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Biosynthesis of α- and β-ionone, prominent scent compounds, in flowers of *Osmanthus fragrans**

Susanne Baldermann[1#], Masaya Kato[2], Peter Fleischmann[3] and Naoharu Watanabe[1]✉

[1]Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, Suruga-ku, Shizuoka, Japan; [2]Faculty of Agriculture, Shizuoka University, Suruga-ku, Shizuoka, Japan; [3]Institute of Food Chemistry, Technische Universität Braunschweig, Braunschweig, Germany

Carotenoid derived volatiles are important fragrance compounds, which contribute to the scents of flowers from diverse taxa. A famous example is represented by the flowers of *Osmanthus fragrans* where apocarotenoids account for more than 20% of all volatiles. In the recent years, bio-degradation of carotenoids has been shown to be an important route for apocarotenoids formation. Here, we report on the contribution the *O. fragrans* carotenoid cleavage dioxygenase 1 to the synthesis of the two predominant C_{13}-apocarotenoids, α- and β-ionone, derived from α-and β-carotene, respectively.

Key words: CCD, carotenoids, apocarotenoids, ionones

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INTRODUCTION

The flower scent of *O. fragrans*, an Oleaceae native to East-Asia, is dominated by carotenoid derived compounds. β- and α-ionone are the most abundant scent constituents and importantly contribute to the scent perception of flowers of *O. fragrans* (Wang *et al.*, 2009; Baldermann *et al.*, 2010). Commercial extracts are of economic importance due to their application in expensive cosmetics or for flavoring of foods, tea, and sweets.

Enzymatic carotenoid cleavage has been discovered in flowers, fruits, and other species and, hence, it was of special interest to elucidate its contribution to apocarotenoid biogenesis in the unique scented flowers of *O. fragrans*.

The carotenoid cleavage dioxygenase (CCD) subfamilies 1 and 4 (CCD1, CCD4) target 9,10 and/or 9',10' double bonds of C_{40}-carotenoids to form important volatile C_{13}-apocarotenoids. CCD1 transcript levels correlate with the emission of β-ionone in petunia flowers (Simkin *et al.*, 2004). CCD4 transcript levels are related to the color of chrysanthemum flowers (Ohmiya *et al.*, 2006) and determine the color of chrysanthemum, as shown by forming white flowers upon suppression of *CmCCD4* (Ohmiya *et al.*, 2009). CCD1 genes and enzymes have been identified and characterized in various flowers and fruits, such as petunia, *R. damascena*, melon, strawberry, star fruit, and tomato (listed in Walter *et al.*, 2011). Common characteristics of CCD1 enzymes are broad substrate specificity *in vitro* and the cleavage of the 9,10 and 9',10' double bonds. Hence, our interest was to identify and characterize a CCD1 enzyme in flowers of *O. fragrans* and clarify its contribution to the formation of α- and β-ionone.

MATERIALS AND METHODS

Plant material. Flowers of *O. fragrans* var. *aurantiacus* Mak. were collected at Shizuoka University ground during the flowering period in autumn 2007. Flowers in full bloom (stage 5, Baldermann *et al.*, 2010) were used in this study.

Analysis of carotenoids. The carotenoids of the flower petals were analysed by HPLC as published previously (Baldermann *et al.*, 2010). Identification was achieved by co-chromatography with authentic reference substances.

Identification and sequence analysis of OfCCD1. Detailed experimental description has been published previously (Baldermann *et al.*, 2010). The sequences were aligned using ClustalW (http://www.genome.jp/). The evolutionary history was inferred using the Neighbor-Joining method and visualized by Tree View.

Expression of OfCCD1 in β-carotene accumulating cells. The cDNA was expressed in *E. coli* XL1Blue harboring the pACCAR plasmid that carries all genes necessary to produce β-carotene (Missawa *et al.*, 1995). A single colony of transformed cells was inoculated in 100 mL YT media containing chloramphenicol and carbenicillin and grown at 37°C over night. One mL overnight culture was inoculated in 100 mL of fresh YT media containing the suitable antibiotics and grown at 27°C until an OD_{550} of 0.5. To this culture ITPG (0.5 mM), ascorbate (6 mM), $FeSO_4$ (5 μM), catalase (200 U/mL) were added to induce the production of the target enzyme and the *in vivo* reaction.

Expression and purification of the recombinant OfCCD1 and *in vitro* assay. Detailed experimental conditions have been published elsewhere (Baldermann *et al.*, 2010).

Analysis of volatile reaction products in the headspace of *E. coli* cultures accumulating β-carotene. The presence of β-ionone, the putative reaction prod-

✉e-mail: acnwata@ipc.shizuoka.ac.jp

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#Present addresses: Leibniz-Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V., Großbeeren, Germany; Institute of Nutritional Science, University of Potsdam, Nuthetal, Germany

Abbreviations: CCD, carotenoid cleavage dioxygenase; HPLC, high-performance liquid chromatography; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

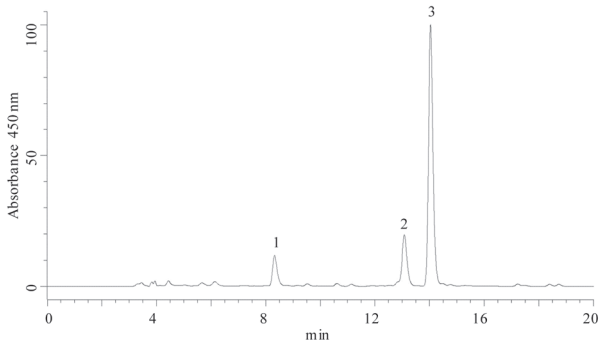

Figure 1. Determination of carotenoids in fully open flowers of *O. fragrans* by HPLC
Peak (1) internal standard β-apo-8′-carotenal, (2) α-carotene (λ_{max} = 419, 447, 475 nm), and (3) β-carotene (λ_{max} = 423, 451, 479 nm).

uct derived from the cleavage of β-carotene, was confirmed after solid phase micro-extraction (SPME) by gas chromatography mass spectrometry (GC-MS). A SPME fiber coated with 100 μm polymethylsiloxane (Supelco, Bellefonte, PA) was introduced into an Erlenmeyer flask containing 100 mL YT liquid culture induced for production of OfCCD1. The volatiles were collected in the headspace for 1 h at 37°C. The reaction products were analyzed by GS-MS using a capillary Suplecowax column (GL Sciences Inc., Japan, 30 m, 0.25 mm ID, 0.25 μm film thickness) using the following temperature program: initial temperature 50°C, maintained for 3 min, ramped to 190°C at 5°C/min, ramped to 240°C at 40°C/min, and held for 3 min. The mass scan range was set to m/z 50–300 and the electric potential to 1.0 kV. Helium was used as carrier gas at a flow rate of 1.7 mL/min.

RESULTS AND DISCUSSION

Yellow flowers often contain carotenoids as major pigments (Ohmiya, 2011), and some of them are also rich in carotenoid derived volatiles.

We elucidated the carotenoid profile of flowers of *O. fragrans* (Fig. 1). The two major pigments are α- and β-carotene. Carotenoids are widely distributed flower pigments and can be found in a broad variety of monocot and eudicot plants (reviewed by Ohmiya, 2011).

Carotenoids serve as possible substrates for carotenoid cleavage enzymes. As β- and α-ionone are the abundant scent compounds emitted by flowers of *O. fragrans*, it was questionable if direct cleavages of the two major carotenes by CCD1 enzymes contribute to their formation. We identified and characterized a CCD1 homologue based on conserved CCD sequences (Baldermann *et al.*, 2010).

Sequence comparison confirmed that *OfCCD1* is am member of the CCD1 family (Fig. 2). CCD1 sequences have been identified in various flowers, among them *Rosa chinensis* and *Petunia hybrida* (Huang *et al.*, 2009a; Simkin *et al.*, 2004). Separately from the CCD1 family the CCD4, CCD7, CCD8, and NCED (9-*cis*-epoxycarotenoid dioxygenase) families form discrete clusters. CCD4 enzymes are correlated with flower color and produce, like CCD1 enzymes, C_{13}-apocarotenoid volatiles (Huang *et al.*, 2009b). They have been localized in the plastids, whereas CCD1 enzymes occur in the cytosol (Bouvier *et al.,* 2003; Rubio *et al.*, 2008). NCED are involved in the cleavage of 9-*cis*-epoxy carotenoids to form the abscisic acid precursor xanthoxin. CCD7 and CCD8 enzymes are

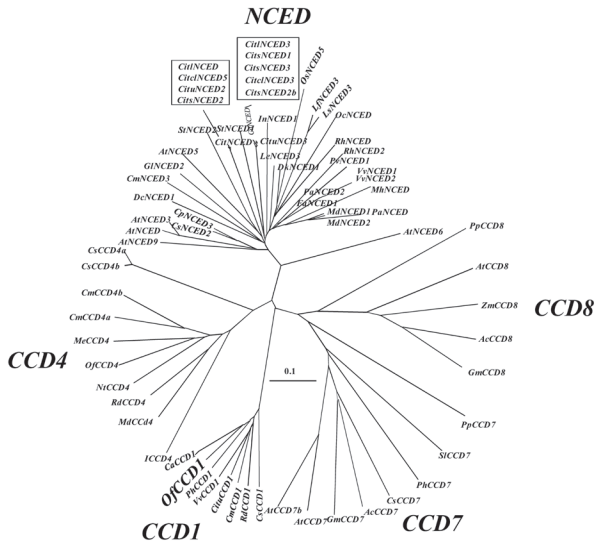

Figure 2. Unrooted phylogenetic tree of cDNA sequences of CCDs involved in the cleavage of carotenoids and apocarotenoids.
NBCI accession numbers: **NCED**: *Arabidopsis thaliana AtNCED* (AB026549), *AtNCED3* (NM_112304), *AtNCED5* (NM_102749), *AtNCED6* (NM_113327), *AtNCED9* (NM_106486); *Citrus clementina CitclNCED3* (DQ309332), *CitclNCED5* (DQ309329); *Citrus limon CitlNCED* (AB219172), *CitlNCED3* (AB219179); *Citrus sinensis CitsNCED1* (DQ028471), *CitsNCED2* (AB219171), *CitsNCED2b* (DQ028472), *CitsNCED3* (AB219177); *Citrus unshiu CituNCED2* (AB219169), *CituNCED3* (AB219174); *Chrysanthemum x morifolium CmNCED3* (AB247159); *Cucurbita pepo CpNCED3* (GU380292); *Cucumis sativus CsNCED2* (EU391615); *Daucus carota DcNCED1* (DQ192200); *Diospyros kaki DkNCED1* (EU925812); *Fragaria x ananassa FaNCED1* (FJ560907); *Gentiana lutea GlNCED2* (AY466118); *Ipomoea nil InNCED1* (HQ641566); *Lycopersicon esculentum LeNCED3* (GQ222384); *Lilium formosanum LfNCED3* (GQ168942); *Lilium speciosum LsNCED3* (GQ168943); *Malus x domestica MdNCED1* (AB593328), *MdNCED2* (AB593329); *Malus hupehensis MhNCED* (EU716329); *Oncidium gower ramsey OgNCED* (FJ859994); *Oryza sativa OsNCED5* (AY838901); *Prunus avium PaNCED* (GQ913652), *PaNCED2* (FJ560910); *Phaseolus vulgaris PvNCED1* (AF190462); *Gladiolus hybrid RhNCED* (JF804768); *RhNCED2 (*JF804768); *Solanum tuberosum StNCED1* (AY662342), *StNCED2* (AY662343); *Vitis vinifera VvNCED1* (AY337613), *VvNCED2* (AY337614); **CCD8**: *Actinidia chinensis AcCCD8* (GU206812); *Arabidopsis thaliana AtCCD8* (NM_119434); *Glycine max GmCCD8* (HM366151); *Physcomitrella patens strain PpCCD8* (HM007803); *Zea mays ZmCCD8* (NM_001197000); **CCD7:** *Actinidia chinensis AcCCD7* (GU206813); *Arabidopsis thaliana AtCCD7* (NM_130064), *AtCCD7b* (AK229864); *Cucumis sativus CsCCD7* (HQ005419); *Glycine max GmCCD7* (HM366150); *Hyscomitrella patens strain PpCCD7* (HM007802); *Petunia x hybrida PhCCD7* (FJ790878); *Solanum lycopersicum SlCCD7* (GQ468555); **CCD1**: *Citrus unshiu CituCCD1* (AB219164); *Coffea arabica CaCCD1* (DQ157170); *Crocus sativus CsCCD1* (AJ132927); *Cucumis melo CmCCD1* (DQ269467); *Osmanthus fragrans OfCCD1* (AB526197); *Petunia hybrida PhCCD1* (AY576003); *Rosa damascena* Rd*CCD1*(ABY47994); *Vitis vinifera VvCCD1* (AY856353); **CCD4:** *Crocus sativus CsCCD4a* (EU523662), *CsCCD4b* (EU523663); *Chrysanthemum morifolium CmCCD4a* (ABY60885), Cm*CCD4b* (BAF36656); *Ipomoea ICCD4* (AB499059); *Malus domestica MdCCD4* (EU327777); *Manihot esculenta MeCCD4* (GU120078); *Nicotiana tabacum NtCCD4* (JF947192); *Osmanthus fragrans OfCCD4* (EU33443); *Rosa damascena RdCCD4* (EU334433).

involved in the biosynthesis of strigolactones (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

To elucidate the enzymatic activity, the full length cDNA of OfCCD1 was cloned into fusion vectors for expression in *E. coli*. The function of OfCCD1 was confirmed by in *in vivo* and *in vitro* experiments (Fig. 3). The purified recombinant OfCCD1 can utilize the two major carotenoids as substrates yielding in α- and β-ionone (Baldermann *et al.*, 2010). In addition, in this study, we carried out *in vivo* experiments using β-carotene accumulating cells. We observed clearly a bleaching of the cells

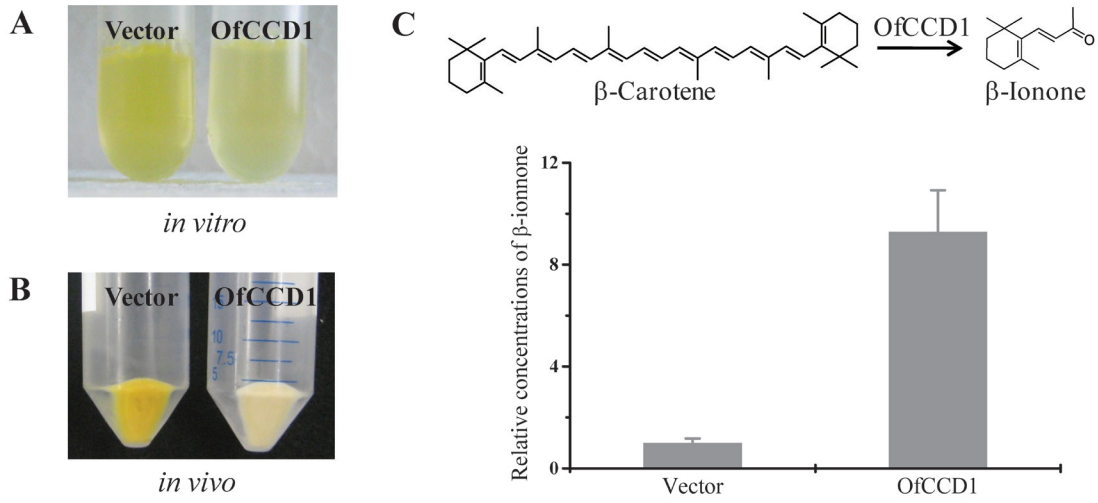

Figure 3. Bleaching of β-carotene by OfCCD1 in:
(A) *in vitro* by purified recombinant enzymes (experimental conditions have been published elsewhere, Baldermann *et al.*, 2010) and **(B)** *in vivo* assays. **(C)** Relative concentrations of β-ionone in the headspace of the *in vivo* assays. The empty vector was utilized for the control experiments.

harboring the *OfCCD1* (Fig. 3B). Moreover, we confirmed the presence of the reaction product β-ionone in the headspace of the liquid cultures accumulating β-carotene (Fig. 3C). The concentrations of β-ionone in the headspace of the OfCCD1 cultures were approximately ten times higher than in the control cultures only harboring the vector.

We verified the involvement of OfCCD1 in the formation of the two major scent compounds α- and β-ionone in flowers of *O. fragrans*.

Detailed analysis during the floral development and effect of photoperiods on transcripts, ionone emission, and carotenoid accumulation has been published elsewhere (Baldermann *et al.*, 2010) and showed that additional studies are needed to clarify the contribution of other CCD enzymes and further biological and chemical formation pathways yielding C_{13}-apocarotenoids in flowers of *O. fragrans*.

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