

# The Biochemical Characterization of Two Carotenoid Cleavage Enzymes from *Arabidopsis* Indicates That a Carotenoid-derived Compound Inhibits Lateral Branching\*<sup>§</sup>

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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 dioxygenase from maize, which catalyzes the rate-limiting step of abscisic acid 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 nine-*cis*-epoxy carotenoid dioxygenases. Five of these proteins appear to be involved in abscisic acid 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  $\beta$ -carotene to produce the 10'-apo- $\beta$ -carotenal (C<sub>27</sub>) and  $\beta$ -ionone (C<sub>13</sub>). When AtCCD7 and AtCCD8 were co-expressed in a  $\beta$ -carotene-producing strain of *E. coli*, the 13-apo- $\beta$ -carotene (C<sub>18</sub>) was produced. The C<sub>18</sub> product appears to result from a secondary cleavage of the AtCCD7-derived C<sub>27</sub> product. The sequential cleavages of  $\beta$ -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.

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 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)<sup>1</sup> catalyze the rate-limiting step in abscisic acid 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  $\beta$ -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 abscisic acid synthesis (15). Four members of this protein family in *Arabidopsis* do not cluster with previously characterized NCEDs and are considered unlikely to be abscisic acid 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 that 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

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1 and 2.

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<sup>1</sup> The abbreviations used are: NCED, nine-*cis*-epoxy-carotenoid dioxygenase; CCD, carotenoid cleavage dioxygenase; HPLC, high performance liquid chromatography; ORF, open reading frame.

TABLE I  
Plasmids used in this study

Plasmid	Derivation and function	Source or reference
pACLYC	Biosynthetic genes from <i>Erwinia herbicola</i> for the synthesis of lycopene <i>chm</i> <sup>r</sup>	30
pACBETA	Biosynthetic genes from <i>E. herbicola</i> for the synthesis of $\beta$ , $\beta$ -carotene <i>chm</i> <sup>r</sup>	19
pACZEAX	Biosynthetic genes from <i>E. herbicola</i> for the synthesis of zeaxanthin <i>chm</i> <sup>r</sup>	30
pBHS1	A full-length clone of <i>AtCCD7</i> 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) <i>amp</i> <sup>r</sup>	
pBHS2	A truncated fragment resulting in a 31-amino acid N-terminal deletion was amplified with primer 3: 5'-GCCGCA-ATATCAATATCTATACC-3' and primer 2 (above). The fragment was subcloned into pGEM-T easy vector <i>amp</i> <sup>r</sup>	
pBHS3	A NotI fragment from pBHS1 was subcloned into the NotI site of pGEX 5x-3 from Amersham Biosciences. For expression of <i>AtCCD7</i> protein as a glutathione <i>S</i> -transferase fusion protein <i>amp</i> <sup>r</sup>	
pBHS4	A NotI fragment from pBHS2 was subcloned into the NotI site of pGEX 5x-3. For expression of a truncated <i>AtCCD7</i> protein as a glutathione <i>S</i> -transferase fusion protein <i>amp</i> <sup>r</sup>	
pBHS5	A cDNA for a $\beta$ -carotene 9–10 cleavage dioxygenase ( $\beta$ -Diox II) from mouse was obtained from the ResGen <sup>TM</sup> clone collection (clone 2536812). The $\beta$ -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 <i>amp</i> <sup>r</sup>	
pBHS6	By partial digestion of pBHS5 with EcoRI, the $\beta$ -Diox II gene was isolated and subcloned into pGEX 5x-3 vector for protein expression as a glutathione <i>S</i> -transferase fusion protein <i>amp</i> <sup>r</sup>	
pHB3-His6	UPS expression vector for histidine-tagged fusions ( <i>Arabidopsis</i> stock center no. CD3–595)	31
pAT1	With <i>Cre-lox</i> site-specific recombination, the <i>AtCCD8</i> ORF was placed into pHB3-His <sub>6</sub> for expression as a histidine-tagged protein under the control of a T7 promoter <i>amp</i> <sup>r</sup>	
pJHS1	With T7 promoter and terminator primers, the promoter and the <i>AtCCD8</i> ORF from pAT1 was amplified and subcloned into the <i>ZraI</i> site of pGEX 5x-3 <i>amp</i> <sup>r</sup>	
pJHS2	With T7 promoter and terminator primers, the promoter and the <i>AtCCD8</i> ORF from pAT1 was amplified and subcloned into the <i>ZraI</i> site of pBHS5 <i>amp</i> <sup>r</sup>	

*AtCCD7* in carotenoid-producing strains of *Escherichia 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  $\beta$ -carotene to produce the 10'-apo- $\beta$ -carotenal ( $C_{27}$ ) and  $\beta$ -ionone ( $C_{13}$ ). The *AtCCD8* protein is able to catalyze a secondary cleavage of the 10'-apo- $\beta$ -carotenal at the 13–14 position to produce the 13-apo- $\beta$ -carotenone ( $C_{18}$ ).

#### EXPERIMENTAL PROCEDURES

**Cloning of *AtCCD7* and *AtCCD8***—A cDNA clone of *AtCCD7* was obtained by reverse transcription-PCR with RNA isolated from 1-week-old *Arabidopsis* seedlings. Total RNA was reverse transcribed with Superscript from Invitrogen and an 18-mer oligo (dT) primer. The *AtCCD7* gene was then amplified with platinum *Taq* polymerase from Invitrogen and the primers listed in Table I. The sequence of the cloned cDNA was identical with the sequence in GenBank<sup>TM</sup> (NM\_130064). The *AtCCD8* ORF in the pUNI51 vector was produced by the Salk/Stanford/PGEC consortium (18) and obtained from the *Arabidopsis* stock center (stock number U19580). Subsequent subcloning of *AtCCD7* and *AtCCD8* for recombinant protein expression is described in Table I.

**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 I). For expression in carotenoid-accumulating strains of *E. coli* (19), 2-ml cultures were grown overnight in LB medium with 100  $\mu$ g ml<sup>-1</sup> ampicillin and 35  $\mu$ g ml<sup>-1</sup> 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 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 liter<sup>-1</sup>.

For quantitative analysis of carotenoid accumulation, 1 ml of culture was centrifuged, and the medium was discarded. The cell pellet was resuspended in 100  $\mu$ l of formaldehyde, and then 1 ml of the ethanol was added. The tubes were placed at 4 °C for 3 h before the cell debris was removed by centrifugation. For  $\beta$ -carotene- and zeaxanthin-accumulating strains of *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 were partitioned into an equal volume of diethyl ether. Both the cell extract and the medium 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- $\mu$ m  $C_{18}$  Adsorbosil column from Alltech (Deerfield, IL) and eluted with 50% acetonitrile and water at 1 ml min<sup>-1</sup> 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 2 $\times$  YT 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 isopropyl  $\beta$ -D-thiogalactopyranoside, 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 MgCl<sub>2</sub>, 100  $\mu$ g lysozyme, and 100 units of endonuclease). The cells were left on ice for 20 min and then frozen in liquid nitrogen. After cells were thawed, Triton X-100 was added to a final concentration of 0.25%, and the cells were shaken on ice for 30 min. The recombinant protein was bound to glutathione-agarose from Sigma, 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 described previously (20). Assays contained 0.1% Triton X-100, 0.5 mM FeSO<sub>4</sub>, 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 N<sub>2</sub>, 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 was used, and cultures were grown as described above. For induction of the *tac* promoter expressing *AtCCD7*, isopropyl  $\beta$ -D-thiogalactopyranoside 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 were extracted and analyzed as described above.

**Characterization of Apocarotenoid Products**—The apocarotenoid products were purified by reverse phase HPLC (described above), re-

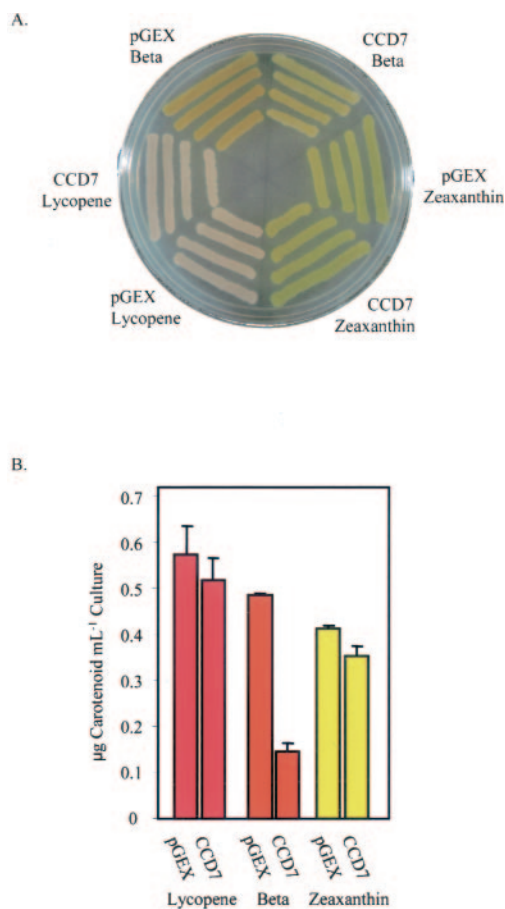


FIG. 1. A, the expression of AtCCD7 or an empty vector control (pGEX) in *E. coli* strains that accumulate lycopene (pACLYC),  $\beta$ -carotene (pACBETA), or zeaxanthin (pACZEAX). B, quantitative analysis of carotenoid accumulation in liquid-grown cultures.

duced with  $\text{NaBH}_4$ , and further purified by normal phase HPLC on an analytical  $\mu\text{porasil}$  column from Waters Corporation (Milford, MA) that was equilibrated with 97:3 (hexane:ethyl acetate) at  $2 \text{ ml min}^{-1}$ . 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  $\text{C}_{27}$  product of AtCCD7, 10'-apo- $\beta$ -carotenol, and the  $\text{NaBH}_4$  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- $\beta$ -carotenol and carotenol were apparent. A high-resolution spectrum for the  $M^+$  of the 10'-apo- $\beta$ -carotenol was also obtained. The trimethylsilyl derivative of the 13-apo- $\beta$ -carotenol was analyzed by gas chromatography-mass spectrometry with a DB5-MS column (30 m with an inner diameter of 0.32 mm, 0.25- $\mu\text{m}$  film, J&W Scientific) and the following temperature program: 100  $^\circ\text{C}$  for 1 min, 100–230  $^\circ\text{C}$  at 40  $^\circ\text{C}/\text{min}$ , 230–280  $^\circ\text{C}$  at 8  $^\circ\text{C}/\text{min}$ , and 280–300  $^\circ\text{C}$  at 20  $^\circ\text{C}/\text{min}$ .

## RESULTS

**Expression of ATCCD7 in Carotenoid-accumulating Strains of *E. coli***—To determine the reactions catalyzed by AtCCD7, a glutathione *S*-transferase 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-producing strain (pACLYC) or zeaxanthin-producing strain (pACZEAX) had little effect on color development (Fig. 1, A and B). Conversely, expression of AtCCD7 in a  $\beta$ -carotene-producing strain (pACBETA) had a significant effect on the accumulation of this carotenoid (Fig. 1, A and B).

The cells and media from the AtCCD7 expression strains

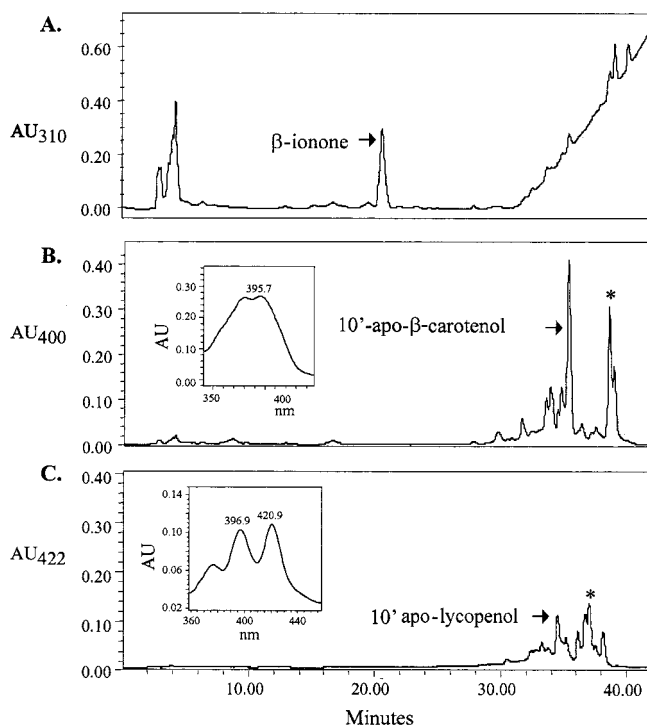


FIG. 2. HPLC analysis of carotenoid-accumulating strains that are expressing AtCCD7. Contour plots, which allow for a range of wavelengths to be monitored simultaneously, are presented in supplemental Fig. 1. A, extracted chromatogram (absorbance at 310 nm) of the pACBETA strain with the  $\beta$ -ionone peak indicated. B, extracted chromatogram (absorbance at 400 nm) of the pACBETA strain with the 10'-apo- $\beta$ -carotenol indicated. Inset, on-line spectrum of the indicated peak. C, extracted chromatogram (absorbance at 422 nm) of pACLYC strain with the 10'-apo-lycopenol indicated. Inset, on-line spectrum of the indicated peak. The  $\beta$ -carotene or lycopene peaks are indicated by asterisks.

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-visible spectrum and retention time that was identical to  $\beta$ -ionone ( $\text{C}_{13}$ ) (Fig. 2A). The second product had a UV-visible spectrum that was consistent with the 10'-apo- $\beta$ -carotenol ( $\text{C}_{27}$ ) (Fig. 2B) (22, 23). Several smaller peaks, which had similar retention times and absorption spectra, are most likely *cis* isomers of the  $\text{C}_{27}$  product. These apocarotenoids would result from the 9–10 cleavage of  $\beta$ -carotene (Fig. 3). The initial cleavage product 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 reported previously (8, 13). When AtCCD7 was expressed in a lycopene-producing strain of *E. coli*, a small amount of a compound with a UV-visible spectrum 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 thin-layer chromatography (Fig. 4) and HPLC (supplemental Fig. 2). No products were detected with lycopene, lutein, zeaxanthin, violaxanthin, or neoxanthin as substrates. A single product was apparent in assays with  $\beta$ -carotene. The UV-visible spectra of the enzyme product and of the  $\text{NaBH}_4$  reduced form were very similar to published



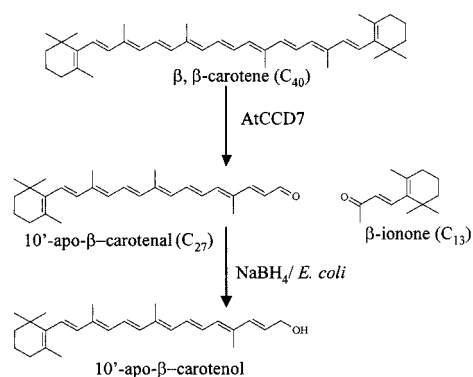


FIG. 3. The 9–10 cleavage of  $\beta$ -carotene catalyzed by the recombinant AtCCD7 protein and the subsequent reduction of the 10'-apo- $\beta$ -carotenal to the corresponding alcohol by *E. coli* or  $\text{NaBH}_4$ .

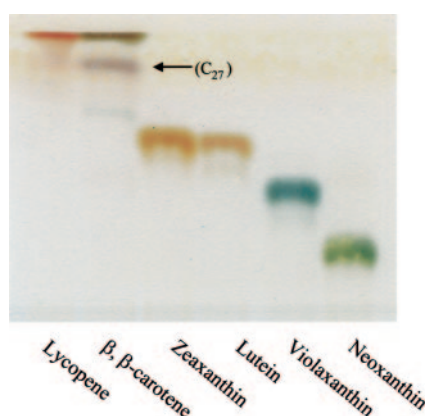


FIG. 4. Thin-layer chromatography analysis of assays with the recombinant AtCCD7 protein and some common plant carotenoids. Enzyme assay products were separated on a thin-layer silica plate that was developed in hexane and 2-propanol (90:10). Following chromatography, the plate was sprayed with 2,4-dinitrophenylhydrazine to detect aldehydes and ketones. The 10'-apo- $\beta$ -carotenal is indicated by an arrow. HPLC analysis of *in vitro* assays with lycopene and  $\beta$ -carotene are presented in supplemental Fig. 2.

spectra for the 10'-apo- $\beta$ -carotenal and the 10'-apo- $\beta$ -carotenol, respectively (supplemental Fig. 2B) (23). The  $[M]^+$  and  $[M + H]^+$  ions for the 10'-apo- $\beta$ -carotenal and 10'-apo- $\beta$ -carotenol were detected by fast atom bombardment-mass spectrometry, and a high-resolution spectrum of the reduced product matched the molecular formula for the 10'-apo- $\beta$ -carotenol,  $\text{C}_{27}\text{H}_{38}\text{O}$ . The calculated mass of the apocarotenol is 378.29230, whereas 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  $\beta$ -carotene (data not shown). The addition of water-miscible organic solvents has been shown to enhance the activity of lipoxygenases (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:  $K_m = 15.2 \mu\text{M}$  and  $V_{\max} = 4.5 \text{ pmol/mg of protein/min}$ . With the addition of 25% methanol, the  $K_m$  and  $V_{\max}$  values increased to  $20.0 \mu\text{M}$  and  $10.1 \text{ pmol/mg of protein/min}$ .

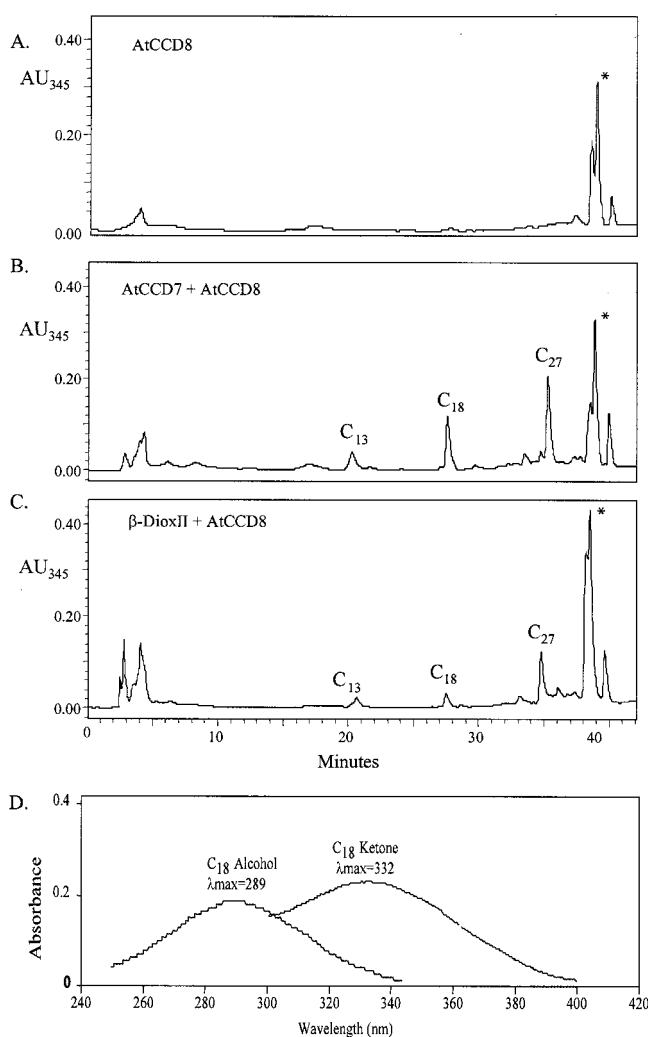


FIG. 5. A–C, HPLC analysis of pACBETA strains with a construct for the expression of AtCCD8 (A), the co-expression of AtCCD7 and AtCCD8 (B), or the co-expression of  $\beta$ -Diox II and AtCCD8 (C). Contour plots for the expression of AtCCD8 and the co-expression of AtCCD7 and AtCCD8 are presented in supplemental Fig. 1, C and D. D, the UV-visible spectra of 13-apo- $\beta$ -carotenone ( $\text{C}_{18}$ ) and the reduced product, 13-apo- $\beta$ -carotenol, in hexane.

**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 of 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  $\text{NaBH}_4$  (Fig. 5B) is similar to the 13-apo- $\beta$ -carotenone and carotenol ( $\text{C}_{18}$ ) (25). A trimethylsilyl derivative of the reduced product was analyzed by gas chromatography-mass spectrometry (Fig. 6). The observed molecular ion of 332 is the expected mass for the trimethylsilyl derivative of the 13-apo- $\beta$ -carotenol. An authentic standard of 13-apo- $\beta$ -carotenone was produced according to a synthesis described previously (26). The chromatography, UV-visible spectra, and mass spectra of the *E. coli* product and the synthetic product were the same.

The absence of the 13-apo- $\beta$ -carotenone when AtCCD8 is

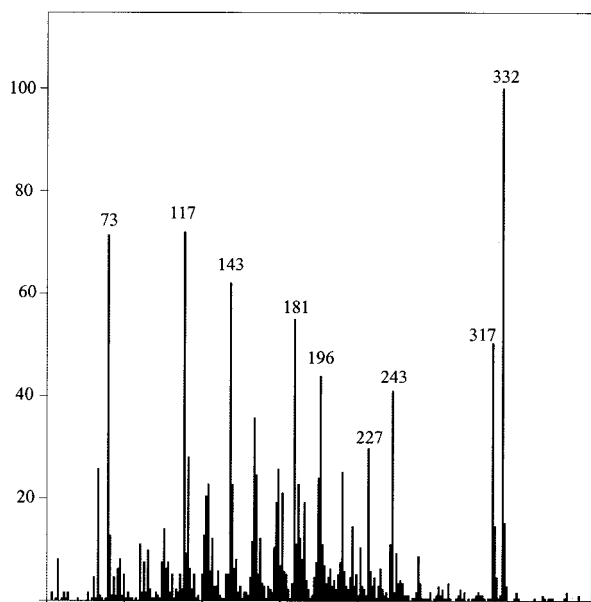


FIG. 6. Mass spectrum of the trimethylsilyl derivative of 13-apo- $\beta$ -carotenol isolated from a  $\beta$ -carotene-accumulating strain of *E. coli* that is co-expressing *AtCCD7* and *AtCCD8*.

expressed alone in the  $\beta$ -carotene strain indicates that *ATCCD8* cleaves the  $C_{27}$  product, the 10'-apo- $\beta$ -carotenol or carotenol (Fig. 7). A  $C_9$  product may also result from this cleavage reaction, but it has not yet been identified. To determine whether 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  $\beta$ -Diox II from mouse and *AtCCD8*. The  $\beta$ -Diox II also catalyzes a 9–10 cleavage of  $\beta$ -carotene (22), but it shares only 13% identity and 23% similarity with *AtCCD7*. Co-expression of  $\beta$ -Diox II and *AtCCD8* also resulted in the production of 13-apo- $\beta$ -carotenone (Fig. 5C).

#### DISCUSSION

The more axillary branching mutants, *max3* and *max4*, result from lesions in the *AtCCD7* and *AtCCD8* genes, respectively (16, 17). Because of an inability to repress the outgrowth of axillary buds, the *max* mutants display an increase in 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 the shoot, it is most likely transported through the xylem. Considering that the *AtCCD7* and *AtCCD8* proteins are similar to carotenoid cleavage dioxygenases characterized previously, it is likely that the inhibitor of axillary bud outgrowth is a carotenoid-derived molecule (2, 8, 9, 10).

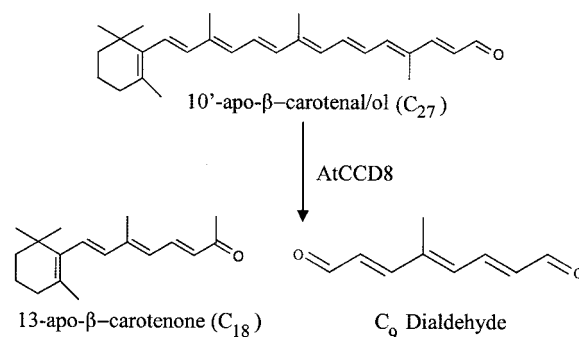


FIG. 7. The proposed cleavage of the 10'-apo- $\beta$ -carotenol catalyzed by *AtCCD8* to form the 13-apo- $\beta$ -carotenone and a  $C_9$  dialdehyde.

In plants, the cyclization of lycopene is a major branch point in carotenoid biosynthesis. The introduction of two  $\beta$ -rings produces  $\beta$ -carotene, which may subsequently be converted to zeaxanthin, violaxanthin, and neoxanthin. The introduction of a  $\beta$ -ring and an  $\epsilon$ -ring produces  $\alpha$ -carotene, which is converted primarily to lutein in most tissues. The *lut2* mutant in *Arabidopsis* is unable to produce  $\alpha$ -carotene and lutein (30) and has no branching phenotype. Therefore, it is unlikely that either of these carotenoids is a precursor of the lateral branch inhibitor. By the same rationale, mutants impaired in epoxy-carotenoid synthesis (20) 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,  $\beta$ -carotene, or an acyclic precursor.

The recombinant *AtCCD7* protein was able to catalyze the 9–10 cleavage of  $\beta$ -carotene to produce the 10'-apo- $\beta$ -carotenol ( $C_{27}$ ) and  $\beta$ -ionone ( $C_{13}$ ). 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- $\beta$ -carotenol.

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  $\beta$ -carotene-accumulating strain, the  $C_{27}$  and  $C_{13}$  products of *AtCCD7* were detected. A third product was also produced by this strain and identified as the 13-apo- $\beta$ -carotenone ( $C_{18}$ ). Because this product was not detected when *AtCCD8* was expressed by itself, it most likely results from a secondary cleavage of the  $C_{27}$  product of *AtCCD7*. A  $C_9$  product may 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 ( $\beta$ -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 suggest that *AtCCD7* and *AtCCD8* are necessary for the synthesis of an apocarotenoid that inhibits axillary bud outgrowth. The cleavage of  $\beta$ -carotene by *AtCCD7* to a  $C_{27}$  product is likely the first committed step in the biosynthetic pathway of this inhibitor.

The C<sub>27</sub> product may subsequently be cleaved by AtCCD8 to form the 13-*apo*-β-carotenone (C<sub>13</sub>) and a C<sub>9</sub> 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.

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**The Biochemical Characterization of Two Carotenoid Cleavage Enzymes from *Arabidopsis* Indicates That a Carotenoid-derived Compound Inhibits Lateral Branching**

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