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Tomato carotenoid cleavage dioxygenases 1A and 1B: Relaxed double bond specificity leads to a plenitude of dialdehydes, mono-apocarotenoids and isoprenoid volatiles

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ABSTRACT

The biosynthetic processes leading to many of the isoprenoid volatiles released by tomato fruits are still unknown, though previous reports suggested a clear correlation with the carotenoids contained within the fruit. In this study, we investigated the activity of the tomato (*Solanum lycopersicum*) carotenoid cleavage dioxygenase (SlCCD1B), which is highly expressed in fruits, and of its homolog SlCCD1A. Using *in vitro* assays performed with purified recombinant enzymes and by analyzing products formed by the two enzymes in carotene-accumulating *Escherichia coli* strains, we demonstrate that SlCCD1A and, to a larger extent, SlCCD1B, have a very relaxed specificity for both substrate and cleavage site, mediating the oxidative cleavage of *cis*- and all-*trans*-carotenoids as well as of different apocarotenoids at many more double bonds than previously reported. This activity gives rise to a plenitude of volatiles, mono-apocarotenoids and dialdehyde products, including *cis*-pseudoionone, neral, geranial, and farnesylacetone. Our results provide a direct evidence for a carotenoid origin of these compounds and point to CCD1s as the enzymes catalyzing the formation of the vast majority of tomato isoprenoid volatiles, many of which are aroma constituents.

1. Introduction

Carotenoids are lipophilic pigments produced by plants, photosynthetic prokaryotes and several heterotrophic bacteria and fungi. Carotenoids play a vital role in photosynthesis, as pigments protecting from photo-oxidation and contributing to the light-harvesting process. Moreover, carotenoids serve as signals in the plant–animal communication, since they are responsible for the color of many fruits and flowers, e.g. tomato fruits and daffodil flowers, where they frequently accumulate in chromoplasts [1–3]. Generally, animals lack the capability to synthesize carotenoids and, hence, they need a dietary source for these pigments that act as antioxidants and, more importantly, as precursors of vitamin A (retinol) and its derivatives retinal and retinoic acid [4,5]. Furthermore, carotenoid derived compounds other than retinoids, like β-apo-13-carotenone and apolycopenals, are supposed to exert different biological activities in animal systems [6,7].

Plant carotenoid biosynthesis occurs in plastids. The first step in this pathway is the condensation of two molecules geranylgeranyl diphosphate, which yields the first carotenoid 15-*cis*-phytoene. This C_{40}-compound contains only three conjugated double bonds and is, therefore, colorless. Desaturation and isomerization reactions lead via specific *cis*-isomers of the intermediates phytofluene, ζ-carotene, neurosporene and lycopene to all-*trans*-lycopene, the red tomato fruit pigment with 11 conjugated double bonds. Cyclization of all-*trans*-lycopene gives rise to β- or α-carotene that can be hydroxylated to form zeaxanthin and lutein, respectively. Zeaxanthin is the precursor of violaxanthin and neoxanthin (for review, see [1–4,8].

Due to the presence of a conjugated double bond system, carotenoids are susceptible to oxidative cleavage that yields carbonyl products, generally referred to as apocarotenoids. Some of the apocarotenoids fulfill important environmental, physiological or developmental functions, immediately or after being structurally modified by other types of enzymes [9–12]. The group of apocarotenoids with known biological function includes retinoids, the

Abbreviations: CCD, carotenoid cleavage dioxygenase

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fungal sexual hormone trisporic acid [13], the plant hormone abscisic acid [14], and strigolactones [15], a group of bioactive compounds that act as phytohormones and signaling molecules inducing the hyphal branching symbiotic arbuscular mycorrhizal fungi and the seed germination of root parasitic plants [16]. Apocarotenoids are also responsible for the yellow pigmentation of roots infected with mycorrhizal fungi [12] and are the pigments accumulated in *Bixa orellana* seeds and saffron (*Crocus sativus*) stigmas [10]. Several volatile organic compounds, e.g. β-ionone, released by plants are carotenoid cleavage products supposed to contribute to plant–animal communication [10].

Carotenoids are cleaved either enzymatically, in general by carotenoid cleavage oxygenases (CCOs) [3,9,11], or non-enzymatically by reactions initiated by ROSs [17]. CCOs constitute a ubiquitous family of enzymes differing in substrate specificity and cleavage site. CCOs from plants are classified as either nine-*cis*-epoxycarotenoid cleavage dioxygenases (NCEDs) that catalyze a stereospecific cleavage of 9-*cis*-violaxanthin or 9′-*cis*-neoxanthin into the ABA precursor xanthoxin (C_{15}) and a C_{25} apocarotenal, or as carotenoid cleavage dioxygenases (CCDs) a term used to describe all other plant members of the enzyme family regardless of their substrate specificities [10,11]. Plant CCDs are divided into the four groups CCD1s, CCD4s, CCD7s and CCD8s. CCD4 enzymes from apple, chrysanthemum and saffron cleave β-carotene in *Escherichia coli* strains that accumulate this carotenoid, releasing β-ionone [18,19], and that CCD4 activity leads to a loss of pigmentation and a decrease in the carotenoid content of Arabidopsis seeds [20,21], chrysanthemum petals [22] and potato tubers [23]. However, *Citrus* CCD4 enzymes have been recently shown to produce the pigments β-apo-8′-carotenal and 3-OH-β-apo-8′-carotenal (β-citraurin) responsible for the orange-reddish tin of the peel of oranges and mandarins, by cleaving the 7′,8′ double bond in β-carotene, β-cryptoxanthin and zeaxanthin [24,25]. CCD7 and CCD8 enzymes are involved in strigolactone biosynthesis [26,27]. Investigations of the Arabidopsis CCD7 activity in carotenoid accumulating *E. coli* cells indicated a cleavage of all-*trans*-configured carotenoids at the 9,10 or 9′,10′ double bond, forming C_{13}-ketones and C_{27}-aldehydes like β-ionone and β-apo-10′-carotenal, respectively [28,29]. However, an *in vitro*-study performed with different carotenoid *cis*-*trans*-isomers and CCD7s from different plant species suggested that these enzymes are stereospecific and catalyze the cleavage of 9-*cis*-β-carotene into β-ionone and 9-*cis*-β-apo-10′-carotenal [15] whose stereo-configuration was very recently unequivocally determined by using NMR [30]. CCD8s are unusual carotenoid cleavage dioxygenases that convert the *cis*-configured intermediate 9-*cis*-β-apo-10′-carotenal into the triple oxygenated compound carlactone, a strigolactone-like compound, by catalyzing a combination of reactions supposedly including isomerization, repeated dioxygenation, cleavage and Baeyer–Villiger like rearrangements [15]. Though at a much lower conversion rate, CCD8 also converts all-*trans*-β-apo-10′-carotenal, however, into the conventional cleavage product β-apo-13-carotenone (C_{18}), by catalyzing a specific single cleavage reaction similar to those mediated by other carotenoid cleavage oxygenases [15,31].

Compared to other plant CCD subfamilies, CCD1 enzymes exhibit a wide substrate and cleavage-site specificity [10–12]. They convert different all-*trans*-carotenoids, like β-carotene, zeaxanthin and lycopene [3], as well as apocarotenoids, like β-apo-8′-carotenal [32,33], β-apo-10′-carotenal and apolycopenals [34], indicating a role in the scavenging of destructed carotenoids. Furthermore, CCD1 enzymes target different double bonds in monocyclic and linear carotenoids. The first cleavage sites reported for CCD1 enzymes are the C9–C10 and C9′–C10′ double bonds in C_{40}-carotenoids, which give rise to two C_{13}-ketones like β-ionone and one C_{14}-dialdehyde [35]. This double cleavage at symmetrical sites was also shown for CCD1s from many other plant species [3]. Later on, CCD1 enzymes from *A. thaliana*, maize and tomato were shown to cleave the 5–6 and/or 5′–6′ double bond in lycopene, leading to the flavor volatile 6-methyl-5-hepten-2-one (MHO; C8) [33]. In addition, a further cleavage site was reported for a cyanobacterial CCD1-homolog and for the rice CCD1, which cleaves the C7–C8 double bonds of apolycopenals, acyclic and monocyclic carotenoids, forming the C_{10}-aldehyde geranial [34,36], one of the many isoprenoid volatiles released by tomato fruits in a pattern correlating with the carotenoid profile [37].

In this work, we report on a comprehensive study on the activities of both CCD1 enzymes (SlCCD1A and SlCCD1B) of tomato. Our data suggest that geranial and many other tomato isoprenoid volatiles such as farnesyl acetone are indeed formed from carotenoids, as a result of the very relaxed double bond and substrate specificities of the tomato SlCCD1A and SlCCD1B enzymes.

2. Results

2.1. SlCCD1A and 1B target several double bonds of apocarotenoids in vitro

To elucidate the enzymatic activities of CCD1A and CCD1B *in vitro*, we produced each of the two enzymes fused to glutathione-S-transferase (GST) in the *E. coli* strain BL21(DE3) equipped with the plasmid pGro7 encoding the groES-groEL-chaperone system. Usage of this expression system had proven to increase solubility and activity of several carotenoid cleavage enzymes [34,38]. Using affinity chromatography, we purified the GST-fusions and released SlCCD1A and SlCCD1B by Factor-X protease treatment. Purification steps were monitored by SDS–PAGE (Fig. S1).

In a first approach, we incubated purified SlCCD1A and SlCCD1B with synthetic apolycopenals (for structures, see Fig. 1 compounds I, II) of different chain lengths, i.e. apo-8′- (C_{30}, I), apo-10′-lycopenal (C_{27}, II), which can arise from lycopene, the major pigment of tomato fruits. HPLC analysis of these *in vitro* assays showed the formation of various products: Incubation of SlCCD1A with I led to the formation of 4 products (products 2, 3, 5, 6; Fig. 1) identified, based on their reported elution pattern and UV–Vis spectra [34], as pseudoionone (C_{13}, 2), 8′,10-diapocarotene-8′,10-dial (C_{17}, 3), 8′,8-diapocarotene-8′,8-dial (C_{20}, 5) and 6,8′-diapocarotene-6,8′-dial (C_{22}, 6). These products suggest that SlCCD1A cleaves the three double bonds C5–C6, C7–C8 and C9–C10 in apo-8′-lycopenal (s. Fig. 1, Substrate I), and imply the formation of the volatiles MHO and geranial, besides pseudoionone. Incubation of SlCCD1B with the substrate apo-8′-lycopenal (I) led to a yet un-described dialdehyde product (product 1) identified by LC–MS analysis (Fig. S2) as 14, 8′-diapocarotene-14, 8′-dial (C_{12}, product 1 in Fig. 1), and to the products 2, 3 and 5, which were also observed with SlCCD1A. We also determined the volatiles released by SlCCD1B from apo-8′-lycopenal, by using a solid-phase micro-extraction (SPME) and analyzing collected compounds by GC–MS. As shown in Fig. 2, the SlCCD1B produced MHO (Fig. 2A), both isomers of citral, i.e. geranial (*trans*-citral, Fig. 2B, peak 2b) and neral (*cis*-citral, Fig. 2B, peak 2a), and *cis*- and *trans*-pseudoionone (Fig. 2, peak 3a, and 3b). This pattern strongly suggests that SlCCD1B, like SlCCD1A, targets the three double bonds C5–C6, C7–C8 and C9–C10 in apo-8′-lycopenal, although the dialdehyde 6,8′-diapocarotene-6,8′-dial (C_{22}; Fig. 1 product 6 of SlCCD1A), which is expected to arise due to the C5–C6 double bond cleavage, was not detected in the corresponding HPLC analysis (see Discussion). In addition, the formation of 14, 8′-diapocarotene-14, 8′-dial (C_{12}, product 1 in Fig. 1, s. also Fig. S2) suggests that SlCCD1B also targets the C13–C14 double bond.

Incubation of SlCCD1A with the substrate apo-10′-lycopenal (Fig. 1, substrate II, C_{27}) resulted in the formation of pseudoionone (product 2) and a dialdehyde identified according to [34] as

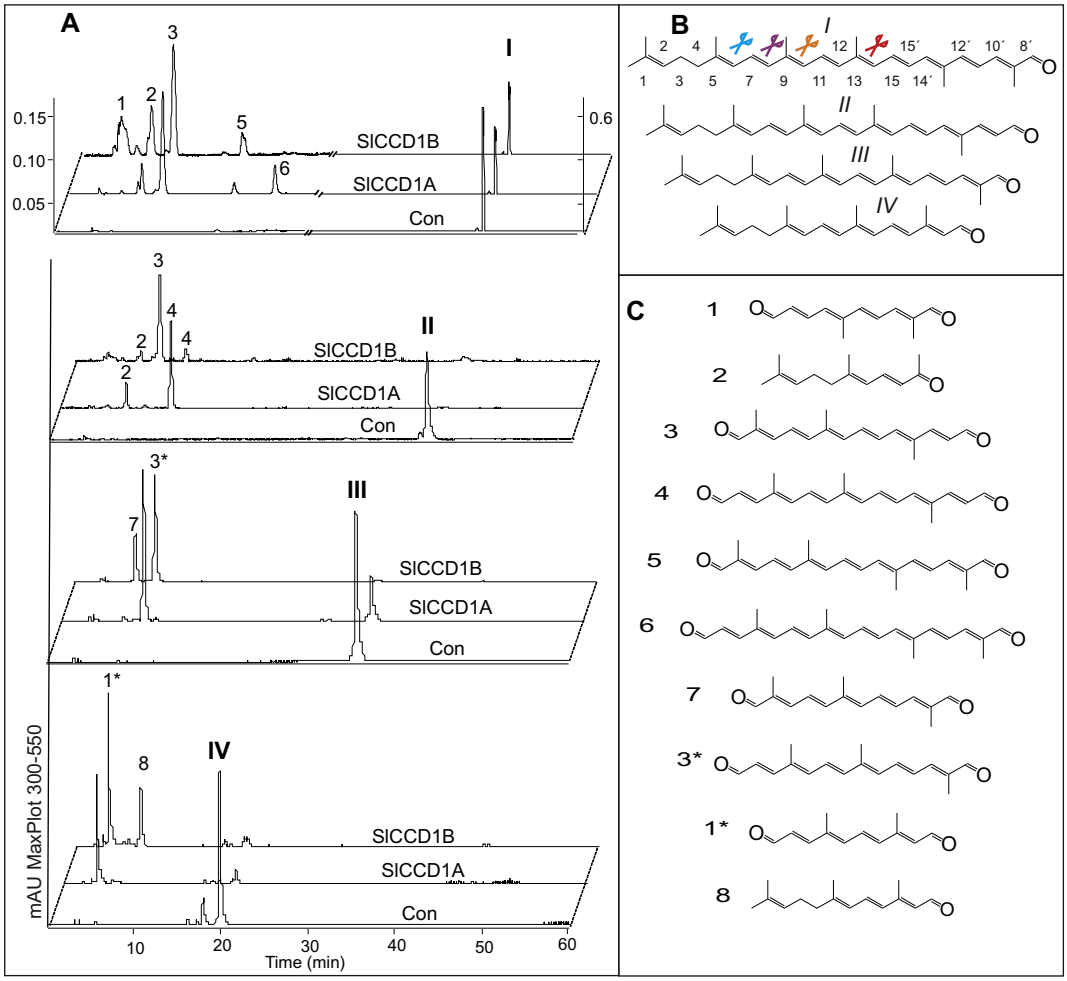

Fig. 1. HPLC analysis of incubations with apolycopenals. The four HPLC chromatograms (A) represent product profiles obtained with the different apolycopenals shown in (B, compound I–IV). The structures of the products formed are depicted in (C). SlCCD1B converted apo-8′-lycopenal (I, C_{30}) into 14, 8′-diapocarotene-14, 8′-dial (1, C_{12}, s. Fig. 3), pseudoionone (2, C_{13}), 8′, 10-diapocarotene-8′, 10-dial (3, C_{17}) and 8′, 8-diapocarotene-8′, 8-dial (5, C_{20}). SlCCD1A converted substrate I into products 2, 3, 5, in addition to 6, 8′-diapocarotene-6, 8′-dial (6, C_{22}). SlCCD1B converted apo-10′-lycopenal (II, C27) into pseudoionone (2, C_{13}), 8′, 10-diapocarotene-8′, 10-dial (3, C_{17}) and 10′, 6-diapocarotene-10′, 6-dial (4, C_{19}). SlCCD1A converted substrate II into products 2, 4. SlCCD1B converted apo-12′-lycopenal (III, C_{25}) into 12′, 6-diapocarotene-12′, 6-dial (3*, C_{17}) and 12′, 8-diapocarotene-12′, 8-dial (7, C_{15}). SlCCD1A converted substrate III into 12′, 6-diapocarotene-12′, 6-dial (3*, C_{17}). SlCCD1B converted apo-15′-lycopenal (IV, C20) into tentatively identified apo-12-lycopenal (8, C_{15}) and 15′, 6-diapocarotene-12′, 6-dial (1*, C_{12}). SlCCD1A converted substrate IV into apo-12-lycopenal (8, C_{15}). Numbering of C atoms in apo-8′-lycopenal and deduced cleavage sites for both enzymes are depicted in B.

10′,6-diapocarotene-10′,6-dial (C_{19}, product 4), besides traces of 10′,10-diapocarotene-10′,10-dial (C_{14}) and 8′,10-diapocarotene-8′,10-dial (C_{17}). Incubation of SlCCD1B with this substrate also led to products 2 and 4, in addition to the major product 8′,10-diapocarotene-8′,10-dial (C_{17}, product 3). These data suggest that both enzymes cleave the three double bonds C5–C6, C7–C8 and C9–C10 of apo-10′-lycopenal. To identify which of the three cleavage sites is preferred by SlCCD1A and SlCCD1B, we measured the relative amounts of the C_{17}- and C_{19}-, and of the C_{17}-, C_{20}- and C_{22}-diladhyde products formed from apo-10′- (C_{27}) and apo-8′-lycopenal (C_{30}). As shown in Table 1, SlCCD1A produced from apo-10′-lycopenal (C_{27}) much more C_{19}- than C_{17}-dialdehyde, pointing to a preference for the C5–C6-double bond. This cleavage would lead to the volatile MHO (C_8). However, this preference was dependent on the chain length of the substrate and changed in apo-8′-lycopenal (C_{30}) towards the C9–C10 double bond, which would result in the formation of the volatile pseudoionone (C_{13}) and a C_{17}-dialdehyde, followed by the C5–C6 and the C7–C8 double bonds whose cleavage leads to the C_{22}-dialdehyde and MHO, and the C_{20}-dialdehyde and geranial, respectively. The preferences of SlCCD1B were clearly different from those of SlCCD1A. SlCCD1B produced from apo-10′-lycopenal (C_{27}) much more C_{17}- than C_{19}-dialdehyde, indicating a preference for the C7–C8-double bond and, accordingly, for the formation of geranial (C_{10}) rather than of MHO (C_8). In apo-8′-lycopenal (C_{30}), the C9–C10 double bond was the preferred site whose cleavage leads to pseudoionone (C_{13}) and a C_{17}-dialdehyde, followed by C7–C8 double bond yielding the C_{20}-dialdehyde and geranial (C_{10}).

We also tested the cleavage of shorter apolycopenals, i.e. apo-12′- (C_{25}, Fig. 1, substrate III) and apo-15′-lycopenal (acycloretinal, C_{20}, Fig. 1, substrate IV). SlCCD1A converted substrate III into a dialdehyde identified based on UV/Vis spectrum and retention time as 12′,6-diapocarotene-12′,6-dial (C_{17}, 3*). The same dialdehyde arose also from incubation with SlCCD1B, besides a 12′,8-diapocarotene-12′,8-dial (C_{15}, 7) that was identified by comparison to a synthetic standard. SlCCD1A converted the shortest apolycopenal tested (substrate IV, apo-15,-lycopenal; C_{20}) into a 15′,6-diapocarotene-12′,6-dial (C_{12}, 1*). Incubation of SlCCD1B with substrate IV yielded the same product (C_{12}, 1*), besides a tentative apo-12-lycopenal (C_{15}, 8).

Apocarotenals are additional substrates of CCD1 enzymes. We incubated purified SlCCD1A and 1B with apocarotenals of different chain lengths, i.e. β-apo-8′- (C_{30}), β-apo-10′- (C_{27}), 3-OH-β-apo-12′- (C_{25}), β-apo-14′- (C_{22}) and β-apo-15′- (C_{20}, retinal), and analyzed

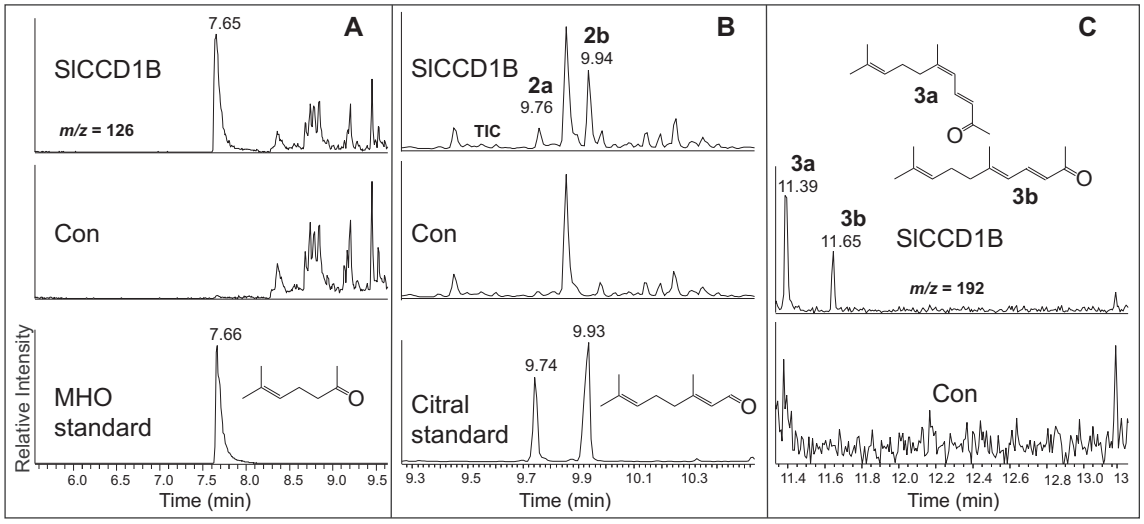

Fig. 2. GC–MS analysis of SlCCD1B incubation with apo-8′-lycopenal. Volatiles were collected by SPME and analyzed. The enzyme released MHO (A), geranial and neral, corresponding to *trans*- and *cis*-citral (B), respectively, and two isomers of pseudoionone. Compounds were identified based on authentic standards and spectral comparison with NIST library.

Table 1
Site preference of SlCCD1A and SlCCD1B in apolycopenals.

Enzyme	Substrate	C_{17} (%)	C_{19} (%)	C_{20} (%)	C_{22} (%)
SlCCD1A	apo-10′-lycopenal	4.2 ± 0.28	95.8 ± 0.28	–	–
	apo-8′-lycopenal	67.31 ± 0.28	–	13.83 ± 0.35	18.86 ± 0.29
SlCCD1B	apo-10′-lycopenal	87.53 ± 0.16	12.47 ± 0.16	–	–
	apo-8′-lycopenal	69.54 ± 11.36	–	30.46 ± 11.3	Not detected

Preference is estimated by calculating amount ratios of dialdehyde products that resulted from the cleavage of apo-10′-lycopenal at the C7–C8 (C_{17}-dialdehyde) and the C5–C6 (C_{19}-dialdehyde) double bonds, and of apo-8′-lycopenal at the C9–C10 (C_{17}-dialdehyde), the C7–C8 (C_{20}-dialdehyde) and the C5–C6 (C_{22}-dialdehyde) double bonds. The values were calculated from the product peak areas of a MaxPlot 300–550 nm of the respective HPLC analyses. Values represent the mean ± SE of four independent incubations.

the assays using HPLC or GC-MS. Both enzymes did not show detectable conversion of retinal (C_{20}) and formed only traces of β-ionone from β-apo-14′-carotenal (C_{22}), indicating a minimal chain length requirement of C_{22} (data not shown). The C9–C10 double bond was the only cleavage site detected with both enzymes in 3-OH-β-apo-12′- (C_{25}) and β-apo-10′-carotenal (C_{27}), leading to the formation of 3-OH-β-ionone and β-ionone, respectively, as well as to the corresponding dialdehyde products (data not shown). Similarly, incubation of SlCCD1A with β-apo-8′-carotenal (C_{30}) led to β-ionone (C_{13}, Fig. 3A, product 2) and the C_{17}-dialdehyde 10′,8-diapocarotene-10′,8-dial (Fig. 3A, product 3), suggesting the C9–C10 double bond as a sole target site. However, SlCCD1B formed, besides the two major products β-ionone (C_{13}) and 10′,8-diapocarotene-10′,8-dial (C_{17}) arising from the cleavage of the C9–C10 double bond, two minor products (Fig. 3A) that indicate further cleavage sites. Product 1 was identical with 14, 8′-diapocarotene-14, 8′-dial (C_{12}) formed by SlCCD1B from apo-8′-lycopenal, while product 4 resembled β-apo-14′-carotenal (C_{22}) in its chromatographic and spectral features. To verify the identity of product 4, we purified and subjected it to LC–MS-analysis that unravelled a $[M+H]^+$ of 311.34 corresponding to the expected mass of β-apo-14′-carotenal (Fig. 3B). This result suggests that SlCCD1B cleaves the three double bonds C9–C10, C13–C14 and C13′–C14′ of β-apo-8′-carotenal (s. Fig. 3C).

2.2. SlCCD1A and B cleave four double bonds of acyclic carotenes in vivo

To check the cleavage activities of SlCCD1A and 1B *in vivo*, we expressed the two enzymes in fusion with thioredoxin in lycopene accumulating *E. coli* cells, collected the volatiles from the head space of the cultures using SPME fibres and analyzed them by GC–MS. As shown in Fig. S3A and 3B, the GC–MS analysis revealed the formation of MHO and pseudoionone, confirming the cleavage of the C5–C6 and C9–C10 double bonds of lycopene. We did not detect geranial in the head space and, therefore, we extracted the culture medium and subjected it to GC–MS analysis. This analysis did not reveal geranial, but showed the corresponding alcohol, geraniol, Fig. S3D), likely formed by the *E. coli* background from the aldehyde cleavage product. In addition to the lycopene-derived MHO and pseudoionone, we also detected geranylacetone (Fig. S3C), which can arise by cleaving the C9–C10 double bond in the lycopene-precursors phytoene, phytofluene or ζ-carotene. In addition, we detected farnesylacetone (C_{18}) as a product of SlCCD1B, formed also in traces by SlCCD1A (Fig. 4). The structure of farnesyl acetone indicates its formation through the cleavage of phytoene or phytofluene at the C13–C14 double bond. Indeed, expression of SlCCD1A and 1B in a ζ-carotene strain that also accumulates phytoene and phytofluene proved that both enzymes produce farnesyl acetone, particularly from phytofluene (data not shown). Moreover, the expression of SlCCD1B in this strain demonstrated that this enzyme cleaves the C13–C14 double bond also in ζ-carotene. As shown in Fig. 5, the activity of SlCCD1B led to two HPLC-detected products (1 and 2) that were identified by LC–MS as apo-13-ζ-carotenone and apo-14′-ζ-carotenal, the two products expected upon the cleavage of C13–C14 double bond.

2.3. SlCCD1A and B cleave different sites of all-trans- and cis-configured carotenoids in vitro

Prolycopene (7,7′,9,9′-tetra-*cis*-lycopene, for structure, s. Fig. 6) is the immediate precursor of all-*trans*-lycopene in plants and cya-

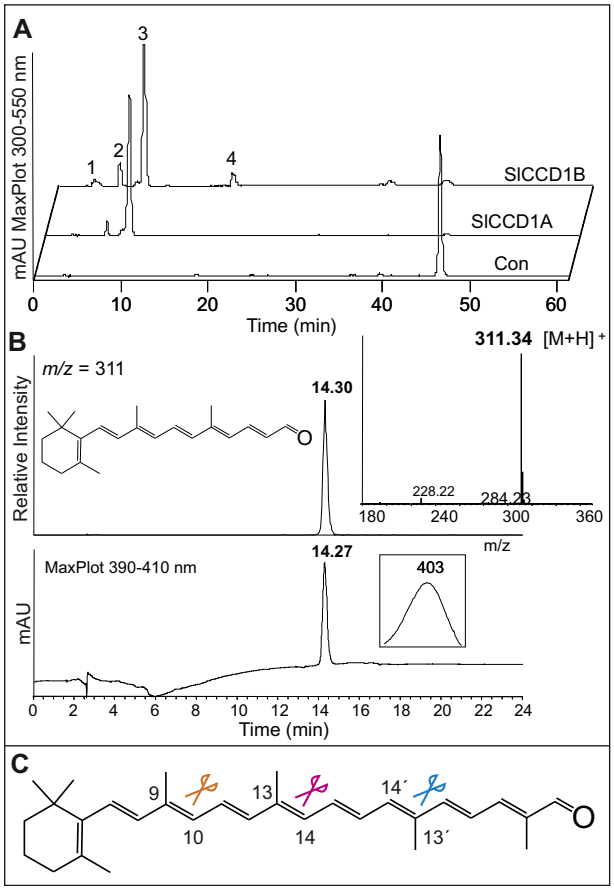

Fig. 3. HPLC analysis of incubations with β-apo-8′-carotenal. (A) Incubation of SlCCD1B with β-apo-8′-carotenal led to β-ionone (2, C_{13}), 10′,8-diapocarotene-10′,8-dial (3, C_{17}) and two minor peaks (1, 4) corresponding to 14, 8′-diapocarotene-14, 8′-dial (1, C_{12}) and β-apo-14-carotenal (4, C_{22}). SlCCD1A converted β-apo-8′-carotenal into β-ionone (2, C_{13}), 10′,8-diapocarotene-10′,8-dial (3, C_{17}). (B) The identity of product 4 was confirmed by LC–MS analysis that unraveled the expected $[M+H]^+$ value of 311.34 co-eluting with the purified product (UV–Vis spectrum depicted in the inset). (C) Deduced cleavage sites of SlCCD1B sites in β-apo-8′-carotenal.

nobacteria. To investigate, whether it is cleaved by SlCCD1A and 1B, we performed *in vitro* assays and analyzed them by HPLC (Fig. 6A). Though the activity of both enzymes was low, we detected in incubations with SlCCD1A the products pseudoionone (Fig. 6A, product 1), a tentative 6′,10-diapocarotene-6′,10-dial (C_{19}, 3), two new compounds (4, 5), as well as tentatively *cis*-configured apo-12′- and apo-10′-lycopenal (6, 7). Incubation with SlCCD1B led to pseudoionone, a tentative apo-12-lycopenal (C_{15}, product 2), the two novel products (4 and 5), apo-12′- (C_{25}, product 6) and apo-10′-lycopenal (C_{27}, product 7). To identify products (4 and 5), we purified both compounds and subjected them to LC–MS analysis (Fig. 6B). The determined $[M+H]^+$ ions of 259.26 and 311.22 as well as the absorption maxima identified the products as apo-13-lycopenone (4, C_{18}) and apo-14′-lycopenal (5, C_{22}). These data suggest that both enzymes cleave prolycopene at the three double bonds C9′–C10′, C11′–C12′ and C13′–C14′ double bond and that SlCCD1A targets the C5–C6 double bond as an additional site (Fig. 6C).

We also tested whether the two enzymes differ in their activity towards monocyclic and bicyclic carotenoids. As shown in Fig. 7, incubation of SlCCD1A with the monocyclic 3-OH-γ-carotene led to 3-OH-β-ionone (Fig. 7A, product 1), pseudoionone (2, *cis-trans*-isomers), 10, 8′-diapocarotene-10, 8′-dial (3, C_{17}) and 10, 6′-diapocarotene-10, 6′-dial (5, C_{19}) identified according to [34]. These products suggest the cleavage of the C9–C10, C9′–C10′, C7′–C8′ C5′–C6′ double bonds. The combined cleavage of the C9–C10, C7′–C8′ and C9–C10, C5′–C6′ led to the dialdehydes 3 and 5, respectively. The dialdehyde 10, 10′-diapocarotene-10, 10′-dial (C_{14}) expected to occur due to the combination C9–C10, C9′–C10′ was not detectable, very likely due to its instability. SlCCD1B converted 3-OH-γ-carotene into the products 3-OH-β-ionone (1), pseudoionone (2, *cis-trans*-isomers), 10, 8′-diapocarotene-10, 8′-dial (3, C_{17}) and traces of 10, 6′-diapocarotene-10, 6′-dial (5, C_{19}). In addition, the enzyme produced two products (4, 6) tentatively identified as apo-13-lycopenone (C_{18}) and 3-OH-β-apo-14′-carotenal (C_{22}), respectively. These products indicate that SlCCD1B cleaves the C13′–C14′ double bond in addition to the three sites already demonstrated for SlCCD1A (Fig. 7C).

Both enzymes cleaved all-*trans*-configured bicyclic substrates like all-*trans*-zeaxanthin at the C9–C10 and C9′–C10′ (data not shown), as previously reported [39]. However, the two enzymes showed different cleavage patterns, when incubated with 9-*cis*-β-carotene (Fig. 7B). SlCCD1A converted 9-*cis*-β-carotene into β-ionone (C_{13}, product 1), presumably 9-*cis*-configured β-apo-10′-carotenal (C_{27}, product 6) and traces of tentatively identified 9-*cis*-β-apo-13-carotenone (C_{18}, product 3). In contrast, SlCCD1B yielded 6 products that included the two major ones (products 1 and 6), β-apo-11-carotenal (C_{15}, product 2) confirmed through comparison with authentic standard and the tentatively identified 9-*cis*-β-apo-13-carotenone (C_{18}, product 3), β-apo-14′-carotenal (C_{22}, product 4) and 9-*cis*-β-apo-12′-carotenal (C_{25}, product 5). To verify the identity of the products 3 and 4, we purified both and subjected them to LC–MS-analysis that unravelled an $[M+H]^+$ of 259.21 and 311.28 corresponding to masses expected for β-apo-13-carotenone and β-apo-14′-carotenal (Fig. 7C). These results suggest that SlCCD1A cleaves the C9′–C10′ and to a much lower extent, the C13–C14 double bond, while SlCCD1B cleaves the C9′–C10′, C13–C14 and C11′–C12′ double bonds of 9-*cis*-β-carotene (s. Fig. 7D).

3. Discussion

A previous study on the activity of CCD1 enzymes from tomato suggested their role in the formation of the C_{13}-volatiles, β-ionone and geranylacetone, which originate from the cleavage of carotenoids at the C9–C10 and/or the C9′–C10′ double bonds. This role was supported by a reduction of these volatiles in the corresponding knock-down tomato lines, and by the very high level of SlCCD1B transcripts in tomato fruits, which constitute 0.11% of total RNA [39]. Later on, it was shown that CCD1 enzymes from different plant species, including tomato, catalyze the cleavage of the C5–C6 double bond in lycopene, leading to MHO, a widespread isoprenoid volatile compound [33]. Investigation of the rice CCD1 suggested the C7–C8 double bond of lycopene as a further target, resulting in geranial formation [34], which is also produced by a cyanobacterial ortholog [36]. Geranial is one of the major norisoprenoids released by tomato fruits and a major volatile of lemon-scented plants [40] with a broad antimicrobial activity [41]. In *Ocimum basilicum*, geranial formation is catalyzed by the geraniol synthase that converts geranyl-diphosphate (GPP) into geraniol, which is oxidized by the geraniol dehydrogenase [42]. Expression of the *O. basilicum* geraniol synthase in tomato fruits led to an improvement of its aroma quality due to the accumulation of monoterpenes [40]. The formation of geranial by the rice OsCCD1 provided the mean for a new route originating from lycopene, which was previously assumed for tomato and watermelon fruits [37] and which was indicated by the release of a higher amount of geranial in transgenic tomato fruits with increased carotenoid content [43]. These finding and the striking correlation between the release of several volatiles of uncertain biosynthetic origin

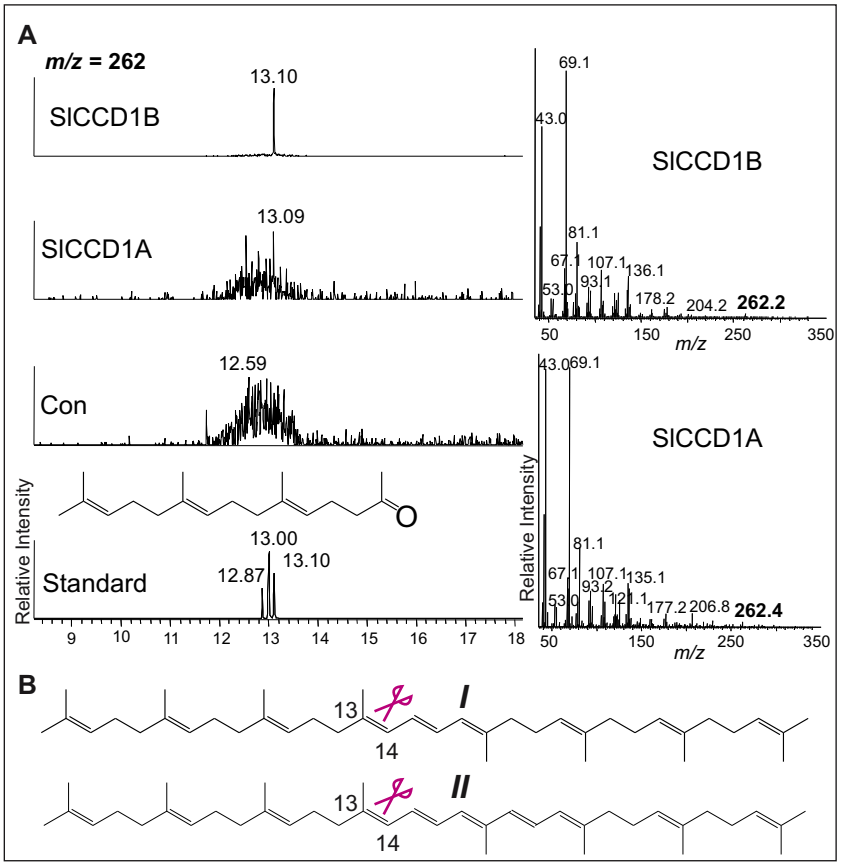

Fig. 4. GC–MS identification of farnesylacetone produced by SlCCD1A and 1B. Volatiles were collected by SPME from the gas space of carotene-accumulating *E. coli* cultures expressing thioredoxin-SlCCD1A, -1B or thioredoxin (Con) and analyzed by GC–MS. SlCCD1B produced farnesylacetone identified based on standard and by comparing the detected molecule ion of 262.2 and detected fragments with the NIST library. Farnesylacetone was also produced by SlCCD1A, but at a much lower level. (B) Farnesylacetone can be produced from either phytoene (I) or phytofluene (II) through the cleavage of the C13–C14 double bond.

and the carotenoid content of different tomato mutants [37,44] prompted us to revisit the two tomato CCD1 enzymes, assuming that their cleavage activity may provide the causal link for this correlation.

We hypothesized that lycopene cleavage products (apolycopenals) and other apocarotenoids, which can arise enzymatically or non-enzymatically, are the likely substrates of both enzymes, particularly of SlCCD1B that is highly expressed in the lycopene accumulating fruits. This hypothesis is based on the assumption that CCD1 enzymes act *in planta* as scavengers of mixtures of destructed carotenoids of different chain lengths rather than being a primary cleaver of intact carotenoids. As shown by the overexpression of OsCCD1 in Golden Rice [45] and by knocking-down SlCCD1A and 1B transcript levels [39], manipulation of the CCD1 activity does not significantly impact the carotenoid content of the corresponding tissue. The role of CCD1 in forming the mycorrhizal pigments in a second cleavage step, acting on the apo-10-carotenoids intermediates produced by CCD7 [46] is also in line with this model. Moreover, the very relaxed double bond specificity observed here with many carotenoid substrates is in favor of a scavenger function shredding apocarotenoids down into a large mix of diverse products.

The two tomato CCD1 enzymes are highly similar to each other (83% identity) and they were considered as identical regarding substrate and double bond specificity [39]. However, in our experiments, the two enzymes showed differences in their activity towards different substrates and in their double bond preferences. For instance, SlCCD1B formed a C_{12}-dialdehyde from apo-8′-lycopenal that was not detected in the corresponding SlCCD1A incubation (Fig. 1). The structure of this product indicates a cleavage of the C13–C14 double bond, an activity that can explain the absence of the C_{22}-dialdehyde product expected to occur due to the cleavage of the C5–C6 double bond, which was confirmed by detecting MHO in the GC–MS analysis. Furthermore, the different relative amounts of the dialdehydes formed by the two enzymes from both apo-10′- and apo-8′-lycopenal indicate that the cleavage sites are targeted with significantly different preferences (Table 1). Taken together, our data indicate that SlCCD1B has a more relaxed specificity and seems to be the more active enzyme, a feature likely related to its high expression in the carotenoid accumulating tomato fruits.

The cleavage activity shown here provides a biosynthetic route for most of the isoprenoids released by tomato (Table 2), including farnesylacetone, neral (*cis*-citral), geranial (*trans*-citral), in addition to the previously reported MHO and C_{13}-volatiles such as β-ionone. Moreover, the CCD1 mediated cleavage of prolycopene at different double bonds, described here, explains the formation of MHO, geranial, neral and pseudoionone in the *tangerine* mutant of tomato that accumulates prolycopene in the fruit, instead of the all-*trans* isomer of the wild type. Finally, our data indicate the presence of several new metabolites that may be bioactive in both plants and animals. In particular, the cleavage of the C13–C14 and C13′–C14′ double bonds leads to different apo-13-carotenones, and in case of β-apo-8′-carotenal, also to β-apo-14′-carotenal. A recent study on apocarotenoids functions in mammal cell lines demonstrates that β-apo-13-carotenone and β-apo-14′-carotenal bind to retinoic acid receptors and act as antagonists of all-*trans*-retinoic acid [7], while farnesylacetone isolated from tobacco smoke is a known selective inhibitor of brain monoamine oxidase B whose activity

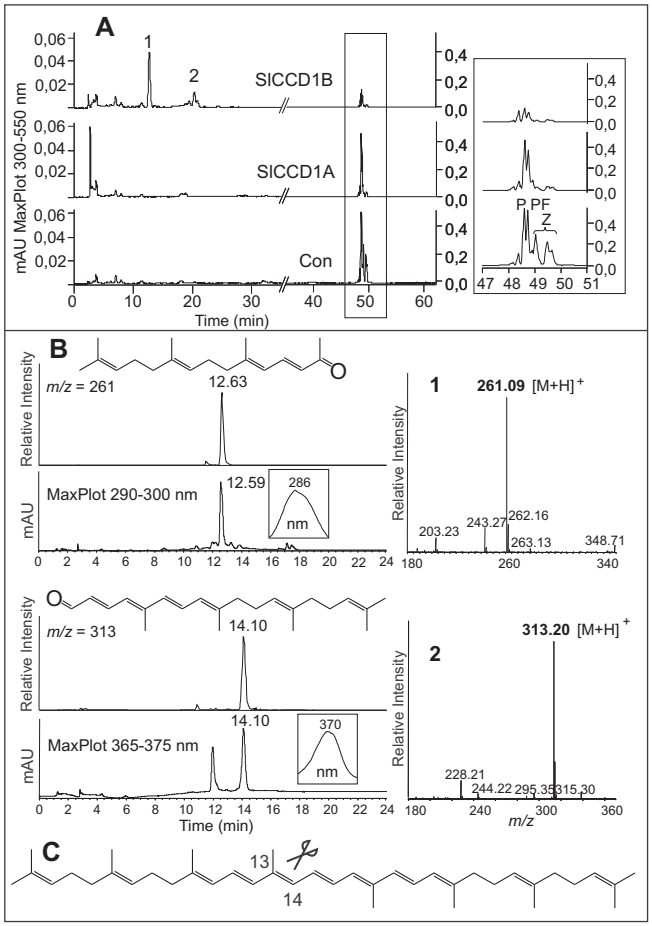

Fig. 5. HPLC analysis of activity in a ζ-carotene-accumulating *E. coli* strain. (A) Expression of thioredoxin-SlCCD1B in ζ-carotene (Z) producing *E. coli* cells that also accumulate phytoene (P) and phytofluene (PF) led to two products (1, 2), that also appeared upon the expression of SlCCD1A, but in traces. (B) LC–MS analysis showed $[M + H]^+$ values of 261 and 313 co-eluting with apocarotenoids with UV–Vis spectra depicted in the insets, identifying product 1 and 2 as apo-13-ζ-carotenone (C_{18}) and apo-14′-ζ-carotenal (C_{22}), respectively. (C) Structure of ζ-carotene indicating the cleavage site leading to product 1 and 2.

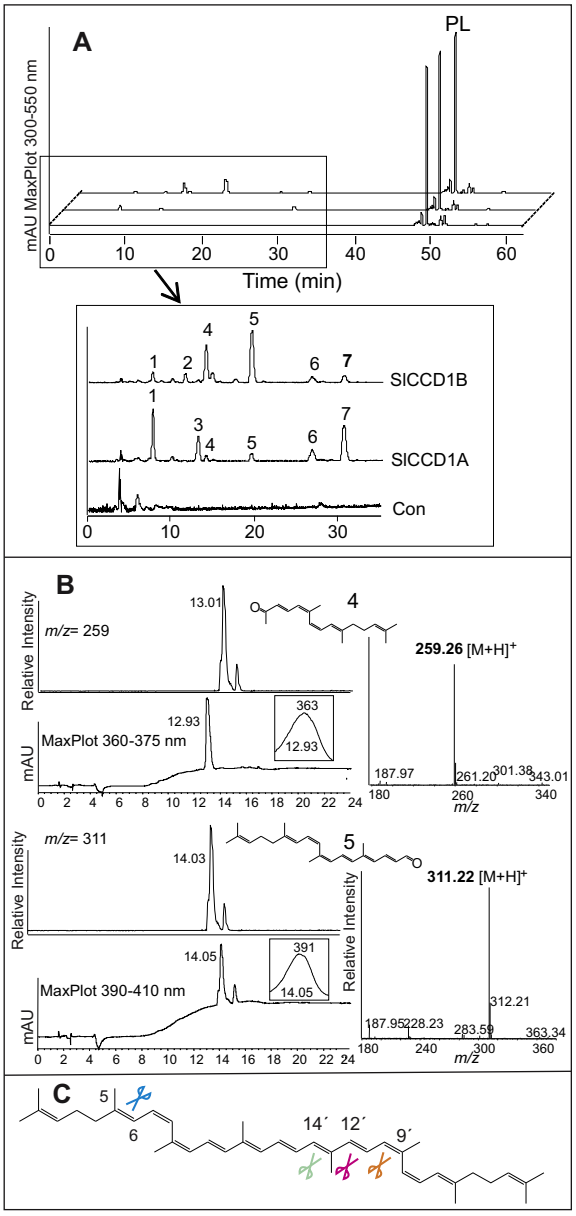

Fig. 6. HPLC analysis of incubations with prolycopene. (A) Incubation with SlCCD1B led to pseudoionone (1, C_{13}), a tentative apo-12-lycopenal (C_{15}, product 2), two new compounds (4 and 5), apo-12′- (6, C_{25}) and apo-10′-lycopenal (7, C_{27}). SlCCD1A converted prolycopene (structure depicted in C) into pseudoionone (1), the products pseudoionone, a tentative 6′,10-diapocarotene-6′,10-dial (C_{19}, 3), the two new compounds (4, 5), and apo-12′- and apo-10′-lycopenal (6, 7). (B) LC–MS analysis of purified product 4 and 5 unraveled $[M + H]^+$ ions of 259.26 and 311.22 and UV–Vis spectra (insets) expected for *cis*-configured apo-13-lycopenone (C_{18}) and apo-14′-lycopenal (C_{22}). (C) Structure of prolycopene indicating the cleavage sites deduced from the products of both enzymes.

is related to Parkinson disease [47]. The C_{18}-ketone β-apo-13-carotenone, designated as d'orenone, is also a bioactive compound in plants, which inhibits the growth of root hairs [48].

4. Materials and methods

4.1. Cloning, expression and purification of SlCCD1A and SlCCD1B

Total RNA was isolated from tomato fruits obtained from the local market, using the Concert™ Plant Reagent Kit (Invitrogen, USA), followed by on-column DNaseI treatment and purification with the RNeasy mini kit (Quiagen, Hilden, Germany). Total cDNA was synthesized from 5 μg total RNA, using SuperScript™ III RnaseH- (Invitrogen, Paisley, UK) and following the manufacturer's instructions. *SlCCD1A* (GenBank: AY576001) and *SlCCD1B* (GenBank: AY576002) were amplified from 2 μL cDNA by the *Phusion* High-Fidelity DNA Polymerase (Finnzymes, Espo, Finland) according to the manufacturer's instructions and using the primers:

SlCCD1A F (5′-ATGGGGAGAAAAGAAGATGATGGA-3′), SlCCD1A R (5′-ATTCAAGAACAAGCCAAACTGTGA-3′), and SlCCD1B F (5′-ATGGGGATGAATGAAGAAGATGGA-3′), SlCCD1B R (5′-ATTCAGGAGCAAGCCAAAATGTGA-3′). Amplified cDNAs were purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, NJ, USA), and inserted into the vectors pJET2.1(Fermentas, St. Leon-Rot, Germany) and pBAD®/Thio-TOPO® (Invitrogen, Paisley, UK), to yield the plasmids pJET-SlCCD1A, pJET-SlCCD1B, pThio-SlCCD1A and pThio-SlCCD1B, respectively. The integrity of the cDNAs was verified by sequencing. To generate pGEX-SlCCD1A, pJET-SlCCD1A was digested with ClaI, treated with T4-DNA polymerase, and the SlCCD1A cDNA was then obtained by XhoI digestion and ligated into accordingly treated pGEX-5X-2 (Amersham Biosciences, NJ, USA). For constructing pGEX-5X-SlCCD1B, the SlCCD1B cDNA was amplified from pJET-SlCCD1B equipped with flanking BamHI and XhoI restriction sites that were then used for cloning into accordingly digested and treated pGEX-5X-2 (Amersham Biosciences, NJ, USA). The pGEX plasmids were trans-

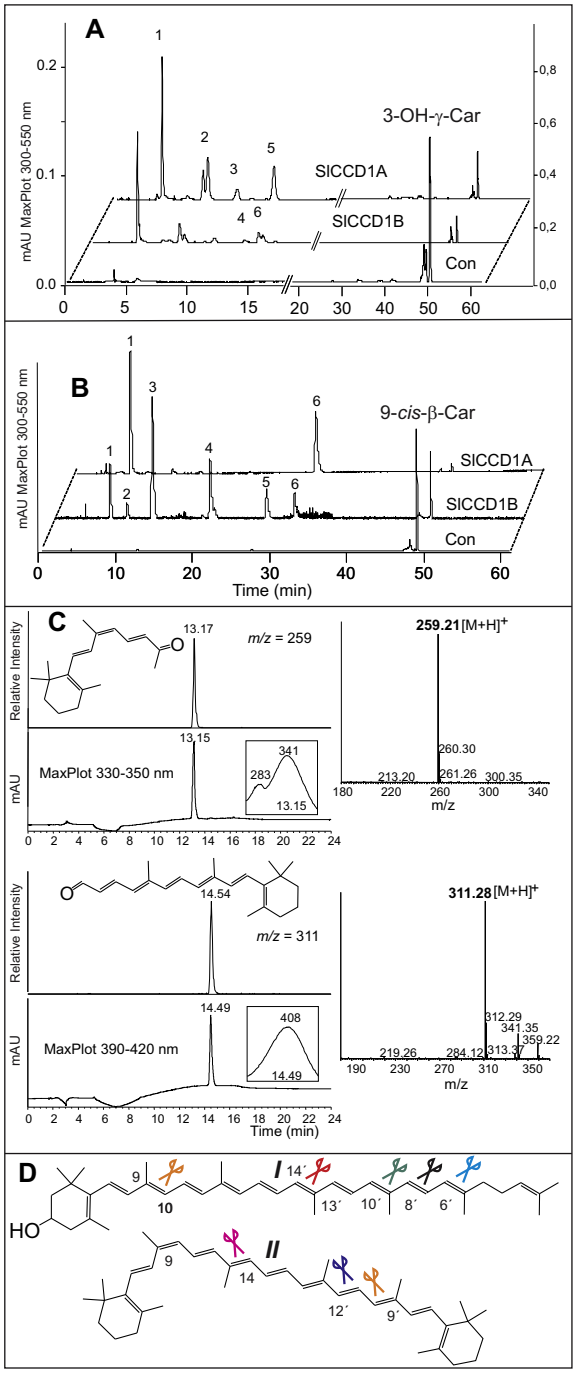

Fig. 7. HPLC analysis of incubations with mono- and bicyclic carotenoids. (A) Incubation of SlCCD1A with 3-OH-γ-carotene (structure I in D) led to 3-OH-β-ionone (1, C_{13}), pseudoionone (2, *cis-trans*-isomers, C_{13}), 10, 8′-diapocarotene-10, 8′-dial (3, C_{17}) and 10, 6′-diapocarotene-10, 6′-dial (5, C_{19}) SlCCD1B converted 3-OH-γ-carotene into 3-OH-β-ionone (1), pseudoionone (2, *cis-trans*-isomers), 10, 8′-diapocarotene-10, 8′-dial (3, C_{17}) and traces of 10, 6′-diapocarotene-10, 6′-dial (5, C19), and two products (4, 6) tentatively identified as apo-13-lycopenone (C_{18}) and 3-OH-β-apo-14′-carotenal (C_{22}), respectively. (B) SlCCD1A converted 9-cis-β-carotene into β-ionone (1, C_{13}) and presumably 9-*cis*-configured β-apo-10′-carotenal (6, C_{27}). Incubation with SlCCD1B yielded 6 products, including 1, 6 the two β-apo-11-carotenal (2, C_{15}) and the tentatively identified 9-cis-β-apo-13-carotenone (C_{18}, product 3), β-apo-14′-carotenal (C_{22}, product 4) and 9-*cis*-β-apo-12′-carotenal (C_{25}, product 5). (C) LC–MS-analysis of purified product 3 and 4 unraveled $[M+H]^+$ values of 259.21 and 311.28 and UV–Vis spectra (insets) corresponding to those expected for β-apo-13-carotenone and β-apo-14′-carotenal. (D) Structure of 3-OH-γ-carotene (I) and 9-*cis*-β-carotene (II) showing the cleavage sites deduced from the products of both enzymes.

Table 2
Tomato volatiles produced by SlCCD1A and SlCCD1B.

Volatile	Described in
6-Methyl-5 hepten-2-one (MHO)	[33], this work
Neral	This work
Geranial	This work
Geranylacetone	[39], this work
Pseudoionone	[39], this work
α-Ionone	[39]
β-Ionone	[39], this work
Farnesyl acetone	This work

Tomato volatiles reported by [37] and identified as products of SlCCD1 enzymes either in this work or in the reference cited.

formed into BL21(DE3) *E. coli* cells transformed with the plasmid pGro7 (Takara Bio Inc.; Mobitec, Göttingen, Germany) that enables an arabinose-inducible co-expression of the groES-groEL-chaperone system. Expression and protein purification were performed according to [38] and monitored by SDS–PAGE.

4.2. Enzyme assays

Synthetic apocarotenoids are a gift of the BASF (Ludwigshafen, Germany). Zeaxanthin and 3-OH-γ-carotene were isolated from accumulating *E. coli* strains expressing the according carotenoid biosynthetic genes (unpublished data). Substrates were purified by TLC according to [49]. Lycopene was purchased from Roth (Karlsruhe, Germany). Prolycopene was isolated from fruits of the *tangerine* tomato mutant. For this purpose, total carotenes were extracted with acetone, purified by TLC and separated by HPLC according to [50]. Fractions containing prolycopene were then collected. Substrates were quantified spectrophotometrically at their individual λ_{max} using extinction coefficients calculated from E1%. Protein concentrations were determined using the BioRad protein assay kit (BioRad, CA, USA).

In vitro incubations with apocarotenoids and carotenoids were performed with 100 ng/μL and 200–250 ng/μl of purified enzyme, respectively, at substrate concentrations of 40 μM (zeaxanthin and apocarotenoids), 50 μM (3-OH-γ-carotene) or 60 μM (prolycopene and β-carotene isomers) in a total volume of 200 μl. Substrate micelles and assays were prepared as described by [34], using a Triton X-100/X-405 mixture for prolycopene and 0.4% octyl-β-glucoside for all other substrates. Incubations were run for 30 min (apocarotenoids), 2 h (zeaxanthin) and 6 h (3-OH-γ-carotene, lycopene and β-carotene isomers), and stopped by adding two volumes of acetone. Extraction was performed using petroleum ether/diethyl ether 1:4 (v/v), and the isolated organic phase was vacuum-dried and dissolved in 40 μl chloroform. 20 μl of the extract was used for HPLC analysis. Volatiles were collected for 30 min, using SPME (solid phase microextraction) fibers (100 μm polydimethylsiloxane, Sigma–Aldrich, Deisenhofen, Germany).

4.3. In vivo test using lycopene-accumulating E. coli cells

Lycopene-accumulating XL1-Blue *E. coli cells* transformed with pFarbeR [51] that encodes the corresponding biosynthetic genes from *Pantoea ananatis* (formerly *Erwinia herbicola*), were transformed with pThio-SlCCD1A, pThio-SlCCD1B and, as a negative control, with the void plasmid pBAD-Thio (Invitrogen, Paisley, UK). Cultures were grown, induced and extracted according to [34]

4.4. Analytical methods

HPLC analysis was performed with a Waters system (Eschborn, Germany) equipped with a photodiode array detector (model 996)

was used. A C30-reversed phase column (YMC Europe, Schermbeck, Germany) and according to [34].

LC–MS analysis was performed using an LC–MS system (Thermo Electron, Waltham, MA, USA) with a Surveyor HPLC system equipped with a PDA detector and an LTQ linear ion trap mass spectrometer. Separation was conducted with a YMC C30-column (150 × 3 mm i.d., 3 μm) and the solvents A: methanol/water/tert.-butylmethylether 50:45:5 (v/v/v) and B: methanol/water/tert.-butylmethylether 27:3:70 (v/v/v), with the water containing 0.1 g/l ammonium acetate. The initial flow rate was set to 0.45 mL/min with 90% A and held for 5 min, followed by a ramp to 5% A in 10 min. Over 4 min, the flow rate was increased to 0.9 mL/min and maintained for another 5 min. The flow rate was decreased to 0.45 mL/min within 0.1 min followed by re-equilibration to initial conditions for 11 min. MS scans were taken using APCI (atmospheric pressure chemical ionization) in the positive mode using nitrogen as sheath- and auxillary gas, with 20 respectively 5 arbitrary units. The vaporizer was set to 225 °C and capillary temperature to 175 °C. The source current was set to 5 μA and capillary voltage to 49 volts. Oximes were produced, isolated and identified according to [34]. C_{17} and C_{19} dialdehydes were separated and identified following [36], and oximes were produced, isolated and identified according to [34].

GC–MS analyses was performed using a ThermoFisher Scientific DSQ II mass spectrometer coupled to a Trace GC gas chromatograph equipped with a 30 m Zebron ZB 5 column (5% phenyl–95% dimethylpolysiloxane, 0.25 mm I.D. and 0.25 μm film thickness; Phenomenex, Aschaffenburg, Germany) according to [34]. Compounds were identified by comparison of mass spectra with the NIST database (National Institute of Standards and Technology Mass Spectral Search Program Version 2.0) and by using synthetic standards.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2014.06.005.

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