Title: The Biochemical Characterization of Two Carotenoid Cleavage Enzymes from Arabidopsis indicates that a Carotenoid-Derived Compound Inhibits Lateral Branching.

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Running title: Biochemical Characterization of AtCCD7 and AtCCD8

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Summary

Enzymes that are able to oxidatively cleave carotenoids at specific positions have been identified in animals and plants. The first such enzyme to be identified was a nine cis-epoxy carotenoid dioxygenases (NCED) from maize, which catalyzes the rate-limiting step of abscisic acid (ABA) biosynthesis. Similar enzymes are necessary for the synthesis of vitamin A in animals and other carotenoid-derived molecules in plants. In the model plant, Arabidopsis, there are nine hypothetical proteins that share some degree of sequence similarity to the NCEDs. Five of these proteins appear to be involved in ABA biosynthesis. The remaining four proteins are expected to catalyze other carotenoid cleavage reactions and have been named carotenoid cleavage dioxygenases (CCDs). The hypothetical proteins, AtCCD7 and AtCCD8, are the most disparate members of this protein family in Arabidopsis. The max3 and max4 mutants in Arabidopsis result from lesions in AtCCD7 and AtCCD8. Both mutants display a dramatic increase in lateral branching and are believed to be impaired in the synthesis of an unidentified compound that inhibits axillary meristem development. To determine the biochemical function of AtCCD7, the protein was expressed in carotenoid accumulating strains of Escherichia coli. The activity of AtCCD7 was also tested in vitro with several of the most common plant carotenoids. It was shown that the recombinant AtCCD7 protein catalyzes a specific 9-10 cleavage of β-carotene to produce the 10'-apo-β-carotenal (C27) and β-ionone (C13). When AtCCD7 and AtCCD8 were co-expressed in a β-carotene producing strain of E. coli, the 13-apo-β-carotenone (C18) was produced. The C18 product appears to result from a secondary cleavage of the AtCCD7 derived C27 product. The sequential cleavages of β-carotene by AtCCD7 and AtCCD8 are likely the initial steps in the synthesis of a carotenoid-derived signaling molecule that is necessary for the regulation lateral branching.
Introduction

Apocarotenoids are a diverse class of compounds that are derived from the oxidative cleavage of carotenoids. These compounds serve important biological functions in a variety of organisms. Vitamin A, for example, is required for vision and development in animals. In plants, abscisic acid (ABA) is necessary for seed development and adaptation to various environmental stresses. The synthesis of these apocarotenoids and others is catalyzed by a class of oxygenases that cleave specific double bonds resulting in two products with carbonyls at the site of cleavage. For many years it was believed that the enzymes that catalyze these reactions were dioxygenases. In a recent study, it was found that oxygen in one of the products comes from water (1); indicating that these enzymes may be monooxygenases. For simplicity, all enzymes will be referred to the name given when originally described.

In plants, nine-cis-epoxy-carotenoid dioxygenases (NCEDs) catalyze the rate-limiting step in abscisic acid (ABA) biosynthesis. An NCED from maize was the first carotenoid cleavage enzyme to be cloned and characterized (2,3). A number of NCEDs (4-7) and similar enzymes that are necessary for the synthesis of other apocarotenoids have since been identified in a variety of plants (8-10). The enzymes that cleave β-carotene to form two molecules of retinal in animals also belong to this family (11-14).

Within the genome sequence of the model plant, Arabidopsis, there are nine hypothetical proteins that share some degree of sequence similarity to the NCEDs. Five of these proteins are believed to be involved in ABA synthesis (15). Four members of this protein family in Arabidopsis do not cluster with previously characterized NCEDs and are considered unlikely to be ABA biosynthetic enzymes. These proteins may, however, catalyze other carotenoid cleavage reactions and are more appropriately referred to as carotenoid cleavage dioxygenases.
(CCDs). The AtCCD1 protein, for example, catalyzes the symmetric 9-10, 9'-10’ cleavage of various carotenoids (8). The specific biochemical functions for the remaining three CCDs from Arabidopsis (AtCCD4, 7, and 8) have not yet been reported.

The max4 mutant in Arabidopsis and the rms1 mutant in pea result from a lesion in AtCCD8 and a pea ortholog (16). Both mutants display an increase in lateral branching and are believed to be impaired in the synthesis of an unknown compound that inhibits axillary meristem development or bud outgrowth. The max3 mutant also displays an increase in lateral branching and results from a lesion in the AtCCD7 gene (17); indicating that AtCCD7 is also involved in the synthesis of this inhibitor. The biochemical characterization of AtCCD7 and AtCCD8 is an important step in identifying the biologically active compound. It has been reported that the expression of AtCCD7 in carotenoid producing strains of E. coli results in a reduced accumulation of carotenoids and the production of some apocarotenoids (17). However, the specific reaction catalyzed by AtCCD7 has not yet been established. It is demonstrated here that the recombinant AtCCD7 protein catalyzes a 9-10 cleavage of β-carotene to produce the 10’-apo-β-carotenal (C27) and β-ionone (C13). The AtCCD8 protein is able to catalyze a secondary cleavage of the 10’-apo-β-carotenal at the 13-14 position to produce the 13-apo-β-carotenone (C18).

**Experimental Procedures**

**Cloning of AtCCD7 and AtCCD8**

A cDNA clone of AtCCD7 was obtained by RT-PCR with RNA isolated from one week old Arabidopsis seedlings. Total RNA was reverse transcribed with Superscript from Invitrogen (Carlsbad, CA) and an 18-mer oligo dt primer. The AtCCD7 gene was then amplified with Platinum Taq polymerase from Invitrogen (Carlsbad, CA) and the primers listed in table one.
The sequence of the cloned cDNA was identical with the sequence in genbank (NM_130064). The \textit{AtCCD8} ORF in the pUNI51 vector was produced by the SSP consortium (18) and obtained from the Arabidopsis stock center (Stock # U19580). Subsequent subcloning of \textit{AtCCD7} and \textit{AtCCD8} for recombinant protein expression are described in table one.

\textit{Expression and Analysis of AtCCD7 in carotenoid accumulating strains of E. coli.}

Most proteins in this study were expressed with an N-terminal Glutathione S-transferase tag (Table 1.). For expression in carotenoid accumulating strains of \textit{E. coli} (19), 2-mL cultures were grown overnight in LB medium with 100 µg mL\(^{-1}\) ampicillin and 35 µg mL\(^{-1}\) chloramphenicol. The overnight cultures were used to inoculate a 30-mL culture of LB with the same antibiotics. After 24 h at 28 °C, 0.1 mM isopropyl \(\beta\)-D-thiogalactopyranoside (IPTG) was added and the cultures were left at room temperature for an additional 48 h. At the same time the recombinant protein was expressed, ferrous sulfate was added to a final concentration of 10 mg L\(^{-1}\).

For quantitative analysis of carotenoid accumulation, one mL of culture was centrifuged and the media was discarded. The cell pellet was resuspended in 100 µL of formaldehyde and then 1 mL of the ethanol was added. The tubes were placed at 4°C for three hours before the cell debris was removed by centrifugation. For \(\beta\)-Carotene and zeaxanthin accumulating strains of \textit{E. coli}, absorbance was measured with a spectrophotometer at 453 nm. For the lycopene accumulating strains, absorbance was measured at 472 nm. The carotenoid content was calculated with known extinction coefficients.

For analysis of apocarotenoid products, a culture was centrifuged and the cell pellet was extracted sequentially with formaldehyde, methanol, and diethyl ether. The media was partitioned into an equal volume of diethyl ether. Both the cell extract and the media partition
were washed with water and the diethyl ether layer was retained. The volume of the diethyl ether was reduced under nitrogen and a solution of 10% KOH in methanol was added to saponify the samples. After 30 min., the samples were partitioned into diethyl ether and washed with water. The diethyl ether layer was dried under a gentle stream of nitrogen and the samples were stored at -80 °C until HPLC analysis.

The *E. coli* extracts were analyzed on a Waters 600 HPLC (Milford, MA) equipped with a Waters 996 photodiode array detector. Samples were injected on a 5 μm C18 adsorbosil column from Alltech (Deerfield, IL) and eluted with the following program: 50% acetonitrile and water at 1 mL min⁻¹ for 4 min; followed by a linear gradient to 100% acetonitrile over 16 min. The gradient was then shifted to 100% acetone over 12 min and left at 100% acetone for an additional 5 min.

*In vitro Assays with recombinant AtCCD7*

For protein expression, 5-mL of an overnight culture was used to inoculate a 100-mL culture in 2XYT medium (per liter: 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl). Cultures were grown at 37 °C until an *A*₆₀₀ of 0.7 was reached. Expression of proteins was induced by the addition of 0.2 mM IPTG, and the cultures were grown at 28 °C for an additional 5 h. The *E. coli* cells were harvested by centrifugation and resuspended in 4 mL of lysis buffer (40 mM Tris pH 7.5, 20 mM NaCl, 2 mM MgCl₂, 100 μg lysozyme, and 100 U of endonuclease). The cells were left on ice for 20 min and then frozen in N₂ (*f*). After thawing, Triton x-100 was added to a final concentration of .25% and the cells were shaken on ice for 30 min. The recombinant protein was bound to glutathione-agarose from Sigma (St Louis, MO), washed three times with Tris-buffered saline and then released by cleavage with Factor Xa from Novagen (Madison, WI) for 7 h at 4 °C. The carotenoid substrates were extracted from plant
tissues and purified by HPLC as previously described (20). Assays contained 0.1% Triton X-100, 0.5 mM FeSO4, 5 mM ascorbate, and the appropriate carotenoid substrate in 100 mM Tris, pH 7.0. The assay products were partitioned into ethyl acetate, dried under N2 and analyzed by HPLC or thin-layer chromatography. To determine the $K_m$ and $V_{max}$ values, the Michaelis-Menten equation was solved by non-linear regression using Sigma Plot 4.01 from Jandel Scientific (San Rafael, CA).

The Co-expression of AtCCD7 and AtCCD8 in carotenoid accumulating strains of E. coli

For expression of AtCCD8 or co-expression of AtCCD7 and AtCCD8, the BL21 (AI) strain from Invitrogen (Carlsbad, CA) was used and cultures were grown as described above. For induction of the $tac$ promoter expressing AtCCD7, IPTG was added to a final concentration of 0.1 mM. Induction of the AtCCD8 protein, under the control of a T7 promoter, was achieved by the addition of 0.02% arabinose. The cells and media was extracted and analyzed as described above.

Characterization of apocarotenoid products

The apocarotenoid products were purified by reverse phase HPLC (described above), reduced with NaBH$_4$ and further purified by normal phase HPLC on an analytical $\mu$porasil column from Waters corporation (Milford, MA) that was equilibrated with 97:3 (hexane:ethyl acetate) at 2 mL min$^{-1}$. The column was eluted with a linear gradient to 50% ethyl acetate over 12 min. Fractions were collected, dried, and dissolved in hexane to determine their absorption spectra. The $C_{27}$ product of AtCCD7, 10’-apo-\(\beta\)-carotenal, and the NaBH$_4$ reduced form were also analyzed by positive ion fast atom bombardment. Samples were introduced by direct insertion probe with 3-nitrobenzyl alcohol as the matrix. The molecular ions (M$^+$ and M$^{+1}$) for the 10’-apo-\(\beta\)-carotenal and carotenol were apparent. A high-resolution spectrum for the M$^+$ of
the 10'-apo-β-carotenol was also obtained. The TMS derivative of the 13-apo-β-carotenol was analyzed by gas chromatography-mass spectrometry with a DB5-MS column (30 m, 0.32 mm inner diameter, 0.25 µm film, J & W Scientific) and the following temperature program: 100 °C for 1 min, 100-230 °C at 40 °C/min, 230-280 °C at 8 °C/min, and 280-300 °C at 20 °C/min.

Results

Expression of ATCCD7 in carotenoid accumulating strains of E. coli

To determine the reactions catalyzed by AtCCD7, a GST-fusion protein was expressed in several carotenoid accumulating strains of E. coli. In previous studies, the expression of a functional carotenoid cleavage enzyme in these strains resulted in a reduced or altered color development (8-10,13,21). A moderate level of AtCCD7 expression in a lycopene (pACLYC) or zeaxanthin (pACZEAX) producing strain had little effect on color development (Fig. 1A and 1B). Conversely, expression of AtCCD7 in a β-carotene producing strain (pACBETA) had a significant effect on the accumulation of this carotenoid (Fig. 1A and 1B).

The cells and media from the AtCCD7 expression strains were extracted and analyzed by reverse phase HPLC. Two major compounds were detected in the pACBETA/AtCCD7 strain that were not detected in the pACBETA strain with an empty vector. One product had a UV-vis spectrum and retention time that was identical to β-ionone (C13) (Fig. 2A). The second product had a UV-vis spectrum that was consistent with the 10'-apo-β-carotenol (C27) (Fig. 2B) (22,23). Several smaller peaks which had similar retention times and absorption spectra are most likely cis isomers of the C27 product. These apocarotenoids would result from the 9-10 cleavage of β-carotene (Fig. 3). The initial cleavage products would be an aldehyde, but it is subsequently reduced to the corresponding alcohol by E. coli. The reduction of the aldehyde cleavage products to alcohols by E. coli has been previously reported (8,13).
When AtCCD7 was expressed in a lycopene producing strain of *E. coli*, a small amount of a compound with a UV-vis spectra similar to the 10’-apo-lycopenol was detected (Fig. 2C). No apocarotenoid products were detected when AtCCD7 was expressed in a zeaxanthin accumulating strain.

*In vitro assays with recombinant AtCCD7*

To further explore the specificity of AtCCD7 and delimit the endogenous substrates, *in vitro* assays were performed with the affinity purified protein and several common plant carotenoids. These assays were analyzed by TLC (Fig. 4) and HPLC (Supplemental Fig. 2). No products were detected with lycopene, lutein, zeaxanthin, violaxanthin or neoxanthin as substrate. A single product was apparent in assays with β-carotene. The UV-vis spectra of the enzyme product and of the NaBH₄ reduced form were very similar to published spectra for the 10’-apo-β-carotenal and the 10’-apo-β-carotenol, respectively (Supplemental Fig. 2B) (23). The [M] and [M+H] ions for the 10’-apo-β-carotenal and 10’-apo-β-carotenol were detected by FAB-MS and a high-resolution spectrum of the reduced product matched the molecular formula for the 10’-apo-β-carotenol, C₂₇H₃₈O. The calculated mass of the apocarotenol is 378.29230, while the experimentally determined mass of the compound was 378.2928 (an error of 1.3 ppm from the calculated).

The AtCCD7 protein contains a probable chloroplast targeting sequence of 31 amino acids (TargetP V1.01), which is consistent with a role in carotenoid metabolism. A truncated AtCCD7 protein (587 amino acids) lacking the N-terminal targeting sequence was also able to catalyze the 9-10 cleavage of β-carotene (data not shown). The addition of water-miscible organic solvents has been shown to enhance the activity of lignostilbene dioxygenases (24), which share sequence similarity to the CCDs and catalyze a similar double bond cleavage...
reaction. The addition of methanol to *in vitro* assays with AtCCD7 had a stimulatory effect on activity at concentrations up to 25% (data not shown). The kinetic values for the standard reaction were: $K_m=15.2 \, \mu M$ and $V_{max} = 4.5$ picoMoles/mg protein/minute. With the addition of 25% methanol, the $K_m$ and $V_{max}$ increased to 20.0 $\mu M$ and 10.1 picoMoles/mg protein/minute.

**Co-expression of AtCCD7 and AtCCD8 in carotenoid accumulating strains.**

No apocarotenoids were detected with the expression of AtCCD8 in the carotenoid accumulating strains of *E. coli* (data not shown). Because mutations in *AtCCD7* and *AtCCD8* result in the same phenotype, it is likely that the two gene products function in the same pathway. So, a construct for the co-expression AtCCD7 and AtCCD8 was transformed into the carotenoid accumulating strains. In addition to the AtCCD7 cleavage products, $\beta$-ionone and the 10’-apo-$\beta$-carotenol, a third product was identified in the $\beta$-carotene co-expression strain (Fig. 5A and Supplemental Fig. 1D). The absorption spectrum of the product in hexane before and after reduction with NaBH$_4$ (Fig. 5B) is similar to the 13-apo-$\beta$-carotenone and carotenol (C$_{18}$) (25). A trimethyl silyl (TMS) derivative of the reduced product was analyzed by GC-MS (Fig. 6). The observed molecular ion of 332 is the expected mass for the TMS derivative of the 13-apo-$\beta$-carotenol. An authentic standard of 13-apo-$\beta$-carotenone was produced according to a previously described synthesis (26). The chromatography, UV-vis spectra and mass spectra of the *E. coli* product and synthetic product were the same.

The absence of the 13-apo-$\beta$-carotenone when AtCCD8 is expressed alone in the $\beta$-carotene strain indicates that ATCCD8 cleaves the C$_{27}$ product, the 10’-apo-$\beta$-carotenal or carotenol (Fig. 7). A C$_{9}$ product would also result from this cleavage reaction, but it has not yet been identified. To determine if the synthesis of the 13-apo-$\beta$-carotenone required a specific interaction between the AtCCD7 and AtCCD8 proteins, a construct was made for the co-
expression of β-Diox II from Mouse and AtCCD8. The β-Diox II also catalyzes a 9-10 cleavage of β-carotene (22), but it shares only 13% identity and 23% similarity with AtCCD7. Co-expression of β-Diox II and AtCCD8 also resulted in the production of 13-apo-β-carotenone (Fig. 5C).

Discussion

The more axillary branching mutants, max3 and max4, result from lesions in the AtCCD7 and AtCCD8 genes, respectively (16,17). Due to an inability to repress the outgrowth of axillary buds, the max mutants display an increase lateral branching and have a bushy appearance. In many plants, the outgrowth of axillary buds is inhibited by the shoot apex. This phenomenon is often referred to as apical dominance and is regulated in large part by the plant hormone, auxin. Auxin is synthesized in the shoot apex and transported to the base of the plant where it inhibits lateral bud outgrowth. If the shoot apex is damaged, auxin levels are reduced and lateral buds may be released from dormancy. There are, however, several lines of evidence to indicate that auxin does not inhibit the outgrowth of axillary buds directly. Grafting experiments with branching mutants in various species have provided strong evidence for the existence of another long-distance signal that inhibits the outgrowth of axillary buds. The phenotype of several branching mutants in pea (rms1, 2, and 5), the max1, 3, and 4 mutants in Arabidopsis, and the decreased apical dominance mutant in petunia (dad1-1) can be rescued by grafting to a wild type rootstock (16,27-29). These results indicate that a compound that is synthesized in the root is capable of inhibiting axillary bud outgrowth. Because the compound moves acropetally from the root to shoot, it is most likely transported through the xylem. Considering that the AtCCD7 and AtCCD8 proteins are similar to previously characterized carotenoid cleavage dioxygenases, it is likely that the inhibitor of axillary bud outgrowth is a carotenoid-derived molecule.
In plants, the cyclization of lycopene is major a branch point in carotenoid biosynthesis. The introduction of two β-rings produces β-carotene, which may subsequently be converted to zeaxanthin, violaxanthin, and neoxanthin. The introduction of a β-ring and an ε-ring produces α-carotene, which is converted primarily to lutein in most tissues. The lut2 mutant in Arabidopsis is unable to produce α-carotene and lutein (30) and has no branching phenotype. Therefore, it is unlikely that either of these carotenoids are a precursor of the lateral branch inhibitor. By the same rationale, mutants impaired in epoxy-carotenoid synthesis (31) indicate that violaxanthin and neoxanthin are not the precursors of the lateral branching inhibitor. The most likely precursor of the branching inhibitor would then be zeaxanthin, β-carotene, or an acyclic precursor.

The recombinant AtCCD7 protein was able to catalyze the 9-10 cleavage of β-carotene to produce the 10'-apo-β-carotenol (C27) and β-ionone (C13). The AtCCD1 protein and homologs from other plants also catalyze a 9-10 cleavage reaction (8,10). There are, however, several key distinctions between the reactions catalyzed by AtCCD1 and AtCCD7. The recombinant AtCCD1 protein cleaves various carotenoids symmetrically at both the 9-10, and 9'-10' positions. Therefore, AtCCD1 does not produce the 10'-apo-β-carotenal.

Because AtCCD7 and AtCCD8 have both been implicated in the synthesis of a lateral branching inhibitor, the two proteins were co-expressed in the carotenoid accumulating strains of E. coli. When co-expressed in the β-carotene accumulating strain, the C27 and C13 products of AtCCD7 were detected. A third product was also produced by this strain and identified as the 13-apo-β-carotenone (C18). Because this product was not detected when AtCCD8 was expressed by itself, it most likely results from a secondary cleavage of the C27 product of AtCCD7. A C9 product would also result from this cleavage reaction, but it has not yet been identified. The
lignostilbene dioxygenases, which share sequence similarity with the CCDs and catalyze a similar cleavage reaction, have been shown to function as homodimers or heterodimers (32).

Direct analysis of AtCCD7 and AtCCD8 dimer formation was complicated, because the majority of the recombinant AtCCD8 protein was insoluble (data not shown). The C_{18} compound was also produced when AtCCD8 was co-expressing with a 9-10 cleavage enzyme from mouse (β-Diox II); providing indirect evidence that dimerization is not essential for the activity of AtCCD8. Formation of an AtCCD7-AtCCD8 heterodimer could, however, increase the rate of the second cleavage reaction.

The phenotype of the max3/atccd7 and max4/atccd8 mutants and the biochemical evidence presented here suggests that AtCCD7 and AtCCD8 are necessary for the synthesis of an apocarotenoid that inhibits axillary bud outgrowth. The cleavage of β-carotene by AtCCD7 to a C_{27} product is likely the first committed step in the biosynthetic pathway of this inhibitor. The C_{27} product may subsequently be cleaved by AtCCD8 to form the 13-apo-β-carotenone (C_{18}) and a C_{9} product. Either of the AtCCD8 cleavage products could give rise to the biologically active inhibitor. Grafting experiments with the max1 mutant in Arabidopsis indicate that this mutant is also impaired in the synthesis of the branching inhibitor (29). Therefore, it is likely that there is one additional step in the pathway. The identification of the biologically active compound will require the characterization of the MAX1 gene product and the development of a bioassay to confirm the role of the inhibitor, in regulating lateral branching.

**Abbreviations**

The abbreviations used are: ABA, abscisic acid; NCED, nine-cis-epoxy-carotenoid dioxygenase; CCD, carotenoid cleavage dioxygenase; FAB-MS, Fast Atom Bombardment-Mass
Spectroscopy; HPLC, high performance liquid chromatography. GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; TMS, trimethyl-silyl.

**Figure Legends**

Figure 1. *A.* The expression of AtCCD7 or an empty vector control (pGEX) in *E. coli* strains that accumulate lycopene (pACLYC), β-carotene (pACBETA) or zeaxanthin (pACZEAX). *A.* Quantitative analysis of carotenoids accumulation in liquid grown cultures.

Figure 2. HPLC analysis of carotenoid accumulating strains that are expressing AtCCD7. Contour plots, which allow for a range of wavelengths to be monitored simultaneously, are presented in supplemental figure 1. *A.*, Extracted chromatogram (absorbance at 310 nm) of the pACBETA strain with the β-ionone peak indicated. *B.*, Extracted chromatogram (absorbance at 400 nm) of the pACBETA strain with the 10’-apo-β-carotenol indicated. *Inset* online spectra of the indicated peak. *C.*, Extracted chromatogram (absorbance at 422 nm) of pACLYC strain with the 10’apo-lycopenol indicated. *Inset* online spectrum of the indicated peak. The β-carotene or lycopene peaks are indicated by an asterisk (*).

Figure 3. The 9-10 cleavage of β-carotene catalyzed by the recombinant AtCCD7 protein and the subsequent reduction of the 10’-apo-β-carotenal to the corresponding alcohol by *E. coli* or NaBH₄.

Figure 4. Thin-layer chromatography analysis of assays with the recombinant AtCCD7 protein and some common plant carotenoids. Enzyme assay products were separated on a thin-layer silica plate that was developed in hexane and 2-propanol (90:10). Following chromatography, the plate was sprayed with 2, 4-dinitrophenylhydrazine to detect aldehydes and ketones. The 10’-apo-β-carotenal is indicated by an arrow. HPLC analysis of in *vitro* assays with lycopene and β-carotene are presented in supplemental figure 2.
Figure 5. HPLC analysis of pACBETA strains with a construct for the expression of AtCCD8 (A), a construct for the co-expression of AtCCD7 and AtCCD8 (B), or a construct for the co-expression of β-Diox II and AtCCD8 (C). Contour plots for the expression of AtCCD8 and the co-expression of AtCCD7 and AtCCD8 are presented in supplemental figure 1C and 1D. D., The UV-vis Spectra of 13-apo-β-carotenone (C_{18}) and the reduced product, 13-apo-β-carotenol, in hexane.

Figure 6. Mass spectrum of the TMS derivative of 13-apo-β-carotenol isolated from a β-carotene accumulating strain of *E. coli* that is co-expressing AtCCD7 and AtCCD8.

Figure 7. The proposed cleavage of the 10’-apo-β-carotenal catalyzed by AtCCD8 to form the 13-apo-β-carotenone and a C_9 dialdehyde.

**Supplemental Figures.**

Supplemental Figure 1. HPLC analysis of β-carotene accumulating strains (pACBETA) that are co-expressing AtCCD7 and/or AtCCD8. The photodiode array data are represented as contour plots, which allows for a range of wavelengths to be monitored. A., pACBETA strain with an empty vector as control. B., pACBETA strain expressing AtCCD7. C., pACBETA strain expressing AtCCD8. D., pACBETA strain co-expressing AtCCD7 and AtCCD8.

Supplemental Figure 2. HPLC, contour plots for *in vitro* assays with AtCCD7 and lycopene (A) or β-carotene (B). C., Extracted chromatogram (absorbance 430) for assays with β-carotene. The 10’-apo-β-carotenal is indicated by an arrow. D., UV-vis Spectra for the 10’-apo-β-carotenal and the 10’-apo-β-carotenol in hexane.

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Table 1.

| Plasmid   | Derivation and Function                                                                 | Source or Reference |
|-----------|-----------------------------------------------------------------------------------------|---------------------|
| pACLYC    | Biosynthetic genes from *Erwinia herbicola* for the synthesis of lycopene *chm*′          | (30)                |
| pACBETA   | Biosynthetic genes from *Erwinia herbicola* for the synthesis of β, β-carotene *chm*′     | (19)                |
| pACZEAX   | Biosynthetic genes from *Erwinia herbicola* for the synthesis of zeaxanthin *chm*′         | (30)                |
| pBHS1     | A full-length clone of AtCCD7 was amplified with the following primer pair:               |                     |
|           | 5′- ATGTCTCTCCCTATCCCGC-3′ (primer #1) and 5′-TCAGTCGCTAGCCCATAAAC-3’ (primer #2). The fragment was subcloned into pGEM-T easy vector from Promega (Madison, WI) *amp*′ |                     |
| pBHS2     | A truncated fragment resulting in a 31 amino acid N-terminal deletion was amplified with primer #3: 5′GCGCAATATCAATCTATCTACC-3′ and primer #2 (above). The fragment was subcloned into pGEM-T easy vector *amp*′ |                     |
| pBHS3     | A *NotI* fragment from pBHS1 was subcloned into the *NotI* site of pGEX 5x-3 from Amersham Biosciences (Piscataway, NJ). For expression of AtCCD7 protein as a |                     |
| **GST fusion protein** | amp<sup>r</sup> |
|-----------------------|------------------|
| **pBHS4**             | A NotI fragment from pBHS2 was subcloned into the NotI site of pGEX 5x-3. For expression of a truncated AtCCD7 protein as a GST fusion protein amp<sup>r</sup> |
| **pBHS5**             | A cDNA for a β-carotene 9, 10 cleavage dioxygenase (β-Diox II) from mouse was obtained from the ResGen™ clone collection (clone i.d.: 2536812). The β-Diox II gene was amplified with a gene specific primer: 5’-ATGTTGGGACCGAAGCAAAG-3’ and a vector primer: 5’-CGACCTGCAGCTCGAGCACA-3’. The fragment was cloned into pGEM T-easy amp<sup>r</sup> |
| **pBHS6**             | By partial digestion of pBHS5 with EcoRI, the β-Diox II gene was isolated and subcloned into pGEX 5x-3 vector for protein expression as a GST-fusion protein amp<sup>r</sup> |
| **pHB3-His6**         | UPS expression vector for Histidine tagged fusions (Arabidopsis stock center # CD3-595) |
| **pAT1**              | With Cre-lox site specific recombination, the AtCCD8 ORF was placed into pHB3-His6 for expression as a histidine tagged protein under the control of a T7 promoter amp<sup>r</sup> |
| **pJHS1**             | With T7 promoter and terminator primers, the promoter and the AtCCD8 ORF from pAT1 was amplified and |
|         |                      |
|---------|----------------------|
| **pJHS2** | With T7 promoter and terminator primers, the promoter and the AtCCD8 ORF from pAT1 was amplified and subcloned into the *ZraI* site of pBHS5 *amp<sup>r</sup>.* |

subcloned into the *ZraI* site of pGEX 5x-3. *amp<sup>r</sup>*
Schwartz et al., Figure 1A and 1B
Schwartz et al., Figure 2
β, β-carotene (C<sub>40</sub>)

AtCCD7

10'-apo-β-carotenal (C<sub>27</sub>)

β-ionone (C<sub>11</sub>)

NaBH<sub>4</sub> / E. coli

10'-apo-β-carotenol

Schwartz et al., Figure 3
Schwartz et al., Figure 6
10’-apo-β–carotenal/ol (C\textsubscript{27})

AtCCD8

13-apo-β–carotenone (C\textsubscript{18})

C\textsubscript{9} Dialdehyde

Schwartz et al., Figure 7
A. pGEX

B. AtCCD7

β-ionone (C₁₃)

C. AtCCD8

D. AtCCD7 + 8

β-ionone (C₁₃)

Schwartz et al., Supplemental Figure 1
Schwartz et al., Supplemental Figure 2
The biochemical characterization of two carotenoid cleavage enzymes from Arabidopsis indicates that a carotenoid-derived compound Inhibits lateral branching

Steven H. Schwartz, Xiaoqiong Qin and Michele C. Loewen

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