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# Isolation and Functional Characterization of Carotenoid Cleavage Dioxygenase-1 from *Laurus nobilis* L. (Bay Laurel) Fruits

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Supporting Information

**ABSTRACT:** Bay laurel (*Laurus nobilis* L.) is an agriculturally important tree used in food, drugs, and the cosmetics industry. Many of the health beneficial properties of bay laurel are due to volatile terpene metabolites that they contain, including various norisoprenoids. Despite their importance, little is known about the norisoprenoid biosynthesis in *Laurus nobilis* fruits. We found that the volatile norisoprenoids 6-methyl-5-hepten-2-one, pseudoionone, and $\beta$-ionone accumulated in *Laurus nobilis* fruits in a pattern reflecting their carotenoid content. A full-length cDNA encoding a potential carotenoid cleavage dioxygenase (*LnCCD1*) was isolated. The *LnCCD1* gene was overexpressed in *Escherichia coli*, and recombinant protein was assayed for its cleavage activity with an array of carotenoid substrates. The LnCCD1 protein was able to cleave a variety of carotenoids at the 9,10 (9′,10′) and 5,6 (5′,6′) positions to produce 6-methyl-5-hepten-2-one, pseudoionone, $\beta$-ionone, and $\alpha$-ionone. Our results suggest a role for LnCCD1 in *Laurus nobilis* fruit flavor biosynthesis.

**KEYWORDS:** *Laurus nobilis, carotenoids, norisoprenoid, 6-methyl-5-hepten-2-one, pseudoionone, β-ionone, α-ionone, carotenoid cleavage dioxygenase-1*

## INTRODUCTION

Bay leaf (*Laurus nobilis*) is a characteristic evergreen Mediterranean tree that belongs to the family Lauraceae, and is one of the most widely used culinary spices in the Mediterranean countries, in Europe, and in the U.S.[1,2] Fresh or dried bay leaves are used extensively in the food industry, and the essential oil traditionally has been used as herbal medicine and has pharmacological activity that includes antibacterial, antifungal, antidiabetes, and anti-inflammatory effects.[3−5] In addition, laurel essential oil was reported to be used by the cosmetic industry in creams, perfumes, and soaps for its antidandruff activity and for the external treatment of psoriasis.[6,7]

The volatile constituents of various *L. nobilis* tissues such as leaves, flower, fruits, buds, and roots have been previously examined and found to be mostly composed of mono- and sesquiterpenes.[8,9] In fresh *L. nobilis* leaves, the monoterpene 1,8-cineole is the main volatile compound, and $\alpha$-terpinyl acetate, terpinene-4-ol, $\alpha$-and $\beta$-pinene, sabinene, and linalool have been reported to occur at appreciable levels.[10,11]

Also, the essential oil obtained from the fruits of *L. nobilis* has been used in soap making, as well as in treatment of rheumatism and dermatitis, gastrointestinal problems, such as epigastric bloating, impaired digestion, eructation, and flatulence.[6,7] In addition, it has been used as insect repellant.[12] The aqueous extract of the *L. nobilis* fruits has been in Turkish folk medicine as an antihemorrhoidal, antirheumatic, as an antidote in snakebites, and for the treatment of stomach ache.[6,7]

Carotenoids are $C_{40}$ isoprenoids essential to photosynthesis that can also accumulate in the plastids of nonphotosynthetic tissues. Characteristic structural features of carotenoids are extended conjugated double bond systems that are responsible for their vivid colors.[13] A number of bioactive compounds found in plants, bacteria, fungi, and animals are derived from carotenoid precursors. Among those are apocarotenoids (norisoprenoids) that result from the cleavage of carotenoids by the action of carotenoid cleavage dioxygenases (CCDs).[13−15] Norisoprenoids are widely distributed in nature and serve in important biological functions. In plants, norisoprenoids act as phytohormones (e.g., abscisic acid[16] and strigolactones[17−20]) and chromophores (e.g., bixin and crocin)[21−23] and more. Of particular interest to agricultural and food chemists are norisoprenoids that exhibit low odor thresholds, as they affect the organoleptic perception of the tissue where they occur. Two prominent examples of such norisoprenoids are $\alpha$-ionone and $\beta$-ionone, whose odor threshold is estimated as 0.007 ppb in water.[24] The presence of even minute amounts of such norisoprenoids has a strong impact on human judgment of the aroma of the fruit.[25,26] While previous studies suggest that there is an interrelationship between carotenoid accumulation patterns and the volatile composition of fruits,[27−29] the existence of such an interrelationship in *L. nobilis* fruits has not been investigated to date.

CCDs are nonheme iron oxygenases that cleave carotenes and xanthophylls to yield apocarotenoids. They often display a high degree of regio-specificity for the position of the double

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bonds of their substrates, thus being capable of giving rise to a variety of analogous volatile norisoprenoids.[13,22,30] Plant CCDs can be classified into six subfamilies according to the cleavage position and/or their substrate preferences: CCD1, CCD2, CCD4, CCD7, CCD8, and 9-cis-epoxy-carotenoid dioxygenases (NCEDs).[13,14,22,31] Plant CCD1 and CCD4 appear to be primarily functioning in the biosynthesis of norisoprenoid components of flavor, aroma, and pigmentation of fruits and flowers.[32] Recently, a novel CCD2 was identified and characterized from *Crocus sativus* that catalyzes the first dedicated step in crocin biosynthesis.[22] Conversely, CCD7 and CCD8 appear to be dedicated to the biosynthesis of strigolactones, plant growth regulators impacting several important physiological processes such as shoot branching and development of arbuscular mycorrhizae.[18−20] Finally, all NCEDs, which are only distantly related to CCDs, play a role in the biosynthesis of abscisic acid by producing its C15-precursor xanthotoxin from the 9-*cis* isomers of epoxy-carotenoids.[33]

The present study describes the carotenoid accumulation, norisoprenoid content, transcript level, and the isolation of a *CCD1* gene from fruits of *L. nobilis* (*LnCCD1*). To characterize the catalytic activity of *LnCCD1*, the gene was isolated and functionally expressed in *Escherichia coli*. The recombinant protein was assayed for cleavage activity with a variety of carotenoid substrates. To study the possible involvement of LnCCD1 in the synthesis of norisoprenoids flavor compounds, the expression pattern of this gene was determined in the fruit of *L. nobilis* by real-time-PCR.

## MATERIALS AND METHODS

**Chemicals.** HPLC-grade acetonitrile, methanol, acetone, $\alpha$-ionone, $\beta$-ionone, lycopene, lutein, and $\alpha$- and $\beta$-carotenes were purchased from Sigma-Aldrich.

**Plant Material.** *Laurus nobilis* plants were grown in the "Newe Yaar" Research Center in northern Israel, under standard field irrigation and fertigation conditions. Pericarps of freshly harvested green and black fruits were crushed in liquid nitrogen and stored at −80 °C for analysis of carotenoids, norisoprenoid volatiles, and transcripts.

**Carotenoid Extraction.** Carotenoids were extracted by grinding fresh *L. nobilis* pericarp of green and black fruits (100 mg) in acetone. The solvent was collected and filtered and the grinding and collecting of solvent repeated until the solvent was colorless. Acetone was dried under a stream of $N_2$ and then redissolved in 1 mL of acetone. 100 $\mu$L of the sample in acetone was diluted 10 times and spared for spectroscopic quantification. The rest of the sample was again dried under a stream of $N_2$ and redissolved in acetone for further analysis by HPLC.

**HPLC Analyses and Carotenoid Quantification.** HPLC analysis was performed on a Waters HPLC system equipped with a Waters 600 pump, a Waters PDA detector 996, and a Waters 717 plus autosampler. A Spherisorb ODS2 C18 column (Waters, 5 $\mu$m, 4.6 × 250 mm) coupled to a guard cartridge system SecurityGuard (Phenomenex) was used. Gradient was applied at a constant flow of 1.6 mL/min with acetonitrile:water (9:1; A) and ethyl acetate (B) as described in Isaacson et al. (2004).[34] Data were collected in the range of 250−600 nm, and analyzed using the Empower software. Quantification of carotenoids was done spectroscopically on the diluted carotenoid sample in acetone.[35] Phytoene quantification was done by normalizing its integrated peak at 286 nm to correct for its specific mass extinction coefficient relative to $\beta$-carotene (factor of 2.074).

**Extraction of Volatile Compounds from Fruits of *L. nobilis*.** Three replicates of fresh *L. nobilis* pericarp of green and black fruits (1 g) were ground into a homogeneous powder under liquid nitrogen using mortar and pestle. The fine powders were placed in a 20 mL DuPont autosampler vial (DuPont Performance Elastomers, http://www.dupontelastomers.com) with a white solid-top polypropylene cap (Alltech, http://www.alltech.com). Samples were overlaid with 5 mL of NaCl (25%) solution and 1 g of NaCl (for inhibition of enzyme activity). To each sample, the internal standard 2-heptenone was added to produce a final concentration of 1 mg/kg (1 ppm). After incubation at room temperature (25 °C) for 1 h, the volatile compounds were collected with a solid phase micro extraction (SPME) device PDMS-100 with a polydimethylsiloxane fiber (Sigma-Aldrich) by inserting the fiber into the tube and leaving it in place for 20 min at room temperature. After this incubation step, the SPME fiber was injected directly into the GC−MS.

**Auto-HS-SPME-GC−MS Analysis of Volatile Compounds.** Volatile compounds were analyzed on a GC−MS apparatus (Agilent Technologies, CA) equipped with an Rtx-5SIL MS (30 m × 0.25 mm × 0.25 $\mu$m) fused-silica capillary column essentially as described by Yahyaa et al. (2015).[36] Briefly, $He_2$ (1 mL $min^{-1}$) was used as a carrier gas. The injector temperature was 250 °C, set for splitless injection. The oven was set to 50 °C for 1 min, and then the temperature was increased to 220 °C at a rate of 5 °C $min^{-1}$. For SPME analysis, thermal desorption was allowed for 40 min. The detector temperature was 280 °C. The mass range was recorded from 41 to 450 $m/z$, with electron energy of 70 eV. Identification of the main components was done by comparison of mass spectra and retention times with those of authentic standards and supplemented with a Wiley GC−MS library.

**Isolation and Characterization of *L. nobilis* Fruit Carotenoid Cleavage Dioxygenase-1.** Putative *L. nobilis* CCD encoding genes were searched for using a homology-based algorithm in the transcriptome database of *L. nobilis*, which has been assembled on the basis of available GenBank sequence data entries. A single full-length gene was tentatively identified as *L. nobilis* carotenoid cleavage dioxygenase-1 (*Ln*CCD1). Two specific primers corresponding to the *LnCCD1* protein-coding sequence 5′-end (5′-ATG GAG AAG GAA AAT GGA A-3′) and 3′-end (5′-CTT TCC CTG TTG TTG AAG TTG TTC C-3′) were designed. RNA from *L. nobilis* pericarp of the green and black fruits was isolated using the Spectrum Plant Total RNA Kit (Sigma-Adlich). For producing a cDNA clone, 5 $\mu$g of total RNA from fruits of *L. nobilis* was reverse transcribed using the SuperScript One-Step RT-PCR with Platinum *Taq* DNA polymerase (Invitrogen) used for amplification, yielding a 1650 bp specific fragment. The cDNA was inserted into the pEXP5-CT/TOPO TA expression vector (Invitrogen Corporation, Carlsbad, CA), yielding pEXP-*LnCCD1* in which the LnCCD1 coding sequence was fused with a His tag-coding extension at the C-terminus. The construct was introduced into *E. coli* Top10 cells. The constructs' fidelity and orientation of the inset in the vector were verified by DNA sequencing.

**Functional Expression Experiments and Determination of Volatiles from Bacterial Headspace.** *E. coli* strains engineered to accumulate phytoene (pBCAR-EB), lycopene (pBCAR-EBI), $\beta$-carotene (pBCAR-EBIY), $\delta$-carotene (pDCAR), and zeaxanthin (pZEAX) were transformed with the expression construct pEXP-*LnCCD1*.[37,38]

Production of the recombinant protein was induced by IPTG, and the volatiles were collected by SPME and analyzed by GC−MS, respectively, as described by Yahyaa et al., 2013.[39]

***LnCCD1* Transcript Analysis.** For real-time RT-PCR analysis of *LnCCD1*, total RNA (5 $\mu$g) from *L. nobilis* pericarp of green and black fruits was extracted (Spectrum Plant Total RNA Kit, Sigma-Aldrich) and reverse transcribed using an oligo$(dT)_{12-18}$ primer and the SuperScript II first-strand system (Invitrogen). RNA quality and quantity were determined by using agarose gel electrophoresis, and the concentrations were measured using the NanoDrop ND-1000 spectrophotometer (Bargal Analytical Instruments). To remove the trace amounts of genomic DNA during RNA purification from RNA preparations, On-Column DNase I Digest Set was applied (Sigma-Aldrich).

Real-time RT-PCR was performed on an Applied Biosystem StepOnePlus Real-Time PCR System (Life technology) using ABsolute Blue qPCR SYBR Green ROX Mix (Tamar Laboratory


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Supplies Ltd., Israel), using 5 ng of reverse-translated total RNA and 100 ng of primers.

Primers for *LnCCD1* were LnCCD1_F_qPCR (5′-AGT CTT ACA CAG GCA GGA AAC-3′) and LnCCD1_R_qPCR (5′-CAA ACT TGA TGA TGC CCT TCA C-3′). A relative quantification of gene expression was performed using actin from *L. nobilis* as a reference gene. The following actin primers were used as positive controls: Actin-F-qPCR (5′- GTG AGC TGA GGC TTG ATT GA-3′) and Actin-R-qPCR (5′- TCG ACC CAA GTA CAC AGA CTA-3′). Differences in relative expression levels of LnCCD1 were calculated from 2-ΔΔCt value after normalization of LnCCD1 data to actin. All analyses were performed using three biological replicates.

## RESULTS AND DISCUSSION

**Carotenoid Pigmentation Patterns of *L. nobilis* Influence Fruit Norisoprenoid Volatile Composition.** We previously demonstrated that variation in terpenoid volatiles accumulation in different *L. nobilis* tissues is organ and gender dependent.[9] Also, variation in total content of *L. nobilis* green and black fruits terpenes has been reported.[8,9] However, to the best of our knowledge, the influence of carotenoid composition on volatile norisoprenoid accumulation in fresh *L. nobilis* pericarp of green and black fruits has never been shown. Therefore, it was of interest to analyze the carotenoid content of fresh *L. nobilis* pericarp of green and black fruits and the norisoprenoid volatiles using HPLC and GC−MS, respectively. The main carotenoid found in *L. nobilis* pericarp of the green and black fruits is lutein (Table 1). The content of β-carotene, zeaxanthin, antherexanthin, violaxanthin, and neoxanthin was different in pericarp of the green and black bay laurel fruits. The total carotenoid level in green fruits of *L. nobilis* was 2-fold higher than that found in the black fruits (Table 1). A drastic and marked decrease of the β-carotene and lutein contents was found during fruit development (in the black fruit). In accordance, our analyses showed that norisoprenoid volatiles such as 6-methyl-5-hepten-2-one (MHO), pseudoionone, and β-ionone are present only in the fresh pericarp of the black *L. nobilis* fruits (Table 1). Of particular interest is the fact that the levels of MHO, pseudoionone, and β-ionone, which could be derived from the breakdown of lycopene, α-carotene, and β-carotene, were correlated with the β-carotene content in the black fruits (Table 1).

**Table 1. Carotenoids and Norisoprenoid Volatiles in *Laurus nobilis* Fruits**

| | green fruits | black fruits |
|---|---|---|
| Carotenoid[a] Content (μg g$^{-1}$ of FW) | | |
| β-carotene | 38.51 ± 0.54 | 13.70 ± 1.4 |
| zeaxanthin | 0.42 ± 0.09 | 0.20 ± 0.05 |
| lutein | 83.25 ± 0.95 | 33.07 ± 3.67 |
| antheraxanthin | 8.88 ± 1.45 | 9.59 ± 2.47 |
| violaxanthin | 6.65 ± 0.50 | 4.92 ± 0.34 |
| neoxanthin | 7.58 ± 0.28 | 7.36 ± 1.05 |
| total | 145.29 ± 0.63 | 68.84 ± 1.49 |
| Norisoprenoid[b] Compounds (ng g$^{-1}$ of FW) | | |
| 6-methyl-5-hepten-2-one | nd | 51.2 ± 5.1 |
| pseudoionone | nd | 48.7 ± 14.9 |
| β-ionone | nd | 31.6 ± 0.9 |

[a]Carotenoid standards were identified by HPLC on the basis of commercial standards. [b]Norisoprenoid compounds were identified by GC−MS on the basis of reference volatiles. The results shown are an average and standard errors of at least three biological replicates. nd = not detected.

Many volatiles are produced in plant tissues at specific developmental stages, for example, during flowering, ripening, or maturation. Although a single fruit synthesizes several hundred volatiles, only a small subset generates the "flavor fingerprint" that helps animals and humans recognize appropriate foods and avoid poor or dangerous food choices.[40]

β-Ionone is widely distributed in plants and is considered an important and potent flavor contributor in many fruits and fruit-based foods, due to its extremely low odor thresholds (0.007 ppb).[28,39,41] MHO is also an important flavor and aroma volatile found in a number of fruits such as tomato.[42] MHO is present as a component of the floral scent of numerous plants, occurring in over 50% of 991 species of flowering plants that had their floral scent analyzed.[43]

Our data presented above indicate that norisoprenoid volatiles found in the fresh *L. nobilis* pericarp black fruits indeed originate from the oxidative degradation of carotenoids as the main route, as it also is in many other plants, for example, in carrots and melon,[28,39] in watermelon and tomato,[29,44] in strawberry,[45] in *Rosa damascena*,[46] in pepper,[27] and in *Crocus sativus*.[22] Also, the function of fruit volatiles as a signal of ripeness and as an attractant for seed-dispersing organisms is supported by the fact that some substances are specifically formed by ripe fruits but are absent in vegetative tissues and nonripe fruit (Table 1). Unlike ripe fruits and flowers, vegetative tissues often produce and release many of the volatiles sensed as flavors only after their cells are disrupted.

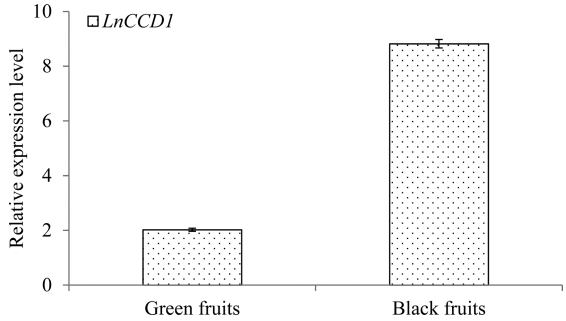


**Figure 1.** Expression patterns of *LnCCD1* in green and black fruits of *L. nobilis*. Quantification of *LnCCD1* transcript levels by real-time RT-PCR analysis normalized to actin transcripts. All analyses were performed using three biological replicates.

**Expression Patterns of *LnCCD1* in Fruits of *L. nobilis*.** The expression pattern of the *LnCCD1* gene along fruit development (green and black fruits) was examined by real-time RT-PCR (Figure 1). The expression level of the *LnCCD1* gene in black fruits was 6-fold higher than that found in the green fruits (Figure 1). Moreover, a significant correlation was observed between increase of the expression level of *LnCCD1* and the accumulation of norisoprenoids during fruits development and the decrease of β-carotene content (Table 1 and Figure 1).

In our analysis of norisoprenoid volatiles in *L. nobilis* green and black fruits, we were not able to detect volatile norisoprenoids in the green fruits (Table 1). Thus, we cannot conclude that the *LnCCD1* gene expression in *L. nobilis* green fruits is related to the production of norisoprenoids. However, a lag between CCD1 gene expression and norisoprenoid volatiles


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production has been reported,[47,48] which could be explained by the different cellular localization of carotenoids and CCD1 enzymes.[21,47,49] Although there is a temporal delay in volatile emission in relation to gene expression, in tomato the silencing of both *LeCCD1A* and *LeCCD1B* genes generates a reduction in $\beta$-ionone and geranylacetone. This provides a cause-and-effect relationship between *LeCCD1* gene expression and the formation of those important flavor volatiles in vivo.[47]

**Identification of LnDcCCD1.** The role of CCDs in the formation of norisoprenoids and their flavor properties in *L. nobilis* fruits has not been reported. In attempts to identify a gene responsible for norisoprenoid formation in *L. nobilis* fruits, we examined the recently created *L. nobilis* transcriptome database[9] for genes exhibiting similarity to known CCD sequences from other plants.

Data mining of the *L. nobilis* database resulted in identification of one full length cDNA clone (Figure S1), displaying high sequence similarity to other plant carotenoid cleavage dioxygenases. The clone, designated *LnCCD1*, originated from a leaves expressed sequence tagged (EST) library of *L. nobilis*. The predicted LnCCD1 protein sequence consists of 550 amino acids, with a calculated molecular mass of 61.9 kDa. The deduced amino acid sequence of LnCCD1 contains two conserved domains, which are typically found in enzymes involved in the biosynthesis of norisoprenoids.[50]

LnCCD1 protein displays a high sequence similarity (86%) to the CCD from *Phoenix dactylifera* (date plum), and (84%) to CCD1 from *Nelumbo nucifera* (sacred lotus). LnCCD1 is also similar to the *Vitis vinifera* VvCCD1 (84%) that cleaves zeaxanthin symmetrically yielding 3-hydroxy-$\beta$-ionone, a C13-norisoprenoidic compound, and a C14-dialdehyde.[51] LnCCD1 is also similar to CCD1 from *Prunus mume* (chinese plum) (82%), to CCD1 from *Cucumis melo* (melon) (82%),[28] to *Malus* × *domestica* (apple) (81%), to CCD1 from *Osmanthus fragrans* (fragrant olive) (80%), and to DcCCD1 from *Daucus carota* (carrot) (80% identity), which encodes enzyme that cleaves only cyclic carotenoids to generate $\alpha$- and $\beta$-ionone.[39] Phylogenetic analyses show that the protein encoded by this cDNA clusters with other plant CCD1 enzymes (Figure 2).

**LnCCD1 Cleaves Multiple Carotenoids To Generate Norisoprenoids Volatiles Derived from 9,10 and 5,6 Double Bond Cleavage.** To determine the substrate specificities and bond cleavage position preferences of the *LnCCD1*, the cDNA was cloned into bacterial expression vector pEXP5-CT/Topo, and the recombinant LnCCD1 protein was expressed in *E. coli* strains that accumulate various carotenoids biosynthesis enzymes.[37,38] Except for phytoene that is colorless, the carotenoids that accumulate in these strains provide a specific color to the cells depending on the carotenoid accumulated, and a loss of color indicates that the carotenoids could be metabolized to colorless compounds. Cultures expressing LnCCD1 and accumulating various carotenoids were grown in darkness, induced with IPTG, and grown for a further 12 h at room temperature. The norisoprenoid volatiles were collected by auto-HS-SPME and analyzed by GC−MS.

No isoprenoid-derived volatiles were identified in the culture synthesizing the acyclic carotenoids phytoene by GC−MS headspace analyses (Figure 3 and Table 2). To ensure that LnCCD1 did not cleave these acyclic carotenoids and generate nonvolatile products, the phytoene-accumulating *E. coli* cultures expressing the LnCCD1 were extracted and analyzed by HPLC. The phytoene carotenoid levels of the LnCCD1-expressing cultures were identical to controls harboring empty vectors and

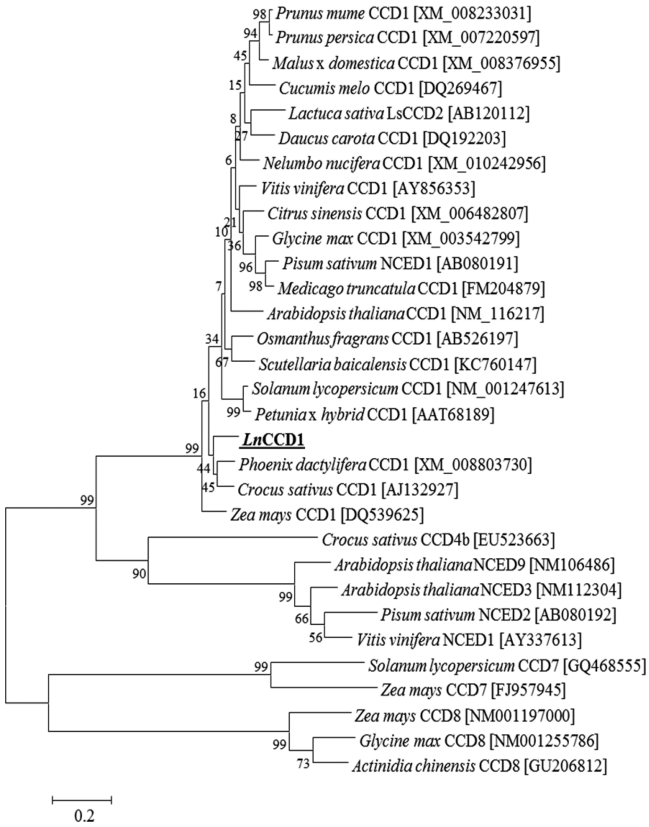


**Figure 2.** Phylogenetic tree of deduced amino acid sequences of carotenoid cleavage oxygenases from *L. nobilis* and other plant species. The tree was generated using Phylogeny Analysis MEGA6.1 program.[54] The resulting tree was bootstrap analyzed with 1000 replicates. The black bold underline indicates the *L. nobilis LnCCD1* gene identified in this study. Accession numbers are indicated in parentheses.

not overexpressing LnCCD1 (data not shown). Moreover, no new nonvolatile products were found, as compared to the control (data not shown), confirming that LnCCD1 did not cleave phytoene at a detectable rate. Similarly, Yahyaa et al. (2013)[39] reported that DcCCD1, a CCD isolated from *Daucus carota* roots, did not cleave the acyclic carotenoids phytoene. Also, similar results were reported by Vogel et al. (2008)[52] that ZmCCD1, a CCD isolated from maize, did not cleave phytoene.

Norisoprenoid volatiles were identified in vitro during coexpression of *LnCCD1* in different carotenoid producing *E. coli* strains (Table 2 and Figure 3). Pseudoionone and MHO were detected in lycopene-accumulating *E. coli* strain (Table 2 and Figure 4A). Pseudoionone is generated by 9,10 or 9′,10′ bond cleavage of lycopene, whereas MHO is generated by 5,6 or 5′,6′ bond cleavage. The presence of MHO and pseudoionone was unequivocally confirmed by a comparison of their retention indices (RI) and a mass spectra to that of authentic MHO and pseudoionone. Both pseudoionone and MHO were not detected in *E. coli* cells transformed with control plasmids devoid of the *LnCCD1* gene (Table 2 and Figure 4B). These results indicate that the *L. nobilis* CCD1 enzyme, like the *Arabidopsis*, maize, tomato, and *Rosa damascena* CCD1, cleaves lycopene at 9,10 (9′,10′) and 5,6 (5′,6′) bonds to generate pseudoionone and MHO.[46,52]

The norisoprenoid volatiles derived from $\delta$- and $\beta$-carotene-accumulating *E. coli* strains revealed a similar pattern (Table 2,


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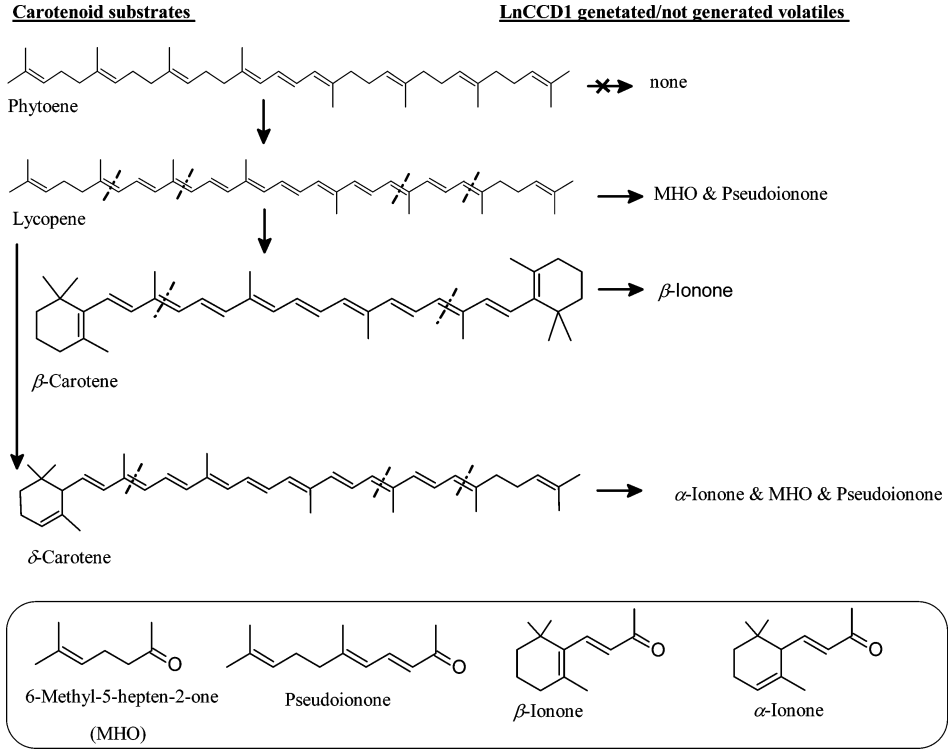


**Figure 3.** Proposed sites of LnCCD1 bond cleavage and the volatiles generated. An abbreviated version of the carotenoid biosynthetic pathway in higher plants is shown. Carotenoid substrates (left) are oxidatively cleaved by LnCCD1 to yield the norisoprenoid derivatives (right).

**Table 2. Norisoprenoids Formed in Vitro during Coexpression of *LnCCD1* in Carotenoid Producing *E. coli* Strains**

| | | norisoprenoids[a] present in headspaces of bacterial cultures (ng/mL) | | | |
|---|---|---|---|---|---|
| plasmid | carotenoid | 6-methyl-5-hepten-2-one | pseudoionone | β-ionone | α-ionone |
| pBCAR-EB | phytoene | | | | |
| pBCAR-EB + *LnCCD1* | | | | | |
| pBCAR-EBI | lycopene | | | | |
| pBCAR-EBI + *LnCCD1* | | 36.05 ± 3.17 | 26.57 ± 1.08 | | |
| pBCAR-EBIY | β-carotene | | | | |
| pBCAR-EBIY + *LnCCD1* | | 16.08 ± 0.91 | 1.36 ± 0.33 | 22.51 ± 4.61 | |
| pZEAX | zeaxanthin | | | | |
| pZEAX + *LnCCD1* | | 21.04 ± 2.12 | 4.92 ± 0.29 | 5.46 ± 0.86 | |
| pDCAR | δ-carotene | | | | |
| pDCAR + *LnCCD1* | | 38.07 ± 1.50 | 11.88 ± 0.64 | | 1.56 ± 0.12 |

[a]Norisoprenoid compounds were identified by GC−MS on the basis of mass spectra matching with the standard NIST-98.L and Wiley 7N.I library (ms), comparison of retention index (RI) with literature data (RI), comparison with standard. The results shown are an average and standard errors of at least three biological replicates.

Figure 5A, and Figure S2). β-Ionone was detected from β-carotene-accumulating culture, and α-ionone was detected in the δ-carotene-accumulating culture, as evidenced by their retention index (RI) and a mass spectrum identical to that of authentic α- and β-ionone (Table 2, Figure 5A, and Figure S2). Both α- and β-ionone are indicative of 9,10 (9′10′) bond cleavage. Additionally, pseudoionone and MHO also were detected in both cultures expressing LnCCD1 enzyme, indicating cleavage of δ- and β-carotene precursor carotenoids at 9,10 (9′,10′) and 5,6 (5′,6′) bonds. Pseudoionone, MHO, and α- and β-ionone were absent in *E. coli* cells transformed with control plasmids devoid of the *LnCCD1* gene (Table 2, Figure 5B, and Figure S2).

*LnCCD1* was also overexpressed in an *E. coli* strain accumulating the yellow-orange colored xanthophyll zeaxanthin, resulting in colorless colonies. The putative volatile enzymatic reaction products of the cleavage of zeaxanthin were further analyzed by auto-HS-SPME-GC−MS. MHO, pseudoionone, and β-ionone were identified in the head space of the *E. coli* strain accumulating zeaxanthin and expressing *LnCCD1* (Table 2 and Figure S3). 3-Hydroxy-β-ionone, the expected oxidative cleavage product of zeaxanthin, could not be detected in the headspace of these cultures (Table 2). MHO, pseudoionone, and β-ionone are breakdown products of lycopene and β-carotene, respectively, which are intermediate in zeaxanthin biosynthesis, suggesting that LnCCD1 may compete for the lycopene and β-carotenoid substrate with the β-carotene cyclase and the β-carotene hydroxylases (respectively) that are expressed in the zeaxanthin accumulating *E. coli* strain carrying that gene, the hydroxylase that catalyzes the formation of zeaxanthin in these cultures.[37,38] Similar observations (presence of β-ionone and not 3-hydroxy β-


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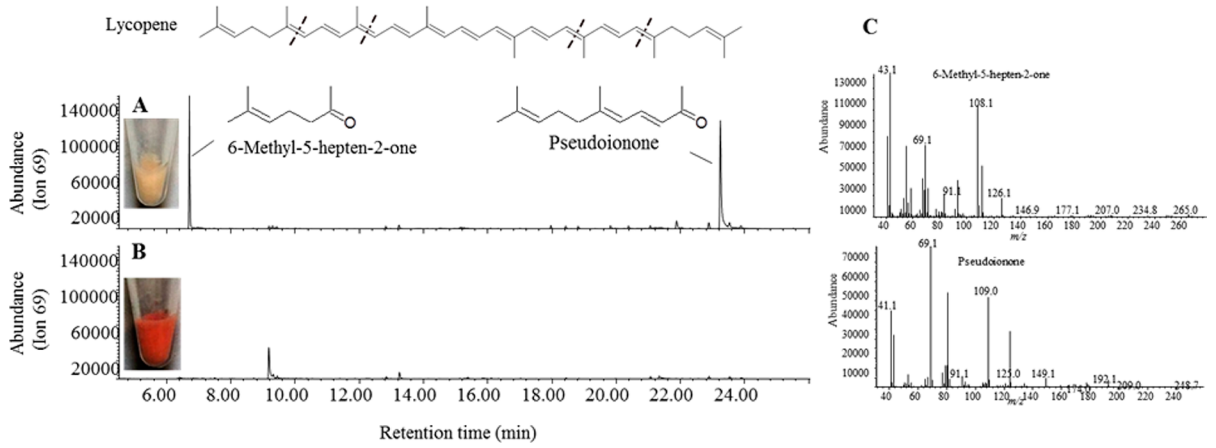


**Figure 4.** Functional expression of *LnCCD1* in *E. coli* cells previously engineered to accumulate lycopene. Dashed lines indicate sites of LnCCD1 cleavage. (A) GC−MS analysis of LnCCD1 activity after transformation in *E. coli* cells previously engineered to accumulate lycopene. (B) GC−MS analysis of bacterial pellets of *E. coli* cells engineered to accumulate lycopene. (C) The inset shows the structure and the mass spectrum of the enzymatic reaction products identified as MHO and pseudoionone by comparison of the retention index and the MS pattern with an authentic standard.

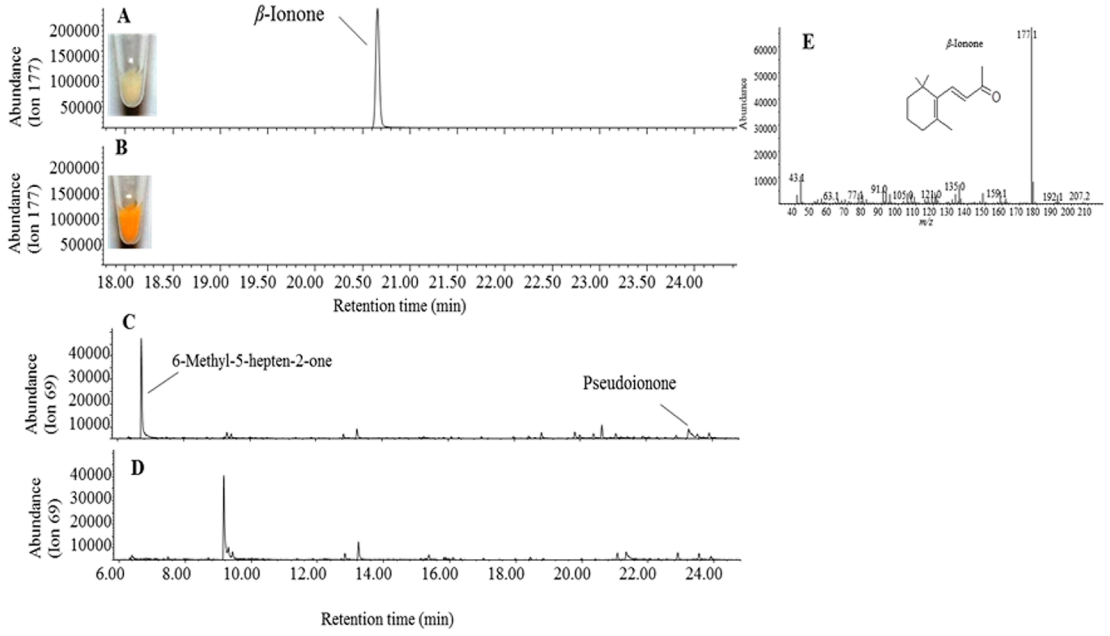


**Figure 5.** Functional expression of *LnCCD1* in *E. coli* cells previously engineered to accumulate *β*-carotene. (A and C) GC−MS analysis of LnCCD1 activity after transformation in *E. coli* cells previously engineered to accumulate *β*-carotene. (B and D) GC−MS analysis of bacterial pellets of *E. coli* cells engineered to accumulate *β*-carotene. 6-Methyl-5-hepten-2-one and pseudoionone are breakdown products of lycopene, which is intermediate in *β*-carotene biosynthesis. (E) The inset shows the structure and the mass spectrum of the enzymatic reaction product identified as *β*-ionone by matching the retention time and the MS pattern with an authentic standard. The identity of the cleavage products (peaks marked) was confirmed using authentic standards.

ionone) have been noted in previous studies of expression of the *DcCCD1* gene from carrot roots,[39] *RdCCD1* and *RdCCD4* genes from *Rosa damascena*, *MdCCD4* gene from *Malus* × *domestica*, *CmCCD4a* gene from *Chrysanthemum* × *morifolium*, and *OfCCD4* gene from *Osmanthus fragrans*.[46,53]

In each carotenoid accumulating *E. coli* strain tested in this study, additional norisoprenoid volatiles consistent with the cleavage of precursor carotenoids were observed, indicating that LnCCD1 cleaves precursor carotenoids before they can be converted into the final product. Similar results were reported by Vogel et al. (2008) and Huang et al. (2009),[46,52] who reported that ZmCCD1 and RdCCD1, CCDs isolated from maize and *Rosa damascena*, respectively, have a broad substrate specificity and cleaved multiple carotenoids at two different double bond positions (9,10, 9′10′, 5,6, and 5′6′).

One of the most significant findings of this work is that LnCCD1 can cleave lycopene at 9,10 (9′,10′) and 5,6 (5′,6′) double bonds to produce pseudoionone and MHO. Cleaving lycopene by LnCCD1 provides a biological route to synthesize MHO, an important flavor and aroma volatile found in a number of fruits such as tomato.[42] Accordingly, the bulk of the volatile norisoprenoid products detected in the recombinant *E. coli* overexpressing the *LnCCD1* gene apparently result from the 9,10 (9′,10′) and 5,6 (5′,6′) cleavage of different carotenoids. We therefore conclude that *LnCCD1* encodes a protein that displays 9,10 and 5,6-carotenoid cleavage


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dioxygenase activity and can accept a wide range of carotenoids as a substrates. LnCCD1 is able to release MHO, pseudoionone, $\beta$-ionone, and $\alpha$-ionone depending on the carotenoid substrate offered.

In conclusion, several carotenoids and norisoprenoid volatile compounds, for example, MHO, pseudoionone, and $\beta$-ionone, were detected in the *L. nobilis* pericarp of the black fruit. LnCCD1 was found to have a broad substrate specificity and to cleave multiple carotenoids at two different double bond positions (9,10, 9′10′, 5,6, and 5′6′). On the basis of the pattern of norisoprenoid volatiles detected by auto-HS-SPME-GC−MS analysis, and their odor thresholds, we suggest that LnCCD1 cleavage products may contribute to *L. nobilis* fruit flavor and aroma.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b02941.

Figure S1: Sequences of open reading frames and encoded protein of LnCCD1 from *Laurus nobilis*. Figure S2: Functional expression of LnCCD1 in *E. coli* cells previously engineered to accumulate $\delta$-carotene. A and C: GC−MS analysis of LnCCD1 activity after transformation in *E. coli* cells previously engineered to accumulate $\delta$-carotene. B and D: GC−MS analysis of bacterial pellets of *E. coli* cells engineered to accumulate $\delta$-carotene. The identity of the cleavage products (peaks marked) was confirmed using authentic standards. Figure S3: Functional expression of LnCCD1 in *E. coli* cells previously engineered to accumulate zeaxanthin. A and C: GC−MS analysis of LnCCD1 activity after transformation in *E. coli* cells previously engineered to accumulate zeaxanthin. B and D: GC−MS analysis of bacterial pellets of *E. coli* cells engineered to accumulate zeaxanthin. 6-Methyl-5-hepten-2-one, pseudoionone, and $\beta$-ionone are breakdown products of lycopene and $\beta$-carotene, respectively, which are intermediate in zeaxanthin biosynthesis. The identity of the cleavage products (peaks marked) was confirmed using authentic standards (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

CCD, carotenoid cleavage dioxygenase; LnCCD, *Laurus nobilis* carotenoid cleavage dioxygenase; FW, fresh weight; GC−MS, gas chromatography mass spectrometry; HPLC, high performance liquid chromatography; IPTG, isopropyl-1-thio-$\beta$-D-galactopyranoside; MHO, 6-methyl-5-hepten-2-one; HS-SPME, head-space-solid phase micro extraction

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