JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Functional Characterization of FaCCD1: A Carotenoid Cleavage Dioxygenase from Strawberry Involved in Lutein Degradation during Fruit Ripening

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A gene encoding a carotenoid cleavage dioxygenase class 1 enzyme (*FaCCD1*) was identified among a strawberry fruit expressed sequence tag collection. The full-length cDNA was isolated, and the expression profiles along fruit receptacle development and ripening, determined by quantitative real time polymerase chain reaction, showed that *FaCCD1* is a ripening-related gene that reaches its maximal level of expression in the red fully ripe stage. *FaCCD1* was expressed in *Escherichia coli*, and the products formed by the recombinant protein through oxidative cleavage of carotenoids were identified by liquid chromatography−mass spectrometry and gas chromatography−mass spectrometry analyses. The FaCCD1 protein cleaves zeaxanthin, lutein, and β-apo-8′-carotenal in vitro. Although β-carotene is not a good substrate for FaCCD1 in vitro, the expression of *FaCCD1* in an engineered carotenoid-producing *E. coli* strain caused the degradation of β-carotene in vivo. Additionally, the carotenoid profile in strawberry was analyzed by high-performance liquid chromatography−photodiode detection, and a correlation between the increase of the expression level of *FaCCD1* during ripening and the decrease of the lutein content suggests that lutein could constitute the main natural substrate of FaCCD1 activity in vivo.

KEYWORDS: CCD1; carotenoids; *Fragaria* × *ananassa* lutein; gene expression; strawberry; ripening

INTRODUCTION

Strawberry (*Fragaria* × *ananassa*) is cultivated in nearly all countries of the world and is one of the most popular fruits that is consumed as a fresh or manufactured product. One of the more valuable organoleptic traits of the strawberry fruit, in both the fresh and processed markets, is its flavor (*1*). The volatile components of strawberry fruit formed during ripening have been intensively studied, and more than 360 volatiles have been identified (*2, 3*). The relative abundance of individual volatiles is a fingerprint of a particular cultivar and species. The relationship between the aroma impact volatiles and strawberry varieties has been the subject of numerous investigations (*4–6*). Different studies have demonstrated that strawberry aroma is the result of the combined perception of fruity (ethyl butanoate, ethyl hexanoate, and methyl 2-methylbutanoate), green ((*Z*)-3-hexenal), sweaty (butanoic acid and 2-methylbutanoic acid), peachlike (γ-decalactone), and caramel-like [4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF; Furaneol) and 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (DMMF)] flavor notes (*6, 7*). Although exhaustive information regarding strawberry volatile chemical composition is available, few detailed biochemical and genetic studies have been done in relation to aroma biosynthesis. Only recently, it was shown that the *SAAT* gene coding a strawberry alcohol acyl-CoA transferase and the *FaNES* gene coding a *Fragaria* × *ananassa* nerolidol synthase are involved in strawberry fruit ester and terpene formation, respectively (*8, 9*), Besides, it has been shown that *FaOMT*, which codes a *Fragaria* × *ananassa* O-methyltransferase, is responsible for DMMF biosynthesis (*10, 11*). Recently, *FaQR* coding a *Fragaria* × *ananassa* quinone reductase has been cloned and functionally characterized. The *FaQR* gene is involved in the biosynthesis of Furaneol, the key flavor compound in strawberries (*12*).

The natural ionones derived from the enzymatic oxidative cleavage of carotenoids have been implicated as crucial components in the full flavor of many fruits (*13*). Apocarotenoids such as β-ionone are of particular interest due to their extremely potent odor thresholds (0.007 ppb). Therefore, although generally they are present at relatively low levels in fruits, they often possess strong effects on the overall human

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10.1021/jf801096t CCC: $40.75 © 2008 American Chemical Society
Published on Web 09/09/2008

appreciation of the aromas. These compounds are biosynthesized by the carotenoid cleavage dioxygenase class 1 (CCD1) enzymes. In *Arabidopsis thaliana*, the AtCCD1 enzyme catalyzes the cleavage of carotenoids at the 9,10 and 9′,10′ positions, yielding one or two ionone molecules or the various oxyfunctionalized derivates depending on the nature of the carotenoid substrate (*14, 15*). *AtCCD1* orthologues have been identified either genetically or biochemically in grape (*Vitis vinifera* (*16*)), nectarine (*Prunus persica* (*17*)), tomato (*Lycopersicum esculentum* (*18*)), petunia (*Petunia hybrida* (*19*)), star fruit (*Averrhoa carambola* (*20*)), melon (*Cucumis melo* (*21*)), and crocus (*Crocus sativus* (*22*)). In some *Fragaria* × *ananassa* cultivars, the presence of β-ionone has been reported (*23, 24*). However, the levels of this compound were low.

Due to the importance of strawberry flavor the aim of this work was the isolation and functional characterization of *FaCCD1*, a gene encoding a carotenoid cleavage dioxygenase isolated from strawberry fruits. Moreover, the carotenoid profile in strawberry was also analyzed to determine the possible role for in vivo carotenoid degradation by FaCCD1 activity.

MATERIALS AND METHODS

Plant Material. Strawberry (*Fragaria* × *ananassa*) fruits, cv. Camarosa, were harvested at the following ripening stages: small-sized green fruits (G1), middle-sized green fruits (G2), full-sized green fruits (G3), white fruits (W), turning stage fruits (T), and fully ripe red fruits (R). The fully open flowers, runners, leaves, and crown were also harvested. Samples were immediately frozen in liquid nitrogen and stored at − 80 °C.

Cloning of the *FaCCD1* Gene. A strawberry fruit EST collection was screened by comparison between mRNA populations from green and red fruit receptacles by microarray approaches. Bioinformatics analysis showed that one of the ESTs selected (*FaCCD1*) presented a high sequence homology with those sequences corresponding to *CCD1* genes of plants. The full-length sequence of *FaCCD1* was obtained by 5′- and 3′-RACE using the Marathon cDNA amplification kit (Clontech). First-strand cDNA synthesis was carried out using 1 μg of poly(A^+) mRNA from red strawberry fruits and the modified lock-docking oligo(dT) primer provided with the kit. Second-strand synthesis and ligation of the double-strand cDNA to the adaptor were achieved following the manufacturer's instructions. RACE reactions were developed with the Advantage 2 polymerase mix (Clontech) using the Marathon cDNA adaptor primer and the 5′ and 3′ gene-specific primers used for QRT-PCR analysis. The PCR products were cloned into pGEM-T-Easy (Promega) and sequenced.

Auxin Treatment. Achenes of G2-stage strawberry fruits were carefully removed from the receptacle using the tip of a scalpel blade. One set of deachened fruits was treated with the synthetic naphthaleneacetic acid auxin (NAA) [1 mM NAA in 1% (v/v) DMSO] as a lanoline paste. Another set of deachened fruits was treated with the same paste but without NAA. Samples were harvested 5 days after treatment, immediately frozen in liquid nitrogen, and stored at −80 °C. Untreated fruits were used as controls.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) Analysis. Total RNA from a pool of six to seven strawberry fruits at different ripening stages, from fruits subjected to auxin treatment and from the flowers, runners, leaves, and crown were extracted according to Manning (*25*). Genomic DNA was eliminated from RNA preparations by treatment with RNase-free DNase I according to the manufacturer's instructions (Amersham Biosciences, Uppsala, Sweden). Genomic DNA contamination of RNA was checked by PCR amplification with the specific primers for *FaCCD1* and internal control genes. A 1 μg portion of DNase I-treated RNA of each sample was reverse transcripted using the iScript cDNA synthesis kit (BioRad). The RT reaction contained 4 μL of 5× iScript reaction mix, and 1 μL of iScript reverse transcriptase in a final volume of 20 μL. The mixture was then incubated at 25 °C for 5 min, at 42 °C for 30 min, and at 85 °C for 5 min. QRT-PCR assays were performed on an iCycler system (BioRad) using SYBR Green (Molecular Probes, Eugene, OR) as the fluorophor in 96-well plates. Specific primers for the *FaCCD1* gene (forward, 5′-GGG GAT TTT TGG TTT ACT CAT GG-3′; reverse, 5′-TTG TCT CCC TCT GAA AGA GCT AGA A-3′) were designed to generate amplicons of 144 bp. The PCR reaction mixture (25 μL total volume) consisted of: 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, a 0.2 μM concentration of each sequence-specific primer, 3 μL of diluted SYBR Green (1:150.000, v/v), 3 μL of transcribed cDNA, and 0.6 U of Taq polymerase (Biotools). The thermal cycling conditions were 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. PCR reactions were performed in triplicate to increase the accuracy and reliability of the procedures. Melting curve analysis parameters were 1 min at 95 °C, 1 min at 55 °C,and 10 s at a temperature range of 55−95 °C (with an increase of 0.5 °C each cycle) for 80 cycles.

In QRT-PCR assays, quantification of the PCR products is based on the threshold cycle (Ct) value, which is defined as the cycle at which fluorescence is first detectable above the background. The Ct value is inversely proportional to the logarithm of the initial copy number. In our system, each 10-fold difference in initial copy number produced a 3.2 cycle difference in Ct. Corresponding Ct values for each PCR reaction were determined. These values were then normalized using the Ct value corresponding to a strawberry (housekeeping) 18S−26S interspacer gene (internal control). The increases in gene expression were determined according to the following expression: fold change $= 2^{-\Delta\Delta Ct}$, where ΔCt = [Ct(target) − Ct(internal control)] of the reference sample − [Ct(target) − Ct(internal control)] of the problem sample (*26*).

Expression of Full-Length *FaCCD1* cDNA in *Escherichia coli* Carotenoid Accumulating Strains. The coding sequence for protein expression was amplified with primers, 5′-GGA TCC AAG CAG AAG CTG GTG-3′ and 5′-TCG ACT CAC TCG AGT AAA TTT GCT TG-3′, and cloned in the *Bam*HI/*Sal*I sites of pGEX-4T-1 (Amersham Biosciences). After sequencing, the construction *FaCCD1*−pGEX was maintained in *E. coli* DH5α.

Plasmid *FaCCD1*−pGEX and empty p-GEX-4T-1 (negative control) were introduced into *E. coli* strain JM101 previously transformed with the plasmids pACCAR25ΔcrtX and pACCAR16ΔcrtX. These plasmids carry genes from *Erwinia uredorova*, allowing the synthesis of zeaxanthin (pACCAR25ΔcrtX) and β-carotene (pACCAR16ΔcrtX), respectively (*27*). Colonies were selected on solid LB plates containing ampicillin (100 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}). An overnight culture of 5 mL was centrifuged, and the pellet was photographed.

Crude Extract Preparation. An overnight culture (2 mL) of *E. coli* BL21 (DE3) transformed with *FaCCD1*−pGEX was used to inoculate 200 mL of LB medium containing 100 μg mL^{-1} ampicillin. The cultures were incubated (37 °C, 150 rpm) until they reached an A_{600} of 0.6−0.8. Expression of the protein was induced by the addition of 0.2 mM isopropyl β-D-thiogalactoside, and the culture was grown at 16 °C for an additional 14 h. *E. coli* cells were harvested by centrifugation (4000*g*, 20 min, 4 °C) and resuspended in 8 mL of phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing 5 mM sodium ascorbate. The cells were disrupted by sonication on ice with an MS73 sonotrode (Bandelin Electronic, Berlin) four times for 30 s at 10% maximal power. After sonication, 0.1% (v/v) Triton X-100 was added, and the cell lysate was gently shaken on ice for 60 min. Cell debris was removed by centrifugation (5000*g*, 30 min, 4 °C). The supernatant was frozen at −80 °C until use. BL21 cells containing the empty pGEX-4T-1 vector were treated in the same manner, and the respective extract was used as a control in all experiments.

In Vitro Assay with Recombinant GST−FaCCD1 Protein. The assay with the different carotenoid substrates was developed as described in ref *14*. A 500 μL portion of ethanol containing 0.05 mg of β-apo-8′-carotenal (zeaxanthin or lutein) and 250 μL of an ethanolic β-octyl glucoside solution (4% w/v) were combined and evaporated to dryness. A 1 mL sample of the crude cell extract described above was added. The solution was shaken vigorously and incubated at 30 °C for 3 h. The reactions were extracted three times with equal volumes of diethyl ether. The organic phase was dried with Na_2SO_4, concentrated

to 100 μL, and analyzed by GC−MS. For the analysis of the C17-dialdehyde, the organic phase was evaporated to dryness, dissolved in methanol, and subjected to LC−MS analysis.

Carotenoid Extraction. For carotenoid extraction 2−10 g of fruits and tissues were first homogenized in liquid N_2 and afterward in 50 mL of 40% (v/v) aqueous methanol containing β-apo-8′-carotenal (0.15 mg) as an internal standard using a homogenizer (polytron) for 5 min at 6000 rpm. After vacuum filtration the pigment in the residue was extracted with 50 mL of diethyl ether/methanol (7:3 v/v) containing 0.1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol (BHT) until the residue was colorless. The extract was transferred to a separatory funnel containing 100 mL of diethyl ether. The ether phase was washed two times with NaCl-saturated water and treated with anhydrous Na_2SO_4. After the ether phase was evaporated, the residue was dissolved in 20 mL of diethyl ether, mixed with 10 mL of 20% methanolic KOH, and placed for 18 h in the dark overnight at room temperature. The alkaline mix was transferred to a separatory funnel containing 100 mL of diethyl ether. The ether phase was then washed five to six times with NaCl-saturated water and treated with anhydrous Na_2SO_4. After the ether phase was evaporated the dry residue was kept at −80 °C until analysis. Before HPLC analysis the sample extracts were first dissolved in 200 μL of tetrahydrofuran (<10%) and afterward diluted with the equal volume of acetonitrile/methanol (54:46, v/v) supplied with 0.2% TFA stabilized with 0.01% BHT.

GC−MS Analysis of Apocarotenoides. GC analyses were performed with a Thermo Finnigan Trace DSQ mass spectrometer coupled to a Thermo Finnigan Trace gas chromatograph with a split injector (1:20) and a 0.25 μm BPX5 20 M fused silica capillary column with a 30 m × 0.25 mm inner diameter. The temperature was held at 40 °C for 3 min and then increased to 250 °C at 5 °C min^{-1} intervals, with a helium flow rate of 3 mL min^{-1}. The electron ionization voltage was 70 eV, and the ion source and interface temperature were both 250 °C. Spectra were recorded and evaluated with Xcalibur software (version 1.4) supplied with the device.

LC−MS Analysis of C_{17}-Dialdehyde. The HPLC system consisted of a quaternary pump and a variable-wavelength detector, all from Agilent 1100 (Bruker Daltonics, Bremen, Germany). The column was a LUNA C18 (2) 100A 150 × 2 mm (Phenomenex, Aschaffenburg, Germany). Coupled to the HPLC instrument was a Bruker Esquire 3000plus mass spectrometer with an electrospray ionization interface that was used to record the mass spectra. The ionization voltage of the capillary was 4000 V, and the end plate was set to −500 V. The capillary exit was 106 V, and the octopole radio frequency amplitude was 112.2 Vpp. The temperature of the dry gas (N_2) was 300 °C, flowing at 9 mL min^{-1}. The full scan mass spectra were measured from *m/z* 50 to *m/z* 600 until the ion charge control target reached 20 000 or for 200 ms, whichever came first. Tandem mass spectrometry was performed using helium as the collision gas, with the collision energy set to 1.20 V. All mass spectra were acquired in the positive ionization mode. The LC parameters went from 100% water (with 0.1% formic acid) to 50% acetonitrile (with 0.1% formic acid)/50% acidic water in 20 min, then in 5 min to 100% acetonitrile, and in 10 min to 70% acetonitrile/30% 2-propanol, which was held for 10 min. The concentration was then changed in 3 min back to 100% acetonitrile and in 5 min back to 100% water, which was held for 7 min. The detection wavelength was 285 nm.

HPLC Analysis of Carotenoids. Samples were analyzed using the HPLC system Agilent 1100 (Agilent Technologies, Waldbronn, Germany) with a photodiode array detector and a Synergi 4 μm hydro-reversed-phase 80A column (250 × 4.6 mm, 4 μm) from Phenomenex (Aschaffenburg, Germany). Separation of carotenoids was carried out at a constant flow rate of 1 mL min^{-1} with an acetonitrile/water (80:20, v/v) mobile phase A and a *tert*-butyl methyl ether/acetonitrile/water (75:21:4, v/v/v) mobile phase B each containing 0.2% TFA. Gradient elution was started with 100% A and performed by changing mobile phase B to 80% (0−7 min to 21% B, 7−26 min to 40% B, 26−36 min to 80% B, 36−40 min 80% B). The injection volume was 10 μL. Carotenoids were identified on the basis of relative retention times and by comparison of spectra with those of pure standard mixtures. Carotenoids were detected at 450 nm and quantified via external calibration.

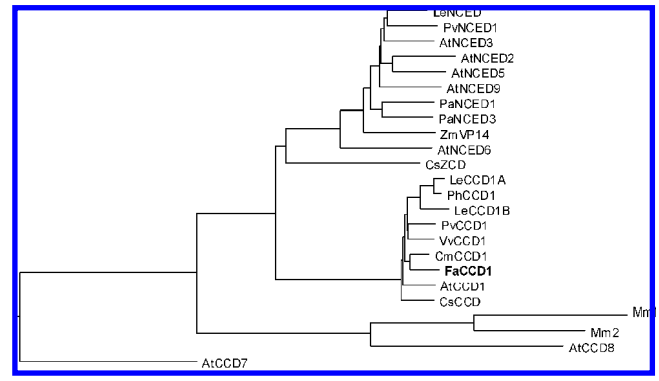

Figure 1. Phylogenetic tree of the deduced amino acid sequences of functionally characterized carotenoid cleavage dioxygenase proteins. The protein sequences were clustered using ClustalW (DNASTAR Lasergene software v.5.0). Accession numbers for the sequences: *A. thaliana* (AtCCD1, NP_19191.1; AtCCD7, NP_182026.1; AtCCD8, NP_195007.2; AtNCED2, NP_193569.1; AtNCED3, NP_188062.1; AtNCED5, NP_174302.1; AtNCED6, NP_189064.1; AtNCED9, NP_177960.1); *C. sativus* (CsZCD, AJ489276); *C. melo* (CmCCD1, DQ269467); *Fragaria* × *ananassa* (FaCCD1, EU314719) *L. esculentum* (LeCCD1A, AY576001; LeCCD1B, AY576002; LeNCED, CAB10168.1); *Mus musculus* (Mm1, AAG33982.1; Mm2, CAC28026.1); *Phaseolus vulgaris* (PvCCD1, AAK38744.1; PvNCED1, AAF26356.1); *Persea americana* (PaCCD1, AAK00622.1; PaNCED1, AAK00632.1; PaNECD3, AAK00623.1); *P. hybrida* (PhCCD1, AY576003); *V. vinifera* (VvCCD1, AY856353); *Zea mays* (ZmVp14, AAB62181.1).

RESULTS

Identification of *FaCCD1* and Sequence Analysis. The full-length sequence of the *FaCCD1* cDNA codes a predicted FaCCD1 protein containing 540 amino acids (1623 bp) with a calculated molecular mass of 60.89 kDa and pI of 6.7. Sequence comparison with other *CCD1* sequences of higher plants showed that *FaCCD1* belongs to the group of genes that encode the carotenoid cleavage dioxygenase class 1 enzymes (**Figure 1**). This class includes the *V. vinifera* VvCCD1 protein and *C. melo* CmCCD1 protein, which share 82% and 80% amino acid sequence homology with the FaCCD1 protein, respectively. The enzymatic characterization of the VvCCD1 recombinant protein demonstrated that the enzyme cleaves zeaxanthin and lutein (*28*). The FaCCD1 protein also presents amino acid sequence similarity with the *L. esculentum* LeCCD1A (78%) and LeCCD1B (74%) proteins (*18*), *P. hybrida* PhCCD1 protein (78%) (*19*), and *A. thaliana* AtCCD1 (79%) (*15*) protein. These enzymes are able to cleave multiple carotenoid substrates at the 9,10 and 9′,10′ positions to produce a C_{14}-dialdehyde and two C_{13}-cyclohexenones (*15*). More recently it has been shown that the AtCCD1 enzyme also cleaves β-apo-8′-carotenal into C_{17}-dialdehyde and β-ionone (*14*). FaCCD1 as a member of the CCD1 family is clearly distinct from other members of the carotenoid cleavage dioxygenase family (*29*). Thus, it shares only 32% homology of the amino acid sequence on average with those sequences corresponding to proteins of the NCED class, whose members are involved in ABA biosynthesis and 16% with AtCCD7 and AtCCD8 (**Figure 1**).

Expression Patterns of *FaCCD1*. The spatial−temporal expression patterns of the *FaCCD1* gene along fruit development and ripening was studied by QRT-PCR. The transcript amount increased throughout the ripening stages, reaching its maximal value at totally mature receptacles (red stage) (**Figure 2A**). In achenes, the expression of the gene remained almost stable throughout all stages of development and ripening (**Figure 2B**). Comparison of *FaCCD1* expression levels in different tissues showed that the highest amount of transcripts were found

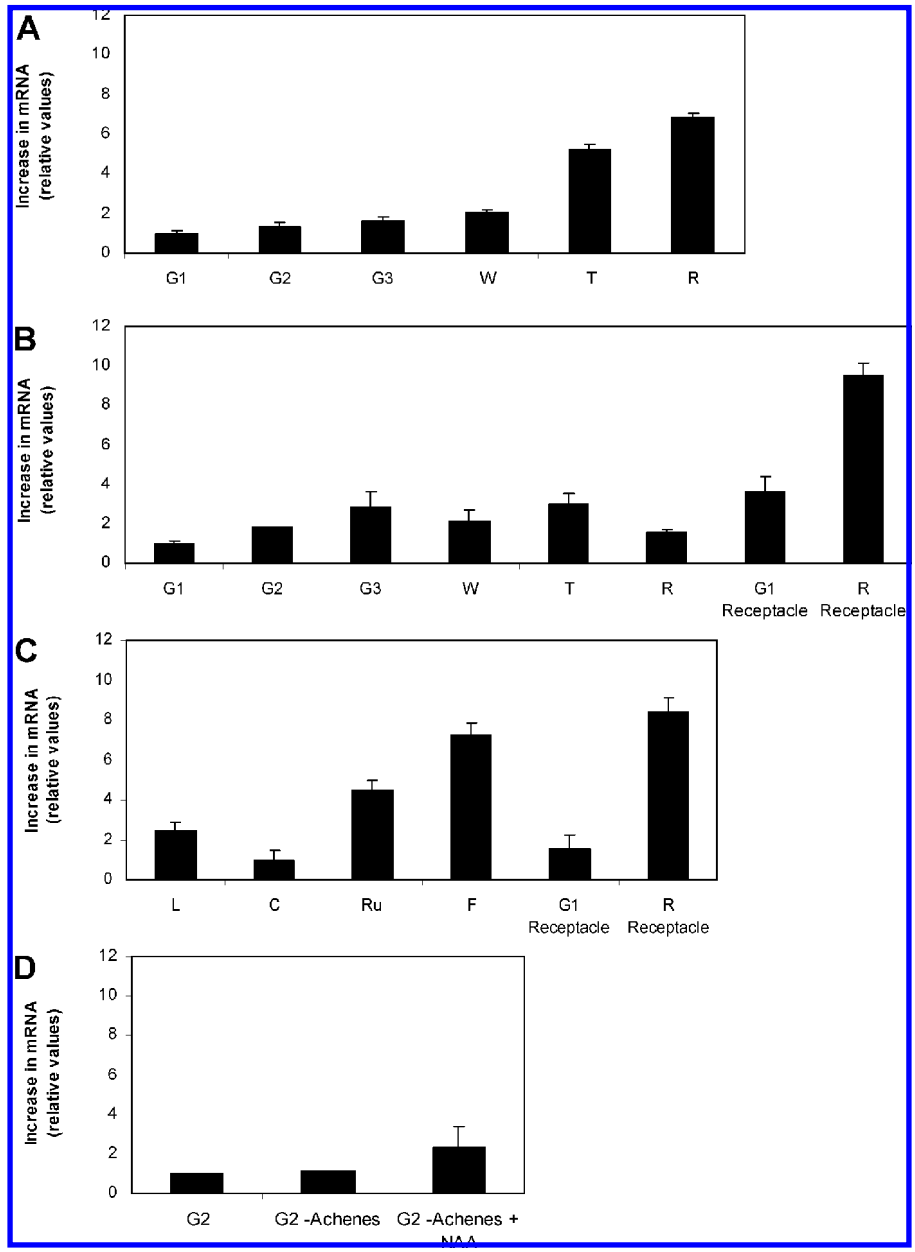

Figure 2. Developmental and spatial expression patterns of the *FaCCD1* gene in strawberry. Relative gene expression analysis was developed by quantitative real-time PCR. Values are expressed in units relative to the strawberry constitutive 18S−26S interspacer gene and to the sample with a lower value of gene expression. (**A**) Changes in mRNA expression in the receptacle at the different fruit ripening stages (G1, G2, G3, W, T, and R; see the Materials and Methods). (**B**) Changes in mRNA expression in achenes at the different fruit ripening stages and in the receptacle corresponding to the G1 and R stages. (**C**) Expression levels in the leaves (L), crown (C), runners (Ru), and flowers (F) and in the receptacle corresponding to the G1 and R stages. (**D**) Expression levels in deachened receptacles at the G2 development stage (G2-achenes) compared with the untreated control (G2) and with deachened receptacles treated with the synthetic auxin NAA. Mean values ± SD of three independent experiments are shown.

in the red receptacles and flowers where the quantity of transcript was comparable (**Figure 2C**).

The expression of the vast majority of strawberry fruit-specific genes has been shown to be under the negative regulatory control of auxins (*30–32, 12*). To determine whether the *FaCCD1* gene expression was negatively regulated by auxins synthesized in achenes, we performed gene expression studies on deachened green fruit (G2 stage). The results showed clearly that no changes in *FaCCD1* gene expression were detected after removal of the achenes of green fruit receptacles (treated or untreated with NAA) kept on the plants (**Figure 2D**). However, the amount of transcript corresponding to a control gene (*FaQR*) whose expression is negatively regulated by auxins (*12*) increased in the same experiment, so no correlation between auxin levels and *FaCCD1* gene expression was found (data not shown). Thus, the increase in the expression levels of *FaCCD1* might be regulated by clues other than the content of auxins in the fruit receptacle.

Activity of the FaCCD1 Protein. To investigate the enzymatic characteristics of the FaCCD1 protein, the full-length *FaCCD1* cDNA gene was cloned into the *E. coli* expression vector pGEX-4T-1. This recombinant plasmid was first introduced into *E. coli* strains previously engineered to accumulate different carotenoid compounds (*27*). The carotenoids accumulated in these strains impart color to the cells. A loss of color in the *E. coli* cells transformed with the plasmid harboring a recombinant protein indicates that the carotenoids are metabolized by this protein to colorless compounds. Thus, when

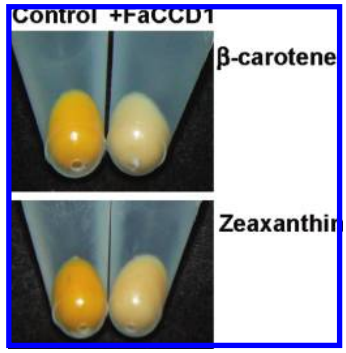

Figure 3. *E. coli* strains engineered to accumulate β-carotene and zeaxanthin were cotransformed with the *FaCCD1*−pGEX construction (+FaCCD1) or empty vector p-GEX-4T-1 (control).

the recombinant protein FaCCD1 was expressed in cells producing β-carotene and zeaxanthin, there was a loss of color in both strains, indicating a potential catabolism of these carotenoid substrates (**Figure 3**).

To determine the potential substrates of the FaCCD1 protein for its enzymatic activity, a crude extract of the recombinant protein was assayed in vitro on a variety of carotenoid substrates. The cleavage products of β-apo-8′-carotenal were identified by GC−MS and LC−MS (for detailed information, see Figures 1 and 2 in the Supporting Information). GC−MS analysis revealed a major peak exhibiting a mass spectrum identical to that of β-ionone. The newly formed β-ionone was only present in crude extracts of *E. coli* cells harboring the *FaCCD1*−pGEX plasmid, while it was absent in the control cells transformed with the empty vector. Moreover, LC−MS analysis showed the presence of a compound eluting at 29.8 min corresponding to a C_{17}-dialdehyde that was not present in the control reaction. When lutein was used as the substrate, the two isoforms, 3-hydroxy-α-ionone (**Figure 4A**) and 3-hydroxy-β-ionone (**Figure 4C**), were detected by GC−MS. In this case we found higher quantities of both in *E. coli* extracts expressing the FaCCD1 protein in comparison to those found in *E. coli* control extracts. Using zeaxanthin as the substrate, the 3-hydroxy-β-ionone was also detected by GC−MS analysis (for detailed information, see Figure 3 in the Supporting Information). However, in our experimental conditions we were not able to detect the C_{14}-dialdehyde when either lutein or zeaxanthin was used as the substrate. Notwithstanding, the analysis of the UV spectrum of the enzymatic reactions corresponding to FaCCD1 samples showed a degradation of both lutein (**Figure 5A**) and zeaxanthin (**Figure 5B**) carotenoids as a result of the cleavage of both substrates. These degradations were not observed in their respective controls.

Analysis of the Carotenoid Content of the Strawberry Fruit Receptacle. The carotenoid content in the strawberry fruits during different developmental and ripening stages and also in the flowers and leaves were determined. The main carotenoid found in the receptacle, flower, and leaf tissues was lutein (**Figure 6B**). The content of zeaxanthin and β-carotene was very similar in all the tissues analyzed. However, β-crypthoxanthin was detected in the leaves but not in the flowers, while green receptacles contained only trace amounts (**Figure 6B**). In fruits the concentration of all the carotenoids analyzed decreased during ripening (**Figure 6A**). The decreasing rate observed for zeaxanthin, β-carotene, and β-crypthoxanthin was very similar among them. However, a drastic and marked decrease of the lutein content was found during the ripening stages. This higher decrease of the lutein content in comparison with the reductions found for the other carotenoids could be due to an additional degradation not related to the normal turnover of carotenoids during ripening.

DISCUSSION

In the present study we report the isolation and characterization of a strawberry gene that encodes a protein which shows high identity of the amino acid sequence with sequences corresponding to CCD1 proteins from other plants. In the putative amino acid sequence of the FaCCD1 protein the four conserved histidines, which are characteristic of the carotenoid cleavage dioxygenase family, are present (*33*). These four histidines are essential for the coordination of the iron cofactor used in catalysis for these dioxygenases.

The recombinant FaCCD1 protein has been assayed with different carotenoid substrates. β-Apo-8′-carotenal appears to be the best substrate for FaCCD1 activity in vitro. Similar results have been found for *A. thaliana* AtCCD1 (*14*). Probably, the higher solubility of β-apo-8′-carotenal in water with respect to lutein and zeaxanthin makes it more easily accessible for the enzyme (*14*).

The FaCCD1 protein as well as its homologues from *A. thaliana* (AtCCD1 (*15*)), *C. sativus* (CsCCD (*22*)) *V. vinifera* (VvCCD1 (*16*)), and *L. esculentum* (LeCCD1A and LeCCD1B (*18*)) catalyzes the symmetrical cleavage of zeaxanthin in the 9,10 and 9′,10′ positions, generating 3-hydroxy-β-ionone. By using *E. coli* carotenoid accumulating strains (*15, 18*) or spectrophotometrical assays of the recombinant activity (*28*), the degradation of lutein by CCD1 enzymes has been shown. Similarly, the FaCCD1 enzyme catalyzed the in vitro cleavage of lutein, rendering both isoforms of ionone (3-hydroxy-α-ionone and 3-hydroxy-β-ionone), which were confirmed by LC−MS for the first time. Moreover, 3-hydroxy-β-ionone and 3-hydroxy-α-ionone are produced in the same amount, which reveals that the enzyme symmetrically cleaves the two 9,10 and 9′, 10′ bonds with the same specificity. In vitro assay of FaCCD1 recombinant activity showed that the enzyme does not use β-carotene as a substrate. However, the *E. coli* expression assays clearly indicated that the enzyme can cleave this carotenoid in vivo. The same result was obtained for the *A. thaliana* AtCCD1 enzyme (*14, 15*). It seems that xanthophylls, i.e., carotenoids that contain oxygen, appeared to be better substrates for CCD1 that β-carotene itself (*14, 15*).

It has been proposed that the auxins are the primary hormones controlling strawberry fruit ripening (*34*). A gradual decline in the fruit receptacle of the auxin supplied by achenes in the stages of fruit ripening has been associated with gene expression profile characteristic of the strawberry fruit ripening processes (*34, 35*). Indeed, this negative and direct correlation has been shown for many strawberry ripening-related genes after removal of achenes from the immature green fruit receptacle kept in planta (*30–32, 12*). However, no up-regulation of *FaCCD1* gene expression was found by removing achenes from the immature green receptacle. Thus, although *FaCCD1* showed a typical fruit ripening-related expression pattern, its expression is not regulated by auxins. This result also suggests that physiological signals other than the auxin content of the fruit receptacle could be involved in the production of the gene pattern profile responsible for the strawberry fruit ripening. In this sense it has been proposed that the content of ABA plays an important role in the strawberry fruit ripening process (*34*). Besides, the oxidative stress produced in the strawberry fruit receptacle has also been related to the strawberry fruit ripening (*30*).

FaCCD1 expression increased continuously throughout the fruit ripening stages. This up-regulation during the fruit

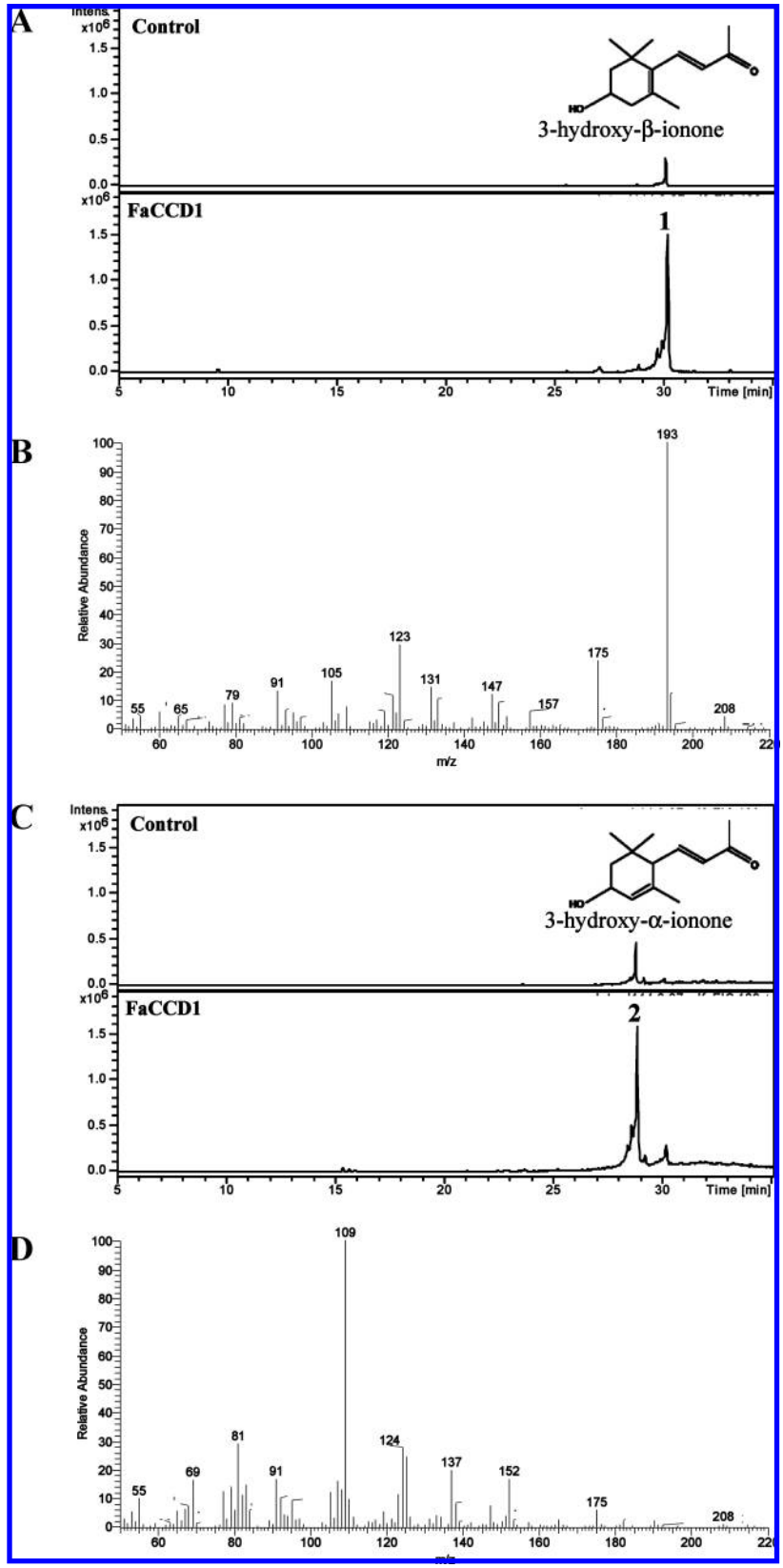

Figure 4. GC−MS analysis of the 3-hydroxy-β-ionone (ion trace *m*/*z* 193) and 3-hydroxy-α-ionone (ion trace *m*/*z* 109) formed by FaCCD1 from lutein. (**A**, **C**) Crude extracts were prepared from *E. coli* BL21 cells expressing the recombinant protein GST−FaCCD1 (bottom panel) and from BL21 transformed with the empty vector pGEX-4T-1 (control, top panel). (**B**) Mass spectrum of peak 1 corresponding to 3-hydroxy-β-ionone. (**D**) Mass spectrum of peak 2 corresponding to 3-hydroxy-α-ionone.

development and ripening has also been observed in *CCD1* genes of other plants such as *C. melo* (*21*), *V. vinifera* (*16*), and *Satsuma mandarin* (*36*) and the *LeCCD1A* gene from *L. esculentum* (*18*). This increase in gene expression during fruit ripening seems to be involved in the volatile apocarotenoid emission in grape (*16*) and tomato (*18*). However, there is a lag between *CCD1* gene expression and apocarotenoid volatile production (*16, 18*), which could be explained by the different

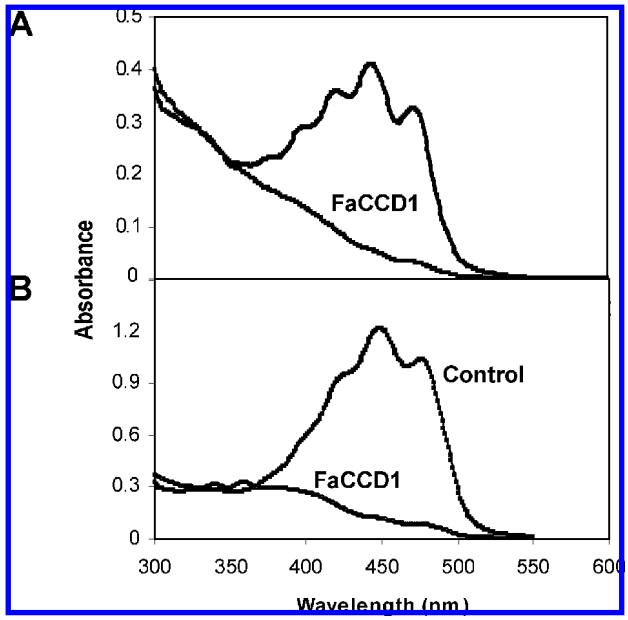

Figure 5. UV spectrum of the reaction assay containing lutein (**A**) or zeaxanthin (**B**) as the substrate. Crude extracts were prepared from *E. coli* BL21 cells expressing the recombinant protein GST−FaCCD1 and from BL21 transformed with the empty vector pGEX-4T-1 (control).

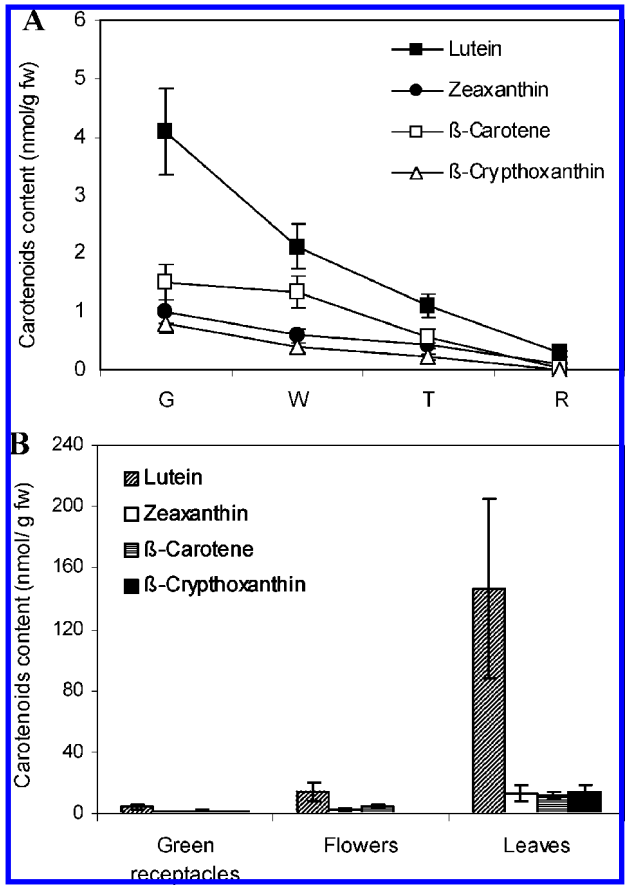

Figure 6. Carotenoid content in strawberry: (**A**) levels of carotenoids in the receptacles during different strawberry ripening stages (G, W, T, and R; see the Materials and Methods); (**B**) levels of carotenoids in the green receptacles, flowers, and leaves. Mean values ± SD of three independent experiments are shown.

cellular localization of carotenoids and CCD1 enzymes (*29, 22, 18*). Although there is a temporal delay in volatile emission in relation to gene expression, in tomato the silencing of both *LeCCD1A* and *LeCCD1B* genes generates a reduction in β-ionone and geranylacetone. This provides a cause-and-effect relationship between *LeCCD1* gene expression and the formation of those important flavor volatiles in vivo (*18*). In our experimental conditions we were not able to detect volatile apocarotenoids in strawberry, although minor amounts of β-ionone have been described in some strawberry varieties (*23, 24*). Thus, we cannot conclude that the increase in *FaCCD1* gene expression during strawberry fruit ripening is related to the production of apocarotenoids. It has also been shown that hydroxy-β-ionone is glycosylated in the strawberry receptacles (*37*). However, there is a correlation between the increase in gene expression and the decrease of the carotenoid content during the different ripening stages. In strawberry fruits the carotenoid content decreases during fruit development (*38*). The decreases observed for the zeaxanthin, β-carotene, and β-crypthoxanthin content along fruit ripening could be explained by the normal catabolism of carotenoids. However, in the case of lutein the high decrease produced in the transition between the green and red stages could be related, at least partially, to the degradation of this carotenoid by FaCCD1 activity. Moreover, lutein is the most abundant carotenoid present in the red fruit stage where the maximal expression of *FaCCD1* was found. Thus, lutein could be the natural substrate for the production of apocarotenoids. In this sense, CCD1 activity seems not to be responsible for the varietal differences in the carotenoid content during citrus fruit maturation (*36*). However, in AtCCD1 loss-of-function mutants an increase in carotenoid content in the mature seed was observed, indicating a role for CCD1 in carotenoid catabolism (*29*).

In addiction to that in the fruits, there is also a significant expression of *FaCCD1* in the leaves, crown, runner, achenes, and flowers. Some of the CCD1 apocarotenoid products have been reported to have antimicrobial activities (*39*). Thus, it is possible that expression of *FaCCD1* in all tissues may have a role in the formation of multiple antimicrobial compounds that may be important for plant defense. Moreover, the high expression in the flower, with levels similar to those in the red fruit stage, could be responsible for the synthesis of floral volatiles for attracting pollinating insects (*19*).

In conclusion, CCD1 enzymes produce a variety of apocarotenoid volatiles that are important for the flavor of agronomically important plants. The cDNA for the strawberry *FaCCD1* gene has been cloned from fully red ripened fruits. The recombinant protein catalyzes the in vitro cleavage of β-apo-8′-carotenal, lutein, and zeaxanthin. Although we cannot conclude the implication of FaCCD1 in apocarotenoid production in vivo, our results suggest that FaCCD1 could be implicated in the catabolism of carotenoids, or more concretely in lutein degradation during fruit ripening.

ABBREVIATIONS USED

ABA, abcisic acid; BHT, 2,6-di-*tert*-butyl-4-methylphenol; CCD1, carotenoid cleavage dioxygenase class 1; Ct, threshold cycle; DMMF, 2,5-dimethyl-4-methoxy-3(2*H*)-furanone; DMSO, dimethyl sulfoxide; EST, expressed sequence tag; G1, small-sized green fruits; G2, middle-sized green fruits; G3, full-sized green fruits; GC, gas chromatography; GC−MS, gas chromatography−mass spectrometry; LB, Luria−Beltrani medium; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LC−MS, liquid chromatography−mass spectrometry; *m/z*, mass to charge ratio; NAA, naphthaleneacetic acid; NCED, 9-*cis* epoxy carotenoid dioxygenase; QRT-PCR, quantitative real time polymerase chain reaction; R, fully ripe red fruits; RT,

reverse transcription; T, turning stage fruits; TFA, trifluoroacetic acid; W, white fruits.

ACKNOWLEDGMENT

We thank Thomas Hoffmann, Beate Arold, and Florian Vitzthum for their useful help with the GC−MS and LC−MS analysis and Nancy Terrier for sending the carotenoid accumulating *E. coli* strains.

Supporting Information Available: GC−MS analysis of the β-ionone formed by FaCCD1 from β-apo-8′-carotenal, LC−MS analysis of the C_{17}-dialdehyde formed by FaCCD1 from β-apo-8′-carotenal, and GC−MS analysis of the 3-hydroxy-β-ionone formed by FaCCD1 from zeaxanthin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review April 7, 2008. Revised manuscript received June 6, 2008. Accepted June 17, 2008. C.G.-L. thanks MEC for a research contract associated with Grant BIO2004-04885-C02-02.

JF801096T