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

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 Baldermann[1#], Masaya Kato[2#], Miwako Kurosawa[3], Yoshiko Kurobayashi[3], Akira Fujita[3], Peter Fleischmann[4], Naoharu Watanabe[1]*

[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, 38106 Braunschweig, Germany

[#]These authors contributed equally to this article.

To whom the correspondence should be addressed:

Prof. Naoharu Watanabe, PhD, Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

Tel, Fax: 81-(0)54-238-4870

E-mail: acnwata@agr.shizuoka.ac.jp

Date of submission: 19. December 2009

Number of tables: 0

Number of figures: 7

Supplementary data: Table S1, Table S2, Fig S1, Fig S2

Abstract

Carotenoids are the precursors for important fragrance compounds in flowers of *Osmanthus fragrans* Lour. var. aurantiacus, which exhibit the highest diversity of carotenoid-derived volatiles among flowering plants investigated (Kaiser, 2002). We identified a cDNA encoding a carotenoid cleavage enzyme OfCCD1 from transcripts isolated from flowers of *O. fragrans* Lour. We showed 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 emission, and *OfCCD1* transcript levels are subjected to photorhythmic changes and increased principally during daylight hours. At the times where *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 emission 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 aroma of model mixtures suggests that the proportionally higher contribution of α-ionone and β-ionone to total volatile emission in the evening is likely the reason for increased perception of scent emission of *Osmanthus* flowers by humans.

Key words: Carotenoids, norisoprenoids, carotenoid cleavage, scent perception, circadian rhythmicity, CCD1, *Osmanthus fragrans*

Introduction

Osmanthus fragrans Lour. is a shrub native to East-Asia, and horticultural varieties can 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 flavoring 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.*, 2004; Mathieu *et al.*, 2005; Ibdah *et al.*, 2006; Kato *et al.*, 2006; Simkin *et al.*; 2008, 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 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 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 9,10 (9'10') double bonds of carotenoids have been identified, such as AtCCD1 from *Arabidopsis thaliana* (Schwartz *et al.*, 2001); PhCCD1 from *Petunia hybrida (*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); CcCCD1 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 9,10 (9'10') double bounds. 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 stimulation of colonization of arbuscular mycorrhizal fungi (Akiyama *et al.*, 2005), 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 bounds, 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 carrying 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; e.g. AtCCD4 from *A. thaliana* and RdCCD4 from *R. damascena* prefer apocarotenoids and CmCCD4a from *Chrysanthemum morifolium* and MdCCD4 from *M. domestica* carotenoids and are suggested to exhibit different biochemical functions (Huang *et al.*, 2009a). Recombinant CCD1 enzymes can utilize either carotenoids or apocarotenoids in *in vitro* (Huang *et al.*, 2009b). However, it was recently suggested that the *in vivo* substrates of CCD1 are C_{27}-apocarotenoids. RNAi-mediated MtCCD1 repression in mycorrhizal roots of *Medicago truncatula* caused an accumulation of C_{27}-apocarotenoids and therefore have been suggested to be the major substrates for CCD1 enzymes in planta (Floss *et al.*, 2008).

Previously we identified α-carotene and β-carotene as the two dominant carotenoids in petals of *O. fragrans* flowers (Baldermann 2008). These two carotenes contribute to more

than 90% to the amount of total 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 color 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), 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). We therefore hypothesized that a member of the CCD1 family might be involved in the C_{13}-norisoprenoid formation in flowers of *O. fragrans,* identified its gene and functional characterized the enzyme it encodes. The determination 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. aurantiacus were collected at Shizuoka University ground, Japan during the flowering period in autumn 2006 and 2008. Flowers releasing the strongest odor during the unfurling process (stages 4 and 5) after changing the color from yellow to orange were used for 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-h light/12-h-dark regime for 48 h or 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 OfCCD1

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 manufacturers' instructions. The cDNA fragments of *OfCCD1* genes were amplified by PCR with the cDNA template and the primers that have been reported previously (Table S1) (Kato *et al.*, 2006). The PCR product was purified by Microspin™ columns (Amersham Bioscience, Piscataway, NJ) and the amplified cDNAs of the 3' and 5' were cloned with TOPO TA-Cloning Kit (Invitrogen, San Diego, CA) and sequenced. End to end PCR was performed with primers designed from the cDNA sequences obtained by RACE-PCR.

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 Table S1. The cDNA fragments were cloned into EcoR I and Xho I/BamH I 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 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 under addition of 1000 µL of 100 mM $FeSO_4$, and 100 µL of 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 buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_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 x 30 sec) (Ultrasonic Homogenizer, SMT Co., LTD, Tokyo, Japan). Subsequently 1 mL 20% Triton X-100 (v/v) was added and the lysate moderatly 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 case of cultivation of XL1-Blue in absence of ferrous iron). The recombinant protein was obtained after digestion with PreScission Protease (Amersham Bioscience) at 5°C over night. The purity of the recombinant protein was analyzed by SDS-PAGE on 12.5% polyacrylamide gel (e-PAGE 12.5%, Tokyo, Japan) using the Precission Plus Protein Dual Colour Standard (BioRad, 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 analyze 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 micro-centrifuge tube containing 350 ng of the internal standard β-apo-8'-carotenal. Firstly, 100 µL of methanol and then 100 µL of 50 mM Tris–HCl (pH 8.0) containing 1 M NaCl were added. The carotenoids were extracted with 400 µL chloroform

(3 x). 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 in the volume ratio 1:4 (v/v).

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 x 250 mm, 5 µm). Mixtures of methanol methyl-*tert*-butyl-ether and water in different volume ratios (solvent A: 81/15/4 and solvent B: 6/90/4) were used as mobile phases at a flow rate of 0.8 mL min^{-1}. The carotenoids were separated in gradient mode from 30% 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 radom 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, Table S1). 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) using the ABI PRISM 7000 instrument (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^{TA} (180 mg) and analyzed 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 1 min, and split ratio of 15:1. The GC was equipped with a capillary TC-WAX column (GL Sciences Inc., Japan), 60 m x 0.25 mm I.D., 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, 3°C min^{-1} to 230°C, and kept at this temperature for 60 min. The mass scan range was *m/z* 29 to 500 and the electric potential was set to EI 70 eV. Under these conditions, α- and β-ionones were detected at 59.6 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.

252 Identification of the volatile reaction products of recombinant proteins

253

254 The volatile reaction products of the assay mixtures were analysed after solid phase micro-
255 extraction (SPME) by gas chromatography mass spectrometry (GC-MS). Therefore a SPME
256 fiber coated with 100 µm polymethylsiloxane (Supelco, Bellefonte, PA) was introduced into
257 a headspace vial containing 2 mL enzymatic reaction mixture and 1 mL saturated sodium
258 chloride solution and stirred for 1 hr at 35°C. The volatiles absorbed to the fiber were
259 analyzed by GS-MS using a capillary Suplecowax column (GL Sciences Inc., Japan, 30 m x
260 0.25 mm I.D., 0.25 µm film thickness). The temperature program of the oven was set as
261 follows: 50°C maintained for 3 min, 5°C min^{-1} to 190°C, 40°C min^{-1} to 240°C, and hold for
262 3 min. The mass scan range was set to m/z 50-300 and the electric potential to 1.00 kV. α-
263 Ionone and β-ionone were detected at 23.1 min and 25.1 min, respectively.

264

265 Sensory evaluation

266

267 For sensory evaluation three model mixtures simulating *O. fragrans* flavor were evaluated
268 by 23 panelists (16 male and 7 female). The three model samples contained different
269 amounts of β-ionone, α-ionone, linalool, linalool oxides (furanoids) and γ-decalactone in
270 ratios and concentrations comparable to the emitted volatiles at 2:00, 10:00, and 18:00
271 o'clock (indicated with 1, 2, and 3 in Fig 7A). The exact compositions of the model
272 mixtures are listed in Table S2. To consider the different amounts of emitted volatiles
273 0.06 g, 2.0 g, and 0.6 g of mixture 1, 2 and 3 were diluted in ethanol (w/w) to give 10 g of
274 stock solutions 1, 2, and 3, respectively. The three concentrates were diluted 1:10 with
275 ethanol (w/w) and subsequently with MilliQ water until their odor intensities were felt to be
276 the same as living flowers (100 ppm). For sensory evaluation 10 g of samples in
277 concentrations of 0.1 ppm, 1 ppm, and 10 ppm in ascending order were presented to the
278 panelist in closed sensory vials (total volume 50 mL) coded by a random three-digit number.
279 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 odor of this concentration was evaluated to be similar to living flowers. The results were averaged and analyzed 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, we designed degenerate oligonucletides based on conserved CCD sequences, and amplified cDNA fragments of RNA isolated from *O. fragrans* flowers. 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). We therefore designated the cDNA 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% acryl amide gel identified a single band with a calculated molecular size of 65 kD (Fig. S2). This was in accordance with a predicted molecular mass of 64 kD.

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, 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 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 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 to active recombinant OfCCD1 enzymes that degraded β-carotene faster and without supplementation 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_m and v_{max} values were so far not determined.

The volatile enzymatic reaction products of the cleavage of β-carotene and α-carotene were analyzed by SPME-GS-MS. β-Ionone was detected in the headspace of the 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-cyclocitral resulting from the cleavage of the7,8 (7',8') double bond have not been 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, 5B).

336 Cleavage activity against α-carotene and β-carotene

337

338 Other CCD1 enzymes from *Arabidopsis thaliana* (Schwartz *et al.*, 2001), tomato (Simkin *et*
339 *al.*, 2004b), melon (Ibdah *et al.*; 2006), maize (Vogel *et al.*, 2008), and roses (Huang *et al.*,
340 2009a) showed a broad substrate specificity against carotenoids and apocarotenoids. All
341 CCD1 enzymes cleave at 9,10 (9',10') double bonds. The widest range of substrates was
342 tested with the rose CCD1, which cleaved symmetric carotenoids at both ends symmetrically
343 (Huang *et al.*, 2009b). The RdCCD1 exhibited different affinities against the end group
344 moieties of pseudo-symmetric molecules, except for the pseudo-symmetric xanthophyl lutein
345 where similar levels of the reaction products 3-hydroxy-α-ionone and 3-hydroxy-β-ionone
346 were observed. So far in no study the symmetric and pseudo-symmetric carotenes β- and α-
347 carotene were used as substrates. The ratio of α- and β-ionone of the reaction by OfCCD1
348 with α-carotene was approximately 1.7:1, indicating that the preferred cleavage site was the
349 α-ionone ring moiety (Fig. 5).

350

351 Changes in *OfCCD1* transcript levels

352

353 To determine the change of the *OfCCD1* transcript levels throughout the days, RNA was
354 isolated from petals harvested in intervals of 4 h over 48 h. In addition, RNA was isolated
355 from petals of cut flowering branches subjected either to 24 h light or 24 h continuous dark
356 periods. *OfCCD1* transcript levels were determined by qRT-PCR. *OfCCD1* steady-state
357 transcript levels increased during the light periods and reached their maximal levels either at
358 12:00 noon or 16:00 o'clock (Fig. 6 A). When the branches were subjected to constant
359 darkness for 24 h, the transcript levels increased over the time (Fig. 6B), even though at a
360 reduced level compared to the *OfCCD1* transcript level changes detected during 12/12 h
361 (dark/light) photoperiods (Fig. 6A). The maximum transcript levels were observed after 20 h
362 incubation in continuous dark, which was somewhat delayed compared to the flowers
363 subjected in parallel to 12/12 h (dark/light) photorhythmic conditions (Figs. 6A, 6B). 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 flowers petals of branches placed into 24 h continuous light were detected after 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 *OfCCD1* peak equal transcript levels during day, the *OfCCD1* transcripts of flowers grown outside and picked equally at 14:00 o'clock were analyzed. The transcript levels in *O. fragrans* flowers picked outside at equal time at various flowering stages (2, 4, 7, and 10; Fig. 2) changed 0.2 fold (2 arbitrary units, Fig. S1), whereas the transcripts varied up to 3.5 fold (21 arbitrary units) between light and dark periods (Fig. 7A).

Changes in the carotenoid content in *Osmathus 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 analyzed in intervals of 4 h. The concentrations of both α- and β-carotene increased in the presence of light (Figs. 6C, 6D), indicating that carotenoid biosynthesis in the flowers of *O. fragrans* is influenced by light. During the dark period, little changes 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 hours, the carotenoid content decreased or remained at a relatively low level, like at 12:00 and 16:00 o'clock of the first and second day, respectively (12/12 h (dark/light) photoperiods, Figs. 6A, 6C).

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 of the carotenoids was obtained inside flowers in the absence of light and only little changes were observed under continues

photoemission (Fig. 6D). Lower carotenoid concentrations in flowers subjected to 24 h continuous light or dark were detected compared to the flowers subjected to 12-h-light/12-h-dark regime. The carotenoid content decreased at the peaks of the *OfCCD1* transcript levels at 4:00 and 12:00 o'clock under continuous illumination (Figs. 6B, 6D).

Volatile emission and α-ionone and β-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 6:00 am (Fig. 6E). To test if the release of volatiles was regulated by circadian mechanisms, we subjected the cut flowering branches 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 volatile release 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 day when the samples were subjected to 12/12 h (dark/light) photoperiods.

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

Compared to the total volatile emission, the emission of ionones was higher in 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 odor detection

thresholds in humans. To evaluate changes in the scent of flowers of *Osmanthus fragrans* at different day times the odor intensities of three model mixtures reflecting the floral scent at 2:00, 10:00, and 18:00 o'clock (1, 2, and 3 Fig. 7A, Table S2) were subjected to sensory evaluation. Although the amount of emitted volatiles were much higher at 10:00 o'clock; the model mixtures 2 (10:00 o'clock) and 3 (18:00 o'clock) were evaluated as similar, but significantly different to model mixture 1 (2:00 o'clock) (Fig. 7B).

Discussion

Isolation and functional analysis of OfCCD1

It is well known that the color of yellow flowers is often caused by the presence of high amounts of carotenoids. Some flowers also have a broad variety of carotenoid-derived scent compounds, like in 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 β-ionone formation was observed in transgenic petunia plants in which *PhCCD1* gene expression was inhibited (Simkin *et al.*, 2004a) we examined the possible role of OfCCD1 on ionone biosynthesis in *O. fragrans.*

Apart from the formation of volatile C_{13}-norisoprenoids through the action of CCD1 enzymatic cleavage of the 9,10 (9',10') double bond has also been demonstrated for CCD4 enzymes from *Crocus sativus, R. damascena, Chrysanthemum 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) we focused in this study on the functional characterization of the OfCCC1 enzyme. The putative amino acid sequence of OfCCD1 exhibited the conserved histidine residues of the active center of CCDs and in the presence

447 of ferrous iron, the recombinant enzymes showed cleavage activity towards the two
448 dominant carotenoids (β-carotene and α-carotene) found in flowers of *O. fragrans*.
449 CCD1 enzymes cleave symmetric and pseudo-symmetric carotenoids differently. Based on
450 the observations of Kloer and Schulz (2006), it was suggested that pseudo-symmetric
451 molecules undergo a two-step cleavage. First the enzyme cleaves the C_{40} substrate once,
452 releasing the products, then it binds to the primary non-volatile apocarotenoid and cleaves it
453 a second time. It should be noted that recent studies suggest that CCD1 enzymes cleave the
454 primary derived cleavage products (C_{27}-apocarotenoids) in the cytosol *in vivo* (Floss *et al.*,
455 2009). In *in vitro* CCD1 can cleave either carotenoids or apocarotenoids (Huang *et al.*,
456 2009b). In roses, the non-volatile reaction products (C_{27}-apaocarotenoids) of the first
457 cleavage were only formed when the substrates contained different moieties at their ends. In
458 this study the symmetric β-carotene and the pseudo-symmetric α-carotene were used as
459 substrates and the cleavage of α-carotene resulted in higher amounts of α-ionone, suggesting
460 that the first site of cleavage is the one with the α-ionone moiety. However, in the case of
461 the rose enzyme (RdCCD1), the same amounts of the reaction products 3-hydroxy-α-ionone
462 and 3-hydroxy-β-ionone were obtained from pseudo-symmetric xanthophyll lutein (Huang *et*
463 *al.*, 2009b).
464
465 Photorhythmic changes of *OfCCD1* transcript levels, carotenoid concentrations, and volatile
466 emission
467
468 Photorhythmic volatile emission in plants has been demonstrated in several flowering plants
469 (Matile and Altenburger 1988, Loughrin *et al.*, 1990, Helsper 1998, Picone *et al.* 2004,
470 Dudavera and Pichersky 2006). In general, nocturnally pollinated flowers tend to have a
471 maximum of scent emission during the dark period, whereas the diurnally pollinated flowers
472 release higher amounts of volatiles during day time. Volatile emission can be regulated
473 either by light or by endogenous circadian mechanisms, mostly controlled at the gene
474 expression transcription level (Hendel-Rahmanim *et al.* 2007). One group of plant enzymes

475 is characterized by an increase in activity in young flowers and a decline during aging, while
476 a second group of enzymes show little or no decline at the end of the flowers life (Dudavera
477 and Pichersky 2006). During the floral development of *Ipomoea* sp., *I. obscura*, and *I. nil*
478 the CCD1 and CCD4 transcript levels decreased (Yamamizo *et al.* 2009). In case of
479 *OfCCD1*, the steady state transcript levels are subjected to circadian mechanisms and have a
480 peak during day.
481 The concentrations of α-carotene and β-carotene also underwent photorhythmic changes. It
482 is interesting to note that there is a negative correlation between the abundance of *OfCCD1*
483 mRNA and the concentrations of the substrates (α-carotene and β-carotene). In *O. fragrans*
484 flowers, the carotenoid levels remained low or decreased if the transcript levels of *OfCCD1*
485 were high. The carotenoid content increased over the experimental interval and reached the
486 maximal concentration under light conditions. The light/dark regulation of carotenoid
487 biosynthesis was investigated in red pepper, where all transcript levels of genes involved in
488 the carotenoid biosynthesis decreased under dark conditions (Simkin *et al.*, 2003). In citrus
489 fruits, the transcript levels of genes encoding enzymes involved in carotenoid biosynthesis as
490 well as CCD transcript levels increased during the fruit maturation (Kato *et al.*, 2007). In
491 chrysanthemums, a negative correlation between CmCCD4a mRNA abundance and
492 carotenoid content was observed. However, recently obtained results during the flower
493 development of *Ipomoea* sp., *I. obscura*, and *I. nil* suggest that the flower color cannot be
494 correlated to carotenoid degradation activity in *Ipomoea* plants (Yamamizo *et al.*, 2009). In
495 *O. fragrans* the OfCCD4 showed very low activity against carotenoids and apocarotenoids
496 (Huang *et al.*, 2009a) and hence, the contribution to the biodegradation of carotenoids is
497 unclear. However, the transcript levels were quite similar to those of *OfCCD1* (unpublished
498 results). Hence, based on the work presented here it might be suggested that in *Osmanthus*
499 flowers the slight decrease in α-carotene and β-carotene levels observed in light periods is at
500 least partly due to high activity of OfCCD1.
501 In petunia corollas, a correlation between mRNA abundance and β-ionone emission was
502 observed (Simkin *et al.*, 2004a). However, emission was still increasing when transcript

503 levels already began decreasing during the afternoon time. This study provides a similar
504 observation in *Osmanthus* flowers, where the β-ionone emission remained at high levels
505 after the transcript levels of *OfCCD1* already decreased during the day time. It was
506 suggested that there might be some limitation due to the substrate availability. Carotenoids
507 are synthesized in the plastids whereas the CCD1 enzymes are located in the cytosol and
508 therefore the cytosolic CCD1 enzymes have access only to those carotenoids distributed on
509 the outer envelope of plastids, where for example significant amounts of β-carotene have
510 been detected in pea chloroplasts (Markwell *et al.*, 1992). In *O. fragrans* flowers the
511 carotenoid concentrations increased over the flowering period and hence, a limiting factor
512 for the reaction of OfCCD1 with the substrates can be the access inside the cell
513 compartments.
514 Another regulatory factor can be the catalytic efficiency of enzymes with their substrates.
515 Carotenoid cleavage enzymes purified from plant tissues exhibit different affinities towards
516 β-carotene. For example the K_m values for β-carotene obtained for carotenoid cleavage
517 enzymes isolated from different fruits varied from 11.0 μM L^{-1} for quince fruit, 6.6 μM L^{-1}
518 nectarine, and 3.6 μM L^{-1} star fruit, respectively (Fleischmann *et al.*, 2002; Fleischmann *et*
519 *al.*, 2003; Baldermann *et al.*, 2005). In *Osmanthus* flowers the carotenoid content increased,
520 steady state maximal transcript levels were observed under light conditions, whereas the
521 emission of ionones, as enzymatic reaction products, decreased over the flowering period. It
522 might be suggested that the catalytic efficiency of the OfCCD1 enzymes with their substrates
523 is another regulatory factor.
524 Our results demonstrate that OfCCD1 in flowers of *Osmanthus fragrans* Lour. is likely
525 involved in the oxidative cleavage of carotenoids to produce the volatile scent compounds α-
526 and β-ionone. However, detailed analysis of carotenoids as putative precursors, transcript
527 levels of *OfCCD1*, and volatile emission indicate that the activity of this enzyme is not
528 sufficient to account for the total emission of these volatiles. Additional work is needed to
529 clarify the contribution of other carotenoid cleavage enzymes to ionone emission and
530 identify the *in vivo* substrates.

Changes in β-ionone and α-ionone emission in relation to sent 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 already began decreasing during the afternoon time. 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 odor 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 2:00, 10:00, and 18:00 o'clock demonstrated that the scent in morning and early evening is considered as similar, although the total volatile emission is decreasing by approximately 3 fold. A similar example is the low amount of C_{13} norisoprenoids in rose, which nonetheless make a strong contribution to the scent; while constituting less than 1% of 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 early evening are likely 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 stage 2, 4, 7, and 10 at 14:00 o'clock.

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 the valuable discussions.

References

Akiyama K, Matsuzaki K, Hayashi H. 2005. Plant sequiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **435**, 824-827.

Auldridge ME, Block A, Vogel DJ, Dabney-Smith C, Mila I, Bouzayen M, Magallanes-Lundback M, DellaPenna D, McCarty DR, Klee HJ. 2006. Characterization of three members of the *Arabidopsis* carotenoid cleavage dioxygenase family demonstrates the diveregent roles of this multifunctional enzyme family. *The Plant Journal* **45**, 982-993.

Baldermann S, Naim M, Fleischmann P. 2005. Enzymatic carotenoid degradation and aroma formation in nectarines (*Prunus persica*). *Food Research International* **38**, 833-836.

Baldermann S. 2008. Carotenoid oxygenases from *Camellia sinensis*, *Osmanthus fragrans*, and *Prunus persica* nucipersica. PhD thesis, Technische Universität Braunschweig, Germany, 51-52.

Bouvier F, Dogbo O, Camara B. 2003. Biosynthesis of food and cosmetic plant pigment Bixin (*Annatto*). *Science* **300**, 2089-2091.

Bouwmeester HJ, Roux C, Lopez-Raez JA, Becard G. 2007. Rhizosphere communication of plants, parasitic plants and AM fungi. *Trends in Plant Science* **12**, 224-230.

Buttery RG, Teranishi R, Ling LC, Turnbaugh JG. 1990. Quantitative and sensory studies on tomato paste volatiles. *Journal of Agricultural and Food Chemistry* **38**, 336-340.

Dudareva N, Pichersky E. 2006. Floral scent metabolic pathways: Their regulation and evolution. In: Dudareva N, Pichersky E, eds *Biology of Floral Scent*, Section II. Biochemistry and Molecular Biology of Floral Scent. Boca Raton, CRC Press Taylor & Francis Group, 55-78.

Fleischmann P, Studer K, Winterhalter P. 2002. Partial purification and kinetic characterization of a carotenoid cleavage enzyme from quince fruit (*Cydonia oblonga*). *Journal of Agricultural and Food Chemistry* **50**, 1677-1680.

Fleischmann P, Watanabe N, Winterhalter P. 2003. Enzymatic carotenoid cleavage in star fruit (*Averrhoa carambola*). *Phytochemistry* **63**, 131-137.

Floss DS, Schliemann W, Schmidt J, Strack D, Walter MH. 2009. RNA interference-mediated repression of MtCCD1 in mycorrhizal roots of *Medicago truncatula* causes accumulation of C_{27} apocarotenoid, shedding light on functional role of CCD1. *Plant Physiology* **148**, 1267-1282.

García-Limones C, Schnäbele K, Blanco-Portales R, Luz Bellido M, Caballero JL, Schwab W, Muňoz-Blanco J. 2009. Functional characterization of FaCCD1: A carotenoid cleavage dioxygenase from strawberry involved in lutein degradation during fruit ripening. *Journal of Agricultural and Food Chemistry* **56**, 9277-9285.

Helsper JPFG, Davies JA, Bouwmeester HJ, Krol AF, van Kampen MH. 1998. Circadian rhythmicity in emission of volatile compounds by flowers of *Rosa hybrida* L. cv. Honesty. *Planta* **207**, 88-95.

Hendel-Rahmanim K, Masci T, Vainstein A, Weiss D. 2007. Diurnal regulation of scent emission in rose flowers. *Planta* **226**, 1491-1499.

Huang F-C, Molnár P, Schwab W. 2009a. Cloning and functional characterization of carotenoid cleavage dioxygenase 4 genes. *Journal of Experimental Botany* **60**, 3011-3022.

Huang F-C, Horváth Gr, Molnár P, Turcsi E, Deli JZ, Schrader J, Sandmann G, Schmidt H, Schwab W. 2009b. Substrate promiscuity of RdCCD1, a carotenoid cleavage oxygenase from *Rosa damascena*. *Phytochemistry* **70**, 457-464.

Ibdah M, Azulay Y, Portnoy V, Wasserman B, Bar E, Meir A, Burger Y, Hirschberg J, Schaffer ArA, Katzir N, Tadmor Y, Lewinsohn E. 2006. Functional characterization of CmCCD1, a carotenoid cleavage dioxygenase from melon. *Phytochemistry* **67**, 1579-1589.

Ikoma Y, Yano M, Ogawa K, Yoshioka T, Xu ZC, Hisada S, Omura M, Moriguchi T. 1996. Isolation and evaluation of RNA from polysaccharide-rich tissues in fruit for quality by cDNA library construction and RT-PCR. *Journal of the Japanese Society for Horticultural Science* **64**, 809-814.

Ilg A, Beyer P, Al-Babili S. 2009. Characterization of the rice carotenoid cleavage dioxygenase1 reveals a novel route for geranial biosynthesis. *The FEBS Journal* **3**, 736-747.

Kaiser R. 2002. Carotenoid-derived aroma compounds in flower scents. In: Winterhalter P, Rouseff L, eds. *Carotenoid-derived aroma compounds*. Washington DC, American Chemical Society, 160-182.

Kato M, Mastumoto H, Ikoma Y, Okuda H, Masamichi Y. 2006. The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. *Journal of Experimental Botany* **57**, 2153-2164.

Kato M, Mastumoto H, Ikoma Y, Kuniga T, Nakajima N, Yoshida T, Yano M. 2007**.** Accumulation of carotenoids and expression of carotenoid biosynthetic genes and carotenoid cleavage dioxygenase genes during fruit maturation in the juice sacs of 'Tamami,' 'Kiyomi' Tangor, and 'Wilking' mandarin. *Journal of the Japanese Society for Horticultural Science* **76**, 103–111.

Kloer DP, Schulz GE. 2006. Structural and biological aspects of carotenoid cleavage. *Cellular and Molecular Life Sciences* **63**, 2291-2303.

Loughrin JH, Hamilton-Kemp TR, Andersen RA, Hildebrand DF. 1990. Volatiles from flowers of *Nicotiana sylvestris, N. otophora* and *Malus domestica:* Headspace components and day/night changes in their relative concentrations. *Phytochemistry* **29**, 2473-2477.

Markwell J, Bruce BD; Keegstr K. 1992. Isolation of a carotenoid-containing sub-membrane particle from the chloroplastic envelope outer membrane of pea (*Pisum sativum*) The *Journal of Biological Chemistry* **267**, 13933-13937.

Mathieu S, Terrier N, Procureur J, Bigey F, Günata Z. 2005. A carotenoid cleavage dioxygenase from *Vitis vinifera* L.: Functional characterization and expression during grape berry development in relation to C_{13}-norisoprenoid accumulation. *Journal of Experimental Botany* **56**, 2721-2731.

Matile P, Altenburger R. 1988. Rhythms of fragrance emission in flowers. *Planta* **174**, 242-247.

Ohloff G, Demole E. 1987. Importance of the odoriferous principle of Bulgarian rose oil in flavor and fragrance chemistry. *Journal of Chromatography* **406**, 181-183.

Ohmiya A, Kishimoto S, Aida R, Yoshioka S, Sumimoto K. 2006. Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in chrysanthemum petals. Plant Physiology **142**, 1193-1201.

Picone JM, Clery RA, Watanabe N, MacTavish HS, Turnbull CGN. 2004. Rhythmic emission of floral volatiles from *Rosa damascena semperflorens* cv. 'Quatre Saisons'. *Planta* **219**, 468-478.

Schwartz SH, Qin XQ, Zeevaart JAD. 2001. Characterization of a novel carotenoid cleavage dioxygenase from plants. *The Journal of Biological Chemistry* **276**, 25208-25211.

Schwartz SH, Qin XQ, Loewen M.C. 2004. The biochemical characterization oft wo carotenoid cleavage enzymes from *Arabidopsis* indicates that a carotenoid-derived compound inhibits lateral branching. *The Journal of Biological Chemistry* **45**, 46940-46945.

Simkin AJ, Underwood BA, Auldridge M, Loucas HM, Shibuya K, Schmelz E, Clark DG, Klee HJ. 2004a. Circadian regulation of the PhCCD1 carotenoid cleavage dioxygenase controls emission of beta-ionone, a fragrance volatile of petunia flowers. *Plant Physiology* **136**, 3504-3514.

Simkin AJ, Schwartz SH, Auldridge M, Taylor MG, Klee HJ. 2004b. The tomato carotenoid cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles beta-ionone, pseudoionone, and geranylacetone. The Plant Journal **40**, 882-892.

Simkin AJ, Moreau H, Kuntz M, Pagny G, Lin C, Tanksley S, McCarthy J. 2008. An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea arabica*. *Journal of Plant Physiology* 165, 1087-1106.

Rubio A, Rambla JL, Santaella M, Gomez MD, Orzaez D, Granell A, Gomez-Gomez L. 2008. Cytosolic and plastoglobule-targeted carotenoid dioxygenases from *Crocus sativus* are both involved in β-ionone release. *The Journal of Biological Chemistry* **283**, 24816-24825.

Taylor KL, Brackenridge AE, Vivier MA, Oberholster A. 2006. High-performance liquid chromatography profiling of the major carotenoids in *Arabidopsis thaliana* leaf tissue. *Journal of Chromatography A* **1121:** 83–91.

Teranishi R, Buttery RG. 1987. The significance of low threshold odor compounds in aroma research. In: Martens M, Dalen GA, Russwurm H, eds. *Flavor Science and Technology*, Weihnheim, John Wiley and Sons, Inc, 513-527.

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K, Junko Kyozuka J, Yamaguchi S. 2008. Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **455**, 195-200.

Vogel JT, Tan B-C, McCarty DR, Klee HJ. 2008. The carotenoid cleavage dioxygenase 1 enzyme has broad substrate specificity, cleaving multiple carotenoids at two different bond positions. *The Journal of Biological Chemistry* **283**, 11364-11373.

Wang L, Li M, Jin W, Li S, Zhang S, Yu L. 2009. Variations in the components of *Osmanthus fragrans* Lour. essential oil at different stages of flowering. *Food Chemistry* **114**, 233-236.

Yamamizo C, Kishimoto S, Ohmiya A. 2009. Carotenoid composition and carotenogenic gene expression during *Ipomoea* petal development. *Journal of Experimental Botany* **61**, 709-719.

Figure legends

Fig. 1

Oxidative enzymatic cleavage of α-carotene by carotenoid cleavage enzymes yielding to α-ionone and β-ionone. In *in vitro* assays both oxidative cleavage steps can be carried out by CCD1 and CCD4 enzymes, respectively.

Fig. 2

Flowering of *Osmanthus fragrans* Lour. var. auranticus. Detailed studies were carried out using flowers during the unfurling period (stage 4 and 5), at the time where the flowers released the strongest odor (Odor intensity: - no ,(+) very weak, + weak, +(+) low, ++ medium, +++ high).

Fig. 3

Phylogenetic tree of deduced amino acid sequences of carotenoid cleavage enzymes involved in the cleavage of carotenoids (C_{40}) or C_{27} apocarotenoids (CCD1, CCD4, CCD7, and CCD8) at 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* CsCCD1 AJ132927, CCD4a EU523662, CCD4b EU523663; *Cucumis melo* CCD1 DQ269467; *Malus domestica* CCD4 EU327777; *Fragaria ananassa* CCD1 ACA13522; *Medicago truncatula* CAR57918; *Osmanthus fragrans* CCD4 EU33443, CCD1 AB526191; *Petunia hybrida* CCD1 AY576003; *Rosa damascena* CCD4 EU334433 RdCCD1

ABY47994; *Solanum lycopersicum* SlCCD1a AY576001, SlCCD1b 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 induction of the protein expression in the presence ferrous iron, whereas the recombinant OfCCD1* was purified after induction of the 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^{-1} initial concentration of β-carotene, 10 µg ml^{-1} protein in 1 mL Tis-HCl buffer (pH = 7, 50 mM Tris-HCl, 125 mM KCl, 5 mM $MgCl_2$, 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 the chemical β-carotene degradation under equal assay conditions. The data are presented as mean ± SD from three replicates.

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 difference of the sample chromatogram and the chromatogram obtained in the control assay).

Fig. 6

Changes in *OfCCD1* transcript levels (A, B), α-carotene and β-carotene concentrations (C, D), volatile emission (E, F), and α-ionone and β-ionone release (G, H) of flowers of *Osmanthus fragrans* Lour.

var. auranticus exposed to constant temperature (22°C), humidity (70 %), and different photo periods (12-h-light/12-h-dark regime or 24 h continuous dark or 24 h light). The photoperiods are indicated by shading of the background. The data of RT-qPCR and analysis of carotenoids are presented as mean ± SD from three replicate. The volatile emission profiles were comparable over different flowering periods (years, data not shown).

Fig. 7

(A), Ratios of selected volatiles emitted by flowers of *Osmanthus fragrans.* Lour. var. auranticus. The emitted volatiles were collected by dynamic headspace sampling over 48 h in intervals of 4 h and the concentrations determined by GC-MS. The cut flowering branches were subjected to constant temperature (22°C), humidity (70 %) and 12/12 h (dark/light) photoperiods as indicated by shading of the background (B), Results of sensory evaluation of model mixtures reflecting the flower scent at 2:00, 10:00, and 18:00 o'clock (marked as 1, 2, and 3 in Fig. A). The model mixtures were prepared by mixing different ratios of α-ionone, β-ionone, linalool, linalool oxides (furanoids), and γ-decalactone (Table S2). The odor intensities of model mixtures were evaluated by 23 panelists according to the procedure described in the experimental part.

Fig. 1

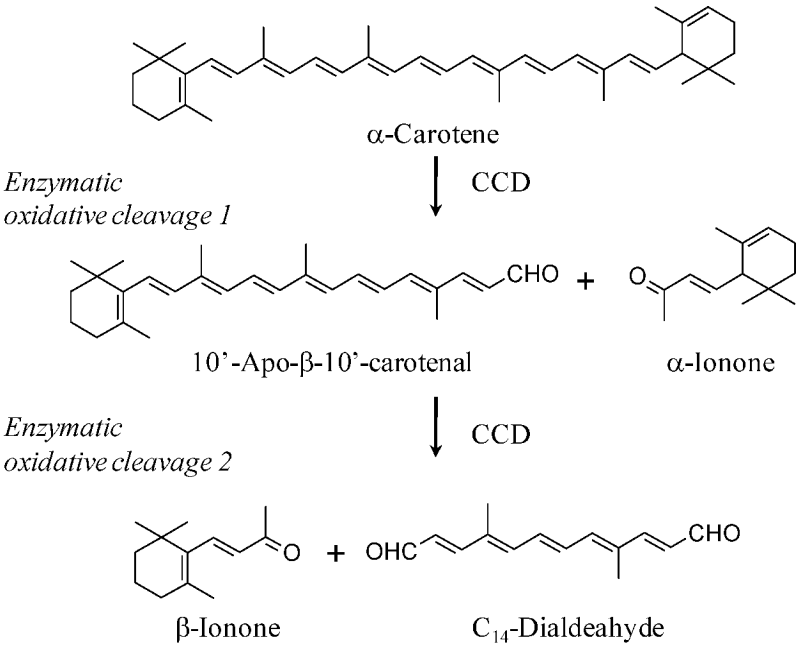

α-Carotene
Enzymatic
oxidative cleavage 1
CCD
CHO
+
O
10'-Apo-β-10'-carotenal
α-Ionone
Enzymatic
oxidative cleavage 2
CCD
O
+
OHC
CHO
β-Ionone
C14-Dialdeahyde

Fig. 2

Stage	1	2	3	4	5	6	7	8	9	10	11
											
Inflorescence	Pale yellow buds	Yellow buds	Pale orange petals	Orange petals, flowers start to open	Orange petals, full flowering	Orange petals, full flowering, flower petal tips lose color	Color intensity of flower petals decrease	Color intensity of flower petals decrease further, first flowers drop-off	Further decrease in color, more flowers drop-off	Further decrease in color flowers drop-off	Flower color changed partly to brownish, end of flowering
Odor Intensity*	-	(+)	+	+ + +	+ + +	+ +	+ +	+ (+)	+	(+)	-

Fig. 3

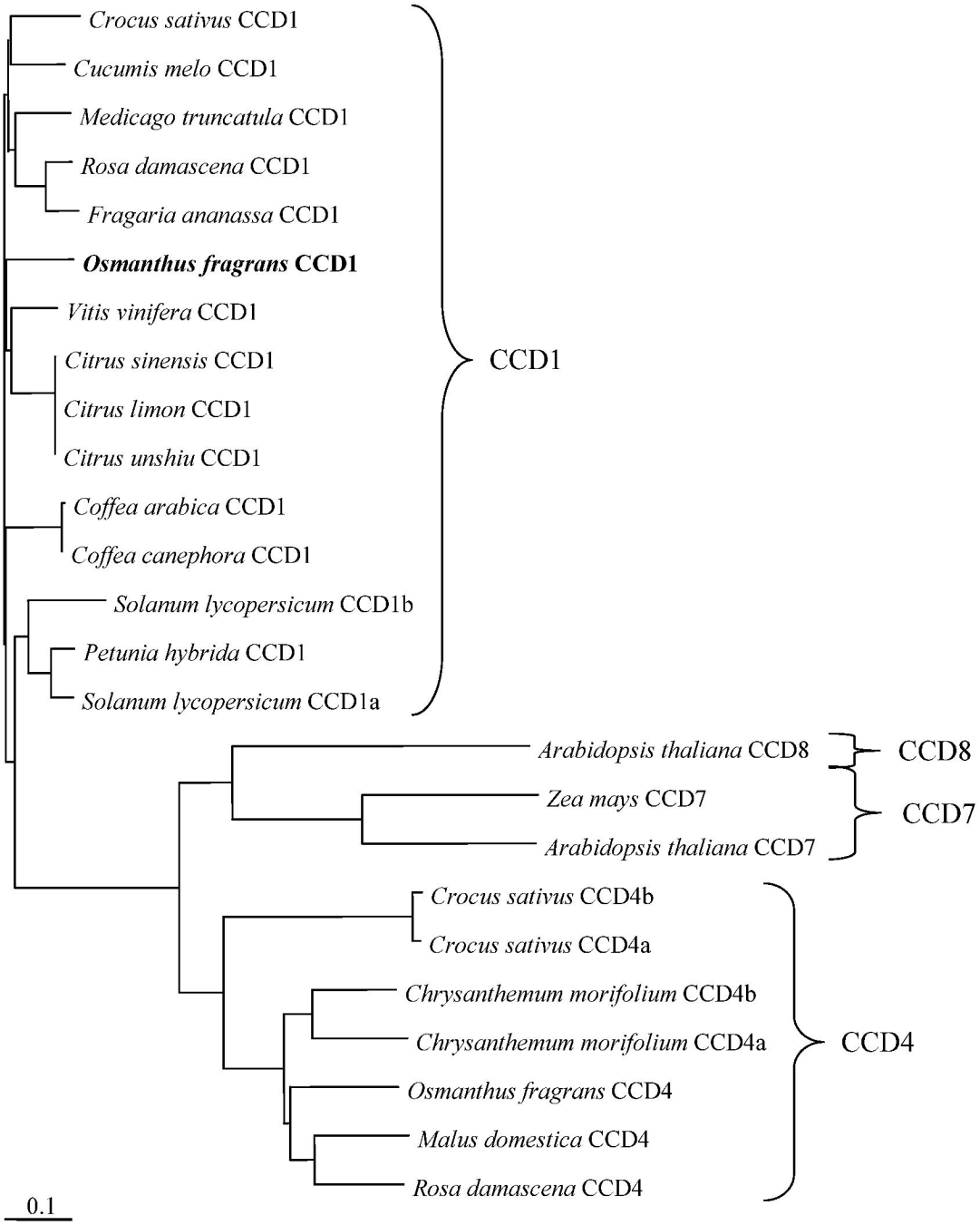

Crocus sativus CCD1
Cucumis melo CCD1
Medicago truncatula CCD1
Rosa damascena CCD1
Fragaria ananassa CCD1
***Osmanthus fragrans* CCD1**
Vitis vinifera CCD1
Citrus sinensis CCD1
Citrus limon CCD1
Citrus unshiu CCD1
Coffea arabica CCD1
Coffea canephora CCD1
Solanum lycopersicum CCD1b
Petunia hybrida CCD1
Solanum lycopersicum CCD1a
CCD1
Arabidopsis thaliana CCD8
CCD8
Zea mays CCD7
Arabidopsis thaliana CCD7
CCD7
Crocus sativus CCD4b
Crocus sativus CCD4a
Chrysanthemum morifolium CCD4b
Chrysanthemum morifolium CCD4a
Osmanthus fragrans CCD4
Malus domestica CCD4
Rosa damascena CCD4
CCD4
0.1

Fig. 4

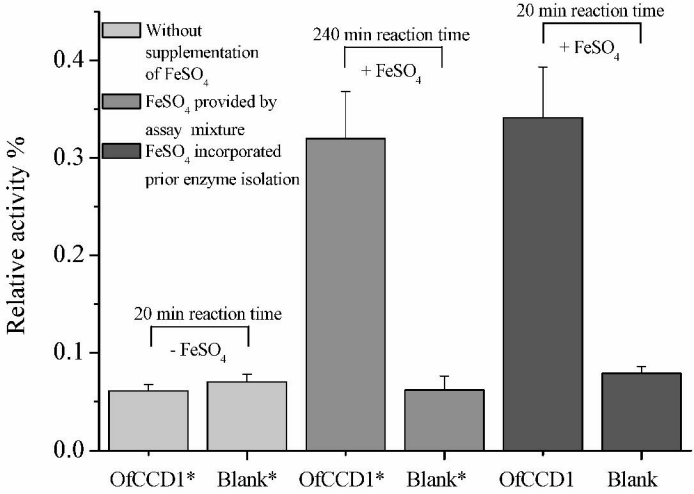

Without supplementation of FeSO4
FeSO4 provided by assay mixture
FeSO4 incorporated prior enzyme isolation
Relative activity %
0.0
0.1
0.2
0.3
0.4
20 min reaction time
- FeSO4
240 min reaction time
+ FeSO4
20 min reaction time
+ FeSO4
OfCCD1*
Blank*
OfCCD1*
Blank*
OfCCD1
Blank

Fig. 5

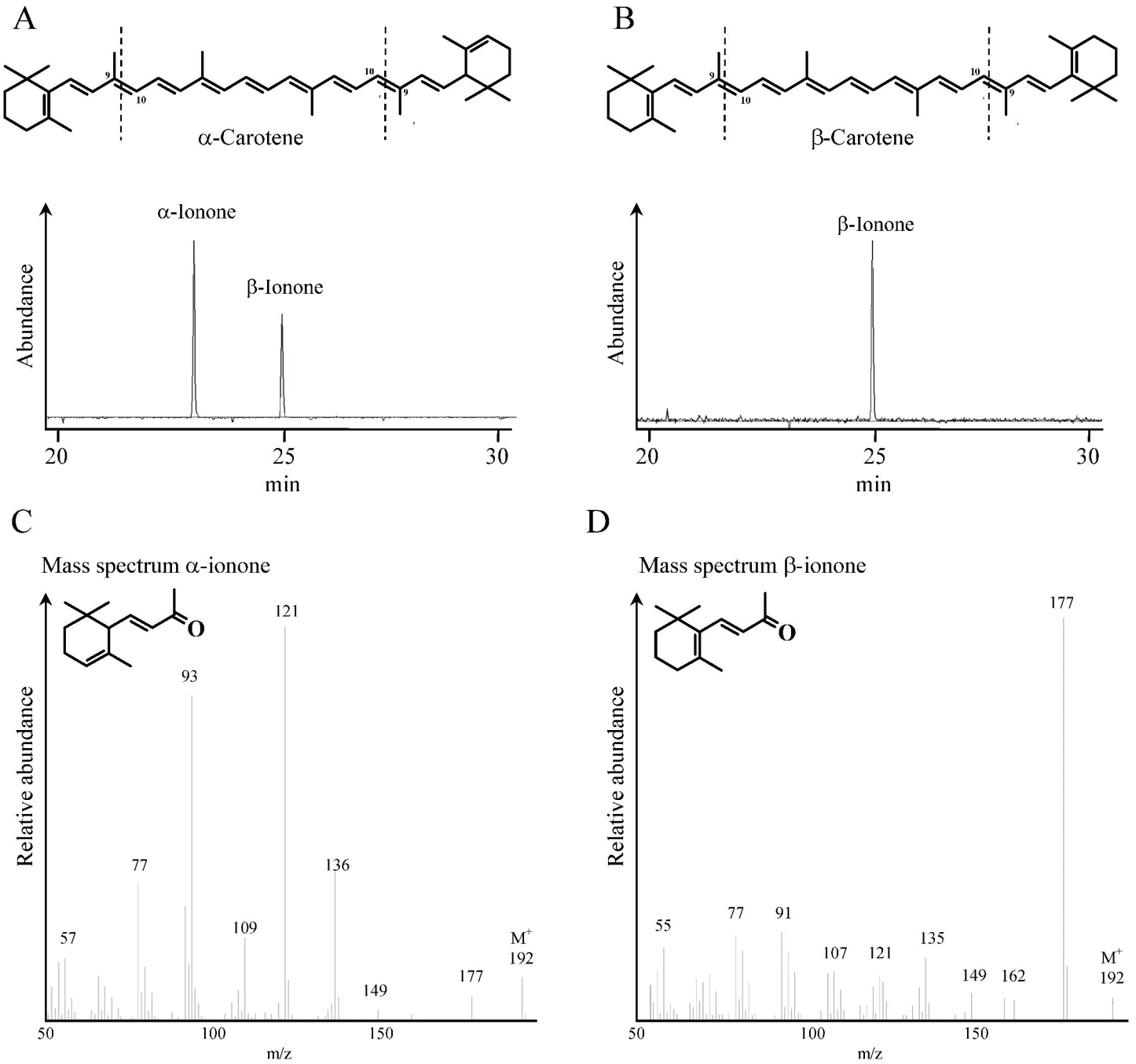
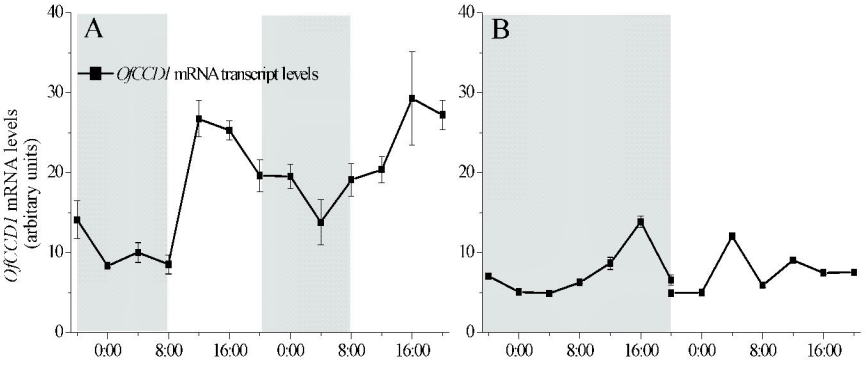
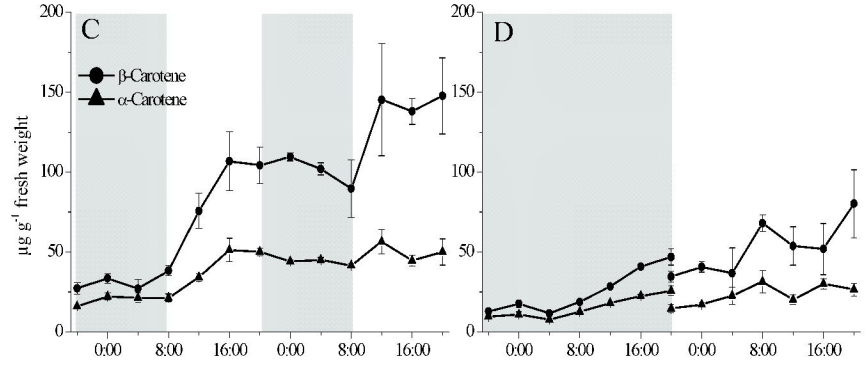

A
α-Carotene
B
β-Carotene
α-Ionone
β-Ionone
Abundance
20
25
30
min
C
Mass spectrum α-ionone
Relative abundance
121
93
77
136
57
109
M+
192
149
177
m/z
D
Mass spectrum β-ionone
177
55
77
91
107
121
135
149
162
M+
192

Fig. 6

Changes in *OfCCD1* transcripts determined by RT-qPCR



Changes in α-carotene and β-carotene concentrations determined by HPLC



Changes in total volatiles and α-ionone and β-ionone concentrations determined by GC-MS

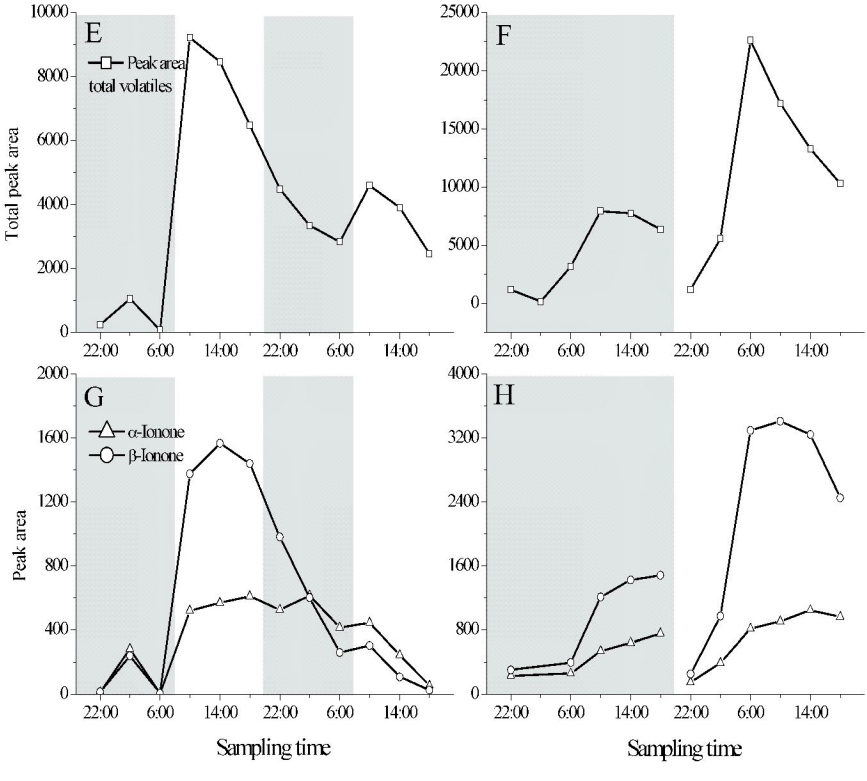
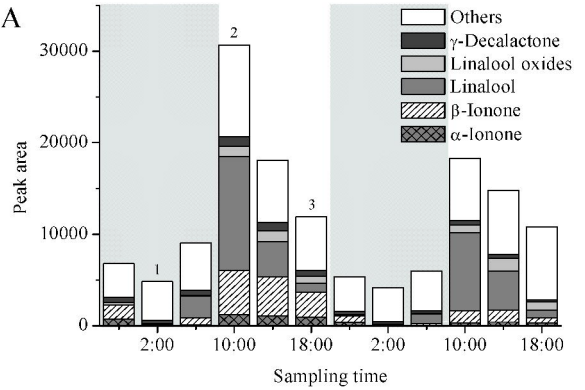


Fig. 7

Volatile emission of flowers of *Osmanthus fragrans*



Results of the sensory evaluation of model mixtures reflecting the flower scent at 2:00 (1), 10:00 (2) and 18:00 (3) o'clock