RESEARCH PAPER

The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit

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Abstract

To investigate the relationship between a carotenoid profile and gene expression for carotenoid cleavage dioxygenases, three citrus varieties that exhibit different 9-cis-violaxanthin levels in their juice sacs, Satsuma mandarin (Citrus unshiu Marc.; a variety accumulating a low level of 9-cis-violaxanthin), Valencia orange (Citrus sinensis Osbeck; variety accumulating a high level of 9-cis-violaxanthin), and Lisbon lemon (Citrus limon Burm.f.; a variety accumulating an undetectable level of 9-cis-violaxanthin) were used. Three cDNAs (CitCCD1, CitNCED2, and CitNCED3) were cloned. The recombinant CitCCD1 protein cleaved β-cryptoxanthin, zeaxanthin, and all-trans-violaxanthin at the 9-10 and 9-9-10 positions and 9-cis-violaxanthin at the 9-9-10 position. The recombinant CitNCED2 and CitNCED3 proteins cleaved 9-cis-violaxanthin at the 11-12 position to form xanthoxin, a precursor of abscisic acid (ABA). The gene expression of CitCCD1 increased in the flavedos and juice sacs of the three varieties during maturation. In Satsuma mandarin, the gene expression of CitNCED2 and CitNCED3 increased noticeably, accompanying a massive accumulation of ABA in the flavedo and juice sacs. In Valencia orange, the gene expression of CitNCED3 increased with a slight elevation of the ABA level in the flavedo, whereas neither the gene expression of CitNCED2 nor the ABA level increased noticeably in the juice sacs. In Lisbon lemon, the gene expression of CitNCED2 increased remarkably, accompanying increases in the ABA level in the flavedo and juice sacs. These results suggest that, in the juice sacs, the efficient cleavage reaction for ABA synthesis reduces the 9-cis-violaxanthin level in Satsuma mandarin and Lisbon lemon, whereas the low cleavage reaction maintains the predominant 9-cis-violaxanthin accumulation in Valencia orange.

Key words: Abscisic acid, carotenoid, carotenoid cleavage dioxygenase, citrus, fruit maturation, xanthophyll.

Introduction

Carotenoids are integral and essential components of the photosynthetic apparatus in all plants, algae, and cyanobacteria, in which they efficiently quench triplet chlorophyll, singlet oxygen, and superoxide anion radicals (Goodwin, 1980; Cunningham and Gantt, 1998). In plants, carotenoids also dissipate excess light energy absorbed by the antenna pigments, harvest light for photosynthesis, and are exploited as colouring agents in flowers and fruits to attract pollinators and agents of seed dispersal (Goodwin, 1980; Cunningham and Gantt, 1998). In addition, epoxycarotenoids, 9-cis-violaxanthin (9c-vio) and 9'-cis-neoxanthin (9c-neo), can be metabolized to a plant hormone, abscisic acid (ABA; Rock and Zeevaart, 1991). Some carotenoids serve as precursors for vitamin A, which plays 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 pathway of carotenoid biosynthesis in plants was shown in a previous report (Kato et al., 2004).
Carotenoids are metabolized to apocarotenoids through the pathway catalysed 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, the 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). NCED (9-cis-epoxycarotenoid dioxygenase) catalyses the cleavage of 9c-vio or 9c-neo to form C25 epoxy-apocarotenal (Schwartz et al., 2003a), a precursor of ABA (Fig. 1; Schwartz et al., 1997, 2003a). This reaction is a limiting step in ABA biosynthesis (Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Schwartz et al., 2003a). 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). CCD1 catalyses the 9–10 and 9′-10′ cleavages of multiple carotenoid substrates to form C14 dialdehyde and two C13 products, 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 flavour, was produced (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 (Lycopersicon esculentum; LeCCD1A and LeCCD1B; Simkin et al., 2004a), and petunia (Petunia hybridra; PhCCD1; Simkin et al., 2004b).

Citrus fruits exhibit bright yellow or orange colours in both flavedos (peels) and juice sacs (pulp). These colours are mainly attributed to β,β-xanthophylls (β-cryptoxanthin β-cry), zeaxanthin [zea], and violaxanthin [vio] accumulation. The carotenoid concentration and composition of citrus fruit vary greatly among varieties and depend on the growing conditions (Gross, 1987). The carotenoid concentration and composition also differ between the flavedo and juice sacs of citrus fruit (Kato et al., 2004). Mandarin varieties, such as Satsuma mandarin (Citrus unshiu Marc.), accumulate β-cry predominantly in the juice sacs (Goodner et al., 2001; Ikoma et al., 2001; Kato et al., 2004). By contrast, mature sweet orange (Citrus sinensis Osbeck) accumulates vio isomers predominantly in the juice sacs (Molnár and Szabolcs, 1980; Lee and Castle, 2001; Kato et al., 2004), in which 9c-vio is found to be the principal carotenoid (Molnár and Szabolcs, 1980; Kato et al., 2004). Mature lemon (Citrus limon Burm.f.) accumulates much lower levels of carotenoids than Satsuma mandarin and Valencia orange in the juice sacs, although lemon fruit accumulates β-cry as a principal carotenoid (Kato et al., 2004). These varieties of citrus fruit, which exhibit different carotenoid profiles, are useful materials to investigate the mechanism of carotenoid accumulation.

In a previous report, the gene expression regulating carotenoid concentration and composition during fruit maturation was investigated using three varieties, namely, Satsuma mandarin (variety accumulating β-cry), Valencia orange (variety accumulating vio), and Lisbon lemon (variety accumulating small amounts of carotenoids; Kato et al., 2004). In this study, the carotenoid concentration and composition in the flavedos and juice sacs of these three varieties were analysed during fruit maturation. Eight carotenoid biosynthetic genes (CitPSY, CitPDS, CitZDS, CitCRTISO, CitLCYe, CitHYb, CitZEP, and CitLCYe) were also cloned from the three varieties in order to analyse their gene expression during fruit maturation. The results showed that, with the transition of peel colour from green to orange, the change from β,ε-carotene (ε-carotene and lutein) accumulation to β,β-carotenoid (β-carotene, β-cry, zea, and vio) accumulation was observed in the flavedos of Satsuma mandarin and Valencia orange, accompanying the disappearance of CitLCYe gene expression and the increase in CitLCYb gene expression (Kato et al., 2004). In both the flavedos and juice sacs of Satsuma mandarin and Valencia orange, simultaneous increases in the expression of the CitPSY, CitPDS, CitZDS, CitLCYb, CitHYb, and CitZEP genes, which make up a set of genes to produce β,β-xanthophylls, occurred with a massive accumulation of β,β-xanthophylls. Furthermore, it was found that the expression balance between upstream carotene synthesis
genes (CitPSY, CitPDS, CitZDS, and CitLCYb) and downstream xanthophyll synthesis genes (CitHYb and CitZEPE) was different in Satsuma mandarin and Valencia orange (Kato et al., 2004). The gene expression of upstream carotenoid synthesis in Satsuma mandarin was higher than that in Valencia orange, whereas the gene expression of downstream xanthophyll synthesis in Satsuma mandarin was lower than that in Valencia orange (Kato et al., 2004). Thus, it was deduced that the mechanism causing this diversity of carotenoid accumulation among the three citrus varieties was closely related to the expression of the carotenoid biosynthetic genes. However, this explanation was insufficient because of the lack of information on CCDs, which are involved in carotenoid catabolism.

In the present study, the cDNAs related to carotenoid catabolism (CitCCD1, CitNCED2, and CitNCED3) were cloned from Satsuma mandarin (a variety accumulating a low level of 9-cis-violaxanthin), Valencia orange (a variety accumulating a high level of 9-cis-violaxanthin), and Lisbon lemon (a variety accumulating an undetectable level of 9-cis-violaxanthin). Furthermore, functional analyses of the affinity-purified recombinant proteins of these CCDs were performed. The expression of these genes and the level of ABA were then analysed in the flavedos and the juice sacs of the three varieties during fruit maturation. The results suggest that the expression of the NCED genes plays an important role in the regulation of the carotenoid profiles in citrus fruit during maturation.

Materials and methods

Plant materials

Satsuma mandarin (Citrus unshiu Marc.), Valencia orange (Citrus sinensis Osbeck), and Lisbon lemon (Citrus limon Burm.f.) cultivated at the National Institute of Fruit Tree Science, Department of Citrus Research, Okitsu (Shizuoka, Japan) were used as materials. Fruit samples were collected periodically from August to January for Satsuma mandarin and from August to February for Valencia orange and Lisbon lemon. Flavedos and juice sacs were separated from sampled fruits, immediately frozen in liquid nitrogen, and kept at −80°C until use. A part of each sample had been used in the previous study for the analyses of carotenoid contents and the expression of carotenoid biosynthetic genes (Kato et al., 2004). The remainder of each sample was used in the present study for the analyses of ABA contents and the expression of CCD genes.

Isolation and sequence analysis of citrus CCD genes

Total RNA was extracted from the orange flavedos of Satsuma mandarin, Valencia orange, and Lisbon lemon according to the method described by Iokma et al. (1996). The first-strand cDNA was synthesized from 5 μg of the total RNA with the Ready-To-Go T-Primed First-Strand Kit (Amersham Bioscience, Little Chalfont, UK). The cDNA fragments of CCD genes were amplified by PCR with the cDNA template of Satsuma mandarin and a set of primers designed by common sequences that have been reported (Table 1). The amplified cDNAs were cloned with a TOPO TA Cloning Kit (Invitrogen, San Diego, CA), and their sequences were determined with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using the ABI PRISM 3100 Genetic Analyser (Applied Biosystems). After sequencing, three types of CCD cDNAs were isolated and designated as CitCCD1, CitNCED2, and CitNCED3. RACE-PCRs for CitCCD1, CitNCED2, and CitNCED3 were performed with the cDNA template of Satsuma mandarin and each set of primers designed from the cDNA sequence using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA; Table 1). The amplified cDNAs of the 5′ and 3′ ends for CitCCD1, CitNCED2, and CitNCED3 were cloned and sequenced. End-to-end PCR was performed for CitCCD1, CitNCED2, and CitNCED3 of Satsuma mandarin, Valencia orange, and Lisbon lemon with each cDNA of the three varieties and each set of primers designed from the cDNA sequences amplified by RACE-PCRs (Table 1). The amplified cDNAs for CitCCD1, CitNCED2, and CitNCED3 of the three varieties were cloned and sequenced. From the amplified amino acid sequences of CitCCD1, CitNCED2, and CitNCED3, subcellular locations were predicted with TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP; Emanuelsson et al., 2000).

Expression and purification of the recombinant protein

The cDNAs of CitCCD1, CitNCED2, and CitNCED3 from Satsuma mandarin, Valencia orange, and Lisbon lemon were amplified by PCR for the expression of recombinant CCD proteins with each cDNA template and each set of primers (Table 1). The primers for CitCCD1, CitNCED2, and CitNCED3 were designed to amplify the CCDs without plastid-targeting sequences predicted by TargetP 1.1 (Kato et al., 2004). The gene expression of upstream CitPSY, CitPDS, and CitZDS was confirmed by RT-PCR with the cDNA template of Satsuma mandarin and each set of primers designed from the cDNA sequence using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA; Table 1). The amplified cDNAs of the CitCCD1, CitNCED2, and CitNCED3 were cloned and sequenced. End-to-end PCR was performed for CitCCD1, CitNCED2, and CitNCED3 of Satsuma mandarin, Valencia orange, and Lisbon lemon with each cDNA of the three varieties and each set of primers designed from the cDNA sequences amplified by RACE-PCRs (Table 1). The amplified cDNAs for CitCCD1, CitNCED2, and CitNCED3 of the three varieties were cloned and sequenced. From the amplified amino acid sequences of CitCCD1, CitNCED2, and CitNCED3, subcellular locations were predicted with TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP; Emanuelsson et al., 2000).

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Table 1. Primer sequences used for the PCRs and TaqMan MGB probes and primer sequences used for the real-time quantitative RT-PCRs of CCD genes

<table>
<thead>
<tr>
<th>Primers for RT-PCRs to amplify cDNA fragments</th>
<th>cDNA</th>
<th>Sequence (5’ to 3’)</th>
<th>Primer or probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CitCCD1</td>
<td>GCBACYCNAARVYBGAYCC</td>
<td>Sense primer</td>
<td></td>
</tr>
<tr>
<td>CitNCED2</td>
<td>GAGATTCTGGTATGTTGTCCAAAGGT</td>
<td>Antisense primer</td>
<td></td>
</tr>
<tr>
<td>CitNCED3</td>
<td>GACGTTGATTCCTTTGGAACGCAAC</td>
<td>Antisense primer</td>
<td></td>
</tr>
<tr>
<td>CitCD1</td>
<td>TCATCGTATGTTGTCCAAAGGT</td>
<td>Primer or probe</td>
<td></td>
</tr>
<tr>
<td>CitNCED2</td>
<td>GAGATTCTGGTATGTTGTCCAAAGGT</td>
<td>Primer or probe</td>
<td></td>
</tr>
<tr>
<td>CitNCED3</td>
<td>GACGTTGATTCCTTTGGAACGCAAC</td>
<td>Primer or probe</td>
<td></td>
</tr>
<tr>
<td>TaqMan MGB probes and primers for real-time quantitative RT-PCRs</td>
<td>CitCCD1</td>
<td>TCCGAAACTATTCTTCTCTCATGCAT</td>
<td>Sense primer</td>
</tr>
<tr>
<td></td>
<td>CitNCED2</td>
<td>GAGATTAATCCTTCTCTTCTCTCATGCAT</td>
<td>Sense primer</td>
</tr>
<tr>
<td></td>
<td>CitNCED3</td>
<td>GAGATTAATCCTTCTCTTCTCTCATGCAT</td>
<td>Sense primer</td>
</tr>
</tbody>
</table>

0.05% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM carotenoid substrate (β-cry, t-cry, or 9c-cry), and 5 μg of the purified 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 a HPLC system (Jasco, Tokyo). The sample (0.5 g dry weight) was extracted in a YMC Carotenoid S-5 column (250 mm × 4.6 mm i.d.) using a linear gradient from 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). Products were isolated by HPLC and identified by spectrophotometry and mass spectrometry. The spectrophotometry analysis was performed using the UV2200 spectrophotometer (220–550 nm; Shimadzu, Kyoto) or a photodiode array detector (MD-910, Jasco) on the HPLC system. Fast atom bombardment mass spectrometry analysis was performed with the JMS-700 mass spectrometer (JEOL, Tokyo). For the identification of β-ionone, a purchased standard was used (Extrasynthese).

**ABA quantification in citrus fruit**

Samples for ABA extraction were lyophilized and ground to a fine powder with a mortar and pestle. The extraction of ABA was performed according to the method described by Okuda (2000) with slight modifications. The sample (0.5 g dry weight) was extracted in 30 ml of acetone overnight at 4 °C in the dark. The extract was homogenized further in a Polytron. The sample was filtered and evaporated to dryness. The residue was dissolved in 3 ml of an ethyl acetate:n-hexane (1:9, v/v) solution. After the addition of 3 ml 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 CitCCD1, CitNCED2, and CitNCED3 were designed on the basis of the common sequences among the three varieties for each gene with the Primer Express software (Applied Biosystems; Table 1). For an 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 7000 (Applied Biosystems) according to the manufacturer’s instructions. Each reaction contained 900 nM primers, a 250 nM TaqMan MGB Probe, and calibrator 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 1 min, and 60 °C for 60 s. The levels of gene expression were analysed with ABI PRISM 7000 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.
Results

Isolation and identification of the cDNA fragments of CCDs

By RT-PCR, three types of cDNA fragments were cloned from Satsuma mandarin. These cDNAs were designated as CitCCD1 (168 bp), CitNCED2 (171 bp), and CitNCED3 (170 bp). Based on the three types of cDNA fragments from Satsuma mandarin, three sets of primers for RACE-PCRs were designed (Table 1). By use of the RACE-PCRs, the full-length cDNAs for CitCCD1 (2031 bp), CitNCED2 (2319 bp), and CitNCED3 (2313 bp) were isolated from Satsuma mandarin (Table 2). For each cDNA, the deduced amino acid sequences of the full-length cDNAs shared high similarities with those from other plant species reported previously (>65.6%; Table 2).

To isolate the regions encoding the amino acid sequences of CitCCD1, CitNCED2, and CitNCED3 from Satsuma mandarin, Valencia orange, and Lisbon lemon, end-to-end PCRs were performed. The isolated cDNAs of CitCCD1 from the three varieties were 1571 bp long and highly similar among the varieties (>99.8% at the deduced amino acid level; Table 2). The isolated cDNAs of CitNCED2 from the three varieties were 1763 bp long and highly similar among the varieties (>98.0% at the deduced amino acid level; Table 2). The isolated cDNAs of CitNCED3 from the three varieties were 1706 bp long and highly similar among the varieties (>97.9% at the deduced amino acid level; Table 2).

Functional analyses of recombinant CCD proteins

Three sets of primers were designed for RT-PCRs to construct the plasmids for the expression of recombinant CitCCD1, CitNCED2, and CitNCED3 proteins (Table 1). Plasmids to produce the recombinant protein for three types of CCDs of Satsuma mandarin, Valencia orange, and Lisbon lemon were constructed. The affinity-purified recombinant CCD proteins from the three varieties were detected as single bands by SDS–PAGE (data not shown). These proteins were used to determine the substrate specificity of the cleavage reaction of CCD enzymes.

The cleaved products catalysed by recombinant CCD proteins were separated by HPLC and identified by spectrophotometry and mass spectrometry. When β-cry was used as a substrate for the cleavage reaction of the recombinant CitCCD1 protein from Satsuma mandarin, three products were detected at wavelengths of 300 nm (peaks 3 and 5) and 400 nm (peak 4; Fig. 2A). These products were isolated, and their mass spectra showed the molecular ion at m/z 209 ([M+H]+) for peak 3, m/z 217 ([M+H]+) for peak 4, and m/z 193 ([M+H]+) for peak 5 (Table 3). The absorption maximum of peak 3 (289 nm in ethanol) was close to that of 3-hydroxy-β-ionone reported previously (Table 3; Kato-Noguchi et al., 1993). The absorption maxima of peak 4 (361, 380, and 402 nm in diethyl ether) were close to those of C14 dialdehyde reported previously.

Table 2. Comparison of full-length CCD cDNAs from Satsuma mandarin

<table>
<thead>
<tr>
<th>cDNA (accession no.)</th>
<th>Total cDNA length (bp)</th>
<th>Open reading frame (bp)</th>
<th>Number of amino acids</th>
<th>Predicted plastid-targeting peptide</th>
<th>Identity on the level of deduced amino acid sequences (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CitCCD1 (AB219164)</td>
<td>2031</td>
<td>1644</td>
<td>547</td>
<td>No</td>
<td>99.8 (CitCCD1, Valencia orange, AB219165)</td>
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<td>100.0 (CitCCD1, Lisbon lemon, AB219168)</td>
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<td>82.7 (AtCCD1, Arabidopsis, At3g63520)</td>
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<td>83.8 (LeCCD1A, tomato, AY579001)</td>
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<td>71.7 (PaCCD1, avocado, AF224670)</td>
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<tr>
<td>CitNCED2 (AB219169)</td>
<td>2319</td>
<td>1830</td>
<td>609</td>
<td>Yes</td>
<td>98.3 (CitNCED2, Valencia orange, AB219171)</td>
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<td>98.0 (CitNCED2, Lisbon lemon, AB219172)</td>
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<td>76.4 (AtNCED3, Arabidopsis, At1g30100)</td>
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<td>67.4 (PaNCED1, avocado, AF224672)</td>
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<td>65.7 (PaNCED3, avocado, AF224671)</td>
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<tr>
<td>CitNCED3 (AB219174)</td>
<td>2313</td>
<td>1821</td>
<td>606</td>
<td>Yes</td>
<td>98.9 (CitNCED3, Valencia orange, AB219177)</td>
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<td>97.9 (CitNCED3, Lisbon lemon, AB219179)</td>
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<td>78.5 (AtNCED3, Arabidopsis, At3g14440)</td>
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<td></td>
<td>66.6 (PaNCED1, avocado, AF224672)</td>
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<td>65.6 (PaNCED3, avocado, AF224671)</td>
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</table>

* Plastid-targeting peptide was predicted with Target P 1.1.
The absorption maximum of peak 5 (300 nm in the eluent) was consistent with that of \(\beta\)-ionone (Table 3). Therefore, peaks 3, 4, and 5 were identified as 3-hydroxy-\(\beta\)-ionone, C14 dialdehyde, and \(\beta\)-ionone, respectively (Table 3). When 9c-vio was used as a substrate for the cleavage reaction of the recombinant CitCCD1 protein from Satsuma mandarin, two products were detected at wavelengths of 245 nm (peak 1) and 400 nm (peak 7; Fig. 2B). These products were isolated, and their mass spectra showed the molecular ion at \(m/z\) 225 ([M+H]\(^+\)) for peak 1 and at \(m/z\) 409 ([M+H]\(^+\)) for peak 7 (Table 3). The absorption maximum of peak 1 (233 nm in ethanol) was consistent with that of 5,6-epoxy-3-hydroxy-\(\beta\)-ionone reported previously (Table 3; Märki-Fischer and Eugster, 1988).

Fig. 2. HPLC analyses of the cleavage products catalysed by recombinant CitCCD1 and CitNCED2 proteins from Satsuma mandarin. (A) Products from the incubation of \(\beta\)-cryptoxanthin with recombinant CitCCD1 protein. (B) Products from the incubation of 9-cis-violaxanthin with recombinant CitCCD1 protein. (C) Products from the incubation of \(\beta\)-cryptoxanthin with recombinant CitNCED2 protein. (D) Products from the incubation of 9-cis-violaxanthin with recombinant CitNCED2 protein. The numbers of the peaks indicated in this figure are the corresponding numbers of the peaks in Tables 3 and 4. Peak 1, 5,6-epoxy-3-hydroxy-\(\beta\)-ionone; peak 2, xanthoxin; peak 3, 3-hydroxy-\(\beta\)-ionone; peak 4, C14 dialdehyde; peak 5, \(\beta\)-ionone; peak 6, C25 epoxy-apocarotenal; peak 7, C27 epoxy-apocarotenal.
Table 3. Identification of cleaved products catalysed by recombinant CCD proteins

The numbers of the peaks indicated in this table are the corresponding numbers of the peaks in Fig. 2 and Table 4.

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Absorption maxima (nm)</th>
<th>Mass (m/z)</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present</td>
<td>Published</td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>233&lt;sup&gt;a&lt;/sup&gt;</td>
<td>233&lt;sup&gt;a&lt;/sup&gt;</td>
<td>225 [M+H]+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 2</td>
<td>282&lt;sup&gt;c&lt;/sup&gt;</td>
<td>282&lt;sup&gt;c&lt;/sup&gt;</td>
<td>251 [M+H]+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 3</td>
<td>290&lt;sup&gt;c&lt;/sup&gt;</td>
<td>290&lt;sup&gt;c&lt;/sup&gt;</td>
<td>209 [M+H]+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 4</td>
<td>359, 380, 401&lt;sup&gt;c&lt;/sup&gt;</td>
<td>359, 380, 401&lt;sup&gt;c&lt;/sup&gt;</td>
<td>217 [M+H]+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 5</td>
<td>300&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(300)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>193 [M+H]+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 6</td>
<td>414&lt;sup&gt;d&lt;/sup&gt;</td>
<td>414&lt;sup&gt;d&lt;/sup&gt;</td>
<td>383 [M+H]+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peak 7</td>
<td>437&lt;sup&gt;d&lt;/sup&gt;</td>
<td>438&lt;sup&gt;d&lt;/sup&gt;</td>
<td>409 [M+H]+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ethanol, <sup>b</sup> methanol, <sup>c</sup> diethyl ether, and <sup>d</sup> benzene were the solvents used in the analyses of the absorption maxima.

<sup>e</sup> The value in parentheses represents the absorption maximum of the purchased β-ionone.

<sup>f</sup> The absorption maxima were measured using a photodiode array detector on an HPLC system.

Taylor and Burden, 1970). The absorption maximum of peak 7 (437 nm in benzene) was close to that of C<sub>27</sub> epoxy-apocarotenal reported previously (Table 3; Parry and Horgan, 1991). Therefore, peaks 1 and 7 were identified as 5,6-epoxy-3-hydroxy-β-ionone and C<sub>27</sub> epoxy-apocarotenal, respectively (Table 3). When β-cry was used as a substrate for the cleavage reaction of the recombinant CitNCED2 protein from Satsuma mandarin, no product peak was detected (Fig. 2C). When 9c-vio was used as a substrate for the cleavage reaction of the recombinant CitNCED2 protein from Satsuma mandarin, two products were detected at wavelengths of 300 nm (peak 2) and 400 nm (peak 6; Fig. 2D). These products were isolated, and their mass spectra showed the molecular ion at m/z 251 ([M+H]+) for peak 2 and at m/z 383 ([M+H]+) for peak 6 (Table 3). The absorption maximum of peak 2 (282 nm in methanol) was consistent with that of xanthoxin reported previously (Table 3; Parry and Horgan, 1991). The absorption maximum of peak 6 (413 nm in benzene) was close to that of C<sub>25</sub> epoxy-apocarotenal reported previously (Table 3; Parry and Horgan, 1991). Therefore, peaks 2 and 6 were identified as xanthoxin and C<sub>25</sub> epoxy-apocarotenal, respectively (Table 3).

To investigate the substrate specificity of recombinant CCD proteins from Satsuma mandarin, Valencia orange, and Lisbon lemon, β-cry, zea, t-vio, and 9c-vio, which are major β,β'-xanthophylls in mature citrus fruit, were tested (Table 4). Among the three varieties, no difference in substrate specificity was observed for each CCD. The recombinant CitCCD1 protein from each of the three varieties cleaved β-cry, zea, and t-vio to yield C<sub>14</sub> dialdehyde (peak 4) and C<sub>13</sub> products (3-hydroxy-β-ionone [peak 3] and β-ionone [peak 5]) for β-cry, 3-hydroxy-β-ionone [peak 3] for zea, and 5,6-epoxy-3-hydroxy-β-ionone [peak 1] for t-vio; Table 4). The recombinant CitCCD1 protein from the three varieties also cleaved 9c-vio to yield 5,6-epoxy-3-hydroxy-β-ionone (peak 1) and C<sub>27</sub> epoxy-apocarotenal (peak 7; Table 4). The recombinant CitNCED2 and CitNCED3 proteins from the three varieties cleaved 9c-vio to yield xanthoxin (peak 2) and C<sub>25</sub> epoxy-apocarotenal (peak 6), and no product peaks were detected when β-cry, zea, and t-vio were tested as substrates (Table 4).

Changes in the expression of CCD genes in the flavedos

Real-time quantitative RT-PCR was performed to investigate the gene expression of CitCCD1, CitNCED2, and CitNCED3 in the flavedos and juice sacs of three citrus varieties during fruit maturation. The green stages of the flavedos were from August to September in Satsuma mandarin and from August to October in Valencia orange and Lisbon lemon (Kato et al., 2004).

During the green stage, the gene expression of CitCCD1 increased in the flavedos of the three varieties (Fig. 3). The gene expression of CitNCED2 remained low in Satsuma mandarin and Valencia orange, but it increased slowly in Lisbon lemon (Fig. 3). The gene expression of CitNCED3 decreased in the three varieties during this stage (Fig. 3). In August, the levels of the gene expression of CitNCED3 were much higher in Valencia orange and Lisbon lemon than in Satsuma mandarin.

After the green stage, increases in the gene expression of CitCCD1 were observed in the flavedos of the three varieties (Fig. 3). In Lisbon lemon, the gene expression of CitCCD1 increased continuously until February. By contrast, no noticeable increase in the gene expression of CitCCD1 was observed in the latter experimental period of Satsuma mandarin (October–January) and Valencia orange (November–February). A distinct difference in the gene expression of CitNCED2 was observed between Valencia orange and the two other varieties (Fig. 3). The gene expression of CitNCED2 increased noticeably in Satsuma mandarin and Lisbon lemon. In Satsuma mandarin, the gene expression of CitNCED2 reached its maximum level in November and was 24.9-fold of that in Valencia orange in November. In Lisbon lemon, the gene expression of CitNCED2 increased remarkably to its maximum level in February and was 150-fold of that in Valencia orange in February. By contrast, the gene expression of CitNCED2...
in Valencia orange remained at an extremely low level, and no noticeable increase in the gene expression was observed throughout the experimental period. The gene expression of *CitNCED3* increased in the three varieties after the green stage (Fig. 3). In Satsuma mandarin and Valencia orange, the gene expression of *CitNCED3* increased rapidly, reaching its peak in November. In Lisbon lemon, the gene expression of *CitNCED3* increased slightly, reaching its peak in December. The peak levels of the gene expression of *CitNCED3* in Satsuma mandarin and Valencia orange in November were much higher than the level in Lisbon lemon in December.
Changes in the expression of CCD genes in the juice sacs

During the green stage of the flavedos, the gene expression of *CitCCD1* increased in the juice sacs of Satsuma mandarin and Lisbon lemon (Fig. 3). In the juice sacs of Valencia orange, the gene expression of *CitCCD1* decreased and subsequently increased. A distinct difference in the gene expression of *CitNCED2* was observed between Valencia orange and the two other varieties during the green stage (Fig. 3). The gene expression of *CitNCED2* increased rapidly in Satsuma mandarin and Lisbon lemon. By contrast, no noticeable increase in the gene expression of *CitNCED2* was observed in Valencia orange. In Lisbon lemon, the gene expression of *CitNCED2* was at its maximum level in October and was 5.9-fold of that of Valencia orange in October. During this stage, the gene expression of *CitNCED3* decreased in the three varieties (Fig. 3).

After the green stage of the flavedos, the gene expression of *CitCCD1* increased in the juice sacs of the three varieties (Fig. 3). Rapid increases were observed in the latter experimental period in Valencia orange (November–January) and Lisbon lemon (December–February), whereas a rapid increase was observed in the early experimental period in Satsuma mandarin (August–October). The gene expression of *CitNCED2* in Satsuma mandarin remained at a high level as of September, reaching its maximum level in November (9.5-fold of that of Valencia orange in November), and subsequently decreased rapidly from November to January (Fig. 3). In Lisbon lemon, the gene expression of *CitNCED2* decreased slightly from the maximum level, but it remained at a high level during this stage. In Valencia orange, the gene expression of *CitNCED2* remained at a low level, and no noticeable increase was observed throughout the experimental period. The gene expression of *CitNCED3* increased in the three varieties, peaking to a maximum level in December (Fig. 3). The maximum level of the gene expression of *CitNCED3* in Satsuma mandarin was higher than those in Valencia orange and Lisbon lemon.

Changes in the ABA level in citrus fruit maturation

Quantifications of the ABA level were performed by ELISA. To confirm the accuracy of the values of the ABA level analysed by ELISA, GC-MS analyses were conducted at several points during the experimental period. The levels of ABA analysed by ELISA were slightly lower than those analysed by GC-MS but showed a changing pattern similar to those shown by GC-MS (data not shown).

During the green stage of the flavedos, the ABA levels remained low in Satsuma mandarin and Valencia orange, but increased slowly in Lisbon lemon (Fig. 4). After the green stage, the ABA levels increased in the three varieties (Fig. 4). In Satsuma mandarin, the ABA level increased rapidly, reaching its maximum in November (1.65 µg g⁻¹ DW) and subsequently decreasing quickly. In Valencia orange, the ABA level also reached its maximum in November (0.48 µg g⁻¹ DW), but the level was much lower than that in Satsuma mandarin. The ABA level in Lisbon lemon increased to its maximum in December (1.10 µg g⁻¹ DW) and remained at a high level in February.

In the juice sacs of the three varieties, the level of ABA increased during the green stage (Fig. 4). The level in Satsuma mandarin was much higher than those in Valencia orange and Lisbon lemon. In Lisbon lemon, the level was at its maximum in October (1.13 µg g⁻¹ DW). After the green stage, the ABA level in Satsuma mandarin increased rapidly to its maximum level in October (3.30 µg g⁻¹ DW; Fig. 4). In Lisbon lemon, the level decreased gradually during this stage. In November, the ABA level in Valencia orange increased slowly to its maximum (0.41 µg g⁻¹ DW), which was much lower than those in Satsuma mandarin and Lisbon lemon. The level of ABA in Satsuma mandarin was much higher than those in the two other varieties throughout the experimental period (Fig. 4).

Discussion

Characteristics of recombinant CCD proteins

In the present study, three CCD cDNAs, *CitCCD1*, *CitNCED2*, and *CitNCED3*, were isolated from Satsuma mandarin, Valencia orange, and Lisbon lemon. In the putative amino acid sequences of the isolated CCDs from the three citrus varieties, four active centre histidines were conserved (Kloer et al., 2005). The recombinant CCD enzymes catalysed the carotenoid cleavage to yield
apocarotenals in the presences of molecular oxygen, ferrous iron, and ascorbate in vitro, indicating that CCD enzymes belong to the dioxygenase family (Schwartz et al., 1997). Recombinant CCD1 proteins were previously characterized by their substrate specificity. In Arabidopsis, the recombinant AtCCD1 protein cleaved β-carotene, lutein, zeaxanthin, and t-violaxanthin at the symmetrical 9-10 and 9′-10′ double bonds to yield two C13 products and C14 dialdehyde (Schwartz et al., 2001). When 9c-violaxanthin and 9c-neoxanthin were used as a substrate, the recombinant AtCCD1 protein produced a C13 product and C27 epoxy-apocarotenal (Schwartz et al., 2001). In the present study, it was also found that the recombinant CitCCD1 proteins showed broad substrate specificity for β,β-xanthophylls (β-cry, zeaxanthin, t-violaxanthin, and 9c-violaxanthin). Previously, the localization of CCD1 proteins was also investigated in some plant species. The CCD1 protein from Arabidopsis, five of which are believed to be NCEDs involved in the biosynthesis of ABA, were imported into chloroplasts (Qin and Zeevaart, 2000). In the present study, two NCED proteins were previously characterized in vitro (Fig. 2D; Table 4). 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). The localization of NCED proteins has been investigated in some plant species. The NCED proteins from other plant species were imported into the chloroplast (Qin and Zeevaart, 1999; Iuchi et al., 2000; Tan et al., 2001, 2003). Similarly, it was thought that both CitNCED2 and CitNCED3 proteins, which contained plastid-targeting peptides in the N-terminal region, were probably imported into plastid and were involved in the 9c-violaxanthin and ABA levels during fruit maturation.

Changes in CitNCED gene expression and ABA level during citrus fruit maturation

In the flavedos, increases in the ABA levels were observed in the three varieties during fruit maturation (Fig. 4). The present study showed that CitNCED genes, which showed different expression patterns, were involved in ABA biosynthesis in the flavedo of each variety. In the flavedo of Satsuma mandarin, the gene expression of CitNCED2 and CitNCED3 increased rapidly with ABA accumulation (Figs 3, 4). 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 (Figs 3, 4). It is noteworthy that the level of the gene expression of CitNCED2 in Lisbon lemon increased remarkably to 150-fold of that in Valencia orange with ABA accumulation, whereas the gene expression of CitNCED3 changed irrespective of ABA accumulation (Figs 3, 4). These results suggested that the gene expression of CitNCED2 and CitNCED3 in Satsuma mandarin, the gene expression of CitNCED2 in Valencia orange, and the gene expression of CitNCED2 in Lisbon lemon were primarily responsible for the ABA accumulation in their flavedos.

In the juice sacs of Satsuma mandarin, the gene expression of CitNCED2 and CitNCED3 increased rapidly with ABA accumulation (Figs 3, 4). In the juice sacs of Lisbon lemon, the levels of the gene expression of CitNCED2 increased rapidly with ABA accumulation, whereas the gene expression of CitNCED3 changed irrespective of it (Figs 3, 4). 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 the ABA accumulation in their juice sacs. In the juice sacs of Valencia orange, the ABA level was much lower than those in the two other varieties throughout the experimental period (Fig. 4). In Valencia orange, no noticeable increase in the gene expression of CitNCED2 was observed (Fig. 3). In addition, the gene expression of CitNCED2 and CitNCED3 in Satsuma mandarin and the gene expression of CitNCED2 in Lisbon lemon were primarily responsible for the ABA accumulation in their juice sacs. In the juice sacs of Valencia orange, the extremely low level of CitNCED2 was primarily responsible for the low level of ABA.

Thus, it was thought that the gene expression of CitNCEDs was a factor in the regulation of the ABA level during citrus fruit maturation. However, other post-transcriptional factors, such as differences in the enzymatic properties among the three citrus varieties, may also be involved in the regulation of the ABA levels in citrus fruit maturation.

In the present study, ABA accumulation was observed in the flavedo and juice sacs. In both tissues, ABA accumulation in Satsuma mandarin, Valencia orange, and Lisbon lemon exhibited different changing patterns during fruit maturation (Fig. 4). A previous report suggested that, in 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 exhibit a delay in the rate of degreening. Although no data are provided in the present study to support these views, the physiological role of ABA accumulation may be involved in chromoplast development and/or the rate of fruit coloration in citrus fruit.
Mechanism of xanthophyll accumulation in citrus fruit

The relationship between carotenoid biosynthesis (Kato et al., 2004) and carotenoid catabolism (the present results) in Satsuma mandarin, Valencia orange, and Lisbon lemon can now be discussed because the plant materials in the present study and previous study were shared (Kato et al., 2004).

In a previous study it was shown that the level of 9c-vio increased more in the flavedos of Satsuma mandarin and Valencia orange than in that of Lisbon lemon (Kato et al., 2004). These increases were probably not only due to higher levels of the expression of the gene set to produce β,β'-xanthophylls (Kato et al., 2004) but also to lower levels of the gene expression of CitNCED2 in Satsuma mandarin and Valencia orange than in Lisbon lemon. By contrast, in the flavedo of Lisbon lemon, the low β,β'-xanthophyll concentration, which is responsible for the distinct yellow colour 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.

A previous study also showed clear differences in β,β'-xanthophyll concentration and composition among the three varieties in the juice sacs (Kato et al., 2004). In mature fruit, the juice sacs of Satsuma mandarin accumulated a low level of 9c-vio, whereas those of Valencia orange accumulated a high level of 9c-vio (Kato et al., 2004). In Valencia orange, it was thought that the 9c-vio accumulated in the juice sacs did not cleave efficiently by CCDs because the gene expression of CitNCED2 remained at an extremely low level (Fig. 3). Therefore, in Valencia orange, the accumulation of 9c-vio was predominant in the juice sacs. In Satsuma mandarin and Lisbon lemon, it was thought that the 9c-vio 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. 3). In fact, the level of ABA noticeably increased (Fig. 4). Therefore, the accumulation of 9c-vio in Satsuma mandarin and Lisbon lemon was much lower than that in Valencia orange (Kato et al., 2004). In addition, in Lisbon lemon, the accumulation of not only 9c-vio but also of 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 that in Satsuma mandarin during maturation (Kato et al., 2004).

The above results clearly show that the oxidative cleavage of 9c-vio catalysed by NCEDs affects the 9c-vio concentration and, consequently, the β,β'-xanthophyll composition of the three citrus varieties during fruit maturation. However, it is possible that 9c-neo, another substrate of NCEDs, is also cleaved in citrus fruit and involved in ABA biosynthesis. The Km for the recombinant PvuNCED1 and VP14 is lower with 9c-neo as a substrate relative to 9c-vio (Qin and Zeevaart, 1999; Schwartz et al., 2003b). In citrus fruit, 9c-neo was not identified in either the flavedos or the juice sacs, probably because of the low concentration. It is unknown whether 9c-neo is used by CitNCEDs to produce ABA during citrus fruit maturation.

In citrus fruit, increases in the gene expression of CitCCD1 were observed in both the flavedos and juice sacs of the three varieties during maturation (Fig. 3). 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, in both the flavedos and juice sacs, no noticeable differences in the levels of the gene expression of CitCCD1 were observed among the three varieties. Recently, in tomato, transgenic plants with a reduced expression of LeCCD1A and LeCCD1B were produced (Simkin et al., 2004a). Their fruits showed significant reductions in the rates of emission of β-ionone and geranylacetone but did not show significant changes in the carotenoid concentration. These results are due to the fact that the CCD1 protein is a non-plastid targeted enzyme and does not have a significant role in carotenoid turnover (Simkin et al., 2004a). In the present study, CitCCD1 proteins from the three citrus varieties were not thought to play an important role in carotenoid accumulation.

In conclusion, three cDNAs for carotenoid cleavage dioxygenase (CitCCD1, CitNCED2, and CitNCED3) were cloned from Satsuma mandarin, Valencia orange, and Lisbon lemon. The recombinant protein of these genes catalysed the cleavage of β,β'-xanthophylls. The present study shows that the level of 9c-vio is low under the high expression of CitNCED genes in the case of the flavedo of Lisbon lemon and the juice sacs of Satsuma mandarin and Lisbon lemon. The present study also shows that the level of 9c-vio is high under the low expression of CitNCED genes in the case of the flavedos of Satsuma mandarin and Valencia orange and the juice sacs of Valencia orange. Thus, it is concluded that CitNCEDs are a determinant of the diversity of the carotenoid profile during maturation in citrus fruit.

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