The Carotenoid Cleavage Dioxygenase 1 Enzyme Has Broad Substrate Specificity, Cleaving Multiple Carotenoids at Two Different Bond Positions

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In many organisms, various enzymes mediate site-specific carotenoid cleavage to generate biologically active apocarotenoids. These carotenoid-derived products include provitamin A, hormones, and flavor and fragrance molecules. In plants, the CCD1 enzyme cleaves carotenoids at 9,10 (9’,10’) bonds to generate multiple apocarotenoid products. Here we systematically analyzed volatile apocarotenoids generated by maize CCD1 (ZmCCD1) from multiple carotenoid substrates. ZmCCD1 did not cleave geranylgeranyl diphosphate or phytoene but did cleave other linear and cyclic carotenoids, producing volatiles derived from 9,10 (9’,10’) bond cleavage. Additionally the Arabidopsis, maize, and tomato CCD1 enzymes all cleaved lycopene to generate 6-methyl-5-hepten-2-one. 6-Methyl-5-hepten-2-one, an important flavor volatile in tomato, was produced by ZmCCD1 cleavage at the 11,12 double bond of neoxanthin and/or violaxanthin. This 9-cis-epoxycarotenoid dioxygenase (NCED) generates abscisic acid via asymmetrical cleavage at the 11,12 double bond of neoxanthin and/or violaxanthin. NCED genes have been identified in a variety of plant species (9–13). Using sequence similarity to VP14, the 15,15'-dioxygenases responsible for vitamin A biosynthesis were identified from Drosophila (14) and humans (15). This and other work reveals that carotenoid-cleaving enzymes are an ancient and highly conserved family with members present in plants, animals, and bacteria.

Apocarotenoids are terpenoid compounds derived from the oxidative cleavage of carotenoids (1). They are generated when double bonds in a carotenoid are cleaved by molecular oxygen, forming an aldehyde or ketone in each product at the site of cleavage. Carotenoids can be cleaved at any of their conjugated double bonds, resulting in a diverse set of apocarotenoids. This structural diversity is the consequence of the large number of carotenoid precursors (more than 600) and subsequent modifications such as oxidation, reduction, and conjugation. Although apocarotenoid formation can also occur via nonspecific oxidation, biologically active forms with regulatory functions are expected to be generated via site-specific cleavage.

Apocarotenoids are widely distributed in nature and serve important biological functions. Examples of biologically active apocarotenoids include retinoids in animals (2), trisporic acid in fungi (3), and abscisic acid in higher plants (4). A variety of other biologically important compounds are believed to be derived by oxidative cleavage of carotenoids. Among these compounds are those associated with mycorrhizal colonization, including mycorradicin (5), blumenin (6), and strigolactone (7).

Various enzymes mediate the site-specific carotenoid cleavage needed to generate biologically active apocarotenoids. The founding member of the carotenoid-cleaving enzymes is maize VIVIPAROUS14 (VP14) (8, 9). This 9-cis-epoxycarotenoid dioxygenase (NCED) generates abscisic acid via asymmetrical cleavage at the 11,12 double bond of neoxanthin and/or violaxanthin. NCED genes have been identified in a variety of plant species (9–13). Using sequence similarity to VP14, the 15,15'-dioxygenases responsible for vitamin A biosynthesis were identified from Drosophila (14) and humans (15). This and other work reveals that carotenoid-cleaving enzymes are an ancient and highly conserved family with members present in plants, animals, and bacteria.

The Arabidopsis thaliana genome contains nine genes similar to VP14, five of which appear to be involved in abscisic acid biosynthesis (16, 17). The four remaining genes have been generically termed carotenoid cleavage dioxygenase-like (CCD) to distinguish them from abscisic acid biosynthetic enzymes. Two CCD enzymes produce an as yet unidentified apocarotenoid hormone involved in shoot branching (AtCCD7/MAX3 and AtCCD8/MAX4) (18–20). The function(s) of the remaining two, CCD1 and CCD4, is less obvious. The present work focuses on the biochemical activity of CCD1.

CCD1 is a non-heme enzyme, requiring only Fe3+ as a cofactor. It contains four conserved histidines that are thought to coordinate iron binding. CCD1 uses oxygen to cleave a variety of carotenoid substrates symmetrically at the 9,10- and 9’,10’-positions, generating (di)aldehydes and ketones (21). Until recently, controversy existed as to whether CCD1 cleavage occurred via a mono- or dioxygenase mechanism. Isotope labeling experiments have now demonstrated a dioxygenase mechanism (22).
The aldehydes and ketones generated by CCD1 are important flavor and fragrance volatiles. Tomato CCD1 generates the flavor volatiles geranylacetone, p-menthaione, and p-ionone (23). In petunia flowers, CCD1 controls emission of the fragrance and pollinator attractant p-ionone (24). CCD1 enzymes have been isolated and shown to generate flavor volatiles from multiple plant species, including grape (25), melon (26), crocus (27), citrus (28), and coffee (29). Additionally, Arabidopsis ccd1 mutants have increased seed carotenoid content, suggesting a role in carotenoid turnover (30). CCD enzymes have also been implicated in generating signaling compounds involved in mediating mycorrhizal relationships (31). Hence, deciphering CCD1 function holds promise for increasing crop carotenoid content, altering volatiles contributing to taste, and understanding arbuscular mycorrhizal symbiosis.

Such an understanding has important implications for human health. Carotenoids with provitamin A activity such as p-carotene, a-carotene, and p-lycopene are essential in the human diet (32), and other carotenoids, such as lycopene, are associated with a decreased risk of prostate cancer (33). Increasing these beneficial carotenoids in the human diet has attracted much attention, and manipulating their metabolism would contribute greatly to this goal.

Despite the growing number of studies, no effort has been undertaken to measure CCD1-generated volatiles in a systematic and quantitative manner mainly because such studies are extremely difficult to perform in vitro. Presenting the enzyme with substrate is problematic as carotenoids are extremely hydrophobic. Although there has been some limited success using natural carotenoids, most in vitro assays have utilized the more soluble synthetic substrate p-apo-8'-carotenal (22, 34, 35). In all cases, water-miscible solvents and/or micellar systems are needed for activity. As such, CCD enzymes have generally been expressed in Escherichia coli engineered to accumulate various carotenoids.

Measuring CCD1-generated volatiles in a systematic and quantitative manner would provide insight into which carotenoids are the preferred substrate in planta. In the work presented, we expressed maize CCD1 (ZmCCD1)3 in E. coli engineered to produce various carotenoids (36–39) and monitored volatile apocarotenoid products. This system provides a flexible method to quickly identify substrates in an environment more closely resembling conditions within plastids. Such work not only sheds light on the function of CCD1 but also provides a foundation for future studies of other carotenoid-cleaving enzymes. There are multiple apocarotenoid flavor and aroma volatiles that are not derived from 9,10 bond cleavage, and the enzyme(s) responsible for their production are not known, including 6-methyl-5-hepten-2-one (MHO) (5,6 cleavage), p-cyclocitral (7,8 cleavage), farnesylacetone (13,14 cleavage), and citral (7,8 cleavage). Identifying the enzymes and substrates that lead to the production of these compounds is essential to understanding carotenoid metabolism and the biological role of apocarotenoid products.

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EXPERIMENTAL PROCEDURES

Cloning of ZmCCD1—A near full-length ZmCCD1 cDNA was amplified by reverse transcription-PCR from developing White cap (Wc) kernels of maize (18 days after pollination) with forward primer 5'-CCCTTCGCTACAAGCCTACA-3' and reverse primer 5'-TTCAATACACGTCTTGCAA-3'. The PCR product was cloned into the pCR4-TOPO vector (Invitrogen) and sequenced. The cDNA contained the complete predicted coding sequence of ZmCCD1. To create a fusion of ZmCCD1 to glutathione S-transferase (GST), the ZmCCD1 coding sequence was first amplified with forward primer 5'-GGATCCATGGGGACGGAGGCGGAGCAGCCG-3' and reverse primer 5'-ATGCCCTGCAATATTCTCA-3' using Pfu DNA polymerase and cloned into the pCR-blunt-TOPO vector (Invitrogen). A BamHI/EcoRI fragment from this vector was ligated into the pGEX-2T vector (Amersham Biosciences/GE Healthcare). The construct was sequenced to confirm that the gene was in the correct reading frame.

Volatile Analysis from E. coli Cultures—pGEX2T-ZmCCD1, pGEX2T empty vector, pDEST15-AtCCD1, pDEST15-LeCCD1A, pDEST15-LeCCD1B, or pDEST15-GUS (23, 30) was transformed into chemically competent BL21-AI (Invitrogen) cells harboring plasmids encoding carotenoid biosynthetic genes (36–39). Three individual colonies for each carotenoid strain were used to inoculate three different 3-ml cultures of LB containing 100 µg/ml carbenicillin and 34 µg/ml chloramphenicol (Sigma). The 3-ml cultures were grown overnight at 37 °C. The following day, 1 ml of overnight culture was used to inoculate 100 ml of LB containing 100 µg/ml carbenicillin and 34 µg/ml chloramphenicol in a 250-ml baffled flask. The cultures were grown at 200 rpm for 2.5–3 h at 33 °C in darkness until an absorbance at 600 nm of 0.4–0.6 was achieved. The flask was then chilled on ice for 5 min and induced with a final concentration of 0.1 mM isopropyl b-D-1-thiogalactopyranoside (Sigma) or 0.1% L-arabinose (Fischer Scientific). The cultures were returned to the shaker and grown for an additional 3 h (200 rpm at 25 °C). The cultures were then placed on a bench and capped with a rubber stopper, and partially purified air (the filter consisted of 0.5 mg of activated charcoal in a 0.6 × 10-cm glass tube) was bubbled through the cultures (~175 ml min⁻¹) for 2 h using a dual diaphragm air pump (General Hydroponics, Sebastopol, CA). The volatile head space was collected on a 30-µg Super Q column (80/100 mesh; Alltech Associates, Inc., Deerfield, IL). After volatile collection, the A500 was recorded. The Super Q columns were eluted with 150 µl of methylene chloride (Fischer Scientific) after addition of 400 ng of nonyl acetate (in 5 µl of methylene chloride) as an internal standard. Alternatively cultures were extracted twice with an equal volume of hexanes. The combined hexane extracts were dried with sodium sulfate. Before injection on the gas chromatograph, nitrogen was blown over the samples to evaporate solvent until they reached a volume of ~300 µl. Separation of volatiles was performed on an Agilent DB-5 column (Palo Alto, CA) and an Agilent 6890N gas chromatograph according to Schmelz et al. (40). The volatiles were compared with known standards (Sigma-Aldrich) and calculated as nmol A500⁻¹ h⁻¹. Any background present in control reactions was subtracted, and values
were normalized for percent recovery of each volatile. Percent recovery was calculated as the average amount of volatiles recovered from flasks with 100 ml of LB treated exactly as described above except that at the point of “induction” a known amount of each volatile compound was added. The identity of each peak was confirmed by gas chromatography/mass spectrometry using an Agilent 6890N gas chromatograph with a DB-5 column and an Agilent 5975 mass detector in electron ionization mode.

**Isoprenoid Isolation**—All solvents were purchased from Fisher Scientific. To analyze isoprenoids present in *E. coli* 100-ml LB cultures in 250-ml baffled flasks with the appropriate antibiotics were grown in darkness (200 rpm at 28 °C) to an A80 of 0.9–1.0. The cultures were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside. After induction, the cultures were grown for 4 h (200 rpm at 25 °C) and centrifuged at 5,000 × g for 15 min. The cells were resuspended in 20 ml of cold phosphate-buffered saline and centrifuged again at 5,000 × g for 15 min at 4 °C. The phosphate-buffered saline was poured off, and the cells were frozen overnight at −20 °C. Frozen cells were thawed on ice for 30 min. The cells were resuspended in 10 ml of cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride. The cells were sonicated on ice for 5 min with 5-s bursts (microtip limit of 5, 50% duty cycle). After sonication, 20% Triton X-100 in phosphate-buffered saline was added to a final concentration of 0.2%. The cell slurry was shaken on ice for 20 min before centrifugation at 12,000 × g for 15 min at 4 °C. The supernatant was filtered through a 0.22-μm filter to obtain a cell free lysate. Glycerol was added to a final concentration of 20%, and the lysate was stored in aliquots at −20 °C. Enzyme assays were performed with 100 μl of lysate unless otherwise noted. GST-tagged ZmCCD1 was purified with glutathione-Sepharose resin (Clontech) according to the manufacturer’s directions.

**Protein Blot Analysis**—Samples of bacteria or protein were mixed in 2× SDS-PAGE buffer and loaded onto 10% Ready Gel Tris-HCl (Bio-Rad). Equal amounts of total bacterial protein were loaded based on culture optical density, whereas protein concentration for all other samples was determined by Bio-Rad protein assay reagent. The proteins present in the gel were stained using Bio-Rad Safe Stain or transferred to a nitrocellulose membrane. Protein blot analysis was performed as described previously (42) using GST antibody (Amersham Biosciences/GE Healthcare).

**Enzyme Assays**—A stock solution of the synthetic substrate β-apo-8′-carotenal (Sigma) was prepared by dissolving 0.01 g in 100 ml of 95% ethanol. The stock solution was stored in the dark under nitrogen at −20 °C. To obtain an aqueous stock solution for enzyme assays, 1 ml of 1% polyoxyethylene sorbitan monopalmitate (Tween 40) (Sigma-Aldrich) in 95% ethanol was added to 3 ml of 0.1 mg/ml β-apo-8′-carotenal. The mixture was vortexed vigorously and dried under a stream of nitrogen. 1.2 ml of 50 mM NaPO4 (pH 7.2) was added and vortexed to obtain a 250 μg/ml solution. *In vitro* assays were performed in a total volume of 1 ml containing 50 mM NaPO4 (pH 7.2), 300 mM NaCl, 5 μM FeSO4, 5 mM ascorbate, 20% methanol, and 30 μg of β-apo-8′-carotenal. The final concentration of Tween 40 in the assay was 0.1%. Water was added to bring the total volume to 0.9 ml, and the assays were mixed and allowed to adjust to room temperature for 5 min. Then 100 μl of lysate or purified protein
was added. Each assay was mixed with gentle swirling and placed in the dark for 30 min at 30 °C.

Assays with lycopene and/or β-carotene were set up in an identical manner except that chloroform was used as the solvent. 1 mg of carotenoid was dissolved in 1 ml of chloroform. The concentration of each stock solution was calculated from known extinction coefficients by measuring the absorbance at 453 or 472 nm (43). Based on the number of assays performed, an appropriate amount of carotenoid was added to 1% Tween 40 diluted in chloroform. This solution was vortexed, and 30 μg of carotenoid was aliquoted into individual glass vials. The carotenoid/Tween-40 solution was dried under a stream of nitrogen. The residue was dissolved in 200 μl of methanol, and the remaining assay components were added. Reactions were incubated in the dark for 16 h at 30 °C. Volatiles were collected from assays on a Super Q column by passing purified air (618 ml min⁻¹) over the assays for 1 h. Volatile elution and analysis were performed as described above.

Values from GC analysis were normalized for percent recovery of each volatile. Percent recovery was calculated as the average amount of volatiles recovered from assays spiked with a known amount of each volatile compound. Non-volatile products were isolated as described for the isoprenoid isolation from E. coli pellets. To determine the concentration of carotenoids dissolved in an assay, the control reactions were centrifuged at 14,000 × g for 30 min to remove any precipitate. The soluble fraction was removed, and 0.5 μg of astaxanthin was added as an internal standard. The carotenoids were isolated, resuspended in ethyl acetate, and analyzed via HPLC. Concentrations were calculated compared with standard curves.

RESULTS

ZmCCD1 Cleaves Multiple Carotenoids to Generate Apocarotenoid Volatiles Derived from 9,10 and 5,6 Double Bond Cleavage—To determine the substrate specificities and bond cleavage preferences of ZmCCD1, we undertook a systematic analysis of the volatile products generated by the enzyme in various carotenoid-accumulating strains of E. coli. The volatile products are indicative of these cleavage activities (Table 1). Plasmids encoding various carotenoid biosynthetic enzymes (36–39) were transformed into E. coli, and carotenoid accumu-
loration was verified by HPLC. Cultures expressing ZmCCD1 and accumulating various carotenoids were grown in darkness, induced, and grown for a further 3 h, and the volatile head space was collected. After addition of an internal standard, volatile products were eluted and analyzed by GC and GC/MS. No isoprenoid-derived volatiles were identified from cultures synthesizing GGPP (data not shown) or phytoene (Fig. 1A), although ZmCCD1 was expressed (Fig. 1B). To ensure that ZmCCD1 did not cleave these isoprenoids and generate non-volatile products, the isoprenoids from GGPP and phytoene cultures were extracted and analyzed by HPLC. No new non-volatile products were found, and compared with the control, the levels of each isoprenoid were unchanged (data not shown), confirming that ZmCCD1 did not cleave GGPP or phytoene.

Apocarotenoid volatiles were identified from cultures producing other carotenoid substrates. Geranylacetone was detected from the ζ-carotene-accumulating strain, indicating 9,10 (9',10') double bond cleavage (Fig. 1A). Geranylacetone was also detected in the lycopene-accumulating cultures. Geranylacetone can, in theory, be generated by cleavage of GGPP, ζ-carotene, or phytoene but not lycopene. Given that no geranylacetone was detected from GGPP- or phytoene-accumulating E. coli, the geranylacetone must come from cleavage of ζ-carotene. This result indicates that ZmCCD1 is able to cleave ζ-carotene before conversion into lycopene.

The other two products detected from lycopene-accumulating E. coli were pseudoionone and MHO (Fig. 1A). 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 existence of MHO was confirmed by comparison with an authentic standard via GC/MS (Fig. 2). This 5,6 bond cleavage of lycopene was an unexpected result and specific to lycopene. MHO can theoretically be generated by the cleavage of several carotenoids (Table 1), but this product was not detected from any lycopene precursor.

The apocarotenoid volatiles derived from δ- and β-carotenone-accumulating strains revealed a similar pattern (Fig. 1A). Geranylacetone, pseudoionone, and MHO were detected in both cultures, again consistent with cleavage of precursor carotenoids. In the case of δ-carotene, MHO can result from 5',6' bond cleavage, and pseudoionone can result from 9',10' bond cleavage. α-Ionone was detected in the δ-carotene culture, and β-ionone was detected in the β-carotene culture. Both α- and β-ionone are indicative of 9,10 (9',10') bond cleavage.

The volatiles produced from a zeaxanthin-accumulating strain were also examined. A pattern of volatile production similar to that from the β-carotene-accumulating strain was found with the notable absence of 3-hydroxy-β-ionone, which is the predicted 9,10 cleavage product (Fig. 1A). Because 3-hydroxy-β-ionone has lower volatility, cultures were extracted with hexanes and dried to ~300 µl before injection into a gas chromatograph/mass spectrometer. 3-Hydroxy-β-ionone was identified in the hexane extraction of the ZmCCD1/zeaxanthin cultures by GC/MS. The major ions of 193 and 175, with a parent ion of 208, are indicative of 3-hydroxy-β-ionone (supplemental Fig. 1).

**CCD1 Cleaves Multiple Carotenoids**

![Image](324x373 to 552x733)

**FIGURE 3.** Carotenoid-accumulating E. coli expressing Arabidopsis or tomato CCD1 enzymes produce volatiles derived from 9,10 and 5,6 bond cleavage. Volatiles were collected from E. coli accumulating the carotenoids listed and harboring the indicated plasmids. The quantity of each compound is the average of three replicates (± S.E.). A, volatiles identified from E. coli accumulating lycopene and expressing AtCCD1, LeCCD1A, or LeCCD1B. B, volatiles identified from E. coli accumulating β-carotene and expressing AtCCD1, LeCCD1A, or LeCCD1B. C, protein blot analysis of CCD1 protein expression postvolatile collection. All three proteins are GST fusions. Protein loading was determined by A400. Error bars, ± S.E.

CCD1 Enzymes from Arabidopsis and Tomato Generate MHO from Lycopene—CCD1 has not been reported previously to cleave lycopene to produce MHO. To determine whether this cleavage activity is specific to the maize enzyme, the activity of the Arabidopsis and tomato CCD1 enzymes was examined. When Arabidopsis or tomato CCD1 enzymes were produced in E. coli accumulating lycopene, MHO, geranylacetone, and pseudoionone were generated (Fig. 3A). This result indicates that 5,6 bond cleavage is not restricted to the monocot enzyme but also occurs with enzymes from dicots.

The activities of the Arabidopsis and tomato enzymes were also examined in phytoene- and β-carotene-accumulating E. coli. Geranylacetone and MHO, the predicted 9,10 and 5,6 cleavage products of phytoene, were not detected (data not shown). β-Ionone, geranylacetone, pseudoionone, and MHO were detected, in roughly equivalent levels, with all three enzymes when expressed in β-carotene-accumulating E. coli (Fig. 3B), indicating cleavage of β-carotene and its precursors.
ZmCCD1 cleaves lycopene in vitro. To accomplish this, we first confirmed isolation of active protein. Similar to previous work (35), experiments monitoring volatiles using native, His-tagged, and GST-tagged CCD proteins indicated that GST enhanced both CCD solubility and activity (data not shown). As such, GST-tagged protein was used for further experiments. Recombinant GST-tagged ZmCCD1 protein was produced in E. coli and purified. Staining and protein blot analysis of the protein at each step in the purification revealed large amounts of enzyme present in the cell debris after cell lysis and centrifugation (Fig. 6A, lane 4) as has been observed for other plant CCD1 enzymes (34, 44). As shown previously, addition of Triton X-100 to lysed cells prior to centrifugation increased soluble protein recovery (22, 44).

To confirm recovery of active enzyme, assays were performed using the synthetic substrate β-apo-8′-carotenal. Addition of ZmCCD1 cell lysate to this substrate resulted in a color change from orange to yellow within 30 min at 30 °C (Fig. 5B). The presence of β-ionone in the head space of the assay was confirmed via GC/MS (supplemental Fig. 2) compared with an authentic standard. The non-volatile products were analyzed via HPLC.

The absorbance spectrum of the new peak in the ZmCCD1 assay matched the expected C 17 dialdehyde (Fig. 5C) (34). The purified enzyme (Fig. 5A, lane 9) displayed greatly reduced activity compared with the cell lysate (Fig. 5A, lane 5) even with greater amounts of purified protein (data not shown). The loss of activity was not due to the buffer used to elute the protein as assays with ZmCCD1 lysate containing the elution buffer retained activity. Other membrane-bound and membrane-associated proteins show a similar loss of activity upon purification (45). Alternatively the GST tag could inhibit activity, but extremely low volatile levels from E. coli expressing native or His-tagged CCD enzymes argues against this possibility (data not shown). Additionally Schilling et al. (35) saw a similar decrease in activity of GST-tagged AtCCD1 postpurification that was not recovered by thrombin cleavage. Subsequent assays were performed using total lysate.

ZmCCD1 Cleaves Multiple Carotenoids

The proteins were expressed to equivalent levels (Fig. 3C). The relative amounts of apocarotenoids produced by LeCCD1A are similar to ZmCCD1. LeCCD1B produced greater levels of β-ionone, whereas apocarotenoid volatiles from precursor carotenoids were low. The levels of apocarotenoids generated by AtCCD1 were similar to those from LeCCD1B.

Isoprenoid Levels Are Greatly Decreased in E. coli Expressing ZmCCD1—To identify non-volatile apocarotenoids, the carotenoid-accumulating E. coli were analyzed by HPLC. Compared with the empty vector, carotenoid levels in the η-carotene, lycopene, δ-carotene, β-carotene, and zeaxanthin cultures were significantly reduced (Fig. 4), indicating near complete carotenoid catabolism. There were no new peaks present in the ZmCCD1 cultures compared with the vector controls, indicating that the predicted dialdehyde products could not be detected in the cells probably due to further catabolism. This result is similar to that reported by Schwartz et al. (21) where the dialdehyde products were not present in E. coli in any appreciable amount.

ZmCCD1 Cleaves the Synthetic Substrate β-Apo-8′-carotenal in Vitro, Generating β-Ionone and a C 17 Dialdehyde—To confirm the 5,6 bond cleavage observed in vivo, we tested
DISCUSSION

Characterization of CCD1 and related enzymes has provided valuable insight into how biologically active apocarotenoids are
synthesized, how carotenoids are catabolized, and how important flavor and aroma compounds are generated. Assaying the volatile apocarotenoids generated by ZmCCD1 in E. coli accumulating various carotenoids found in higher plants has provided new and valuable information concerning CCD1 cleavage activity and substrate preference.

ZmCCD1 did not cleave every isoprenoid substrate tested. No volatile apocarotenoids were generated from E. coli accumulating GGPP or phytoene, indicating that ZmCCD1 did not cleave either isoprenoid. This was confirmed by HPLC analysis showing no decrease in either compound. ZmCCD1 did cleave other carotenoids as volatiles indicative of 9,10 (9\',10\') bond cleavage were observed from ζ-carotene, lycopene, δ-carotene, β-carotene, and zeaxanthin. In each strain tested, additional volatiles consistent with cleavage of precursor carotenoids were observed, indicating that ZmCCD1 cleaves precursor carotenoids before they can be converted into the final product.

The high levels of volatiles from precursor carotenoids highlights the limitations of these assays. E. coli cultures are dynamic systems. At any given time, carotenoids are being synthesized and degraded. Hence volatile production per unit of substrate cannot be accurately determined especially over time. Background expression of the recombinant protein only exacerbates this problem. Due to these limitations, it is difficult to infer substrate preference. Instead complementary in vitro assays were performed to address substrate preferences. Because the combined levels of MHO and pseudoionone were equivalent to β-ionone levels, we conclude that the enzyme does not have a strong substrate preference in vitro. However, because the levels of MHO were significantly greater than those of pseudoionone, ZmCCD1 may have a preference for the 5,6 bond of lycopene. These data support a model of an enzyme that cleaves multiple linear and cyclic carotenoids (Fig. 8).

The volatile cleavage products derived from the β-carotene LeCCD1A strain were similar to those produced in the ZmCCD1-expressing host. In contrast to ZmCCD1 and LeCCD1A, data for AtCCD1 and LeCCD1B cleavage of β-carotene are consistent with substrate preference for β-carotene. However, given the caveats of the in vivo assays, these results should be confirmed with in vitro assays. Still it is interesting to hypothesize that CCD1 enzymes from some species might cleave carotenoids indiscriminately, although in other species, CCD1 may have evolved to cleave certain carotenoids preferentially.

One of the most significant findings from this work is that CCD1 can cleave 5,6 double bonds. When expressed in strains accumulating lycopene, the 5,6 cleavage product MHO was generated. This product is specific to lycopene. MHO can theoretically be produced by cleavage of the 5,6 or 5\',6\' double bonds present in GGPP, phytoene, or ζ-carotene. However, MHO was not detected in strains accumulating these isoprenoids. The cleavage activity observed in vivo was confirmed.

### FIGURE 8. The proposed sites of CCD1 bond cleavage and the volatiles generated. An abbreviated version of the carotenoid biosynthetic pathway in higher plants is shown. Dashed lines indicate sites of CCD1 cleavage. The volatiles generated from each carotenoid are listed to the right of the arrows.
in vitro. When ZmCCD1 was added to assays containing lycopene, MHO and pseudoionone were produced. This 5,6 cleavage of lycopene was also observed with the arabidopsis and tomato CCD1 enzymes. It should be noted that we obtained no evidence for 7,8 bond cleavage with any of the CCD1 enzymes.

Analysis of ZmCCD1-generated volatiles has provided information regarding substrate recognition. Geranylacetone was produced from ζ-carotene but not GGPP or phytoene, although cleavage of GGPP or phytoene at their 9,10 (9’,10’) bonds could generate geranylacetone. Similarly although GGPP, phytoene, ζ-carotene, and lycopene all possess 5,6 double bonds, MHO was only generated from lycopene. What contributes to site-specific cleavage of the same bonds present in certain carotenoids but not others? One obvious structural difference is additional double bonds present in the cleaved carotenoids. Two desaturation events are needed to convert phytoene to ζ-carotene, introducing double bonds at the 11,12- and 11’,12’-positions. Two additional desaturation reactions (7,8- and 7,8’-positions) produce lycopene from ζ-carotene. Only in the presence of these additional double bonds are ζ-carotene cleaved to generate geranylacetone and lycopene cleaved to produce MHO and pseudoionone (Fig. 8).

Another characteristic of CCD1 cleavage sites is the presence of a methyl group immediately adjacent to the cleaved double bond (C-5 and C-9 positions). An additional methyl group is found four carbons central to the cleavage site (on C-9 for 5,6 cleavage and on C-13 for 9,10 cleavage). If CCD1 only recognized the level of desaturation, the enzyme could theoretically cleave the 13,14 double bond in phytoene to generate farnesylacetone, but this was not the case. These methyl groups flank-ing the cleavage site, together with the conjugated double bond system, could be the structural features recognized by CCD1.

CCD1 cleavage of lycopene provides a biological route to synthesize MHO, an important flavor and aroma volatile found in a number of fruits, including tomato. In tomato, MHO is associated with tomato-like flavor (46). MHO is also present as a component of the floral scent of numerous plants, occurring in over 50% of 991 species of flowering plants that have had their floral scent analyzed (47). Knowing which enzyme generates MHO allows for manipulation of this volatile in economically important species, and CCD1 is now a candidate enzyme for accomplishing this goal.

MHO is also a biologically active compound, functioning as a fungal spore germination stimulant (48) and an insect pheromone. MHO functions as a “panic-alarm”-inducing pheromone in the ant Lasius fuliginosus (49). It also functions as an aphid spacing hormone. The aphid Rhopalosiphum padi attacks wheat seedlings, but during infestation, levels of MHO increase and repel the aphid (50). MHO is also released during infestation of the aphid Uroleucon jaceae, attracting a parasitoid wasp (Apodius ervi) (51). These data make MHO a target for increased emission in crop plants, and CCD1 could be used in this endeavor. In fact, if CCD1 is responsible for generating the MHO observed during insect infestation, this could provide an elegant explanation as to why the enzyme is located outside of the plastid (30), whereas its carotenoid substrate is located inside. When a plant is attacked by an insect, cell damage disrupting plastid integrity would lead to carotenoid release into the cytoplasm. CCD1 would then have access to the liberated carotenoids and be able to generate defense-related compounds, such as MHO. This hypothesis is speculative as plant damage caused by aphid feeding (i.e. sucking phloem sap) is substantially different from chewing insects. Still testing this hypothesis could lead to important insights into plant/insect defense responses.

In conclusion, ZmCCD1 was observed to cleave multiple iso-prenoids but not GGPP or phytoene. CCD1 enzymes from multiple species cleave lycopene to generate pseudoionone and MHO. In vitro, ZmCCD1 cleaved linear and cyclic carotenoids with equal efficiency. Based on the pattern of apocarotenoid volatiles produced, we propose that CCD1 substrate recognition is determined by both the saturation status between carbons 7 and 8 and carbons 11 and 12 (11 and 12‘) as well as the methyl groups on carbons 5, 9, and 13 (5‘, 9’, and 13‘).

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