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Formation of Norisoprenoid Flavor Compounds in Carrot (*Daucus carota* L.) Roots: Characterization of a Cyclic-Specific Carotenoid Cleavage Dioxygenase 1 Gene

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Supporting Information

ABSTRACT: Carotenoids are isoprenoid pigments that upon oxidative cleavage lead to the production of norisoprenoids that have profound effect on flavor and aromas of agricultural products. The biosynthetic pathway to norisoprenoids in carrots (*Daucus carota* L.) is still largely unknown. We found the volatile norisoprenoids farnesylacetone, α-ionone, and β-ionone accumulated in Nairobi, Rothild, and Purple Haze cultivars but not in Yellowstone and Creme de Lite in a pattern reflecting their carotenoid content. A cDNA encoding a protein with carotenoid cleavage dioxygenase activity, *Dc*CCD1, was identified in carrot and was overexpressed in *Escherichia coli* strains previously engineered to produce different carotenoids. The recombinant *Dc*CCD1 enzyme cleaves cyclic carotenes to generate α- and β-ionone. No cleavage products were found when *Dc*CCD1 was co-expressed in *E. coli* strains accumulating non-cyclic carotenoids, such as phytoene or lycopene. Our results suggest a role for *Dc*CCD1 in carrot flavor biosynthesis.

KEYWORDS: *Daucus carota, norisoprenoid, farnesylacetone, α-ionone, β-ionone, carotenoid cleavage dioxygenase 1*

INTRODUCTION

The aroma and flavor of fruits and vegetables are determined by combinations of volatile organic compounds (Figure 1).[1] Carrots (*Daucus carota* L.) are popular vegetables not only because of their health benefits but also because of their pleasant flavor.[2,3] Carrot quality is determined by several traits that affect taste, aroma, color, and nutritional value.[4,5] Among the natural products biosynthesized during carrot root development and which directly affect root quality and flavor, terpenes are key players, constituting important pigments (carotenoids) and aroma chemicals (monoterpenes, sesquiterpenes, and norisoprenoids).[6−8] Indeed, the attractive color of carrots results mostly from the accumulation of anthocyanins and several types of carotene compounds, such as α-, β-, γ-, and ζ-carotenes, lycopene, and β-zeacarotene.[9] Carrot flavor is influenced by carotenoid degradation products as well as other compounds, such as organic acids, phenolics, and alkynes.[10,11]

Carotenoids are tetraterpenoid pigments and normally accumulate in the plastids of leaves, flowers, fruits, and roots, where they contribute to the red, orange, and yellow colors.[12] Carotenoids constitute an important precursor reservoir for the biosynthesis of bioactive compounds in plants, bacteria, fungi, and animals. Carotenoids are cleaved into apocarotenoids (norisoprenoids) by regiospecific oxidative enzymes targeting different double bounds on the carotenoid backbone.[12−14] The broad diversity of norisoprenoids can be attributed to a large number of carotenoid precursors and the variation of the cleavage sites. In plant cells, apocarotenoids serve as growth regulators (e.g., abscisic acid),[15] strigolactone-signaling molecules,[15−18] chromophores (e.g., bixin and crocin),[19] etc. Of particular interest are norisoprenoids, which contribute to the flavor and aroma of many of our foods because they exhibit low odor thresholds. These include, for example, farnesylacetone, α-ionone, β-ionone (0.007 μg/kg in water), and β-cyclocitral (5 μg/kg in water).[20] Such norisoprenoids contribute to fruit flavor even at low levels because they often possess strong effects on the overall human appreciation of the aroma of the fruit.[20] Previous studies have indicated that carotenoid pigmentation patterns affect the volatile composition of fruits (Figure 1),[21−23] but little is known on how carotenoid patterns affect the norisoprenoid aroma of vegetable roots.

The oxidative cleavage of plant carotenoids is mainly catalyzed by carotenoid cleavage dioxygenases (CCDs).[13,24] CCDs constitute a large enzyme family and typically exhibit a high degree of regiospecificity to the double bond positions of

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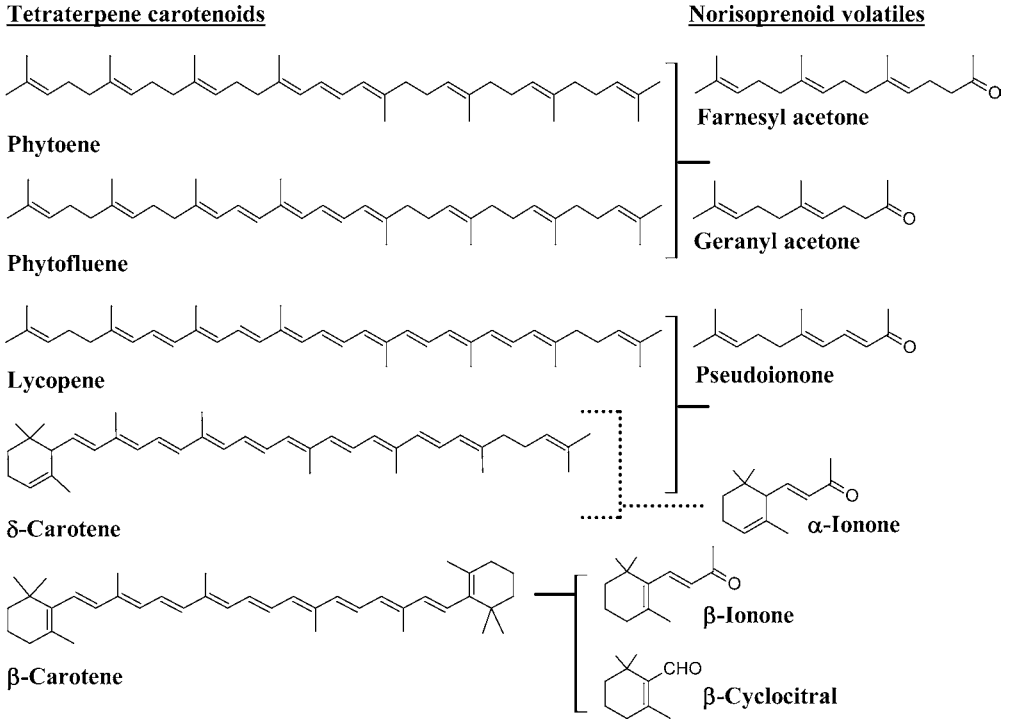

Figure 1. Tetraterpene carotenoid pigments and norisoprenoid products in plants. Tetraterpene carotenoids (left) are apparently cleaved into norisoprenoid volatiles (right).

their substrates. The CCD enzyme can also cleave multiple carotenoid substrates while producing various norisoprenoid volatile compounds.[13,14] Previous studies have unveiled the presence of large families of CCD-encoding genes in plants, bacteria, fungi, and animals.[12,13,25] In general, plant CCD1 and CCD4 are mostly involved in the biosynthesis of norisoprenoids that contribute to the flavor and aroma of fruits and flowers.[26] Conversely, CCD7 and CCD8 are involved in the biosynthesis of strigolactone growth regulators known to be involved in shoot branching.[16,18,27] All 9-*cis*-epoxycarotenoid dioxygenases cleave only 9-*cis* isomers of epoxy carotenoids at the 11 and 12 positions to yield xanthoxin as a C15 product, which is the precursor of abscisic acid.[28]

Despite the presence of large amounts of carotenoids in carrot roots, CCDs and their encoding genes have not been isolated. Moreover, the role of CCDs in the formation of norisoprenoids and their flavor properties in carrots has not been studied. The aim of our project is primarily to study the biochemical and molecular factors that determine the production of norisoprenoid volatile aroma compounds in five carrot cultivars of different colors to assess if carotenoid composition affects the norisoprenoid profile. We show here that different carrot cultivars with distinct carotenoid profiles exhibited different norisoprenoid content patterns reflecting carotenoid composition. In this paper, a carotenoid cleavage dioxygenase from *D. carota* was expressed in *Escherichia coli*. We show here that the recombinant *Dc*CCD1 protein catalyzes the carotenoid cleavage reaction in a different fashion for other known *CCD1* genes, indicating that the *Dc*CCD1 enzyme has a narrower substrate specificity than other plant CCDs.

MATERIALS AND METHODS

Chemicals. High-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, dichloromethane, hexane, acetone, ethanol, triethylamine, isopropyl-1-thio-*β*-D-galactopyranoside (IPTG), dichloromethane (Ch_2Cl_2), methyl *tert*-butyl ether (MTBE), *α*-ionone, *β*-ionone, *β*-cyclocitral, lycopene, lutein, *α*-carotene, and *β*-carotene were purchased from Sigma-Aldrich.

Plant Material. Commercial-colored carrot cultivars, orange "Nairobi", orange "Rothild", purple "Purple Haze", yellow "Yellowstone", and white "Creme de Lite" (Kiepenkerl Profi-Line, www.kiepenkerl.com), were grown in the "Newe Ya'ar" Research Center in northern Israel, under standard field irrigation and fertigation conditions. Freshly harvested 7−12-week-old carrot roots were crushed in liquid nitrogen and stored at −80 °C for carotenoid, norisoprenoid, and transcript analyses.

Carotenoid Analyses. Carotenoids were extracted by grinding fresh carrot roots (1 g) in hexane/acetone/ethanol (EtOH) (2:1:1, v/v/v), followed by 5 min of saponification in 8% (w/v) KOH. The saponified material was extracted twice with hexane, which was then evaporated *in vacuo*. The solid pellet was resuspended in 400 *μ*L of acetonitrile (MeCN)/MeOH/CH_2Cl_2 (45:5:50, v/v/v) and passed through a 0.2 *μ*m Nylon filter before HPLC analyses. Samples for carotenoid extraction were taken from three carrot roots of each of the five different colored varieties.[22]

HPLC Analyses. HPLC analysis was performed according to Ibdah et al.[22] Briefly, 40 *μ*L of filtered extracts were injected into 2996 Waters HPLC equipped with a Waters PDA detector 996, a C18 Nova-Pak (Waters, Milford, MA) column (250 × 4.6 mm inner diameter; 4 mm), and a Nova-Pak Sentry Guard cartridge (Waters, Milford, MA), operated at a flow rate of 1.5 mL min^{-1} at 30 °C, with the following solvent acetonitrile/methanol/dichloromethane (75:20:5, v/v/v) containing 0.05% (v/v) triethylamine. Detection was performed between 260 and 600 nm. Data were analyzed using the Millenium software.

Volatile Norisoprenoid Analyses. Six 12-week-old mature fresh carrot roots were cut into ca. 0.5 cm^3 pieces. Three replicates, each of 5 g cubes, for each cultivar, were vigorously shaken for 16 h with 50 mL of MTBE containing 1 mg/kg in water of 2-heptanone as an internal standard.[29] The upper phase (MTBE) was dried with Na_2SO_4 and concentrated under a gentle stream of nitrogen to 1.0 mL. A total of 1 *μ*L of each sample was analyzed by gas chromatography−mass spectrometry (GC−MS).

Table 1. Carotenoid Pigments and Norisoprenoid Volatiles in Carrot Varieties at 10 Weeks after Sowing

	carotenoid content (μg g^{-1} of FW)				
color	Nairobi, orange	Rothild, orange	Purple Haze, purple−orange	Yellowstone, yellow	Creme de Lite, white
phytoene	1.36 ± 0.06	1.83 ± 0.12	5.93 ± 0.21	0.03 ± 0.01	0
β-carotene[a]	23.40 ± 0.92	16.2 ± 1.14	26.20 ± 0.87	0.73 ± 0.12	0.23 ± 0.06
α-carotene[a]	16.3 ± 0.62	15.3 ± 0.96	23.86 ± 0.76	0.20 ± 0.62	0.10 ± 0.1
lutein[a]	2.76 ± 0.09	1.68 ± 0.09	3.57 ± 0.11	4.93 ± 0.62	0.48 ± 0.05
total	43.82 ± 0.42	35.01 ± 0.57	59.56 ± 0.48	5.89 ± 0.34	0.81 ± 0.07
	Norisoprenoid Compounds (ng g^{-1} of FW)				
farnesylacetone[b]	2.35 ± 0.76	2.53 ± 0.39	2.17 ± 0.48	nd[c]	nd
β-ionone[b]	0.14 ± 0.34	0.12 ± 0.19	0.21 ± 0.32	nd	nd
α-ionone[b]	0.09 ± 0.62	0.08 ± 0.72	0.12 ± 0.53	nd	nd
β-cyclocitral[b]	0.015 ± 0.19	0.013 ± 0.86	0.03 ± 0.71	nd	nd

[a]Carotenoid standards were identified on the basis of commercial standards. [b]Norisoprenoid compounds were identified by GC−MS based on reference volatiles. [c]nd = not detected.

Isolation and Characterization of Carrot Carotenoid Cleavage Dioxygenase 1. Putative carrot CCD encoding genes were searched using a homology-based algorithm in the RoBuST root and bulb genome database of Apiaceae (http://robust.genome.com), which has been assembled on the basis of available GenBank sequence data entries.[30] In this study, a single full-length gene was putatively identified as *D. carota* carotenoid cleavage dioxygenase 1 (*Dc*CCD1). Two specific primers corresponding to the *Dc*CCD1 coding sequence 5′ end (5′-ATG GGG GTG ACA GAG CAC GA-3′) and 3′ end (5′-CAG TTT GGC TTG TTC TTG AAG TTG TTC C-3′) were designed. RNA from carrot root was isolated using the Spectrum Plant Total RNA Kit (Sigma-Adlich). To produce a cDNA clone, 5 μg of total RNA from the Nairobi (orange) cultivar was reverse-transcribed using the SuperScript One-Step reverse transcription polymerase chain reaction (RT-PCR). The DNA molecule was then amplified using Platinum *Taq* DNA polymerase (Invitrogen), yielding a ∼1644 base pair (bp)-specific fragment. The cDNA was ligated into the pEXP5-CT/TOPO TA expression vector (Invitrogen Corporation, Carlsbad, CA), producing pEXP-*DcCCD1*, in which the *Dc*CCD1 coding sequence was fused with a His-tag-coding extension at the C terminus and transformed into *E. coli* Top10 cells. The constructs were verified by DNA sequencing.

Functional Expression Experiments and Determination of Volatiles from Bacterial Headspace. The expression construct pEXP-*DcCCD1* was introduced into *E. coli* strains engineered to accumulate phytoene (pBCAR-EB), lycopene (pBCAR-EBI), β-carotene (pBCAR-EBIY), δ-carotene (pDCAR), and zeaxanthin (pZEAX).[31,32] An overnight *E. coli* starter culture (0.5 mL) was used to inoculate 3 mL of Luria-Bertani (LB) medium containing the appropriate antibiotics in 20 mL tightly closed screw cap vials for solid-phase microextraction (SPME). The vials were incubated at 37 °C with shaking (250 rpm) until an OD_{600} of 0.6 was reached. After 0.5 mM IPTG was added, the vials were incubated at 20 °C for 20 h and shaken (250 rpm). A SPME probe was introduced to the vial through a septum, and the headspace volatiles were allowed to adsorb to the fiber at 45 °C for 30 min. Subsequently, the SPME fiber was introduced into GC and analyzed.[22]

GC−MS Analyses. The volatile compounds collected from the headspace or root MTBE extracts were analyzed on a Hewlett Packard (HP) gas chromatography distillation (GCD) apparatus equipped with a HP-5 (30 m × 0.25 mm) fused-silica capillary column. He (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 200 °C at a rate of 4 °C min^{-1}. The detector temperature was 280 °C. The mass range was recorded from m/z 45 to 450, with electron energy of 70 eV. Identification of the main components was performed by comparison of mass spectra and retention time data to those of authentic standards and supplemented with a Wiley GC−MS library.

***Dc*CCD1 Transcript Analysis.** For RT-PCR and real-time RT-PCR analysis of *DcCCD1*, total RNA (5 μg) from different colored carrot cultivars was extracted (Spectrum Plant Total RNA Kit, Sigma-Adlich) and reverse-transcribed using an oligo primer and the SuperScript II first-strand system (Invitrogen).

For RT-PCR analysis, a *Dc*CCD1 fragment was specifically amplify using gene-specific forward (5′-ATG GGG GTG ACA GAG CAC GA-3′) and reverse (5′-CAG TTT GGC TTG TTC TTG AAG TTG TTC C-3′) primers. The following tubulin primers were used as positive controls: tubulin forward primer (5′-GCT CTT TAC GAT ATC TGC TTC AGG AC-3′) and tubulin reverse primer (5′-ATT CCC TCT CCG GTA TAC CAA TGC-3′).

Real-time RT-PCR was performed on an Applied Biosystem StepOnePlus Real-Time PCR Systems (Life Technology) using ABsolute Blue qPCR SYBR Green ROX Mix (Tamar Laboratory Supplies, Ltd., Israel), using 5 ng of reverse-translated total RNA and 100 ng of primers. Primers for *DcCCD1* were *Dc*CCD1_F_qPCR (5′-CTTTGCGCCTGTGGATGAG-3′) and *Dc*CCD1_R_qPCR (5′-TGATGCGTAATCCATGAATCATG-3′). A relative quantification of gene expression was performed using the housekeeping gene tubulin from carrot as a reference gene. The primers used for tubulin were Tubulin_F_qPCR (5′- TCTTGGAGGTGGCACAGGAT-3′) and Tubulin_R_qPCR (5′-ACCTTAGGAGACGGGAACACAGA-3′). The difference in relative expression levels of *Dc*CCD1 was calculated from the $2^{-\Delta\Delta C_T}$ value after normalization of *Dc*CCD1 data to tubulin. All analyses were performed using three technical replicates.

RESULTS AND DISCUSSION

Carotenoid Pigmentation Patterns of Carrot Roots Influence Norisopernoid Volatile Composition. Previous studies have indicated that carotenoid composition affects

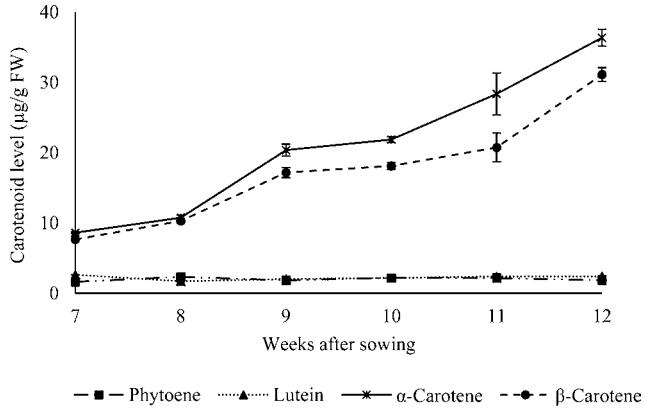

Figure 2. Carotenoid accumulation during carrot root development in the orange cultivar Nairobi. Values are means ± standard error (SE) (n = 4).

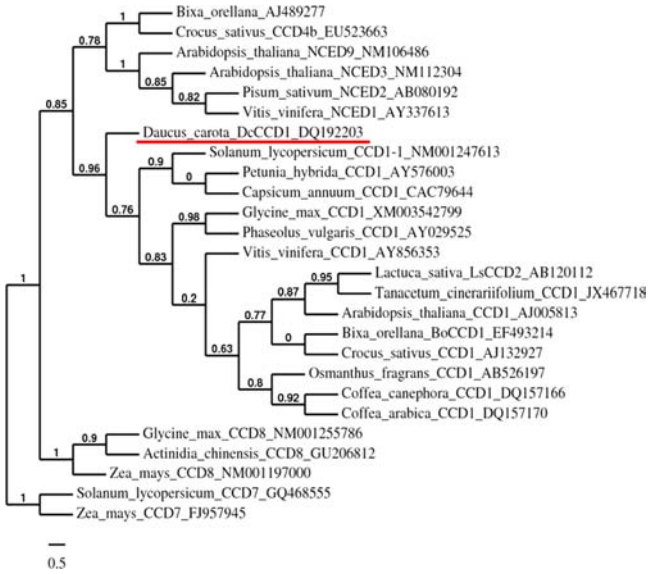

Figure 3. Phylogenetic tree of deduced amino acid sequences of carotenoid cleavage enzymes involved in the cleavage of carotenoids (C_{40}) or C_{27} apocarotenoids (CCD1, CCD4, CCD7, and CCD8) at the 9 and 10 (9′ and 10′) double bond. The sequences were aligned using phylogeny analysis (http://www.phylogeny.fr). The evolutionary history was inferred using the Neighbor-Joining methods and drawn by TreeView. The red bold underline indicates the carrot *DcCCD1* gene identified in this study.

norisoprenoid volatiles in tomato, watermelon, melon, and pepper.[21−23] However, to the best of our knowledge, the influence of carotenoid composition on volatile norisoprenoid accumulation in carrot roots has never been shown. Therefore, we analyzed the norisoprenoid volatiles and the carotenoid content of five carrot cultivars of various colors using GC−MS and liquid chromatography−mass spectrometry (LC−MS), respectively.

The total carotenoid levels of the orange carrots, represented here by Nairobi and Rothild cultivars, ranged from 35 to 43 μg g^{-1} of fresh weight (FW) (Table 1). The yellow carrot variety, Yellowstone, accumulated predominantly lutein (4.9 μg g^{-1} of FW) along with much lower amounts of β- and α-carotenes, while the white cultivar, Creme de Lite, accumulated only traces of carotenoids (0.8 μg g^{-1} of FW) (Table 1). Conversely, the Purple Haze variety tested in this study contained the highest level of total carotenoids (59.56 μg g^{-1} of FW) (Table 1). Although carrot roots differing in colors often contain different carotenoids as major pigments (Table 1), these differences in carotenoid content can only partially explain the different colors displayed by the carrot roots. It has been reported that Purple Haze accumulates, in addition to carotene pigments, a purple anthocyanin[33,34] that likely contributes to its purple color. We nevertheless conclude that there is a clear difference in the carotenoid accumulation patterns between the different colored carrot varieties (Table 1).

Our analyses unveiled the presence of different norisoprenoids in carrot roots according to the variety analyzed and apparently associated with their carotenoid compositions (Table 1). The highest levels of norisoprenoid volatiles are found in Rothild, which contains a high carotenoid content (Table 1). Nairobi also has a high carotenoid content. Farnesyl acetone, a norisoprene derived from the colorless carotenoid biosynthetic intermediates phytoene or phytofluene (Figure 1),[1] was identified primarily in Nairobi, Rothild, and Purple Haze cultivars, which accumulate relatively high levels of carotenoids (Table 1).

Of particular interest is the fact that the levels of α-ionone, β-ionone, and β-cyclocitral, which were derived from the breakdown of α- and β-carotenes (Table 1 and Figure 1), were more plentiful in Nairobi, Rothild, and Purple Haze carrots and correlate with higher β-carotene concentrations in these cultivars. In contrast, the Yellowstone and Creme de Lite carrots that are yellow and white accumulate much lower norisoprenoid levels in accordance with their carotenoid pigment contents (Table 1). α- and β-ionone are widely found in plants and often contribute to flower and fruit aromas.[35,36] While the α-ionone odor threshold is higher (20−40 μg/kg in water) than its isomer β-ionone (0.007 μg/kg in

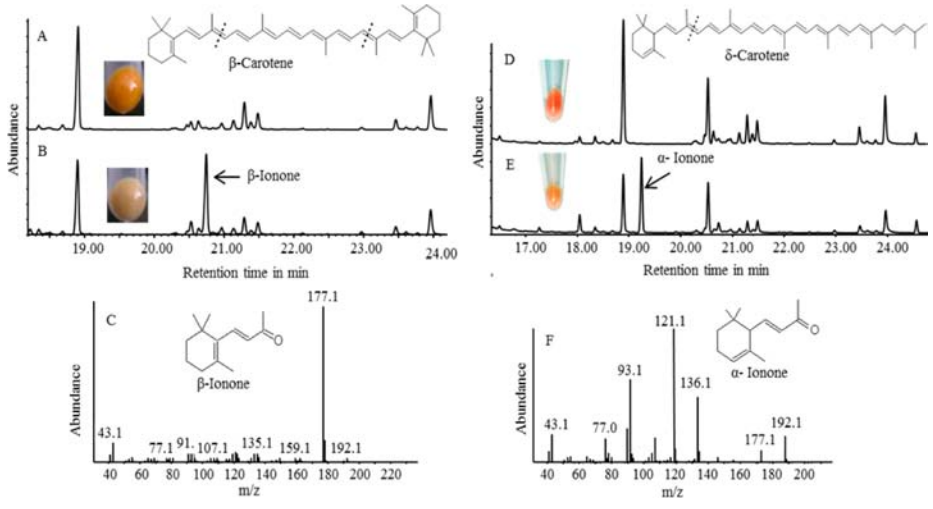

Figure 4. Functional expression of *DcCCD1* in *E. coli* cells previously engineered to accumulate β- or δ-carotene. GC−MS analysis of *Dc*CCD1 activity after transformation in *E. coli* cells previously engineered to accumulate β- and δ-carotene. (A and D) GC−MS analysis of bacterial pellets of *E. coli* cells engineered to accumulate α- and δ-carotene. (B and E) GC−MS analysis of *Dc*CCD1 activity after transformation in *E. coli* cells previously engineered to accumulate β- or δ-carotene. (C and F) Mass spectra of peaks are shown to be identical to those of authentic β- and α-ionone.

Table 2. Norisoprenoids Formed *in Vitro* during Co-expression of *DcCCD1* in Carotenoid Producing *E. coli* Strains

		norisoprenoids present in headspaces of bacterial cultures			
plasmid	carotenoid	geranyl acetone	pseudoionone	β-ionone	α-ionone
pBCAR-EB	phytoene	–	–	–	–
pBCAR-EB + *DcCCD1*		–	–	–	–
pBCAR-EBI	lycopene	–	–	–	–
pBCAR-EBI + *DcCCD1*		–	–	–	–
pBCAR-EBIY	β-carotene	–	–	–	–
pBCAR-EBIY + *DcCCD1*		–	–	++	–
pZEAX	zeaxanthin	–	–	–	–
pZEAX + *DcCCD1*		–	–	+	–
pDCAR	δ-carotene	–	–	–	–
pDCAR + *DcCCD1*		–	–	–	+

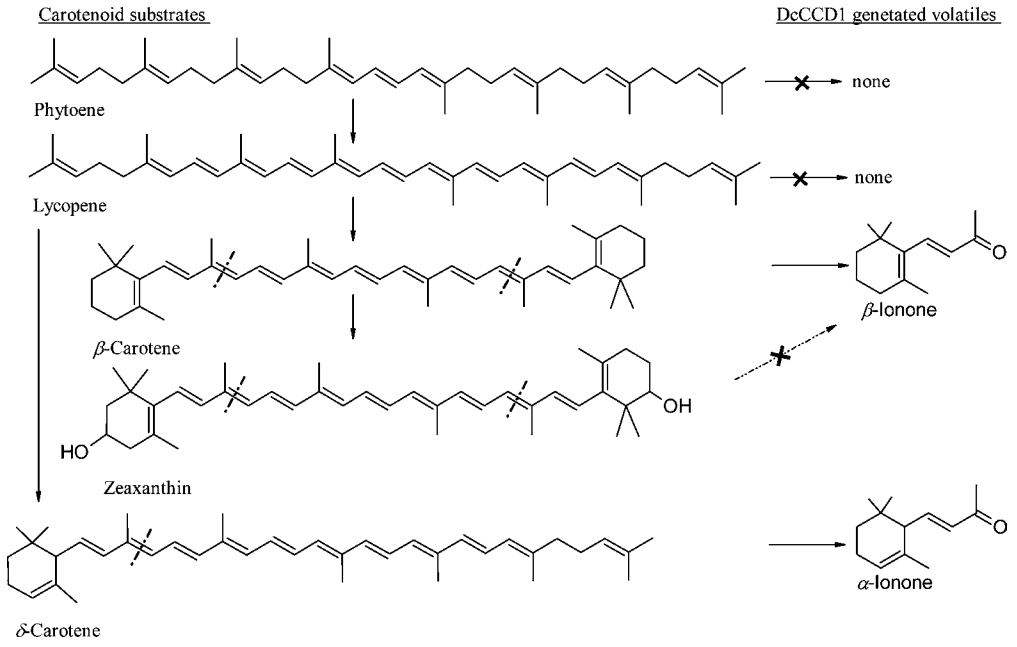

Figure 5. Proposed sites of *Dc*CCD1 bond cleavage and the volatiles generated. An abbreviated version of the carotenoid biosynthetic pathway in higher plants is shown. Carotenoid substrates (left) are oxidatively cleaved by *Dc*CCD1 to yield the norisopernoid derivatives (right). Dashed lines indicate sites of the *Dc*CCD1 cleavage. In cultures producing zeaxanthin, we found the release of β-ionone and not 3-hydroxy-β-ionone probably because β-carotene is an intermediate to zeaxanthin and cleaved before its hydroxylation. *Dc*CCD1 cleaves only the cyclic moiety of δ-carotene and the β rings of β-carotene and is apparently unable to cleave acyclic carotenoids, such as phytoene and lycopene.

water),[20] α-ionone is inferred to have a minor flavor impact in carrots compared to β-ionone.

Although carotenoid levels were ca. 1000-fold higher than their norisoprenoid derivatives, our results demonstrate an association between the β-carotene content and structurally related norisoprenoids. This further corroborates previous observations in melon,[22] watermelon, tomato,[1,23] and pepper,[21] indicating an association between carotenoid pigmentation and aroma volatile content. Buttery et al.[20] reported β-ionone to account for only 0.03% of the total raw carrot volatile content, but given its very low odor threshold (i.e., 0.007 μg/kg in water), they inferred that it may still contribute to the overall flavor of raw carrots. The relatively lower odor threshold for β-cyclocitral (Table 1) suggests a lower flavor impact for these norisoprenoids in carrot overall flavor.

Our above-described data suggest that norisoprenoid volatiles found in the different colored carrot roots are indeed derived from the oxidative degradation of carotenoids, further corroborating that the carotenoid degradation pathway is a key route for the formation of aroma compounds in carrots and many other plants.[12,13]

Analysis of Carotenoid Levels during Root Development. To better understand the patterns of carotenoid accumulation during carrot root development, we analyzed the carotenoid content of the orange variety Nairobi from the 7th and 12th weeks after sowing (Figure 2). At the 7th week, the Nairobi cultivar exhibited relatively small roots (about 5−7 cm in length). HPLC measurements at this stage show significant yet low total carotenoid levels (Figure 2), i.e., 8.6 μg g^{-1} of FW for α-carotene, 7.6 μg g^{-1} of FW for β-carotene, 2.63 μg g^{-1} of FW for lutein, and 1.62 μg g^{-1} of FW for phytoene. Carotenoid accumulation is steady during development. On the 12th week after sowing, the levels of α- and β-carotene contents rose to 36 and 31 μg g^{-1} of FW, respectively (Figure 2). These findings indicate that the levels of α- and β-carotenes consistently increase during carrot root development (Figure 2), while the levels of lutein and phytoene accumulation observed remain unchanged.

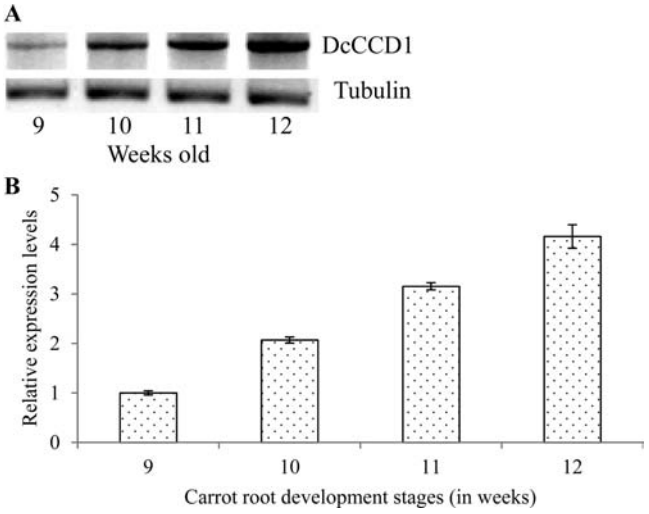

Figure 6. Expression patterns of *DcCCD1* during carrot root development from 9 to 12 weeks after sowing in the orange carrot cultivar Nairobi. (A) RT-PCR analysis of RNA isolated from different developmental stage of carrots. Tubulin was used as a positive control. (B) Quantification of *DcCCD1* transcript levels by real-time RT-PCR analysis normalized to equal levels of tubulin transcripts. All analyses were performed using three technical replicates.

Identification of *Dc*CCD1. The oxidative cleavage of carotenoids is mainly catalyzed by CCDs.[12,13] Despite the presence of large amounts of carotenoids in carrot roots, CCDs and their encoding genes had not been isolated and characterized. Moreover, the role of CCDs in the formation of norisoprenoids and their flavor properties in carrots has not been reported. To identify a gene responsible for norisoprenoid formation in carrot root, we examined the RoBuST root and bulb genome database for Apiaceae for genes exhibiting similarity to known CCD sequences from other plants.

Data mining of the RoBuST root and bulb genome database for Apiaceae (http://robust.genome.com) resulted in identification of clone DAC_79200_005, displaying high sequence similarity to other plant carotenoid cleavage dioxygenases (NCBI Genebank accession number DQ192203). The clone, designated *Dc*CCD1, originated from a root expressed sequence tagged (EST) library of *D. carota* subspecies *sativus*. The predicted *Dc*CCD1 protein sequence consists of 547 amino acids, with a calculated molecular mass of 61.5 kDa. The deduced amino acid sequence of *Dc*CCD1 contains two conserved domains, which are typically found in enzymes involved in the biosynthesis of norisoprenoids.[37]

*Dc*CCD1 protein displays a high sequence similarity (82.8% identity) to the *Cc*CCD1 from *Coffea canephora* and a high sequence similarity (82.2% identity) to *Ca*CCD1 from *Coffea arabica*, which encode enzymes that cleave several types of carotenoids at the 9 and 10 (9′ and 10′) positions, forming various types of norisoprenoids.[38] *Dc*CCD1 is also similar to *Osmanthus fragrans Of*CCD1 (82.2%),[39,40] *Lactuca sativa Ls*CCD1,[41] *Solanum lycopersicum Le*CCD1A (81.2%),[42] *Petunia hybrida Ph*CCD1 (80.9%),[43] *Arabidopsis thaliana At*CCD1 (78.2%),[44] *Crocus sativus Cs*CCD1 (78.8%),[19] and *Vitis vinifera Vv*CCD1 (83.7%),[45] which encode enzymes capable of cleaving several carotenoids at the 9 and 10 (9′ and 10′) positions to form norisoprenoids. Phylogenetic analyses showed the protein encoded by this cDNA cluster with other plant CCD1 enzymes (Figure 3).

Cloning and Expression of *Dc*CCD1 Encoding cDNA. To determine whether *DcCCD1* encodes a functional CCD, the cDNA was cloned into bacterial expression vector pEXP5-CT/Topo and the recombinant *Dc*CCD1 protein was expressed in an *E. coli* strain that accumulates β-carotene.[31,32] Colonies of this β-carotene-accumulating *E. coli* strain develop an orange color (Figure 4A). Loss of color upon induction of *Dc*CCD1 indicates that β-carotene is metabolized to colorless compounds (Figure 4B). This discoloration did not occur when an empty vector was transformed into a β-carotene-producing *E. coli* (Figure 4A). To characterize the cleavage products of the reaction, the volatile enzymatic product of the cleavage of β-carotene was further analyzed by SPME−GC−MS. β-Ionone could be detected in the headspace of the culture (Table 2 and Figure 4C), as evidenced by its retention index (RI) and a mass spectrum identical to that of authentic β-ionone (Figure 4C). β-Ionone was absent in *E. coli* cells transformed with control plasmids devoid of the *DcCCD1* gene (Table 2 and Figure 4C). These results are consistent with previously reported *in vitro* results for the orthologous enzyme *At*CCD1, which cleaves carotenoids (β-carotene, lutein, zeaxanthin, and *trans*-violaxanthin) at the same position as *Dc*CCD1,[44] and other homologues to *Dc*CCD1, such as *C. canephora* and *C. arabica*,[38] *O. fragrans*,[39,40] *S. lycopersicum*,[42] and *Cucumis melo*.[22]

Substrate Specificity of Recombinant *Dc*CCD1. To further characterize the substrate specificity of *Dc*CCD1, we overexpressed it in *E. coli* cells harboring plasmids conferring various carotenoid-biosynthesizing capacities, including phytoene, lycopene, δ-carotene, and zeaxanthin.[31,32] No isoprenoid-derived volatiles were identified in the culture synthesizing acyclic carotenoids, such as phytoene and lycopene, by GC−MS headspace analyses (Table 2 and Figure 5). To ensure that *Dc*CCD1 did not cleave these acyclic carotenoids and generate non-volatile products, the phytoene- and lycopene-accumulating *E. coli* cultures expressing *Dc*CCD1 were extracted and analyzed by HPLC. The phytoene and lycopene carotenoid levels of the *Dc*CCD1-expressing cultures were identical to controls harboring empty vectors and not overexpressing *Dc*CCD1 (see Supplemental Table 1 of the Supporting

Table 3. Norisoprenoid Volatile Accumulation during Carrot Root Development (9-12 Weeks after Sowing) in the Orange Cultivar Nairobi[a]

	norisoprenoid compounds (ng g^{-1} of FW)			
weeks old after sowing	9	10	11	12
farnesyl acetone	2.17 ± 0.19	2.35 ± 0.35	2.50 ± 0.30	2.52 ± 0.42
β-ionone	0.135 ± 0.21	0.141 ± 0.34	0.154 ± 0.29	0.23 ± 0.14
α-ionone	0.081 ± 0.12	0.091 ± 0.62	0.115 ± 0.18	0.144 ± 0.11
β-cyclocitral	0.013 ± 0.09	0.015 ± 0.19	0.017 ± 0.11	0.025 ± 0.08

[a]Values are means ± SE (n = 3).

Information). Moreover, no new non-volatile products were found. Our results confirm that *Dc*CCD1 did not cleave phytoene or lycopene at a detectable rate. Similarity, Vogel et al.[46] reported that *Zm*CCD1, a CCD isolated from maize did not cleave some acyclic carotenoids, such as phytoene, but did cleave other acyclic carotenoids, e.g., lycopene. These results are different from the previously reported *in vitro* and *in vivo* results for the orthologous rice CCD1 enzyme that converts lycopene into three different volatiles, pseudoionone, 6-methyl-5-heptene-2-one, and geranial.[47]

Nevertheless, a comparison of the maize VP14 structure with *Dc*CCD1 and other plant CCD1 models suggests that substitution of some amino acids could lead to the difference in substrate specificity.[37] Substitution of H-493 and A-504 of *Dc*CCD1 by T-553 and L-505 of VP14, respectively, could lead to that specificity for cyclic carotenoids (see Supplemental Figure 1 of the Supporting Information), whereas CCD1 of the other plants used for this alignment have either Val or Phe at these positions. This is in contrast to the carrot CCD characterized here that is inactive toward both phytoene and lycopene (see Supplemental Figure 1 of the Supporting Information).

There are multiple norisoprenoid volatiles that are not derived from 9 and 10 bond cleavage, and the enzyme(s) responsible for their production are not known, including farnesyl acetone. Farnesyl acetone is only present in substantial levels in carotenoid-rich cultivars and could probably be formed from phytoene or phytofluene by a yet unidentified additional CCD enzyme or a substrate not tested here. Identifying the enzymes and substrates that lead to the production of these compounds is essential to understand carotenoid metabolism and the biological role of norisoprenoid production in carrots.

To further investigate the substrate specificity of *Dc*CCD1, we co-expressed the *DcCCD1* gene in a strain of *E. coli* accumulating the asymmetric monocyclic carotenoid δ-carotene. As expected, colonies of the δ-carotene-accumulating *E. coli* cell strain develop a yellow−orange pigment, as determined visually and by GC−MS analysis (Figure 4D). Loss of the yellow−orange color attributed to δ-carotene upon induction of *Dc*CCD1 expression in *E. coli* indicates that δ-carotene is metabolized to a reduced color compound (Figure 4E). Interestingly, the GC−MS data demonstrated that a compound identified as α-ionone was detected in the headspace of these cells (Figure 4E), exhibiting RI and a mass spectrum identical to that of authentic α-ionone (Figure 4F), whereas a corresponding pseudoionone peak (a putative cleavage product of the acyclic end of δ-carotene) was not observed (Table 2). Both pseudoionone and α-ionone were not detected in *E. coli* cells transformed with control plasmids devoid of the *DcCCD1* gene (Figure 4D). These results indicated that *Dc*CCD1 seems only to be capable of cleaving the 9 and 10 positions from the cyclic moiety of carotenoids to form α-ionone but not from the acyclic moiety of δ-carotene to form pseudoionone.

DcCCD1 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 SPME−GC−MS. Interestingly, 3-hydroxy-β-ionone, the expected oxidative cleavage product of zeaxanthin, could not be detected in the headspace of these cultures, but instead, β-ionone was prominent (Table 2 and Figure 5). This β-ionone could result from cleavage of β-carotene formed in the way to zeaxanthin, suggesting that *Dc*CCD1 may compete for the β-carotenoid substrate with the β-carotene hydroxylases from *E. coli* strain carrying that gene, the hydroxylase that catalyzes the formation of zeaxanthin in these cultures.[31,32] Similar observations (presence of β-ionone and not 3-hydroxy-β-ionone) have been noted in previous studies of expression of the *MdCCD4* gene from *Malus* × *domestica*, *CmCCD4a* gene from *Chrysanthemum* × *morifolium*, *RdCCD4* gene from *Rosa damascene*, and *Of CCD4* gene from *O. fragrans*,[48] as well as from *RdCCD1* gene from *R. damascene*[49] and *CmCCD1* gene from melon, in zeaxanthin-producing *E. coli* cells (Ibdah et al., unpublished). In conclusion, our substrate specificity analysis indicated that *Dc*CCD1 can apparently cleave only β-carotene and the cyclic moiety of δ-carotene comparable to non-cyclic carotenoids, such as phytoene and lycopene.

Expression Patterns of *Dc*CCD1 in Developing Carrot Roots. The expression pattern of the *DcCCD1* gene was examined by semi-quantitative RT-PCR and real-time RT-PCR in developing 9−12-week-old root tissues of Nairobi cultivar. *DcCCD1* transcript was detected in all developmental root stages, with increased levels during root maturation, reaching maximum levels in 12-week-old roots (Figure 6A). The results were further quantified by real-time RT-PCR analyses (Figure 6B). *DcCCD1* was upregulated during carrot root development in the orange carrot cultivar Nairobi. The expression level in developmental carrot roots were 2−4-fold higher than the level found in the early stage of carrot root development (9 weeks) (Figure 6B).

To determine whether *Dc*CCD1 expression patterns correlate to the norisoprenoid volatile accumulation in developing carrot roots, the total norisoprenoid levels of the orange carrots, represented here by Nairobi cultivar, were analyzed in developing roots at 9−12 weeks old after sowing. The four norisoprenoid volatile compounds, farensyl acetone, β-ionone, α-ionone, and β-cyclocitral, were detected in all developmental root stages, with consistently increasing levels during carrot root maturation, reaching maximum levels in 12-week-old roots (Table 3). To conclude, our expression patterns of *Dc*CCD1 analysis indicated that *Dc*CCD1 gene expression clearly correlates well with the release norisoprenoid volatiles in developing carrot roots.

In conclusion, five norisoprenoid volatile compounds showed variation among the orange (Nairobi), orange (Rothild), purple (Purple Haze), yellow (Yellowstone), and white (Creme de Lite) carrot genotypes. *Dc*CCD1 was observed to cleave cyclic carotenoids, such as β-carotene and the cyclic moiety of δ-carotene, but not acyclic carotenoids, such as phytoene and lycopene, as well as the acyclic moiety of δ-carotene. On the basis of the pattern of norisoprenoid volatiles detected by GC−MS analysis and their odor thresholds, we suggest that CCD1 cleavage products, namely, norisoprenoids, may contribute to carrot flavors and aroma.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Lycopene content (g g^{-1} of FW) *in vitro* during co-expression of *Dc*CCD1 in lycopene-producing *E. coli* strains (Supplemental Table 1) and translated sequence alignment of VP14 with *Dc*CCD1 from carrot and other plant CCDs (Supplemental Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

CCD, carotenoid cleavage dioxygenase; *Dc*CCD, *Daucus carota* carotenoid cleavage dioxygenase; EtOH, ethanol; FW, fresh weight; GC−MS; gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MeCN, acetonitrile; MTBE, methyl *tert*-butyl ether; SPME, solid-phase microextraction

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