Jasco). The standard for each carotenoid were prepared according to the methods described by Kato et al. (2004). The
carotenoid concentration was estimated by the standard curves and expressed as milligrams per gram fresh weight. Total carotenoid is the sum of the content of various carotenoids identified in this study. Carotenoid quantification was performed in three replicates.

Isolation and Identification of β-Citraurin

The carotenoids extracted from the flavedo of ‘Yamashitabeni-wase’ were loaded on a column of silica gel (2 cm diameter and 52 cm length) using a hexane:ethyl ether: isopropyl alcohol (7:3:1 [v/v]) solution as eluent. The red pigment was collected and evaporated to dryness. The collection was subjected to HPLC and identified by spectrophotometry and mass spectrometry. The UV-visible spectra were taken with a spectrophotometer. Fast atom bombardment mass spectrometry (FAB-MS) analysis was performed with the API 2000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA).

Isolation and Sequence Analysis of CitCCD4

Total RNA was extracted from the flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ according to the method described by Ikoma et al. (1996). First-strand cDNA was synthesized from 2 μg of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). The cDNA fragment of CitCCD4 was amplified by PCR using the degenerate PCR primers designed according to the common sequences that have been reported previously (Supplemental Table S1). The amplified cDNAs were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). After sequencing, RACE-PCR was performed using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA; Supplemental Table S1). The amplified cDNAs of the 5’ and 3’ ends for CitCCD4 were cloned and sequenced. End-to-end PCR was performed with the cDNAs of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ using the primers designed from the cDNA sequences amplified by RACE-PCR (Supplemental Table S1).

The alignment of CitCCD4 between ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ was
created using the Genetyx Analysis Program (Genetyx Corp., Tokyo, Japan). The information regarding gene structure was obtained from the Citrus Genome Database (http://www.citrusgenomedb.org). The sublocation of CitCCD4 was predicated by WoLF PSORT (Horton et al., 2007, http://wolfpsort.org/). Predictions of transit peptides of CitCCD4 were carried out using TargetP. The transmembrane helices of CitCCD4 were predicted using the online prediction server TMpred (Hofmann and Stoffel, 1993).

**Isolation of CitCCD4 Genomic DNA**

Genomic DNA was isolated from leaf tissues of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ using a Qiagen DNA mini kit (Qiagen, Hilden, Germany). Genomic DNA fragment from the start codon to the stop codon of CitCCD4 was amplified by PCR. The PCR program for CitCCD4 was as described above. The amplified DNA was separated by electrophoresis on 1.5% agarose gels, and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

**Total RNA Extraction and Real-time Quantitative RT-PCR**

Total RNA was extracted from the flavedos and juice sacs of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ at different stages according to the method described by Ikoma et al. (1996). The total RNA was cleaned up using the RNeasy Mini Kit (Qiagen) with on-column DNase digestion. The reverse transcription (RT) reaction was performed with 2 μg of purified RNA and a random hexamer at 37 °C for 60 min using TaqMan Reverse Transcription Reagents (Applied Biosystems).

TaqMan MGB probes and sets of primers for genes related to carotenoid metabolism (CitPSY, CitPDS, CitZDS, CitLCYb1, CitLCYb2, CitLCYe, CitHYb, CitZEP, CitCRTISO, CitVDE, CitCCD1, CitNCED2, CitNCED3, and CitCCD4) were designed on the basis of sequences conserved between the two varieties for each gene with the Primer Express software (Supplemental Table S2). For the endogenous control, the TaqMan Ribosomal RNA
Control Reagents VIC Probe (Applied Biosystems) was used. TaqMan real-time PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using ABI PRISM 7300 (Applied Biosystems) according to the manufacturer’s instructions. Each reaction contained 900 nM of the primers, 250 nM of the TaqMan MGB Probe, and template cDNA. The thermal cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The levels of gene expression were analyzed with ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems) and normalized with the results of 18S ribosomal RNA. Real-time quantitative RT-PCR was performed in three replicates for each sample.

**Transient expression of CitCCD4-GFP fusion protein in tobacco leaves**

The full length of *CitCCD4* without the stop codon was added to the 4 bp sequences (CACC) on the N-terminal and subcloned into pENTR/D-TOPO (Invitrogen). The *CitCCD4* sequences were subcloned into new pUGW5 consisting of a synthetic green fluorescent protein (GFP) under the control of cauliflower mosaic virus 35S promoter by using the Gateway LR recombination reaction system (Invitrogen). Plasmid DNA (0.8 μg) was introduced into *Nicotiana tabacum* SR1 leaves using a pneumatic particle gun (PDS-1000/He; Bio-Rad, Richmond, CA). The conditions for bombardment were a vacuum of 28 inches of Hg, a helium pressure of 1300 psi, and a 6-cm target distance using 1-μm gold microcarriers. After the bombardment, the tobacco leaves were incubated for 16–18 h at room temperature. Green fluorescence detected with a band-pass 500~530-nm filter and chlorophyll autofluorescence detected with a long-pass 560-nm filter was observed using a Leica TCS-SL confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany).

**Functional Analysis of the CitCCD4 Enzyme in vivo**

The *CitCCD4* cDNA from ‘Yamashitabeni-wase’ was cloned into the pRSF-2 Ek/LIC vector. The recombinant plasmid was transformed into lycopene-accumulating *Escherichia coli* BL21 (DE3) cells, α-carotene and β-carotene-accumulating *E. coli* BL21 (DE3) cells, as
well as zeaxanthin-accumulating *E. coli* BL21 (DE3) cells (Misawa and Shimada, 1997; Zhang et al., 2012b). The transformants were plated in LB medium supplemented with chloramphenicol (50 µg ml⁻¹), carbenicillin (50 µg ml⁻¹) and kanamycin (50 µg ml⁻¹), and incubated at 37 °C for 20 h. The colonies were incubated in 100 ml of 2×YT medium with chloramphenicol (50 µg ml⁻¹), carbenicillin (50 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) at 37 °C for 16 h. Then, 2 ml of culture solution was inoculated into 200 ml of 2×YT medium with chloramphenicol (50 µg ml⁻¹), carbenicillin (50 µg ml⁻¹) and kanamycin (50 µg ml⁻¹). After 8 h at 27 °C, 200 µl of 0.1 M isopropyl β-D-thiogalactoside (IPTG) was added and cultured overnight at 27 °C.

Cultures of *E. coli* cells were centrifuged at 5,000 g for 10 min and the bacterial pellet was washed twice with Tris-HCl (pH 8.0). The pellet was dried using vacuum freeze drying and stored at -20 °C until the HPLC analysis. The freeze-ground material was extracted with a mixture of chloroform and methanol (2:1 [v/v]) until all the color was removed from the *E. coli* cells. The carotenoid extracts were reduced to dryness by rotary evaporation, and then dissolved in the methyl tertbutyl ether: methanol (1:1 [v/v]) solution containing 0.1% butylated hydroxyl-toluene. The identification and quantification of carotenoids were conducted according to the methods described by Kato et al. (2004).

**Functional Analysis of CitCCD4 Enzyme in vitro**

The *CitCCD4* cDNA from ‘Yamashitabeni-wase’ was cloned into the pCold I vector. The recombinant plasmid was transformed into XL1-Blue cells. The transformants were plated in LB medium supplemented with carbenicillin (50 µg ml⁻¹), and incubated at 37 °C for 20 h. The colonies were incubated in 100 ml of 2×YT medium with carbenicillin (50 µg ml⁻¹) at 37 °C for 16 h. Then, 2 ml of culture solution was inoculated into 200 ml of 2×YT medium with carbenicillin (50 µg ml⁻¹). Cultures were grown at 37 °C until an OD₆₀₀ of 0.7 was reached. The culture solution was quickly refrigerated at 15 °C and left to stand for 30 minutes. The expression of proteins was induced by the addition of 200 µl of 500 mM IPTG, and the cultures were grown at 15 °C for an additional 24 h. The cells were harvested by centrifugation, frozen in liquid nitrogen, and then resuspended in 2.5 ml of extraction buffer
(0.1 M Tris-HCl, pH 7.2, 30 mM Na-ascorbate, 5 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.05% (v/v) Triton X-100). After sonication and centrifugation, the complex was subjected to gel filtration using a PD-10 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) column. The recombinant proteins were analyzed by SDS-PAGE with a 12% (w/v) polyacrylamide gel. The concentration of recombinant proteins was determined using the Bradford Assay. Five μg of the recombinant protein was used for each the cleavage reaction.

For the substrates of carotenoids, β-cryptoxanthin and zeaxanthin were obtained from Extrasynthese (Genay, France), and all-trans-violaxanthin and 9-cis-violaxanthin were prepared from the flavedo of Valencia orange (Kato et al., 2006). The enzymatic activities of the recombinant CitCCD4 protein were assayed in a reaction mixture consisting of 0.1 M Tris-HCl, pH 7.2, 30 mM Na-ascorbate, 50 μM FeSO₄, 20 μg catalase, 0.05% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM carotenoid substrate (β-cryptoxanthin, zeaxanthin, all-trans-violaxanthin and 9-cis-violaxanthin) and 5 μg of the recombinant protein in a total volume of 200 μl at 27 °C for 3 h. After the incubation, 1 ml of water was added to the reaction mixture. The reaction products were partitioned three times into 1.2 ml of ethyl acetate, evaporated to dryness, and dissolved in methanol. An aliquot (20 μl) was separated by a reverse-phase HPLC system (Jasco) fitted with a YMC Carotenoid S-5 column of 250 mm 34.6 mm i.d. (Waters, Milford, MA) at a flow rate of 1 ml min⁻¹. The eluent was detected with a photodiode array detector (MD-2015, Jasco). The product was analyzed using the following elution schedule: 20% (v/v) methanol and 80% (v/v) water (0–5 min), a linear gradient to 100% (v/v) methanol (5–25 min), and 100% (v/v) methanol (25–40 min). For the identification of trans-β-apo-8’-carotenal, a purchased standard was used (Sigma, USA).

Treatment with Ethylene and Red LED light

Fruits of ‘Yamashitabeni-wase’ harvested in October were used as materials. For the ethylene treatment, fruits were treated with 50 μl L⁻¹ ethylene for three days at 20 °C. For the red LED light treatment, fruits were irradiated with red LED lights (660 nm) at an intensity of 150 μmol m⁻² s⁻¹ for three days at 20 °C. Fruits stored at 20 °C (RH 75%) in the dark were used as the control. After each treatment, the flavedos were immediately frozen in liquid
nitrogen, and kept at -80˚C until used.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: AB114648, *CitPSY*; AB114649, *CitPDS*; AB114650, *CitZDS*; AB114652, *CitLCYb1*; AB719392, *CitLCYb2*; AB114655, *CitLCYe*; AB114653, *CitHYb*; AB114654, *CitZEP*; AB114651, *CitCRTISO*; AB781688, *CitVDE*; AB219164, *CitCCD1*; AB219169, *CitNCED2*; AB219174, *CitNCED3*; AB781691, *CitCCD4*.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Changes in the carotenoids contents in juice sacs of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the ripening process.

**Supplemental Figure S2.** Alignment of deduced amino acid sequences of *CitCCD4* in ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’. The alignments were created using the Genetyx Analysis Program (Genetyx Corp., Tokyo, Japan). Underlined amino acids indicate the two transmembrane helices.

**Supplemental Figure S3.** The result of an ethidium bromide gel showing PCR amplification of *CitCCD4* from genomic DNA and cDNA of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’. M, DNA size markers; A, PCR product from genomic DNA of ‘Yamashitabeni-wase’; B, PCR product from genomic DNA of ‘Miyagawa-wase’; C, PCR product from cDNA of ‘Yamashitabeni-wase’; D, PCR product from cDNA of ‘Miyagawa-wase’.

**Supplemental Figure S4.** Alignment of deduced amino acid sequences of CitCCD4, CcCCD4a and CcCCD4b. The alignments were created using the Genetyx Analysis Program (Genetyx Corp., Tokyo, Japan).

**Supplemental Table S1.** Primers sequences used for amplification of CitCCD4.

**Supplemental Table S2.** Primer sequences and TaqMan MGB Probes used for the quantitative RT-PCRs of the genes related to carotenoid metabolism.
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LITERATURE CITED


Cual AL (1965) The occurrence of beta-citraurin and of beta-apo-8’-carotenal in the peels of
california tangerines and oranges. J Food Sci 30: 13-18


FIGURE LEGENDS

Figure 1. Carotenoid and apocarotenoid metabolic pathway in plants. GGPP, geranylgeranyl diphosphate. Enzymes are named according to the designation of their genes. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; ZISO, 15-cis-ζ-carotene isomerase; CRTISO, carotenoid isomerase; LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; HYe, ε-ring hydroxylase; HYb, β-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NCED, 9-cis-epoxycarotenoid dioxygenase; CCD, carotenoid cleavage dioxygenase.

Figure 2. Isolation and identification of β-citraurin from the flavedo of ‘Yamashitabeni-wase’. (A) HPLC analysis of β-citraurin. (B) UV-visible light spectra of β-citraurin. (C) FAB-MS spectra of β-citraurin.

Figure 3. Carotenoids accumulation in flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the ripening process. (A) Changes in the external colors of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the ripening process. (B) Changes in the carotenoids contents in flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the ripening process. The results shown are the mean ± SE for triplicate samples. S, September; O, October; N, November; D, December; J, January. Phy, phytoene; ζ-Car, ζ-carotene; β-Car, β-carotene; β-Cry, β-cryptoxanthin; Zea, zeaxanthin; T-vio, all-trans-violaxanthin; C-vio, 9-cis-violaxanthin; α-Car, α-carotene; Lut, lutein; β-Cit, β-citraurin; Total, total carotenoids.

Figure 4. Changes in the expression of genes related to carotenoid metabolism in flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the ripening process. The results shown are the mean ± SE for triplicate samples. S, September; O, October; N, November; D, December; J, January. The mRNA levels were analyzed by TaqMan real-time quantitative RT-PCR. Real-time RT-PCR amplification of 18S rRNA was used to normalize the expression of the genes under identical conditions.

Figure 5. Changes in the expression of CitCCD4 in flavedos and juice sacs of ‘Yamashitabeni-wase’ (A) and ‘Miyagawa-wase’ (B) during the ripening process. The results shown are the mean ± SE for triplicate samples. S, September; O, October; N, November; D, December; J, January. The mRNA levels were analyzed by TaqMan real-time quantitative...
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**Figure 6.** Subcellular localization of CitCCD4-GFP fusion protein. A: *Nicotianatabacum* SR1 leaves were transfected with the control vector (35S-GFP). B *Nicotianatabacum* SR1 leaves were transfected with the recombinant vector (35S-CitCCD4-GFP).

**Figure 7.** Functional analysis of CitCCD4 enzyme in vivo. (A) HPLC analysis of the cleavage products in lycopene-accumulating *E. coli* BL21 (DE3) cells transformed with pRSF-2 Ek/LIC-CitCCD4. (B) HPLC analysis of the cleavage products in α-carotene and β-carotene-accumulating *E. coli* BL21 (DE3) cells transformed with pRSF-2 Ek/LIC-CitCCD4. (C) HPLC analysis of the cleavage products in zeaxanthin-accumulating *E. coli* BL21 (DE3) cells transformed with pRSF-2 Ek/LIC-CitCCD4.

**Figure 8.** Functional analysis of the CitCCD4 enzyme in vitro. (A) HPLC analysis of the cleavage products from the incubation of β-cryptoxanthin with recombinant CitCCD4. (B) HPLC analysis of the cleavage products from the incubation of zeaxanthin with recombinant CitCCD4. (C) HPLC analysis of the cleavage products from the incubation of all-trans-violaxanthin with recombinant CitCCD4. (D) HPLC analysis of the cleavage products from the incubation of 9-cis-violaxanthin with recombinant CitCCD4.

**Figure 9.** Effect of ethylene and red LED light on β-citraurin content (A) and gene expression of *CitCCD4* (B) in flavedo of ‘Yamashitabeni-wase’. The results shown are the mean ± SE for triplicate samples. The mRNA levels were analyzed by TaqMan real-time quantitative RT-PCR. Real-time RT-PCR amplification of 18S rRNA was used to normalize the expression of the genes under identical conditions.

**Figure 10.** The pathway from β-cryptoxanthin and zeaxanthin to β-citraurin catalyzed by CitCCD4 in citrus fruits.
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Figure 3

A

Yamashitabeni-wase

Miyagawa-wase

B

Carotenoid content (μg gFW⁻¹)

Months
Figure 3. Carotenoids accumulation in flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the ripening process. (A) Changes in the external colors of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the ripening process. (B) Changes in the carotenoids contents in flavedos of ‘Yamashitabeni-wase’ and ‘Miyagawa-wase’ during the ripening process. The results shown are the mean ± SE for triplicate samples. S, September; O, October; N, November; D, December; J, January. Phy, phytoene; ζ-Car, ζ-carotene; β-Car, β-carotene; β-Cry, β-cryptoxanthin; Zea, zeaxanthin; T-vio, all-trans-violaxanthin; C-vio, 9-cis-violaxanthin; α-Car, α-carotene; Lut, lutein; β-Cit, β-citraurin; Total, total carotenoids.
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Figure 7

A

Absorbance at 510 nm (mAU)

Control

Lycopene

CitCCD4

Lycopene

B

Absorbance at 510 nm (mAU)

Control

β-Carotene

α-Carotene

CitCCD4

β-Carotene

α-Carotene

C

Absorbance at 510 nm (mAU)

Control

Zeaxanthin

CitCCD4

β-Citraurin

Zeaxanthin
Figure 7. Functional analysis of CitCCD4 enzyme in vivo. (A) HPLC analysis of the cleavage products in lycopene-accumulating E. coli BL21 (DE3) cells transformed with pRSF-2 Ek/LIC-CitCCD4. (B) HPLC analysis of the cleavage products in α-carotene and β-carotene-accumulating E. coli BL21 (DE3) cells transformed with pRSF-2 Ek/LIC-CitCCD4. (C) HPLC analysis of the cleavage products in zeaxanthin-accumulating E. coli BL21 (DE3) cells transformed with pRSF-2 Ek/LIC-CitCCD4.
Figure 8

A

![Absorbance at 510 nm (mAU) over minutes for Control, β-Cryptoxanthin, and CitCCD4 samples](image)

B

![Absorbance at 510 nm (mAU) over minutes for Control and Zeaxanthin](image)

C

![Absorbance at 510 nm (mAU) over minutes for Control, All-trans-violaxanthin, and CitCCD4 samples](image)

D

![Absorbance at 510 nm (mAU) over minutes for Control and 9-cis-Violaxanthin](image)
Figure 8. Functional analysis of the CitCCD4 enzyme *in vitro*. (A) HPLC analysis of the cleavage products from the incubation of β-cryptoxanthin with recombinant CitCCD4. (B) HPLC analysis of the cleavage products from the incubation of zeaxanthin with recombinant CitCCD4. (C) HPLC analysis of the cleavage products from the incubation of all-trans-violaxanthin with recombinant CitCCD4. (D) HPLC analysis of the cleavage products from the incubation of 9-cis-violaxanthin with recombinant CitCCD4.
Figure 9

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