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 (SICCD1B), which is highly expressed in fruits, and of its homolog SICCD1A. 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 SICCD1A and, to a larger extent, SICCD1B, 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-pseudoio-
none, 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.

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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 chloroplasts [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 apolycopene, 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-phytene. This compound contains only three conjugated double bonds and, therefore, colorless. Desaturation and isomerization reactions lead via specific cis-isomers of the intermediates phytofluene, θ-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. Zea-xanthin 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
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 (C15) and a C25 apocarotenal, or as carotenoid cleavage oxygenases (CCDs) a term used to describe all other plant members of the enzyme family regardless of their substrate specificity [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-ΟH-β-apo-8′-carotenal (β-citrurin) responsible for the orange-reddish tint 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 stirlingia 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 C13-ketones and C27-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 9cisβ-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 (C13), by catalyzing a specific single cleavage reaction similar to those mediated by other carotenoid cleavage oxygenases [15,31].

Compared to other plant CCB 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 apocarotenals [34], indicating a role in the scavenging of destructed carotenoids. Furthermore, CCD1 enzymes target different double bonds in monomeric and linear carotenoids. The first cleavage sites reported for CCD1 enzymes are the C9–C10 and C9′–C10′ double bonds in C40-caroteno- noids, which give rise to two C13-ketones like β-ionone and one C14-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 apocarotenals, acyclic and monocyclic carotenoids, forming the C10-carotenal geranyl [34,36], one of the many isoproenyl 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 (SICCD1A and SICCD1B) of tomato. Our data suggest that geranyl and many other tomato isoproenyl volatiles such as farnesyl acetone are indeed formed from carotenoids, as a result of the very relaxed double bond and substrate specific- ities of the tomato SICCD1A and SICCD1B enzymes.

2. Results

2.1. SICCD1A 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-1-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 cleaving enzymes [34,38]. Using affinity chromatography, we purified the GST-fusions and released SICCD1A and SICCD1B by Factor-X protease treatment. Purification steps were monitored by SDS–PAGE (Fig. S1).

In a first approach, we incubated purified SICCD1A and SICCD1B with synthetic apocarotenals (for structures, see Fig. 1 compounds I, II) of different chain lengths, i.e. apo-8′- (C30, I), apo-10′-lycopenal (C27, 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 SICCD1A 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 (C13, 2), 8′-10′-diapocarotenone-8′,10′-dial (C17, 3), 8′-10′-diapocarotenone-8′,10′-dial (C15, 5) and 6,8′-diapocarotenone-6,8′-dial (C22, 6). These products suggest that SICCD1A cleaves the three double bonds C5–C6, C7–C8 and C9–C10 in apop-8′-lycopenal (s. Fig. 1, Substrate I), and imply the formation of the volatiles MHO and geranyl, besides pseudoionone. Incubation of SICCD1B 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′-diapocarotenone-14,8′-dial (C12, product 1 in Fig. 1), and to the products 2, 3 and 5, which were also observed with SICCD1A. We also determined the volatiles released by SICCD1B 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 SICCD1B 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 SICCD1B, like SICCD1A, targets the three double bonds C5–C6, C7–C8 and C9–C10 in apo-8′-lycopenal, although the dialdehyde 6,8′-diapocarotenone-6,8′-dial (C22; Fig. 1 product 6 of SICCD1A), 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′-diapocarotenone-14, 8′-dial (C12, product 1 in Fig. 1, s. also Fig. S2) suggests that SICCD1B also targets the C13–C14 double bond.

Incubation of SICCD1A with the substrate apo-10′-lycopenal (Fig. 1, substrate II, C27) resulted in the formation of pseudoionone (product 2) and a dialdehyde identified according to [34] as...
10',6-diapocarotene-10',6-dial (C19, product 4), besides traces of 10',10-diapocarotene-10',10-dial (C14) and 8',10-diapocarotene-8',10-dial (C17). 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 (C17, 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 C17 and C19', and of the C17', C20' and C22-dialdehyde products formed from apo-10'-lycopenal (C27) and apo-8'-lycopenal (C30). As shown in Table 1, SlCCD1A produced from apo-10'-lycopenal (C27) much more C19' than C17'-dialdehyde, pointing to a preference for the C5–C6-double bond. This cleavage would lead to the volatile MHO (C8). However, this preference was dependent on the chain length of the substrate and changed in apo-8'-lycopenal (C30) towards the C9–C10 double bond, which would result in the formation of the volatile pseudoionone (C13) and a C17'-dialdehyde, followed by the C5–C6 and the C7–C8 double bonds whose cleavage leads to the C22-dialdehyde and MHO, and the C20'-dialdehyde and geranial, respectively. The preferences of SlCCD1B were clearly different from those of SlCCD1A. SlCCD1B produced from apo-10'-lycopenal (C27) much more C17'- than C19'-dialdehyde, indicating a preference for the C7–C8-double bond and, accordingly, for the formation of geranial (C10) rather than of MHO (C8). In apo-8'-lycopenal (C30), the C9–C10 double bond was the preferred site whose cleavage leads to pseudoionone (C13) and a C17'-dialdehyde, followed by C7–C8 double bond yielding the C10'-dialdehyde and geranial (C10).

We also tested the cleavage of shorter apolycopenals, i.e. apo-12'-lycopenal (C25, Fig. 1, substrate III) and apo-15'-lycopenal (acycloretinal, C20, 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 (C17, 3'). The same dialdehyde arose also from incubation with SlCCD1B, besides a 12,8-diapocarotene-12,8-dial (C15, 7') that was identified by comparison to a synthetic standard. SlCCD1A converted the shortest apolycopenal tested (substrate IV, apo-15'-lycopenal; C20) into a 15,6-diapocarotene-15,6-dial (C19, 1'). Incubation of SlCCD1B with substrate IV yielded the same product (C12, 1'), besides a tentative apo-12'-lycopenal (C15, 8).

Apolycopenals are additional substrates of CCD1 enzymes. We incubated purified SlCCD1A and 1B with apocarotenals of different chain lengths, i.e. β-apo-8'-lycopenal (C30), β-apo-10'-lycopenal (C27), 3-OH-β-apo-12'-lycopenal (C25), β-apo-14'-lycopenal (C22) and β-apo-15'-lycopenal (C20, retinal), and analyzed...
The C9–C10 double bond of lycopene is the immediate precursor of all-trans- and cis-lycopene in plants and consequently of all-trans- and cis- 

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

Table 1

| Enzyme | Substrate       | \( C_{17} \) (%) | \( C_{19} \) (%) | \( C_{20} \) (%) | \( C_{22} \) (%) |
|--------|-----------------|------------------|-----------------|-----------------|-----------------|
| SlCCD1A | apo-10'-lycopenal | 4.2 ± 0.28       | 95.8 ± 0.28     | –               | –               |
| SlCCD1B | apo-10'-lycopenal | 67.31 ± 0.28     | –               | 13.83 ± 0.35    | 18.86 ± 0.29    |
| SlCCD1B | apo-8'-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. The data shown correspond to the mean ± SE of four independent incubations.

2.2. SlCCD1A and B cleave four double bonds of acyclic carotenoids 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 headspace of the cultures using SPME fibres and analyzed them by GC–MS. As shown in Fig. 3A 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 headspace 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. 3D, 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. 3C), which can arise by cleaving the C9–C10 double bond in the lycopene-precursors phytoene, phytofluene or \( \zeta \)-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 \( \zeta \)-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 \( \zeta \)-carotene. As shown in Fig. 5, the activity of SlCCD1B led to two HPLC-detected products \( (1 \text{ and } 2) \) that were identified by LC–MS as apo-13\( \zeta \)-carotene and apo-14\( \zeta \)-carotene, 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-
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 (C14) expected to occur due to the combination C9–C10, C9′–C10′ was not detectable, very likely due to its instability. SICCD1B 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, C17) and traces of 10, 6′-diapocarotene-10, 6′-dial (5, C19). In addition, the enzyme produced two products (4, 6) tentatively identified as apo-13-lycopenone (C18) and 3-OH-β-apo-14-carotenal (C22), respectively. These products indicate that SICCD1B cleaves the C13′–C14′ double bond in addition to the three sites already demonstrated for SICCD1A (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). SICCD1A converted 9-cis-β-carotene into β-ionone (C3, product 1), presumably 9-cis-configured β-apo-10′-carotenal (C27, product 6) and traces of tentatively identified 9-cis-β-apo-13-carotenone (C19, product 3). In contrast, SICCD1B yielded 6 products that included the two major ones (products 1 and 6), β-apo-11-carotenal (C15, product 2) confirmed through comparison with authentic standard and the tentatively identified 9-cis-β-apo-13-carotenone (C19, product 3), β-apo-14-carotenal (C22, product 4) and 9-cis-β-apo-12′-carotenal (C25, 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]+ value of 311.34 co-eluting with the purified product (UV–Vis spectrum depicted in the inset). (C) Deduced cleavage sites of SICCD1B sites in β-apo-8′- carotenal.

Fig. 3. HPLC analysis of incubations with β-apo-8′-carotenal. (A) Incubation of SICCD1B with β-apo-8′-carotenal led to β-ionone (2, C13), 10,8′-diapocarotene-10,8′-dial (3, C17) and two minor peaks (1, 4) corresponding to 14,8′-diapocarotene-14,8′-dial (1, C12) and β-apo-14-carotenal (4, C14). SICCD1A converted β-apo-8′-carotenal into β-ionone (2, C13), 10,8′-diapocarotene-10,8′-dial (3, C17). (B) The identity of product 4 was confirmed by LC-MS analysis that unravelled 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 SICCD1B sites in β-apo-8′-carotenal.
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 (apolycopeneals) and other apocarotenoids, which can arise enzymatically or non-enzymatically, are the likely substrates of both enzymes, particularly of SICCD1B 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 SICCD1A 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, SICCD1B formed a C12-dialdehyde from apo-8'-lycopenal that was not detected in the corresponding SICCD1A 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 C22-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 SICCD1B 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 C13-volatiles such as 4-or-ß-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 ß-apo-8'-carotenal, also to ß-apo-14'-carotenal. A recent study on apocarotenoids functions in mammal cell lines demonstrates that ß-apo-13-carotenone and ß-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
was synthesized from 5 μg total RNA, using the RNeasy mini kit (Qiagen, Hilden, Germany). Total cDNA was synthesized from 5 μg total RNA, using SuperScript™ III Rna-seh- (Invitrogen, Paisley, UK) and following the manufacturer’s instructions. SICCD1A (GenBank: AY576001) and SICCD1B (GenBank: AY576002) were amplified from 2 μL cDNA by the Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) according to the manufacturer’s instructions and using the primers: SICCD1A F (5'-ATGGGGAGAAAAGAAGATGGA-3'); SICCD1A R (5'-ATTCCAAGACAAAAAGAGATGGA-3'), SICCD1B F (5'-ATGGGGAGATTGAAGAAGATGGA-3'), SICCD1B R (5'-ATTCAAGACAAAAAGAGATGGA-3'). Amplified cDNAs were purified using GFXTM 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-SICCD1A, pJET-SICCD1B, pThio-SICCD1A and pThio-SICCD1B, respectively. The integrity of the cDNAs was verified by sequencing. To generate pGEX-SICCD1A, pJET-SICCD1A was digested with Clal, treated with T4-DNA polymerase, and the SICCD1A cDNA was then obtained by XhoI digestion and ligated into accordingly treated pGEX-5X-2 (Amersham BioSciences, UK), to yield the plasmids pJET-SICCD1A, pJET-SICCD1B, pThio-SICCD1A and pThio-SICCD1B, respectively. The integrity of the cDNAs was verified by sequencing. To generate pGEX-SICCD1A, pJET-SICCD1A was digested with Clal, treated with T4-DNA polymerase, and the SICCD1A cDNA was then obtained by XhoI digestion and ligated into accordingly treated pGEX-5X-2 (Amersham Biosciences, NJ, USA). For constructing pGEX-5X-SICCD1B, the SICCD1B cDNA was amplified from pJET-SICCD1B equipped with flanking BamH1 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-

is related to Parkinson disease [47]. The C18-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 SICCD1A and SICCD1B

Total RNA was isolated from tomato fruits obtained from the local market, using the Concert™ Plant Reagent Kit (Invitrogen, USA), followed by on-column DNase treatment and purification with the RNaseh mini kit (Qiagen, Hilden, Germany). Total cDNA was synthesized from 5 μg total RNA, using Superscript™ III Rnaseh- (Invitrogen, Paisley, UK) and following the manufacturer’s instructions. SICCD1A (GenBank: AY576001) and SICCD1B (GenBank: AY576002) were amplified from 2 μL cDNA by the Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) according to the manufacturer’s instructions and using the primers: SICCD1A F (5'-ATGGGGAGAAAAGAAGATGGA-3'); SICCD1A R (5'-ATTCCAAGACAAAAAGAGATGGA-3'), SICCD1B F (5'-ATGGGGAGATTGAAGAAGATGGA-3'), SICCD1B R (5'-ATTCAAGACAAAAAGAGATGGA-3'). Amplified cDNAs were purified using GFXTM 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-SICCD1A, pJET-SICCD1B, pThio-SICCD1A and pThio-SICCD1B, respectively. The integrity of the cDNAs was verified by sequencing. To generate pGEX-SICCD1A, pJET-SICCD1A was digested with Clal, treated with T4-DNA polymerase, and the SICCD1A cDNA was then obtained by XhoI digestion and ligated into accordingly treated pGEX-5X-2 (Amersham Biosciences, NJ, USA). For constructing pGEX-5X-SICCD1B, the SICCD1B cDNA was amplified from pJET-SICCD1B equipped with flanking BamH1 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. 5. HPLC analysis of activity in a -carotene-accumulating E. coli strain. (A) Expression of thioredoxin-SICCD1B in -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 SICCD1A, 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-carotene (C18) and apo-14-carotenal (C22), respectively. (C) Structure of -carotene indicating the cleavage site leading to product 1 and 2.

![Fig. 6. HPLC analysis of incubations with prolycopene. (A) Incubation with SICCD1B led to pseudoionone (1), a tentative apo-12-lycopenal (C18, product 2), two new compounds (4 and 5), apo-12'- and apo-10'-lycopenal (7, C22). SICCD1A converted prolycopene (structure depicted in C) into pseudoionone (1), the products pseudoionone, a tentative 6,10-diapocarotene-6,10-dial (C22, 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 (C18) and apo-14'-lycopenal (C22). (C) Structure of prolycopene indicating the cleavage sites deduced from the products of both enzymes.]
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 λmax 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 (lycopene 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-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 Pantocea annanatis (formerly Erwinia herbicola), were transformed with pThio-SICCD1A, pThio-SICCD1B 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 auxiliary 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], C17- and C19 dialdehydes were separated and identified following [36], and oximes were produced, isolated and identified according to [34].

GC–MS analyses were 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–methylsilicone film thick- ness; Phenomenex, Aschaffenburg, Germany) according to [34].

Compounds were identified by comparison of mass spectra with the NIST database (National Institute of Standards and Technology). Compounds were identified by comparison of mass spectra with the NIST database (National Institute of Standards and Technology) and according to [34]. Oximes were produced, isolated and identified following [36].

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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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