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Functional characterization of *CmCCD1*, a carotenoid cleavage dioxygenase from melon

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This paper is dedicated to Prof. Rodney Croteau in occasion of his 60th birthday, in appreciation for his teachings and support.

Abstract

Carotenoids are nutritionally important tetraterpenoid pigments that upon oxidative cleavage give rise to apocarotenoid (norisoprene) aroma volatiles. β-Carotene is the predominant pigment in orange-fleshed melon (*Cucumis melo* L.) varieties, reaching levels of up to 50 μg/g FW. Pale green and white cultivars have much lower levels (0–10 μg/g FW). In parallel, β-ionone, the 9,10 cleavage product of β-carotene, is present (12–33 ng/g FW) in orange-fleshed melon varieties that accumulate β-carotene, and in much lower levels (0–5 ng/g FW) in pale green and white fleshed varieties. A search for a gene putatively responsible for the cleavage of β-carotene into β-ionone was carried out in annotated melon fruit EST databases yielding a sequence (*CmCCD1*) highly similar (84%) to other plant carot enoid cleavage dioxygenase genes. To test its function, the clone was overexpressed in *Escherichia coli* strains previously engineered to produce different carotenoids. We show here that the *CmCCD1* gene product cleaves carotenoids at positions 9,10 and 9′,10′, generating geranylacetone from phytoene; pseudoionone from lycopene; β-ionone from β-carotene, as well as α-ionone and pseudoionone from δ-carotene. *CmCCD1* gene expression is upregulated upon fruit development both in orange, pale-green and white melon varieties, despite the lack of apocarotenoid volatiles in the later. Thus, the accumulation of β-ionone in melon fruit is probably limited by the availability of carotenoid substrate.
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Keywords: *Cucumis melo* L.; Cucurbitaceae; Melon; Functional expression; Carotenoids; Apocarotenoids; β-Carotene; β-Ionone; Carotenoid cleavage dioxygenase; *CmCCD1*

1. Introduction

Fruit quality is determined by numerous traits that affect taste, aroma, texture, color, nutritional value and shelf life (for reviews, see White, 2002; Adams-Phillips et al., 2004; Giovannoni, 2004). Among the many natural products biosynthesized during fruit development and affecting fruit quality, terpenes play major crucial roles, constituting important pigments (carotenoids) and aroma chemicals (mono- and sesqui-terpenes and norisoprenoids) (Croteau and Krap, 1991; Croteau et al., 2000). Carotenoids are tetraterpenoid pigments, accumulated in the plastids of leaves, flowers and fruits, where they contribute to the red, orange and yellow colours. In addition to their roles in plants as photosynthetic accessory pigments and colorants, carotenoids have fundamental contributions to

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human health (reviewed in Fraser and Bramley, 2004; Galili et al., 2002).

Melon (*Cucumis melo* L.) is a highly polymorphic species that comprises a broad array of wild and cultivated genotypes differing in fruit traits such as ripening physiology (climacteric and non-climacteric), sugar and acid content, and secondary metabolites associated with taste and aroma (Seymour and McGlasson, 1993; Morales et al., 2004). The aroma of melons, as is the case with most fruits, consists of complex mixtures of volatile compounds (Shalit et al., 2001; Beaulieu and Grimm, 2001; Jordan et al., 2001; Aubert and Bourger, 2004). In aromatic melon varieties, volatile esters, mainly acetate derivatives, are prominent, together with lower amounts of sesquiterpenes, norisoprenes, short-chain alcohols and aldehydes (Shalit et al., 2000, 2001). Non-aromatic varieties often have much lower levels of total volatiles, and especially lack the volatile esters.

During the last decade, the role of carotenoids as precursors of volatile aroma molecules, namely: the apocarotenoids (norisoprenes) has become evident (Winterhalter and Rouseff, 2002; Giuliano et al., 2003; Winterhalter and Schreier, 1995). Apocarotenoids are widely distributed in nature. The assortment of apocarotenoids, results from the large number of carotenoid precursors (more than 600 carotenoids have been identified), variations in the site of cleavage and subsequent modifications.

Plant apocarotenoids comprise growth regulators, pigments, flavors, aromas and defense compounds and also serve as ecological cues, having roles as insect attractants or repellant, and also have anti-bacterial and fungicidal properties (Giuliano et al., 2003). Among the better known apocarotenoid compounds are abscisic acid (ABA) (Zeevart and Creelman, 1988) and the retinoids, i.e. vitamin A and related compounds (Nagao, 2004). Apocarotenoid pigments are also economically important. For example, bixin is commonly used as a natural food coloring (Bouvier et al., 2003a), and crocin is the major pigment in saffron (Bouvier et al., 2003b). Of particular interest are apocarotenoids that possess extremely potent odor thresholds, for example β-ionone **8** with threshold of 0.007 ppb (see Fig. 1). Therefore, apocarotenoids, although generally present at relatively low levels in fruits, often possess strong effects on the overall human appreciation of the aromas. Apocarotenoids have been implicated as crucial components in the full flavor of many fruits (Winterhalter and Rouseff, 2002) including tomato (Baldwin et al., 2000), carrots (Kjeldsen et al., 2003) and *Averrhoa carambola* (Winterhalter and Schreier, 1995).

Evidence, based on comparative genetics, has indicated that carotenoid pigmentation patterns have profound effects on the apocarotenoid and monoterpene aroma volatile compositions of tomato and watermelon fruits (Lewinsohn et al., 2005a,b). This work has indicated that the different flavors and aromas perceived by otherwise similar fruit but of different colors have a sound chemical basis and are not solely due to psychological preconception. Enzymes

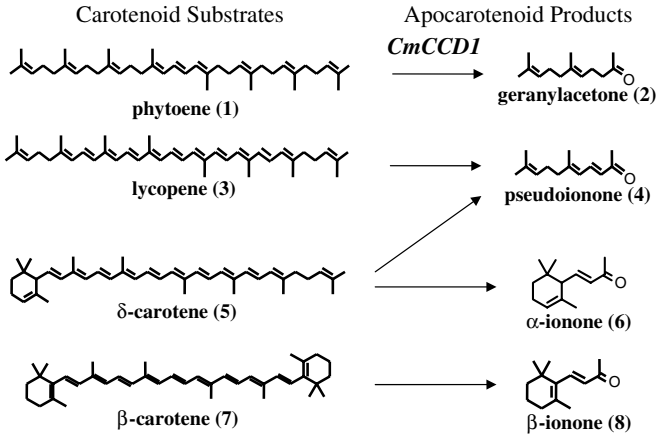


Fig. 1. Carotenoids and their degradation products. Carotenoid substrates (left) are oxidatively cleaved to yield the apocarotenoid derivatives (right).

capable of cleaving carotenoids at specific sites are believed to be involved in the synthesis of a number of apocarotenoids. Carotenoid cleavage dioxygenase enzymes from quince (*Cydonia oblonga*), star fruit (*Averrhoa carambola*), and nectarines (*Prunus persica*) have recently been partially purified and characterized (Fleischmann et al., 2002, 2003; Baldermann et al., 2004). The *Vp14* gene, which encodes an ABA biosynthetic enzyme in maize (Schwarz et al., 1997), was the first carotenoid cleavage enzyme to be cloned from plants. Bouvier et al. (2003b) identified a zeaxanthin-specific 7,8 (7′,8′)-cleavage dioxygenase gene from *Crocus sativus* (*CsZCD*) encoding an enzyme capable of forming crocetin dialdehyde and 3-hydroxy-β-cyclocitral in vitro. A lycopene-specific 5,6 (5′,6′)-cleavage dioxygenase (*BolCD*) responsible for the formation of bixin dialdehyde, a precursor of annatto, has also been identified from *Bixa orellana*, (Bouvier et al., 2003a). An arabidopsis carotenoid cleavage dioxygenase, *AtCCD1*, that in vitro symmetrically cleaves the 9,10 (9′,10′) double bonds of multiple carotenoid substrates into a C14 dialdehyde and two C13 cyclohexane derivates has been also identified (Schwarz et al., 2001). Orthologs of *AtCCD1* have been found in a variety of species including *Crocus sativus* (Bouvier et al., 2003b), *Petunia hybrida* (Simkin et al., 2004a), tomato (Simkin et al., 2004b) and grapevine (*Vitis vinifera*) (Mathieu et al., 2005).

Recently, the utilization of genomic tools, such as DNA microarrays and annotated EST databases, coupled to functional expression of candidate genes have provided vast information on the patterns of gene expression throughout fruit development and have aided in the identification of novel genes involved in the formation of phenolic, esters, terpene and other aroma volatiles (Lange et al., 2000; Aharoni et al., 2000; Gang et al., 2001; Guterman et al., 2002; Lavid et al., 2002; Shalit et al., 2003; Iijima et al., 2004; Beekwilder et al., 2004; Ringer et al., 2005; Davis et al., 2005). The aim of our project was to identify candidate genes that affect the aroma of melons, as part of a broader genomic program aimed at identifying key genes

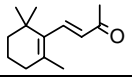
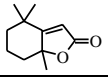
that affect melon quality and marketability (Yariv et al., 2004; Lewinsohn et al., 2004). We hereby report on the discovery of *CmCCD1*, a gene encoding a carotenoid cleavage dioxygenase isolated from the fruits of *C. melo* var. "Tam Dew". Despite the apparent lack of apocarotenoid volatiles in such fruits, functional characterization of *CmCCD1* shows that its product cleaves β-carotene **7** into β-ionone **8** in carotenoid-producing *Escherichia coli*. *CmCCD1* seems upregulated during fruit development and the *CmCCD1* gene product is also able to cleave other carotenoids, normally absent in melons (such as phytoene **1**, lycopene **3** and δ-carotene **5**) into their respective apocarotenoid derivatives **2**, **4** and **6**. Our findings suggest that apocarotenoid content is probably limited by the availability of carotenoid precursors in melon fruits.

2. Results and discussion

2.1. The carotenoid pigmentation patterns of melons affect their aroma volatile composition

Previous studies have indicated that carotenoid pigmentation patterns might affect volatile composition of fruits. In an earlier study, the volatile profiles of tomato lines differing in flesh color were found to be closely related to their fruit carotenoid composition (Stevens, 1970). The use of advanced genetic material with near isogenic backgrounds enabled us to exclude linkage drag and varietal effects and we concluded that in tomato fruit, genes that affect carotenoid pigmentation have pleiotropic effects on aroma volatiles, influencing the apocarotenoid and monoterpene compositions (Lewinsohn et al., 2005a,b). Interestingly, similar associative findings between carotenoid and volatile terpene compositions were observed in watermelon, a fruit that is botanically and physiologically distant from tomato, but similar in its carotenoid pigmentation patterns (Tadmor et al., 2005; Lewinsohn et al., 2005a,b). In order to validate the generality of these findings, we extended our studies to melon, a carotenoid pigmented fruit that also displays varietal variability in flesh color. There is a clear difference in flavor and aroma between the different pigmented melon cultivars and it was of interest to determine their apocarotenoid composition as related to their carotenoid content (Table 1). The carotenoid levels of the orange-fleshed melons, represented here by "Dulce", "Dalton" and the experimental lines HM56, "Ananas 311", HMS54-1, and HMS54-2 ranged from 20 to 50 μg/g FW (Table 1), and consisted mostly of β-carotene **7** (95%) and lutein (ca. 5%) (not shown). In accordance, these melons accumulate relatively high levels of β-ionone **8**, dihydroactinolide and β-cyclocitral (Fig. 1, Table 1). Pale-green and white fleshed varieties such as "Tam Dew", "Arava", "Ein-Dor", "Noy-Yizre'el" "Noy-Amid", "Piel de Sapo", and the exotic aromatic variety "Dudaim" accumulated no or much lower levels of carotenoids as well as the corresponding β-carotene **7** breakdown derivatives (Table 1). Although carotenoid levels are ca. 1000 fold higher than the apocarotenoid derivatives, our results still show a strong association between β-carotene **7** content and structurally related apocarotenoids. This further corroborates our previous observations in tomato and watermelon, indicating a strong association between carotenoid pigmentation and aroma volatile content (Lewinsohn et al., 2005a,b). Although there is a clear difference in the flavor and aroma of watermelon, tomato, and melon, in all three cases, varieties that accumulate β-carotene **7** also contain high levels of β-ionone **8**, while varieties that lack carotenoids, such as the tomato *yellow flesh* (*r*) mutant, the canary yellow watermelon varieties and the pale-green melons analyzed here all, lack apocarotenoid volatiles (Lewinsohn et al., 2005a,b and Table 1).

Table 1
Carotenoids and apocarotenoid volatiles in melon varieties

Variety	Flesh color	Total carotenoids (μg/g FW)	Apocarotenes (ng/g FW)		
			β-Cyclocitral 	β-Ionone 	Dihydroactinolide 
Piel de Sapo	White	0	0	0	0
Dudaim	White	0	0	0	0
Noy Amid	White	Traces	0	0	0
Tam Dew	Green	Traces	0	0	0
Noy Yizre'el	Green	5.7 (1.3)	0	5.1 (2.5)	3.1 (0.8)
Ein Dor	White	6.3 (1.8)	0	0.9 (0.2)	1.9 (0.6)
Arava	Green	10 (2.7)	0	1.3 (0.4)	1.3 (0.5)
Ananas 311	Orange	19.6 (4.2)	2.5 (0.5)	15.7 (2.8)	32.3 (7.2)
HM54-2	Orange	26.7 (4.5)	3.5 (0.4)	16.6 (6.0)	33.1 (11.7)
HM56	Orange	35.3 (4.2)	5.0 (2.0)	33.7 (23.5)	25.7 (8.1)
Dalton	Orange	37.3 (5.2)	3.5 (0.4)	12.7 (2.5)	80.7 (11.8)
Dulce	Orange	45.4 (2.6)	4.57 (0.5)	29.4 (8.5)	61.7 (8.2)
HM54-1	Orange	51.3 (5.9)	5.28 (0.1)	20.4 (5.7)	51.1 (2.0)

Averages and standard errors of at least three replicates are shown.

The above findings strongly suggest that the apocarotenoid volatiles found in these fruits are derived from the oxidative degradation of carotenoids. However, the biochemical mechanisms (whether enzymatic or non-enzymatic) mediating these oxidative degradations are still to be elucidated for each particular case. It was reported that the final reactions can efficiently be catalyzed in vitro by peroxidases, lipoxygenases, dioxygenases (Wu et al., 1999; Schwarz et al., 2001; Zorn et al., 2003; Giuliano et al., 2003) or may be non-enzymatic (Caris-Veyrat et al., 2003; Wache et al., 2003). In attempts to identify a gene responsible for apocarotenoid formation in melon fruit, we examined a recently established melon gene database consisting of several annotated EST libraries derived from several melon varieties at different ripening stages (see below).

2.2. Isolation and functional identification of CmCCD1, a gene encoding a melon carotenoid cleavage dioxygenase

Data mining of the Melon Database (http://melon.bti.cornell.edu/) led to the clone (FR17M11) displaying high sequence similarity to other plant carotenoid cleavage dioxygenase genes. The clone, designated *CmCCD1*, originated from a fruit EST library of "Tam Dew", a pale-green fleshed melon cultivar that lacks carotenoids and apocarotenoids in its fruit. Full length sequence of the *CmCCD1* was obtained and the predicted CmCCD1 protein sequence consists of 543 amino acids, with a calculated molecular mass of 60.8 kDa. (1632 bp). CmCCD1 displays a high sequence similarity (a.a. identity 84%) to the *VvCCD1* gene from *Vitis vinifera*, which encodes an enzyme that cleaves zeaxanthin yielding 3-hydroxy-β-ionone and a C_{14}-dialdehyde (Mathieu et al., 2005), and 83% identity to the carotenoid 9,10 (9′,10′)-cleavage *CsCCD* dioxygenase gene from *Crocus sativus* (Bouvier et al., 2003b). *CmCCD1* is also similar to the *Lycopersicon esculentm LeCCD1A* (81%) (Simkin et al., 2004b), to the *Petunia hybrida PhCCD1* (81%) (Simkin et al., 2004a), and *Arabidopsis thaliana AtCCD1* (81%) (Schwarz et al., 2001) that code for enzymes able to cleave multiple carotenoid substrates at the 9,10 (9′,10′) positions to produce a C14 dialdehyde and two C13 cyclohexones. Based on phylogenetic analysis the CmCCD1 deduced protein belongs to the clad of carotenoid cleavage dioxygenases distinct from the NCED class, a family of proteins involved in the cleavage of carotenoids *en route* of ABA formation (Fig. 2).

2.3. Functional expression of the CmCCD1 in E. coli

Heterologous overexpression of carotenogenesis gene in *E. coli* has proven to be a powerful tool for identifying many of the genes and enzymes involved in carotenoid biosynthesis (Hirschberg et al., 1997). *E. coli* strains have been engineered to produce different carotenoids including phytoene **1**, lycopene **3**, β-carotene **7** and δ-carotene **5**, depending on the carotenogenesis genes overexpressed (Hirschberg, 2001).

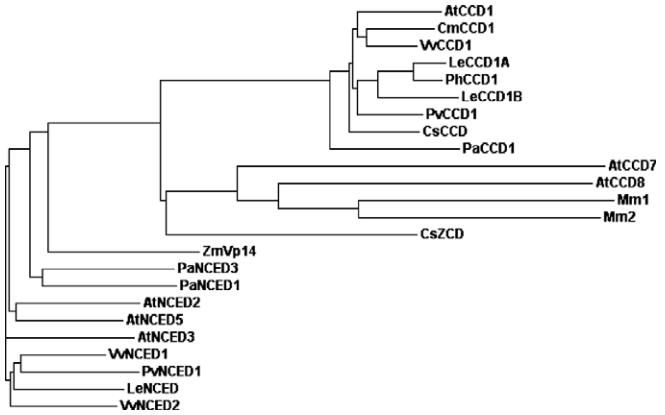


Fig. 2. Phylogenetic tree of the deduced amino acid sequences of functionally characterized CCD genes. The protein sequences were clustered using ClustalW WWW Service at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). Accession numbers for sequences used from the following species are as follows: *Arabidopsis thaliana* (AtCCD1, NP_191911, AtCCD7, NP182026.1, AtCCD8, NP195007.2, AtNCED2, NP_193569, AtNCED3, NP_188062.1, AtNCED5, NP_174302); *Cucumis melo* (CmCCD1, DQ269467); *Vitis vinifera* (VvCCD1,AY856353, VvNCED1,AY337613, VvNCED2,AY337614); *Crocus sativus* (CsCCD,AJ132927, CsZCD,AJ489276); *Lycopersicon esculentum* (LeCCD1A,AY576001, LeCCD1B,AY576002, LeNCED,-CAB10168.1); *Petunia hybrida* (PhCCD1,AY576003); *Persea americana* (PaCCD1,AAK00622, PaNCED3,AAK00623, PaNCED1,AAK00632); *Phaseolus vulgaris* (PvCCD1,AAK38744, PvNCED1,AAF26356); *Mus musculus* (Mm1,AAG33982, Mm2,CAC28026,); *Zea mays* (ZmVp14,AAB62181).

Except for phytoene **1** that is colorless, the carotenoids that accumulate in these strains impart a specific color upon the cells depending on the carotenoid accumulated, and a loss of color indicates that the carotenoids could be metabolized to colorless compounds. The plasmid pBCAR-EIBY harbours functional GGDP synthase (*Crt*B), phytoene synthase (*Crt*E), bacterial phytoene desaturase (*Crt*I) and lycopene β-cyclase (*Crt*Y) genes. *E. coli* cells carrying the plasmid pBCAR-EIBY produced the yellow-orange pigment β-carotene **7** as expected, while co-transformation of these cells with pBK-CMV, a plasmid harboring *CmCCD1*, led to a loss of the orange color, indicating possible catabolism of β-carotene **7** (Fig. 3A). Reversed-phase HPLC analyses of β-carotene **7** producing *E. coli* indicated that when *CmCCD1* was overexpressed, >97% of the β-carotene **7** accumulated in the controls was lost (Fig. 3B). GC–MS analyses of the headspace of these cells revealed a major peak co-eluting with β-ionone and exhibiting a mass spectrum identical to that of β-ionone **8** (Fig. 3C). The newly formed β-ionone **8** was only present in cells harboring the pBK-CMV plasmid, overexpressing *CmCCD1*, and was absent in control cells harboring the pBCAR-EIBY plasmid only (Fig. 3C) or a control pBK- plasmid vector (not shown). Lower levels of pseudoionone **4** and geranylacetone **2** (1/100 of that of β-ionone **8**) were also detected in the headspace of *E. coli* expressing both β-carotene genes and *CmCCD1* genes (not shown). These non-cyclic apocarotenoids are probably derived from the 9,10 and 9′,10′ cleavage of tetraterpene precursors that are intermediates in the formation of β-carotene and therefore

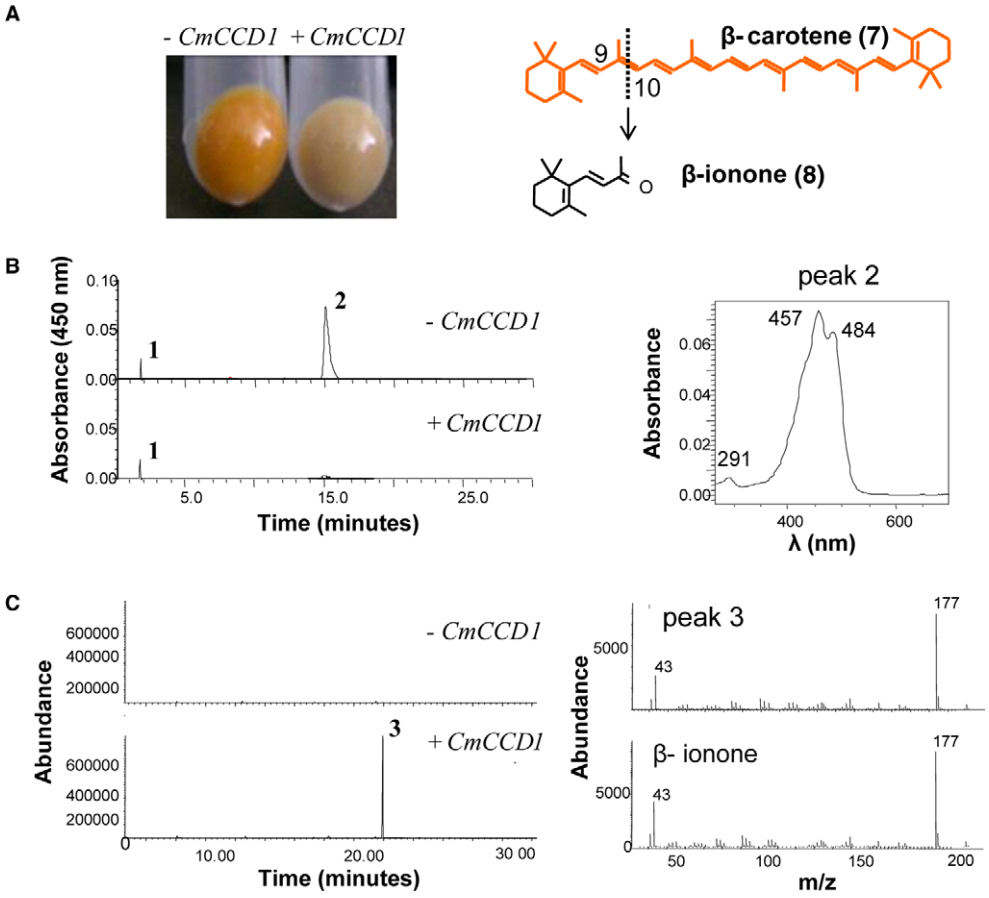


Fig. 3. Functional analysis of *CmCCD1*. *E. coli* strains harboring pBCAR-EBIY and thus producing β-carotene **7**, were cotransformed with pBK-CMV-*CmCCD1*. (A) Bacterial pellets of cells harboring pEBIY and expressing *CmCCD1* (right) and controls harboring pBCAR-EBIY and the pBK vector (left). (B) HPLC-UV–Vis analyses of control cells (top) and *CmCCD1* expressing cells (bottom). The UV–Vis spectrum of β-carotene produced by control cells is shown in the right panel. (C) GC–MS analyses of the bacterial headspace. Left top panel, controls, bottom left *CmCCD1* expressing cells showing a prominent peak that coelutes with β-ionone (not shown). The mass spectrum of peak 3 is shown to be identical to that of authentic β-ionone **8** (right).

less abundant in the cells. Thus, lycopene **3** is probably cleaved into pseudoionone **4** and either phytofluene, ζ-carotene or neurosporene that are cleaved into geranylacetone **2**. Additionally, much lower levels (about 1/1000 of β-ionone **8** level) of β-cyclocitral were detected in the headspace of the cultures overexpressing *CmCCD1* and absent in control cultures (not shown). This β-cyclocitral could be derived directly from the 7,8 cleavage of β-carotene **7** if the CmCCD1 enzyme specificity would allow cleavage at positions other than the 9,10 and 9′,10′ or it could result from a unrelated modification of β-ionone **8** by the *E. coli* cells. The enzyme encoded by the *Crocus CsZCD* gene is able to specifically cleave zeaxanthin at the 7,8 position, but in our experiments, β-cylocitral occurred only as a minor product of carotenoid, as compared to β-ionone **8**, and prevented us from reaching unequivocal conclusions regarding its biosynthetic origin. We thus cannot exclude the possibility that β-cyclocitral is formed both in the β-carotene **7** producing *E. coli* and in the melon tissues by conversion from β-ionone **8**. Nevertheless, our results do indicate that the *CmCCD1* gene product efficiently cleaves β-carotene **7** into β-ionone **8**, and thus we conclude that *CmCCD1* is a novel carotenoid cleavage dioxygenase from melon fruit.

2.4. Substrate specificity of the recombinant CmCCD1 gene product

Several of the carotenoid cleavage dioxygenases isolated from plants can accept a variety of carotenoids as substrates while others seem more specific regarding the substrate and cleavage position (Winterhalter and Rouseff, 2002; Bouvier et al., 2003a,b; Giuliano et al., 2003). It was therefore of interest to assess the substrate specificity of CmCCD1. For that, *CmCCD1* was overexpressed in *E. coli* cells engineered to generate either phytoene **1**, lycopene **3**, or δ-carotene **5**. The accumulation of the specific carotenoids was verified by HPLC-UV–vis spectroscopy and the volatiles released were assessed by SPME–GC–MS.

E. coli cells harboring the plasmid pBCAR-EB produced phytoene **1** as expected. When the recombinant CmCCD1 protein was overexpressed in these cells, phytoene **1** levels diminished to below detectable levels, and geranylacetone **2** (6,10-dimethyl-5,9-undecadien-2-one) was detected by GC–MS (Fig. 1 and Table 2). When *CmCCD1* was overexpressed in *E. coli* harboring pBCAR-EBI and thus producing the red pigment lycopene **3**, the levels of the later compound diminished only by 50%, still enough to be

Table 2
Apocarotenoids formed in vitro during coexpression of *CmCCD1* in carotenoid producing *E. coli* strains

Plasmids	Main carotenoid produced	Apocarotenoids present in headspaces of bacterial cultures			
		Geranyl acetone **2**	Pseudoionone **4**	β-Ionone **8**	α-Ionone **6**
pBCAR-EB	Phytoene **1**	–	–	–	–
pBCAREB + *CmCCD1*		++	–	–	–
pBCAR-EBI	Lycopene **2**	–	–	–	–
pBCAR-EBI + *CmCCD1*		+	++	–	–
pBCAR-EBIY	β-Carotene **7**	–	–	–	–
pBCAR-EBIY + *CmCCD1*		+	+	++	–
pDCAR	δ-Carotene **5**	–	–	–	–
pDCAR + *CmCCD1*		+	++	–	++

evident as judged by color loss. In the headspace of those cells, a prominent peak corresponding to pseudoionone **4** (6,10-dimethyl-3,5,9-undecatrien-2-one) was evident by GC–MS (Fig. 1, Table 2). Interestingly, lower levels (about 1/100 of those of pseudoionone **4**) of geranyl acetone **2** were also evident. Geranyl acetone **2** is probably a breakdown product of phytoene **1**, phytofluene, ζ-carotene or neurosporene, intermediates in lycopene **3** biosynthesis. Additionally, when cells harboring the pBCAR-EBIY plasmid producing the asymmetric carotenoid δ-carotene **5** were challenged with *CmCCD1* expression, a diminution of color intensity was noted, indicative of δ-carotene **5** breakdown. In such cultures, the levels of δ-carotene **5** dramatically diminished as determined by HPLC, and both pseudoionone **4** and α-ionone **6** were present in the headspace (Fig. 1, Table 2). This indicates that CmCCD1 is able to efficiently cleave δ-carotene **5** both at the 9,10 and at the 9′,10′ positions, giving rise to two distinct derivatives: α-ionone **6** derived from the cyclic moiety, and pseudoionone, from the non-cyclic moiety. Also, it seems that the enzyme is more efficient towards β-carotene **7** or phytoene **1** cleavage as compared to lycopene **3**. The isolation of the active protein is essential for the accurate determination of the kinetic properties and the substrate specificity of this enzyme.

Accordingly, the bulk of the volatile apocarotenoid products detected in the recombinant *E. coli* overexpressing the *CmCCD1* gene apparently result from 9,10 and 9′,10′ cleavage of carotenoids. We therefore conclude that *CmCCD1* codes a protein that displays 9,10-carotenoid cleavage dioxygenase activity and can accept a wide range of carotenoids as substrates. CmCCD1 is able to release geranylacetone **2**, pseudoinone **4**, β-ionone **8** and α-ionone **6** depending on the carotenoid substrate offered. Interestingly, most of these carotenoids do not normally accumulate in melon fruits.

Most other plant carotenoid cleavage dioxygenases involved in the formation of aromas, symmetrically cleave carotenoids at 9,10 and 9′,10′ double bonds releasing two molecules of apocarotenoids as well as a central C14 dialdehyde. Attempts to identify the corresponding central C14 dialdehyde released by CmCCD1 using our HPLC method failed, as well as attempts to identify the putative C27 apocarotenoid resulting from asymmetrical cleavage at positions 9,10 or 9′,10′. We therefore cannot decisively conclude whether CmCCD1 is able to cleave carotenoids only at one end, releasing a C27 apocarotenoid or symmetrically at both ends, releasing a C14 dialdehyde. However, both cyclic and acyclic moieties are apparently accepted by the dioxygenase, as indicated by the efficient cleavage of δ-carotene **5** into α-ionone **6** and pseudoionone **4**, two distinct C13 apocarotenoid derivatives.

2.5. Expression patterns of CmCCD1 in developing fruit

Expression profiles of *CmCCD1* were obtained by real-time PCR analysis of developing fruits. The results for "Tam Dew" (green flesh) and "Dulce" (orange flesh) are shown in Fig. 4. *CmCCD1* was upregulated during fruit maturation in both genotypes. The expression levels in mature fruit were 6–20-fold higher than the levels found in early stages of fruit development. The orange variety "Dulce", displayed higher levels of upregulation upon maturation than "Tam Dew", pale-green variety. Upregulation of *CmCCD1* in "Tam Dew" was also prominent (Fig. 4). In leaves, basal expression levels, similar to those found in early stages of fruit development, were detected (data not

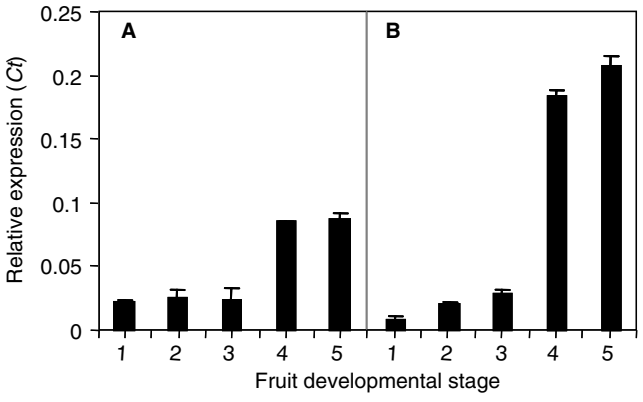


Fig. 4. Expression patterns of *CmCCD* genes during development of melon fruit. (A) Real-time PCR analysis of RNA extracted from "Tam Dew" melons. Fruits at the following stages of development were sampled: 1, very young green fruits; 2, young green fruits; 3, full size mature green fruits; 4, ripe fruits; 5, over- ripe fruits. (B) Similar analyses performed on "Dulce" fruits. Values are means ± SEM of three different evaluations carried out in duplicates with one set of cDNA.

shown). These findings were corroborated by Northern analyses (not shown), and by real time PCR analyses of other varieties such as the orange-fleshed "Verdantrais", (a "Charantais" type melon, an heirloom orange variety), "Tendral Verde" (a pale green variety) as well as "PI 414723" (a cream-fleshed variety), that displayed comparable patterns of upregulation as "Tam Dew" (not shown). Sequence analyses of the amplicon area from different melon varieties indicated a very high degree of identity (>99.9%) among them (not shown), but we cannot rule out the upregulation of additional CCD-like genes in "Tam-Dew" or other varieties. Seemingly, the potential to release apocarotenoid volatiles is prominent in all varieties, although in reality the different varieties display different levels of apocarotenoid volatiles (Table 1). This further indicates that the apocarotenoid levels and compositions are defined by the level and composition of carotenoids available, and not solely by the levels of carotenoid cleavage dioxygenase expression, and further corroborates the apparent pleiotropic effects of carotenogenesis genes on the compositions and levels of aroma volatiles (Lewinsohn et al., 2005a,b).

3. Concluding remarks

The familiar flavors of fruits and vegetables is the consequence of a complex interaction between sugars, acids and a large number of volatile compounds accumulated during fruit ripening (Baldwin et al., 2000; Seymour and McGlasson, 1993). These volatiles are believed to be derived from catabolism of a diverse set of precursors, including amino acids, fatty acids and carotenoids (Buttery and Ling, 1993; Croteau and Krap, 1991). β-Ionone **8**, β-cyclocitral, pseudoionone **4** and geranyl acetone **2** have been postulated to be products of oxidative cleavage of carotenoids (Winterhalter and Rouseff, 2002). We report here that by data mining of an annotated EST database from "Tam Dew" melons, we have successfully isolated a cDNA similar to other plant carotenoid cleavage dioxygenases. Based on nucleotide sequence comparison and functional expression in *E. coli* strains that accumulate phytoene, lycopene **1**, β-carotene **7** and δ-carotene **5**, we were able to determine that this cDNA, termed *CmCCD1*, is a carotenoid cleavage dioxygenase with a broad substrate specificity for the carotenoid precursor, but apparently specific for the site of cleavage.

Interestingly, our experiments indicate that melon cultivars express carotenoid-cleavage dioxygenase like genes during ripening. They include orange-fleshed carotenoid rich melons, as well as pale green and white-fleshed cultivars such as "Tam Dew" that do not normally accumulate norisoprenoids in the fruit. Therefore it seems that melons potentially possess the ability to cleave β-carotene **7** and other carotenoids into their respective apocarotenoid derivatives such as β-ionone **8**, geranyl acetone **2**, and pseudoionone **4**, but the presence of the apocarotenoid derivatives is dependant on the availability of carotenoid substrates. We further reiterate that carotenoids are key compounds in affecting fruit quality by imparting color and nutritional value traits as well as being key precursors for aroma molecules. In view of the marked upregulation of *CmCCD1* upon fruit maturation, and its coding for a protein possessing carotenoid cleavage dioxygenase activity in *E. coli*, it is still unclear what could be its biological role in "Tam Dew" melons, a variety that does not accumulate β-carotene **7** nor β-ionone **8** in its flesh. It could be that *CmCCD1* is a remnant of an ancient gene that had an important role in the parental lineage of this melon or that *CmCCD1* possesses a yet unknown additional role in fruit development.

4. Experimental

4.1. Plant material

Several melon cultivars were grown in the Newe Ya'ar Research Center in northern Israel, under standard field conditions during the summers of 2003, 2004 and 2005. They belong to the following groups: (1) *C. melo* var. *inodorus*: including "Tam Dew", "Tendral Verde", "Noy Amid" and "Piel De Sapo". (2)*C. melo* var. *reticulates*: including "Dulce", "Noy Yizre'el", "Arava", "AN311' and "Ein Dor", and the experimental breeding lines "HM56", "HM54-2", "HM54-1" and "Dalton". (3) *C. melo* var. *cantalupensis* "Vedrantaise" (4) PI 414723 of subsp. *melo* var. *momordica*; and (5) 'Dudaim', subsp. *melo* var. *dudaim*. Fruits were sliced by cross-section and equal quantities from three different fruits were pooled prior to freezing in liquid nitrogen. Mature fruits were sampled for carotenoids and apocarotenoid volatiles (Table 1). Fruits at the following stages of development (based on days post anthesis (DPA), fruit weight and sugar accumulation specific to each variety) were sampled for the *CmCCD1* expression analysis: (1) very young, green fruits (12 DPA); (2) young green fruits (25 DPA); (3) full size mature green fruits ("Tam Dew" – 35 DPA and "Dulce" – 38 DPA); (4) ripe fruits ("Tam Dew" – 38 DPA and "Dulce" – 40 DPA); (5) over-ripe fruits ("Tam Dew" – 42 DPA and "Dulce" – 42 DPA). Mature leaves were also sampled.

4.2. Carotenoid analyses

Melon fruits. Samples for carotenoid extraction were taken from at least three fruits of each accession. Carotenoids were extracted by grinding fresh fruit (0.5 g) in hexane:acetone:EtOH (2:1:1 v/v), followed by 5 min of saponification in 8% (w/v) KOH. The saponified material was extracted twice with hexane, which was then evaporated in vacuo. The solid pellet was resuspended in 400 μl of MeCN:MeOH:CH_2Cl_2 (45:5:50 v/v) and passed through a 0.2-μm Nylon filter for HPLC (see below) and spectrophotometric analyses. Since β-carotene **1** was found to be

the major carotenoid of melon fruits (ca. 95% of total carotenoids), we determined total carotenoid content of the sample spectrophotometrically at OD_{450} nm using the Lambert–Beer equation, with the ε and M of β-carotene ($\varepsilon = 134{,}000$ l/mol cm; $M = 537$ g/mol).

Bacteria. We followed the method of Ronen et al. (1999) with slight modifications. Briefly, bacterial cells (1 ml aliquots) of suspension cultures were harvested by centrifugation at 13,000 rpm for 10 min, washed once with H_2O and resuspended in acetone (1 ml). Cells were lysed by subsequent sonication. The samples were centrifuged again at 13,000 rpm for 5 min and the acetone supernatant containing the pigments was placed in a clean tube. The pigment extract was evaporated to dryness by the use of a speed vac and dissolved in 1 ml MeCN:MeOH:CH_2Cl_2 (45:5:50 v/v) and passed through a 0.2-μm nylon filter. The carotenoids were analyzed by reversed phase liquid chromatography as described below.

HPLC analyses. We essentially followed the method described by Tadmor et al. (2000). Forty microliters of the filtered extract were injected into a 2996 Waters HPLC equipped with Waters PDA detector 996, C18 Nova-Pak (Waters, Milford, MA, USA) column (250 × 4.6 mm i.d.; 60 Å ;4 mm), and a Nova-Pak Sentry Guard cartridge (Waters). Detection of was performed between 260 and 600 nm. Data were analyzed using the MILLENIUM software.

4.3. Volatile apocarotenoid analyses

Melon fruit. At least six fully ripe fresh melon fruits were cut into small (ca. 0.5 cm^3) pieces. Three replicates, each of 50 g of cubes, were vigorously shaken for 16 h with 50 ml of methyl *tert*-butyl ether (MTBE) containing 50 μg of *iso*-butyl benzene as an internal standard (Lewinsohn et al., 2001). The upper phase (MTBE) was dried with Na_2SO_4 and concentrated under a gentle stream of nitrogen to 1.0 ml. One microliter of each sample was analyzed by GC–MS (see below).

4.3.1. Determination of volatiles from bacterial headspace

Sample preparation. An overnight starter culture (0.5 ml) was used to inoculate 3 ml of LB medium containing the appropriate antibiotics and 1 mM IPTG in a tightly closed 20 ml tube. The vials were incubated at 37 °C for 20 h and gently shaken (250 rpm). A SPME probe was introduced to the vial through a septum and the headspace volatiles were allowed to adsorb to the fiber at 45 °C for 30 min. Subsequently the SPME fiber was introduced into the GC and analyzed (see below).

GC–MS analyses. The volatile compounds collected from the headspace or from fruit MTBE extracts were analyzed on an HP-GCD apparatus equipped with an HP-5 (30 m × 0.25 mm) fused-silica capillary column. He (1 ml min^{-1}) was used as a carrier gas. The injector temperature was 250 °C, set for splitless injection. The oven was set to 50 °C for 1 min, and then the temperature was increased to 200 °C at a rate of 4 °C min^{-1}. The detector temperature was 280 °C. Mass range was recorded from 45 to 450 m/z, with electron energy of 70 eV. Identification of the main components was done by comparison of mass spectra and retention time data with those of authentic standards and supplemented with a Wiley GC–MS library (Lewinsohn et al., 2001).

4.4. Bacterial strains and plasmids

The following plasmids were transformed into *E. coli* strain JM 109, conferring carotenoid-biosynthesizing ability: pBCAR-EB, pBCAR-EBI and pBCAR-EBIY, carriying the *crtE* (geranylgeranyl pyrophosphate synthase) and *crtB* (phytoene synthase) genes for phytoene synthesis, the *crtE*, *crtB* and *crtI* (phytoene desaturase) genes for lycopene **1** synthesis, and the *crtE*, *crtB*, *crtI* and *crtY* (lycopene β-cyclase) genes for β-carotene **7** synthesis, respectively, as previously described (Misawa et al., 1990). The plasmid pDCAR expressing *crtE*, *crtB*, *crtI* and *CrtL-e* producing δ-carotene in transformed *E. coli* was also used (Ronen et al., 1999).

4.5. CmCCD1 isolation

4.5.1. Isolation of RNA and construction of melon fruit cDNA libraries

Total RNA was isolated from mature fruit from slices containing both rind and flesh tissues of ''Tam Dew'' melons using a modification to the method of La Claire and Herrin (1997). Poly $(A)^+$ mRNA was purified from 1 mg of total RNA by use of Oligotex® mRNA purification kit according to manufacturer's recommendations (Qiagen, Hilden, Germany). An EST library was constructed using ZAP cDNA Synthesis Kit and ZAP Express® cDNA Gigapack® III Gold Cloning Kit according to manufacturer's recommendations (Stratagene, USA). Poly $(A)^+$ mRNA (5 μg) were used for preparation of the library. Phage clones were converted to pBK-CMV phagemid expression-vector following the manufacturer's instructions (Stratagene, USA). Over 1000 clones from this library were sequenced and annotated. The sequence information is available in the Melon Database (http://melon.bti.cornell.edu/,).

4.5.2. Isolation and characterization of carotenoid cleavage dioxygenase

In silico screening of the Melon Database (http://melon.bti.cornell.edu/) resulted in the detection of a clone (FR17M11) exhibiting a high sequence similarity to other plant carotenoid cleavage dioxygenases. Plasmid DNA of the FR17M11 clone was prepared using Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, USA). Full length sequence of the clone (1632 bp) was obtained using primers of the vector (T3 and T7)

and an internal specific primer 5′-CGCTTGGTCTGCTGGATTATG-3′. DNA sequencing was performed by Macrogen, Inc. (Seoul, Korea). The sequence has been deposited in the GenBank database under the Accession No. DQ269467.

4.6. Functional expression experiments

The pBK-CMV-*CmCCD1* plasmid was introduced into the different carotenoid-accumulating strains of *E. coli* and coexpressed with the carotenoid producing genes described above. The pBK-CMV vector has an IPTG inducible promoter for expression in bacteria. Volatiles and carotenoids of stationary phase cultures were determined as described above.

4.6.1. cDNA synthesis and real-time PCR conditions and analysis

Total RNA from leaves, flowers and various developmental stages of the fruit was used as template for cDNA synthesis. RNA samples (20 μg) were treated with RNAse free DNAse I (20 U) (EPICENTRE®, USA) for 10 min at 37 °C. First strand complementary DNA was synthesized from 1μg of total RNA by use of iScript™ cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad laboratories, USA). cDNA (2μl) were used for each real-time PCR reaction.

Real-time PCR was performed on an ABI PRISM® 7000 Sequence Detection System using SYBR® Green PCR Master Mix (Applied Biosystems, USA). To monitor dsDNA synthesis data was analyzed with ABI Prism® 7000 SDS software. A relative quantification of gene expression was performed using the housekeeping gene *cyclophilin* from melon as a reference. Primers for the amplification of *cyclophilin* were planned based on contig # MU245 from the Melon EST Database as follows: F 5′-GATGGAGCTCTACGCCGATGTC-3′ and R 5′-CCTCCCTGGCACATGAAATTAG-3′. The primers used for the target gene *CmCCD*1 were: F 5′-GATTGGTGATCTGAAAGGGTTCTT-3′ and R 5′-GGTGATATATGAGAGCCGTGTTACC-3′ based on the initial acquisition (FR17M11) exhibiting a high sequence similarity to other plant carotenoid cleavage dioxygenases, using the Primer Express® Software v2.0 (Applied Biosystems, Foster City, CA, USA). All reactions where run three times in duplicates with one set of cDNA. A standard curve composed of five points was constructed from 10-fold dilutions of cDNA's of both *CmCCD1* and *cyclophilin*. The thermal cycling conditions consisted of 50 °C for 2 min followed by an initial denaturation step at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, then 60 °C for 1 min. Threshold cycles for each of the target genes were adjusted manually. The specificity of the PCR amplification was checked with a melting curve analysis following the final step of the PCR. For each sample, threshold cycles (Ct, cycle at which the increase of fluorescence exceeded the threshold setting) were determined. Real-time efficiencies (E) were calculated from the slopes of standard curves for each gene ($E = {}^{10[-1/\text{slope}]}$) (Ramakers et al., 2003). *CmCCD1* gene expression was normalized to that of *cyclophilin* by substracting the C_T value of the *CmCCD1* to that of *cyclophilin*. Relative expression ratio was established using the equation ratio $R = (2)^{-\Delta\Delta Ct}$ where ΔΔCt represents $\Delta C_{\text{TCmCCD1}} - \Delta C_{\text{Tcyclophilin}}$ (Pfaffl, 2001).

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