RESEARCH PAPER

Functional characterization of a carotenoid cleavage dioxygenase 1 and its relation to the carotenoid accumulation and volatile emission during the floral development of *Osmanthus fragrans* Lour.

Susanne Baldermann1,†, Masaya Kato2,†, Miwako Kurosawa3, Yoshiko Kurobayashi3, Akira Fujita3, Peter Fleischmann4 and Naoharu Watanabe1,*

1 Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan
2 Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan
3 Technical Research Center, T. Hasegawa Co. Ltd., 335 Kariyado, Nakahara-ku, Kawasaki-shi 211-0022, Japan
4 Institute of Food Chemistry, Technische Universität Braunschweig, Schleinitzstrasse 20, D-38106 Braunschweig, Germany

† These authors contributed equally to this article.
* To whom correspondence should be addressed: E-mail: acnwata@agr.shizuoka.ac.jp

Received 19 December 2009; Revised 7 April 2010; Accepted 14 April 2010

Abstract

Carotenoids are the precursors of important fragrance compounds in flowers of *Osmanthus fragrans* Lour. var. *aurantiacus*, which exhibit the highest diversity of carotenoid-derived volatiles among the flowering plants investigated. A cDNA encoding a carotenoid cleavage enzyme, OfCCD1, was identified from transcripts isolated from flowers of *O. fragrans* Lour. It is shown that the recombinant enzymes cleave carotenes to produce α-ionone and β-ionone in *in vitro* assays. It was also found that carotenoid content, volatile emissions, and *OfCCD1* transcript levels are subjected to photorhythmic changes and principally increased during daylight hours. At the times when *OfCCD1* transcript levels reached their maxima, the carotenoid content remained low or slightly decreased. The emission of ionones was also higher during the day; however, emissions decreased at a lower rate than the transcript levels. Moreover, carotenoid content increased from the first to the second day, whereas the volatile release decreased, and the *OfCCD1* transcript levels displayed steady-state oscillations, suggesting that the substrate availability in the cellular compartments is changing or other regulatory factors are involved in volatile norisoprenoid formation. Furthermore, the sensory evaluation of the aroma of the model mixtures suggests that the proportionally higher contribution of α-ionone and β-ionone to total volatile emissions in the evening is probably the reason for the increased perception by humans of the scent emission of *Osmanthus* flowers.

Key words: Carotenoid cleavage, carotenoids, CCD1, circadian rhythmicity, *Osmanthus fragrans*, norisoprenoids, scent perception.

Introduction

*Osmanthus fragrans* Lour. is a shrub native to East Asia, and horticultural varieties can be found from Japan through China, Indo-China, Thailand, and India, to the Caucasus region. The petals of the evergreen Oleaceae flowers show by far the highest diversity of carotenoid-derived aroma compounds among the flowering plants (Kaiser, 2002). Because of its unique scent, commercial extracts are in high demand for use in the production of expensive perfumes and cosmetics. In China, the essential oils are used for flavouring tea, wine, and foods. The dominant compound in the essential oils of
O. fragrans is β-ionone (Wang et al., 2009), however, how it is synthesized in these flowers is not known.

The contribution of CCD1 enzymes in norisoprenoid formation in flowers and fruits of other species has been demonstrated (Schwartz et al., 2001; Simkin et al., 2004a, b, 2008; Mathieu et al., 2005; Ibidah et al., 2006; Kato et al., 2006; Vogel et al., 2008; García-Limones et al., 2009; Huang et al., 2009b). Carotenoid cleavage dioxygenases (CCDs) typically exhibit a high degree of regio-specificity for double bond positions and can cleave multiple substrates. There are examples of CCDs that can cleave multiple double bonds, i.e. enzymes of the CCD1 family are involved in the cleavage of the 5,6 (5',6'); 7,8 (7',8'); and 9,10 (9'10') double bonds to produce divergent volatiles. LCD from Bixa orellana, ZmCCD1 from Zea mays, AtCCD1 from Arabidopsis thaliana, and LeCCD1 from Lycopersicon esculentum cleave lycopene at the 5,6 (5',6') double bonds (Bouvier et al., 2003, Vogel et al., 2008). OsCCD1 enzymes from rice can cleave the 7,8 (7',8') double bonds of the non-cyclic carotenoid lycopene (Ilg et al., 2009). A substantial number of enzymes involved in the cleavage of the 9,10 (9'10') double bonds of carotenoids have been identified, such as AtCCD1 from Arabidopsis thaliana (Schwartz et al., 2001); PhCCD1 from Petunia hybridica (Simkin et al., 2004a); LeCCD1 from Lycopersicon esculentum (Simkin et al., 2004b); VvCCD1 from Vitis vinifera (Mathieu et al., 2005); CmCCD1 from Cucumis melo (Ibdah et al., 2006); CitCCD1 from Citrus limon, Citrus sinensis, and Citrus unshiu (Kato et al., 2006); CeCCD1 from Coffea canephora and CaCCD1 from Coffea arabica (Simkin et al., 2008); ZmCCD1 from Zea mays (Vogel et al., 2008); FaCCD1 from Fragaria ananassa (García-Limones et al., 2009); or RdCCD1 from Rosa damascena (Huang et al., 2009b). Moreover, CCD4 enzymes from Crocus sativus, Rosa damascena, Osmanthus fragrans, Malus domestica, Chrysanthemum morifolium (Rubio et al., 2008; Huang et al., 2009a) and CCD7 and CCD8 from Arabidopsis thaliana (Schwartz et al., 2004) can cleave their carotenoid or apocarotenoid substrates at the 9,10 (9'10') double bonds. The role of CCD7 and CCD8 in the production of downstream metabolites involved in branching was known (Schwartz et al., 2004) before the carotenoid-derived strigolactones were identified to be involved in the stimulation of the colonization of arbuscular mycorrhizal fungi (Akiyama et al., 2005), the germination of parasitic plant seeds (Bouwmeester et al., 2007), and bud outgrowth (Umehara et al., 2008).

Although both CCD1 and CCD4 cleave their substrates at the same 9,10 (9'10') double bonds, CCD4 enzymes only cleave cyclic non-polar carotenoids and apocarotenoids such as β-carotene and do not cleave xanthophylls and non-cyclic carotenoids such as zeaxanthin and lycopene (Rubio et al., 2008; Huang et al., 2009a). Moreover CCD1 enzymes are cytoplasmic enzymes, whereas CCD4 enzymes carry a targeting sequence and are located in the plastids (Auldridge et al., 2006; Rubio et al., 2008). Hence, CCD4 enzymes have access to carotenoids located in the plastids. However, recombinant CCD4 isoforms oxidize different substrates; for example, AtCCD4 from A. thaliana and RmCCD4 from R. damascena prefer apocarotenoids and CmCCD4a from Chrysanthemum morifolium and MdCCD4 from M. domestica prefer carotenoids and are suggested to exhibit different biochemical functions (Huang et al., 2009a). Recombinant CCD1 enzymes can utilize either carotenoids or apocarotenoids in vitro (Huang et al., 2009b). However, it was recently suggested that the in vivo substrates of CCD1 are C17-apatocarotenoids. RNAi-mediated MtCCD1 repression in mycorrhizal roots of Medicago truncatula caused an accumulation of C17-apatocarotenoids and, therefore, have been suggested to be the major substrates for CCD1 enzymes in planta (Floss et al., 2009).

Previously α-carotene and β-carotene were identified as the two dominant carotenoids in petals of O. fragrans flowers (Baldermann, 2008). These two carotenes contribute to more than 90% of the total amount of carotenoids in flowers of O. fragrans. β-ionone and α-ionone, two major ionones emitted from flowers of O. fragrans (Wang et al., 2009), are the proposed reaction products of the cleavage of the 9,10 (9'10') double bond of α-carotene and β-carotene (Fig. 1).

In petunia flowers, β-ionone emission was correlated with the transcript levels of PhCCD1 and in chrysanthemum flowers the white colour was associated with the transcript levels of CmCCD4a (Simkin et al., 2004a; Ohmiya et al., 2006). None of these studies investigating the enzymatic carotenoid cleavage in flowers included the determination of the relative levels of the substrates (carotenoids) or reaction products (ionones), in addition to the analysis of the transcript levels.

The OFCCD4 from O. fragrans showed only very low activity with carotenoids and apocarotenoids and it is suggested that isoforms of CCD4 enzymes probably possess different biological functions (Huang et al., 2009a). It is therefore hypothesized that a member of the CCD1 family might be involved in the C13-norisoprenoid formation in flowers of O. fragrans. Its gene was identified and the enzyme it encodes was functionally characterized. The determination

![Fig. 1. Oxidative enzymatic cleavage of α-carotene by carotenoid cleavage enzymes yielding to α-ionone and β-ionone.](image-url)
of the relative levels of the substrates and reaction products in addition to the analysis of the transcript levels of *OfCCD1* by quantitative real-time PCR over the flowering period provided detailed information regarding the role of *OfDDC1* in fragrance formation in flowers of *O. fragrans*.

### Materials and methods

#### Plant materials

The flowers of *Osmanthus fragrans* Lour. var. *auranticus* were collected from the grounds of Shizuoka University, Japan, during the flowering period in autumn 2006 and 2008. Flowers releasing the strongest odour during the unfurling process (stages 4 and 5), after changing colour from yellow to orange, were used for the detailed studies (Fig. 2).

Freshly cut flowering branches without leaves (stage 4) were exposed to constant temperature (22 °C) and relative humidity (70%). Samples were either subjected to a 12/12 h light/dark regime for 48 h or to continuous light or dark periods for 24 h. The light intensity inside the incubator was set to 80 μmol m$^{-2}$ s$^{-1}$. At least 4 g of *O. fragrans* flowers (8.4±3.2 mg and 6.8±0.8 mm per flower) were collected in intervals of 4 h and directly frozen with liquid nitrogen. All samples were stored at –80 °C prior to analysis.

#### Isolation and sequence analysis of *OfCCD*

The first strand cDNA was synthesized from 1 μg total RNA using the SMART RACE cDNA Amplification Kit (Clontech, Laboratories, Palo Alto, CA) according to the manufacturer’s instructions. The cDNA fragments of *OfCCD* genes were amplified by PCR with the cDNA template and the primers that have been reported previously (see Supplementary Table S1 at *JXB* online) (Kato et al., 2006). The PCR product was purified by Microspin$^{	ext{TM}}$ columns (Amersham Bioscience, Piscataway, NJ) and the amplified cDNAs of the 3′ and 5′ were cloned with the TOPO TA-Cloning Kit (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. The cDNAs obtained were sequenced in detail (Taylor et al., 2006). Briefly, at least 4 g flowers petals were ground in liquid nitrogen and 20 mg were transferred to a microcentrifuge tube containing 350 ng of the internal standard β-apo-8′-carotenal. Firstly, 100 μl methanol and then 100 μl 50 mM TRIS-HCl (pH 8.0) containing 1 M NaCl were added. The carotenoids were extracted three times with 400 μl chloroform. The samples were stored under argon atmosphere at –80 °C prior to analysis. For HPLC analysis the samples were dissolved in 50 μl chloroform-methanol (1:4 v/v).

#### Expression and purification of the recombinant protein

The cDNA of *OfCCD1* for the expression of recombinant proteins was amplified by PCR with the primers shown in Supplementary Table S1 at *JXB* online. The cDNA fragments were cloned into *EcoRI* and *XhoI/BamHI* sites of the pGEX-6P-1 plasmid (Amersham Bioscience). The plasmids were transformed into *E. coli* strain XL1-Blue cells. For protein expression, 2 ml of an overnight culture was used to inoculate 200 ml of YT medium (8 g l$^{-1}$ tryptone, 10 g l$^{-1}$ yeast extract, 5 g l$^{-1}$ NaCl) containing the appropriate antibiotics. The cultures were grown at 27 °C until an OD$_{600}$ of 0.6 was reached. The expression of the proteins was induced by addition of 200 μl of 100 mM isopropyl-β-D-thiogalactoside (IPTG).

To simplify the enzyme assay, cultures were alternatively grown with the addition of 1000 μl 100 mM FeSO$_4$, and 100 μl 100 mM IPTG at 16 °C for 18 h. The *E. coli* cells were harvested by centrifugation and immediately frozen in liquid nitrogen. The cells were suspended in 20 ml phosphate buffer saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4) and 5 μl (7.5 U ml$^{-1}$ suspension) lysozyme (rLysozyme, Novagen, Darmstadt, Germany) and 25 μl (20 U ml$^{-1}$ suspension) cold active nuclease (Cryonase, Takara Bio Inc, Shiga, Japan) were added. After incubation for 30 min at room temperature the lysate was sonicated (6×30 s) (Ultrasonic Homogenizer, SMT Co., Ltd, Tokyo, Japan). Subsequently 1 ml 20% Triton X-100 (v/v) was added and the lysate moderately shaken on ice for 30 min. The cell debris was removed by centrifugation at 20 000 g for 60 min and the recombinant protein bound to Sepharose 4B (Amersham Bioscience). The column was washed with 10 ml of phosphate buffered saline and 10 ml cleavage buffer (50 mM TRIS-HCl, 150 mM NaCl, 1 mM dithiothreitol, 0.05% Triton X-100 (v/v), and 1 mM ETDA in the case of cultivation of XL1-Blue in the absence of ferrous iron). The recombinant protein was obtained after digestion with PreScission Protease (Amersham Bioscience) at 5 °C overnight. The purity of the recombinant protein was analysed by SDS-PAGE on 12.5% polyacrylamide gel (e-PAGE 12.5%, Tokyo, Japan) using the PreCission Plus Protein Dual Colour Standard (Bio-Rad, Tokyo, Japan) as marker. The proteins were stained with Bio-Safe Coomassie Blue G-250 Stain (BioRad) following the manufacturer’s instructions.

#### Analysis of carotenoids

The method used to analyse the carotenoids was previously published in detail (Taylor et al., 2006). Briefly, at least 4 g flowers petals were ground in liquid nitrogen and 20 mg were transferred to a microcentrifuge tube containing 350 ng of the internal standard β-apo-8′-carotenal. Firstly, 100 μl methanol and then 100 μl 50 mM TRIS-HCl (pH 8.0) containing 1 M NaCl were added. The carotenoids were extracted three times with 400 μl chloroform. The samples were stored under argon atmosphere at –80 °C prior to analysis. For HPLC analysis the samples were dissolved in 50 μl chloroform-methanol (1:4 v/v).

#### Table

<table>
<thead>
<tr>
<th>Stage</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflorescence</td>
<td>Pale yellow buds</td>
<td>Yellow buds</td>
<td>Pale orange petals</td>
<td>Orange petals, flowers start to open</td>
<td>Orange petals, full flowering</td>
<td>Orange petals, full flowering</td>
<td>Color intensity of flower petals decrease</td>
<td>Color intensity of flower petals decrease</td>
<td>Further decrease in color, more flowers drop-off</td>
<td>Further decrease in color, flowers drop-off</td>
<td>Flower color changed partly to brownish, end of flowering</td>
</tr>
<tr>
<td>Odor Intensity*</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
<td>++</td>
<td>+(*)</td>
<td>+</td>
<td>(*)</td>
<td>-</td>
</tr>
</tbody>
</table>

**Fig. 2.** Flowering of *Osmanthus fragrans* Lour. var. *auranticus*. Detailed studies were carried out using flowers during the unfurling period (stages 4 and 5), at the time where the flowers released the strongest odour [Odour intensity*: − no, (+) very weak, + weak, +(+) low, ++ medium, +++ high].
The carotenoids were analysed on a Jasco HPLC-PDA system (Tokyo, Japan) and separated on a C_{30}-column (YMC Co. Ltd Japan, 4.6×250 mm, 5 μm). Mixtures of methanol methyl-terti-butyl-ether and water in different volume ratios (solvent A: 81/15/4 and solvent B: 69/10/4) were used as the mobile phases at a flow rate of 0.8 ml min^{-1}. The carotenoids were separated in gradient mode from 50% to 100% solvent B within 20 min. Quantification was achieved from dose-response curves and identification by co-chromatography with references substances.

Total RNA extraction, reverse transcription, and real-time quantitative PCR
The total RNA was extracted from at least 4 g flower petals according to the method described by Ikoma et al. (1996). The genomic DNA was removed by on-column DNA digestion during the purification of the RNA using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the specifications given by the manufacturer.

The first strand cDNA was synthesized from 200 ng purified RNA using random hexamers at 37 °C for 60 min and TaqMan reverse transcription reagents (Applied Biosystems, Tokyo, Japan). TaqMan MGB probes and primers were designed on based on common sequences using the Primer express software (Applied Biosystems; see Supplementary Table S1 at JXB online). For endogenous control, the TaqMan ribosomal RNA control reagent VIC probe (Applied Biosystems) was used. TaqMan real-time PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR program included an initial step of 50 °C for 2 min, a 10 min denaturation step at 95 °C and then 40 cycles of 15 s of denaturation at 95 °C and 1 min of hybridization/polymerization at 60 °C. The relative expression ratios were calculated using the ABI PRISM 7000 sequence detection software (Applied Biosystems) and normalized using the 18S ribosomal RNA results. Real-time quantitative PCR was performed in three replicates for each sample.

Headspace sampling and analysis of volatiles
The volatiles emitted by O. fragrans flowers were collected by dynamic headspace sampling. Freshly cut flowering branches, after removal of the leaves, were placed into headspace sampling units and filtered air (Charcoal filter) was pumped at a flow rate of 100 ml min^{-1} through the sampling unit. The volatiles were trapped on Tenax<sup>®</sup> (180 mg) and analysed by GC-MS equipped with a thermal desorption system (TDS, Gerstel GmbH and Co. KG) under the following operating conditions: desorption temperature 260 °C, desorption time for 1 min, and split ratio of 15:1. The GC was equipped with a capillary TC-WAX column (GL Sciences Inc., Japan), 60 m×0.25 mm ID, and 0.5 μm film thickness. Helium was used as a carrier gas at a flow rate of 1.7 ml min^{-1}. The temperature program of the oven was set to 40 °C for 5 min, then 3 °C min<sup>-1</sup> up to 230 °C, and kept at this temperature for 60 min. The mass scan range was m/z 29–500 and the electric potential was set to EI 70 eV. Under these conditions, α- and β-ionones were detected at 59.6 min and 62.7 min, respectively.

Enzyme assays of recombinant proteins
The enzymatic activity of the recombinant OfCCD1 enzyme was assayed according to the method by Kato et al. (2006). For the enzymes obtained after cultivation of the E. coli cells in the presence of ferrous iron the activities were screened following the method described by Fleischmann et al. (2002).

Identification of the volatile reaction products of recombinant proteins
The volatile reaction products of the assay mixtures were analysed after solid phase micro-extraction (SPME) by gas chromatography mass spectrometry (GC-MS). Therefore a SPME fibre coated with 100 μm polymethylsiloxane (Supelco, Bellefonte, PA) was introduced into a headspace vial containing 2 ml enzymatic reaction mixture and 1 ml saturated sodium chloride solution and stirred for 1 h at 35 °C. The volatiles absorbed onto the fibre were analysed by GC-MS using a capillary Supelcowax column (GL Sciences Inc., Japan, 30 m×0.25 mm ID, 0.25 μm film thickness). The temperature program of the oven was set as follows: 50 °C maintained for 3 min, 5 °C min<sup>-1</sup> up to 190 °C, 40 °C min<sup>-1</sup> up to 240 °C, and held for 3 min. The mass scan range was set to m/z 50–300 and the electric potential to 1.00 kV. α-Ionone and β-ionone were detected at 23.1 min and 25.1 min, respectively.

Sensory evaluation
For sensory evaluation, three model mixtures simulating O. fragrans odour were evaluated by 23 panelists (16 male and 7 female). The three model samples contained different amounts of β-ionone, α-ionone, linalool, linalool oxides (furanoids), and γ-decalactone in ratios and concentrations comparable to the emitted volatiles at 02.00 h, 10.00 h, and 18.00 h (indicated by 1, 2, and 3 in Fig 7A). The exact compositions of the model mixtures are listed in Supplementary Table S2 at JXB online. To consider the different amounts of emitted volatiles, 0.06 g, 2.0 g, and 0.6 g of mixtures 1, 2, and 3 were diluted in ethanol (w/w) to give 10 g of stock solutions 1, 2, and 3, respectively. The three concentrates were diluted 1:10 with ethanol (w/w) and subsequently with MilliQ water until their odour intensities were felt to be the same as living flowers (100 ppm). For sensory evaluation, 10 g of samples in concentrations of 0.1 ppm, 1 ppm, and 10 ppm in ascending order were presented to the panelists in closed sensory vials (total volume 50 ml), coded by a random three-digit number. The panelists were asked to evaluate the intensity of the samples from 1 (none) to 5 (very strong). 10 g of Milli-Q water (intensity 1) and model mixture 2 in a concentration of 100 ppm (intensity 5) were provided as reference samples. Model mixture 2 (100 ppm) was used because it simulates the aroma of O. fragrans flowers at the time of highest volatiles emission and the odour of this concentration was evaluated to be similar to living flowers. The results were averaged and analysed by ANOVA (analysis of variance) and Tukey’s multiple comparison test. A probability level of 5% (P<0.05) was considered as significant.

Results
Isolation and functional characterization of OfCCD1
To identify CCD homologues in O. fragrans flowers, de-generate oligonucleotides based on conserved CCD sequences and amplified cDNA fragments of RNA isolated from O. fragrans flowers were designed. A full-length cDNA was subsequently obtained by RACE-PCR using gene-specific primers. The nucleotide sequence of this cDNA encodes a predicted protein of 563 residues. Phylogenetic analyses showed that the protein encoded by this cDNA clusters with other plant CCD1 enzymes (Fig. 3). The cDNA was therefore designated as OfCCD1.

To determine whether OfCCD1 encodes a functional CCD, the cDNA was transferred into a glutathione pGEX-6P-1 fusion vector for expression in E. coli. The recombinant protein was then purified using affinity chromatography. SDS-PAGE analysis on a 12.5% acrylamide gel identified a single band with a calculated molecular size of 65 kDa (see Supplementary Fig. S2 at JXB online). This was in accordance with a predicted molecular mass of 64 kDa.
Two in vitro assays were used to determine the cleavage activity of the recombinant protein. The first assay utilized ferrous iron, catalase, and ascorbic acid, and OfCCD1* purified from *E. coli* cells grown and induced under standard conditions (20 μM isopropyl β-d-thiogalactoside (IPTG), and 27 °C for 6 h). In the second assay, OfCCD1 was purified from *E. coli* cells induced by the addition of reduced amounts of IPTG (10 μM) and the bacteria were grown at 16 °C for an additional 18 h in the presence of ferrous iron (100 μM). The second enzymatic reaction buffer did not contain additional compounds (ferrous iron, catalase, and ascorbic acid) and the enzyme assay was carried out according to Fleischmann *et al.* (2002). After cultivation of the *E. coli* cells in the absence of ferrous iron, the isolated enzymes (OfCCD1*) showed no activity due to the lack of ferrous iron (Fig. 4). The rate of β-carotene degradation was similar to the chemical degradation of β-carotene under our experimental conditions (blank). After the addition of ferrous iron, catalase, and ascorbic acid to the buffer-substrate mixture, OfCCD1* activity could be detected, however, to obtain a comparable decrease of the initial amount of β-carotene for OfCCD1 and OfCCD1* longer reaction times were necessary (Fig. 4). OfCCD1 isolated from liquid cultures containing ferrous iron yielded active recombinant OfCCD1 enzymes that degraded β-carotene faster and without the supplement of additional ferrous iron (Fig. 4). However, high activities were only obtained directly after isolation and a stabilization of the enzymes with glycerol and ascorbate was necessary for storage. Because ascorbate also protects carotenoids against oxidation *K*<sub>m</sub> and *V*<sub>max</sub> values were not determined.

The volatile enzymatic reaction products of the cleavage of β-carotene and α-carotene were analysed by SPME-GS-MS. β-Ionone was detected in the headspace of the

### Fig. 3.
Phylogenetic tree of deduced amino acid sequences of carotenoid cleavage enzymes involved in the cleavage of carotenoids (C<sub>40</sub>) or C<sub>27</sub> apocarotenoids (CCD1, CCD4, CCD7, and CCD8) at the 9,10 (9',10') double bonds. The sequences were aligned using ClustalW (http://www. genome.jp/). The evolutionary history was inferred using the Neighbor–Joining method and drawn by Tree View (accession numbers: Arabidopsis thaliana CCD7 AK229864, CCD8 Q8VY26; Chrysanthemum morifolium CCD4a ABY60885, CCD4b BAF36656; Citrus limon CCD1 AB219168; Citrus sinensis CCD1 AB219165; Citrus unshiu CCD1 AB219164; Coffea arabica CCD1 DQ157170; Coffea canephora CCD1 DQ157166; Crocus sativus CCD1a AJ132927, CCD4a EU523662, CCD4b EU523663; Cucumis melo CCD1 DQ269467; Malus domestica CCD4 EU334433; Rosa damascena CCD1a AY576003; Rosadamasenac CCD4 EU334433 RdCCD1 ABY47994; Solanum lycopersicum SICCD1a AY576001, SICCD1b AY576002; VvCCD1 Vitis vinifera AY856353; Zea mays ACR33784).

### Fig. 4.
Relative enzymatic activities of purified recombinant OfCCD1 enzymes. OfCCD1 was purified from *E. coli* cells after the induction of protein expression in the presence of ferrous iron, whereas the recombinant OfCCD1* was purified after the induction of protein expression in the absence of ferrous iron. The recombinant OfCCD1* showed carotenoid cleavage ability if ferrous iron, catalase, and ascorbic acid were added to the assay mixture, however, prolonged reaction times were necessary. The assays were carried out at room temperature, 0.93 μmol l<sup>-1</sup> initial concentration of β-carotene, 10 μg ml<sup>-1</sup> protein in 1 ml TRIS-HCl buffer (pH 7, 50 mM TRIS-HCl, 125 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol) for 20 min or 240 min, respectively. The relative activities were calculated setting the initial β-carotene concentration to 1. The blank values represent β-carotene degradation under identical assay conditions. The data are presented as mean ± SD from three replicates.
reaction mixtures after the addition of β-carotene as substrate, and both α- and β-ionone were detected as volatiles in the headspace after applying α-carotene as the substrate (Fig. 5). Other putative volatile reaction products derived from the carotenoid cleavage, such as β-cyclocitrinal resulting from the cleavage of the 7,8 (7′,8′) double bond were not detected. These results indicate that the activity of OfCCD1 is similar to that of other CCD1 enzymes involved in the cleavage of the 9,10 (9′,10′) double bonds of cyclic carotenoids (Fig. 5A, B).

**Cleavage activity against α-carotene and β-carotene**

Other CCD1 enzymes from *Arabidopsis thaliana* (Schwartz et al., 2001), tomato (Simkin et al., 2004b), melon (Ibdah et al., 2006), maize (Vogel et al., 2008), and roses (Huang et al., 2009a) showed a broad substrate specificity against carotenoids and apocarotenoids. All CCD1 enzymes cleave at the 9,10 (9′,10′) double bonds. The widest range of substrates was tested with the rose CCD1, which cleaved symmetric carotenoids at both ends simultaneously (Huang et al., 2009b). The RdCCD1 exhibited different affinities against the end group moieties of pseudo-symmetric molecules, except for the pseudo-symmetric xanthophyll lutein where similar levels of the reaction products 3-hydroxy-α-ionone and 3-hydroxy-β-ionone were observed. So far, in no study were the symmetric and pseudo-symmetric carotenoids β- and α-carotene used as substrates. The ratio of α- and β-ionone of the reaction by OfCCD1 with α-carotene was approximately 1.7:1, indicating that the preferred cleavage site was the α-ionone ring moiety (Fig. 5).

**Changes in OfCCD1 transcript levels**

To determine the change in the *OfCCD1* transcript levels over time, RNA was isolated from petals harvested in intervals of...

---

**Fig. 5.** SPME-GC-MS analysis of volatiles formed in *in vitro* assays with the purified OfCCD1, structures and cleaving sites of the substrates. GC-chromatograms (A, B) of volatiles formed in an assay mixture containing α-carotene (A) and β-carotene (B) as substrates. α-ionone and β-ionone were identified as reaction products (the GC-chromatograms are presented as the difference between the sample chromatogram and the chromatogram obtained in the control assay).
4 h over 48 h. In addition, RNA was isolated from petals of cut flowering branches subjected either to 24 h light or to 24 h continuous dark periods. OfCCD1 transcript levels were determined by qRT-PCR. OfCCD1 steady-state transcript levels increased during the light periods and reached their maximal levels either at 12.00 h (noon) or 16.00 h (Fig. 6A). When the branches were subjected to constant darkness for 24 h, the transcript levels increased over time (Fig. 6B), even though at a reduced level compared with the OfCCD1 transcript levels detected during the 12/12 h (dark/light) photoperiods (Fig. 6A). The maximum transcript levels were observed after 20 h incubation in continuous darkness, which was somewhat delayed compared with the flowers subjected in parallel to 12/12 h (dark/light) photorhythmic conditions (Fig. 6A, B). When the flowers were placed in constant light, lower steady-state transcript levels and changes at lower amplitude were observed (Fig. 6B). The maximal OfCCD1 transcript levels in flower petals of branches placed into 24 h continuous light were detected after an 8 h incubation period (Fig. 6B). At this time the flowers which were subjected to 12/12 h (dark/light) photoperiods exhibited the lowest transcript levels during the dark period (Fig. 6A).

To confirm that the OfCCD1 peak equals the transcript levels during day, the OfCCD1 transcripts of flowers grown outside and picked at 14.00 h were analysed. The transcript levels in O. fragrans flowers picked outside at the same times at various flowering stages (2, 4, 7, and 10; Fig. 2) changed 0.2-fold (2 arbitrary units; see Supplementary Fig. S1 at JXB online), whereas the transcripts varied up to 3.5-fold (21 arbitrary units) between the light and dark periods (Fig. 7A).

Changes in the carotenoid content in Osmanthus fragrans flowers

To determine the changes in the concentrations of α- and β-carotene, previously identified as the two major carotenoids (Baldermann, 2008), cut flowering branches were subjected to controlled environmental conditions and the concentrations of α- and β-carotene were analysed at intervals of 4 h. The concentrations of both α- and β-carotene increased in the presence of light (Fig. 6C, D), indicating that carotenoid biosynthesis in the flowers of O. fragrans is influenced by light. During the dark period, little change in carotenoid concentrations were observed and the levels remained nearly at the values reached during the previous light period.

Although OfCCD1 transcript levels and carotenoid concentrations peak with an offset of 4 h, the carotenoid content decreased or remained at a relatively low level, or example, at 12.00 h and 16.00 h of the first and second days, respectively (12/12 h (dark/light) photoperiods, Fig. 6A, C).

To test the effect of light on the carotenoid content, flowers were incubated under continuous 24 h dark or light (Fig. 6D). A nearly steady increase in carotenoids was obtained inside the flowers in the absence of light and only small changes were observed under continuous photoemission (Fig. 6D). Lower carotenoid concentrations in flowers subjected to 24 h continuous light or dark were detected compared with the flowers subjected to 12/12 h light/dark regime. The carotenoid content decreased at the peak of the
Volatile emission and a-ionone and b-ionone release in flowers of O. fragrans

The cut flowering branches subjected to 12/12 h (dark/light) photoperiods released maximum amounts of volatiles shortly after the beginning of the light period, following by a decrease until the lowest release during the dark period at 06.00 h (Fig. 6E). To test if the release of volatiles was regulated by circadian mechanisms, the cut flowering branches were subjected to a regime of 24 h constant light or constant dark. Flowers subjected to constant dark (Fig. 6F) showed a similar emission pattern to those flowers subjected to 12/12 h (dark/light) photoperiods (Fig. 6E). In both cases, the maximum levels of released volatiles were detected after 12 h. The results indicate that the release of volatiles is regulated by both light and circadian mechanisms. Flowers subjected to continuous light reached their maximum emission after 12 h, followed by a decrease in the emission over the rest of the experimental period. The scent emission decreased strongly between the first and second days when the samples were subjected to 12/12 h (dark/light) photoperiods.

The emission of the two primary cleavage products of the major carotenoids of Osmanthus flowers, a- and b-ionones (Fig. 1) were next examined under the different photorhythmic conditions (Fig. 6G, H). As with the total emission of volatiles, the release of ionones was higher during the light periods. Flowers subjected to a 24 h continuous light regime emitted more ionones compared with those flowers treated in parallel under 12/12 h (dark/light) photoperiods (Fig. 6G, H).

Compared with the total volatile emission, the emission of ionones was higher in the early evening, which means that the contribution of the ionones to the total volatiles increases during the day (Fig. 7A). Volatile norisoprenoids are characterized by extremely low odour detection thresholds in humans. To evaluate changes in the scent of flowers of Osmanthus fragrans at different day times, the odour intensities of three model mixtures reflecting the floral scent at 02.00, 10.00, and 18.00 h (1, 2, and 3 in Fig. 7A; see Supplementary Table S2 at JXB online) were subjected to sensory evaluation. Although the amount of emitted volatiles were much higher at 10.00 h, the model mixtures 2 (10.00 h) and 3 (18.00 h) were evaluated as similar, but significantly different from model mixture 1 (02.00 h) (Fig. 7B).

Discussion

Isolation and functional analysis of OfCCD1

It is well known that the colour of yellow flowers is often caused by the presence of large amounts of carotenoids. Some flowers also have a broad variety of carotenoid-derived scent compounds, as in the case of O. fragrans which has the highest diversity of carotenoid-derived scent compounds among 1250 flowering plants investigated (Kaiser, 2002). Hence, it was of special interest to elucidate the biosynthesis of these compounds in O. fragrans. Since a previous report indicated that a 75% decrease in b-ionone formation was observed in transgenic petunia plants in which PhCCD1 gene expression was inhibited (Simkin et al., 2004a), the possible role of OfCCD1 on ionone biosynthesis was examined in O. fragrans.

Apart from the formation of volatile C13-norisoprenoids through the action of CCD1, enzymatic cleavage of the 9,10 (9',10') double bond has also been demonstrated for CCD4 enzymes from C. sativus, R. damascena, C. morifolium,
M. domestica, and A. thaliana (Rubio et al., 2008; Huang et al., 2009a).

Because the O. fragrans CCD4 showed very low activity against carotenoids and apocarotenoids (Huang et al., 2009b) this study focused on the functional characterization of the OfCCD1 enzyme. The putative amino acid sequence of OfCCD1 exhibited the conserved histidine residues of the active centre of CCDs and in the presence of ferrous iron, the recombinant enzymes showed cleavage activity towards the two dominant carotenoids (β-carotene and α-carotene) found in flowers of O. fragrans.

CCD1 enzymes cleave symmetric and pseudo-symmetric carotenoids differently. Based on the observations of Kloer and Schulz (2006), it was suggested that pseudo-symmetric molecules undergo a two-step cleavage. First, the enzyme cleaves the C₄₀ substrate once, releasing the products, and then it binds to the primary non-volatile apocarotenoid and cleaves it for a second time. It should be noted that recent studies suggest that CCD1 enzymes cleave the primary derived cleavage products (C₂₇-apocarotenoids) in the cytosol in vivo (Floss et al., 2009). In vitro, CCD1 can cleave either carotenoids or apocarotenoids (Huang et al., 2009b). In roses, the non-volatile reaction products (C₂₇-apocarotenoids) of the first cleavage were only detected when the substrates contained different moieties at their ends. In this study, the symmetric β-carotene and the pseudo-symmetric α-carotene were used as substrates and the cleavage of α-carotene resulted in higher amounts of α-ionone, suggesting that the first site of cleavage is the one with the α-ionone moiety. However, in the case of the rose enzyme (RdCCD1), the same amounts of the reaction products 3-hydroxy-α-ionone and 3-hydroxy-β-ionone were obtained from pseudo-symmetric xanthophyll lutein (Huang et al., 2009b).

**Photorhythmic changes of OfCCD1 transcript levels, carotenoid concentrations, and volatile emission**

Photorhythmic volatile emission in plants has been demonstrated in several flowering plants (Matile and Altenburger, 1988; Loughrin et al., 1990; Helsper, 1998; Picone et al., 2004; Dudavera and Pichersky, 2006). In general, nocturnally pollinated flowers tend to have maximum scent emission during the dark period, whereas the diurnally pollinated flowers release higher amounts of volatiles during the day. Volatile emission can be regulated either by light or by endogenous circadian mechanisms, mostly controlled at the gene expression transcription level (Hendel-Rahmanim et al., 2007). One group of plant enzymes is characterized by an increase in activity in young flowers and a decline during ageing, while a second group of enzymes show little or no decline at the end of the flower’s life (Dudavera and Pichersky, 2006). During the floral development of Ipomoea sp., I. obscura, and I. nil, the CCD1 and CCD4 transcript levels decreased (Yamamizo et al., 2009). In the case of OfCCD1, the steady-state transcript levels are subjected to circadian mechanisms and have a peak during the day.

The concentrations of α-carotene and β-carotene also underwent photorhythmic changes. It is interesting to note that there is a negative correlation between the abundance of OfCCD1 mRNA and the concentrations of the substrates (α-carotene and β-carotene). In O. fragrans flowers, the carotenoid levels remained low or decreased if the transcript levels of OfCCD1 were high. The carotenoid content increased over the experimental interval and reached the maximal concentration under light conditions. The light/dark regulation of carotenoid biosynthesis was investigated in red pepper, where all transcript levels of genes involved in the carotenoid biosynthesis decreased under dark conditions (Simkin et al., 2003). In citrus fruits, the transcript levels of genes encoding enzymes involved in carotenoid biosynthesis as well as CCD transcript levels increased during fruit maturation (Kato et al., 2007). In chrysanthemums, a negative correlation between Cin CCD4a mRNA abundance and carotenoid content was observed. However, recently obtained results during the flower development of Ipomoea sp., I. obscura, and I. nil, suggest that the flower colour cannot be correlated to carotenoid degradation activity in Ipomoea plants (Yamamizo et al., 2009). In O. fragrans, the OfCCD4 showed very low activity against carotenoids and apocarotenoids (Huang et al., 2009a) and hence, the contribution to the biodegradation of carotenoids is unclear. However, the transcript levels were quite similar to those of OfCCD1 (M Kato, unpublished results). Hence, based on the work presented here, it might be suggested that, in Osmanthus flowers, the slight decrease in α-carotene and β-carotene levels observed in the light periods is at least partly due to the high activity of OfCCD1.

In petunia corolla, a correlation between mRNA abundance and β-ionone emission was observed (Simkin et al., 2004a). However, emission was still increasing when transcript levels began decreasing during the afternoon. This study provides a similar observation in Osmanthus flowers, where the β-ionone emission remained high after the transcript levels of OfCCD1 had already decreased during the day. It was suggested that there might be some limitation due to the substrate availability. Carotenoids are synthesized in the plastids whereas the CCD1 enzymes are located in the cytosol and therefore the cytosolic CCD1 enzymes have access only to those carotenoids distributed on the outer envelope of plastids, where, for example, significant amounts of β-carotene have been detected in pea chloroplasts (Markwell et al., 1992). In O. fragrans flowers, the carotenoid concentrations increased over the flowering period and, hence, a limiting factor for the reaction of OfCCD1 with the substrates could be the access inside the cell compartments.

Another regulatory factor could be the catalytic efficiency of enzymes with their substrates. Carotenoid cleavage enzymes purified from plant tissues exhibit different affinities towards β-carotene. For example the Keq values for β-carotene obtained for carotenoid cleavage enzymes isolated from different fruits varied from 11.0 μM⁻¹ for quince fruit, 6.6 μM⁻¹ for nectarine, and 3.6 μM⁻¹ for star fruit, respectively (Fleischmann et al., 2002, 2003; Baldermann et al., 2005). In Osmanthus flowers, the carotenoid content increased and steady-state maximal transcript levels were observed under light conditions, whereas the emission of ionones, as enzymatic
reaction products, decreased over the flowering period. It might be suggested that the catalytic efficiency of the OfCCD1 enzymes with their substrates is another regulatory factor.

Our results demonstrate that OfCCD1 in flowers of *Osmanthus fragrans* Lour. is probably involved in the oxidative cleavage of carotenoids to produce the volatile scent compounds α- and β-ionone. However, detailed analysis of carotenoids as putative precursors, transcript levels of *OfCCD1*, and volatile emissions indicate that the activity of this enzyme is not sufficient to account for the total emission of these volatiles. Additional work is needed to clarify the contribution of other carotenoid cleavage enzymes to ionone emission and identify the in vivo substrates.

**Changes in β-ionone and α-ionone emission in relation to scent perception**

*Osmanthus* flowers release their volatiles under light conditions. The analysis showed that the highest total volatile emission occurs in the morning, and total emission is lower in the afternoon. The release of β-ionone and α-ionone also strongly increased in the presence of light in the morning, and remained at a high level when the total volatile emission began decreasing during the afternoon. Because β-ionone (0.007 µg l⁻¹; Buttery *et al.*, 1990) and α-ionone (0.4 µg l⁻¹; Teranishi and Buttery, 1987) have very low odour perception thresholds for humans, in water those compounds exhibit a strong impact on floral scents. The sensory evaluation of model mixtures reflecting the floral scent of *O. fragrans* flowers at 02.00, 10.00, and 18.00 h demonstrated that the scent in the morning and early evening is considered as similar, although the total volatile emission had decreased by approximately 3-fold. A similar example is the low amount of C₁₃ norisoprenoids in rose, which nonetheless make a strong contribution to the scent; while constituting less than 1% of the total volatiles, they contribute to more than 90% to the scent impression by humans (Ohloff and Demole, 1987). Hence, the increasing amounts of α-ionone and β-ionone in relation to the total volatiles in the early evening are likely to be responsible for the stronger smell in the afternoon or early evening.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Table S1.** Primer sequences used for *OfCCD1* gene cloning and analysis by TaqMan® real time quantitative PCR assay.

**Supplementary Table S2.** Composition of model mixtures for sensory evaluation of *O. fragrans* aroma.

**Supplementary Fig. S1.** *OfCCD1* transcript levels of flowers at stages 2, 4, 7, and 10 at 14.00 h.

**Supplementary Fig. S1.** SDS-PAGE of purified OfCCD1.

**Acknowledgements**

This work was supported by the Japan Society for the Promotion of Science (JSPS 07434). We thank Dr Eran Pichersky for useful comments on the manuscript and Dr Toshiyuki Ohnishi for valuable discussions.

**References**


