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Carotenoid oxygenases involved in plant branching catalyze a highly specific, conserved apocarotenoid cleavage reaction

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SYNOPSIS

Recent studies with the *high-tillering* mutants in *Oryza sativa*, the *more axillary growth* (*max*) mutants in *Arabidopsis thaliana* and the *ramosus* (*rms*) mutants in *Pisum sativum* have indicated the presence of a novel plant hormone that inhibits branching in an auxin-dependent manner. The synthesis of this inhibitor is initiated by the two carotenoid cleaving oxygenases (CCDs) OsCCD7/OsCCD8b, MAX3/MAX4 and RMS5/1 in rice, Arabidopsis and pea, respectively. MAX3 and MAX4 are thought to catalyze the successive cleavage of a carotenoid substrate yielding an apocarotenoid that, possibly after further modification, inhibits the outgrowth of axillary buds. To elucidate the substrate specificity of OsCCD8b, MAX4 and RMS1, we investigated their activities *in vitro* using naturally accumulated carotenoids and synthetic apocarotenoid substrates, and *in vivo* using carotenoid-accumulating *E. coli* strains. The results obtained suggest that these enzymes are highly specific, converting the C₂₇-compounds β -apo-10'-carotenal and its alcohol to yield β -apo-13-carotenone *in vitro*. Our data suggest that the second cleavage step in the biosynthesis of the plant branching inhibitor is conserved in monocot and dicot species.

Key words: Apical Dominance, Apocarotenoids, β -Apo-13-Carotenone, Carotenoid Cleavage, Plant Branching Inhibitor, Plant Development.

Abbreviations used: CCD, carotenoid cleavage dioxygenase; max, more axillary growth; rms, ramosus; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry.

INTRODUCTION

Apart from their established roles as photoprotective and light-harvesting pigments in photosynthesis, carotenoids fulfill more ubiquitous functions as precursors for an ever-increasing number of physiologically important compounds, termed apocarotenoids. Examples of such carotenoid derivatives are represented by the opsin chromophore retinal, the morphogen retinoic acid, the phytohormone abscisic acid (ABA) and the fungal pheromone trisporic acid. In general, apocarotenoids are synthesized through oxidative cleavage of their precursors mediated by carotenoid cleaving (di)oxygenases (CCD; the mono- or dioxygenase mechanism is still under debate) [for reviews, see 1-4].

The research on carotenoid oxygenases was paved by the identification of VP14 (*viviparous14*) from maize. VP14 is a 9-*cis*-epoxy-carotenoid dioxygenase catalyzing the formation of the ABA-precursor xanthoxin [5], which represents the rate-limiting step in ABA-biosynthesis [6, 7]. Subsequently, the occurrence of homologs in all taxa was discovered *in silico*, which allowed to elucidate the biosynthesis of several carotenoid-derived compounds, such as retinal in animals [8, 9] and fungi [10], the pigments bixin [11], saffron [12] and neurosporaxanthin [13]. In addition, carotenoid oxygenases mediating the formation of volatile compounds, such as β -ionone, were identified from several plants [14, 15]. Moreover, the characterization of homologous enzymes unveiled novel reaction mechanisms, such as the specific cleavage of apocarotenoids instead of bicyclic carotenoids and their conversion into retinal in cyanobacteria [16-18].

Apart from ABA, additional carotenoid-derived signals occur in plants, awaiting their molecular identification [19]. It was shown, for instance, that the *Arabidopsis bypass1* mutant, affected in several developmental processes, can be partially rescued by inhibitors of carotenoid biosynthesis [20]. Moreover, apical dominance mutants of several plant species caused by lesions in two divergent carotenoid oxygenase genes indicated the involvement of an apocarotenoid signal required for maintaining normal plant architecture [21-24].

Apical dominance is mediated by apically derived auxin, which is transported basipetally. However, several lines of evidence excluded a direct mode of action by auxin [25], rather it is thought to exert its function through second messengers, like cytokinin [26-28]. However, the analysis of the *more axillary growth* (*max1-4*) mutants from *Arabidopsis* [29], the *ramosus* (*rms1, 2, 5*) mutants from pea [30-32], and the *decreased apical dominance 1* (*dad1*) mutant from petunia [33], all impaired in apical dominance, provided new insights. Grafting studies showed that wild-type rootstocks were able to rescue the branching phenotype of mutant shoots and suggested the involvement of an entirely novel, upwardly mobile signal acting as a relay between the auxin message and the response of the axillary buds [21, 22]. It was shown that the synthesis of this signal required the activities of the genes *MAX1*, *MAX3*, *MAX4* and *RMS1*, *RMS5* in *A. thaliana* and *P. sativum*, respectively [21, 34].

The link between apocarotenoids and plant branching was uncovered through the identification of MAX4 from *Arabidopsis*, RMS1 from *Pisum*, and DAD1 from *Petunia* [24, 35]. These enzymes constitute a subgroup of the plant carotenoid oxygenase family (carotenoid cleavage dioxygenase 8, CCD8), while MAX3 [23] and RMS5 [34] represent members of the CCD7 subfamily. It has been shown that AtCCD7 (MAX3) mediated the cleavage of several carotenoids at the 9-10 and/or 9'-10' double bond [23, 36] and that the co-expression of AtCCD7 and AtCCD8 (MAX4) in β -carotene-accumulating *E. coli* cells led to the formation of a C₁₈-ketone, β -apo-13-carotenone [36]. These data led to the hypothesis that the synthesis of the branching inhibitory signal is initiated by two sequential cleavage reactions, where AtCCD7 converts a carotenoid into an apocarotenoid, which represents the substrate for AtCCD8. However, this presumed apocarotenoid specificity of AtCCD8 contradicts recent observations indicating that this enzyme can cleave carotenoids [37]. The enzymatic activities of the pea enzymes PsCCD8 (RMS1) and PsCCD7 (RMS5) have not been described so far.

Monocots and dicots share similarities with respect to branching [25]. During the vegetative growth of rice, grain-bearing branches, called tillers, are formed on the unelongated basal internodes and develop their own adventitious roots. The tillering of rice involves the formation of axillary buds and their subsequent outgrowth [38]. The number of tillers is an important agronomic breeding trait for grain production and many rice tillering mutants have been reported. For instance, the *monoculm1* mutant does not develop any tillers due to a lesion in the *MONOCULM1* (*MOC1*) gene. *MOC1* is expressed in the axillary buds and encodes a nuclear protein, which initiates the formation of axillary buds [39]. In contrast, the rice mutant *fc1* (*Os teosinte branched 1*, *Os tb1*) exhibits a high-tillering phenotype combined with dwarfism. The gene *OsTB1* encodes a putative transcription factor, which acts as a negative regulator of tillering by inhibiting the outgrowth of axillary buds [40]. Recently, a closely related Arabidopsis gene, *BRANCHED1* (*BRC1*), has been identified and shown to arrest the development of axillary buds [41]. Adding to these similarities, the characterization of the *High Tillering Dwarf 1* (*HTD1*) and *DWARF10* (*D10*) rice mutants led to the discovery of the putative carotenoid oxygenase genes *OsCCD7* [42] and *OsCCD8b* [43]. It was supposed that these are orthologs of the dicot enzymes AtCCD7 /AtCCD8 or PsCCD7/PsCCD8, mediating the synthesis of a carotenoid-derived branching inhibitor.

In the current work, we investigated the biosynthesis of the branching inhibitor *in vitro*, placing emphasis on the second proposed cleavage reaction. Using heterologously expressed enzymes, we show that the rice *OsCCD8b*, the Arabidopsis AtCCD8 and the pea PsCCD8 are highly specific apocarotenoid cleavage enzymes mediating a common reaction leading to the production of β -apo-13-carotenone (C₁₈), which may be a precursor of the branching inhibitor in monocot and dicot plants.

EXPERIMENTAL PROCEDURES

Cloning of cDNAs and generation of expression plasmids

A synthetic cDNA encoding OsCCD8b (Accession No. NM_191187) deduced from the corresponding gene *OSJNBa0014K08* was obtained from Epoch Biolabs, Inc.; TX, USA and cloned into pBluescript-SK to yield pBSK-OsCCD8b. For the cloning of *PsCCD8*, five microgram of total RNA, isolated from two weeks-old auxin treated pea seedlings, was used for cDNA synthesis using SuperScript™ RnaseH⁻ reverse transcriptase (Invitrogen, Paisley, UK) according to the instructions of the manufacturer. Two microliter of the cDNA was then used for amplification with the following primers: RMS1-I: 5'-atggtttcatagcctcaccaactc-3' and RMS1-II: 5'-ttactgttttggaccagcatcc-3'. The PCR reaction was performed using 500 nM of each primer, 150 μM dNTPs and 2.5 U *OptiTa* Polymerase (Roboklon, Berlin, Germany) in the buffer provided, as follows: 2 min initial denaturation at 94 °C, 32 cycles (20 s 95 °C, 30 s 63 °C, 105 s 72 °C) and 5 min final polymerization at 72 °C. For the cloning of *AtCCD8*, total cDNA was synthesized from RNA isolated from *A. thaliana* two weeks-old seedlings as above. Five microliter cDNA containing oligo-dT, 100 ng of the 5'-specific primer AtCCD8-I: 5'-atggtcttcttgatcacaacc-3' and 100 μM dNTPs were used in a PCR reaction performed with 5 U *Taq*-polymerase (Eppendorf, Germany) in the buffer provided, as follows: 2 min initial denaturation at 94 °C, 32 cycles (30 s 94 °C, 30 s 50 °C, 2 min 72 °C) and 10 min final polymerization at 72 °C. The obtained *PsCCD8* and *AtCCD8* PCR fragments were purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, NJ, USA), and cloned into the pCR2.1®-TOPO® to yield pCR-*PsCCD8* and pCR-*AtCCD8*, respectively.

For *in vitro* assays, the three cDNAs were amplified from the obtained plasmids using the primers Os 8-I: 5'-atgtctcccgetatgctgcaggcgt-3' and Os 8-II: 5'-ttactgtctgttctcttctctggca-3' for *OsCCD8b*, AtCCD8-I and AtCCD8-II: 5'-tcattcaacagctttctccaactc-3' for *AtCCD8*, and RMS1-I, -II for *PsCCD8*. The PCR reactions were performed with *OptiTa* Polymerase (Roboklon, Berlin, Germany) according to the instructions of the manufacturer. The *OsCCD8b*, *AtCCD8* and *PsCCD8* fragments were then purified as described above and cloned into pBAD/THIO-TOPO® TA (Invitrogen, Paisley, UK) to yield pThio-Os8b, pThio-At8 and pThio-Ps8, respectively. The identity of the obtained plasmids was verified by sequencing.

Protein expression and purification

For protein purification, the *OsCCD8b*-cDNA was excised from pBSK-OsCCD8b with the restriction enzymes *Kpn*I and *Eco*RI and ligated into pET32a (Novagen, Darmstadt, Germany) to yield pET32a-Os8b. This plasmid was then transformed into BL21(DE3) *E. coli* cells. 4 ml of overnight cultures grown in LB-medium were inoculated into 50 ml 2 * YT-medium. The cultures were induced at an OD₆₀₀ (0.5) with 0.5 mM isopropyl β-D-thiogalactopyranoside and grown for 24 h at 18 °C. Cells were

then harvested and the thioredoxin-OsCCD8b fusion protein was purified using TALON-resin (Clontech, CA, USA) according to the instructions of the manufacturer.

For crude assays, the plasmids pThio-At8, pThio-Ps8 and pThio-Os8b were transformed into BL21(DE3) *E. coli* cells harboring the plasmid pGro7 (Takara Bio Inc.; Mobitec, Göttingen, Germany), which encodes the groES-groEL-chaperone system under the control of an arabinose-inducible promoter. 2.5 ml of overnight cultures of transformed cells were then inoculated into 50 ml of 2*YT-medium, grown at 28 °C to an OD₆₀₀ of 0.5 and induced with arabinose (0.2%, w/v) for 4h. Cells were harvested and resuspended in 1 ml of the following buffer: 50 mM NaH₂PO₄, 300 mM NaCl, 1 mg/ml lysozyme, 1 mM dithiothreitol and Triton X-100 (0.1%, v/v); pH 8.0. After incubation for 30 min on ice, cells were sonicated and centrifuged at 12000 g at 4 °C for 30 min. The isolated supernatant was then used for *in vitro* assays.

Enzymatic assays

Substrates were purified using thin-layer silica gel plates (Merck, Darmstadt, Germany). Plates were developed in light petroleum/diethylether/acetone (40:10:10, v/v/v). Substrates were scraped off in dim daylight and eluted with acetone. Synthetic apocarotenals were kindly provided by BASF (Ludwigshafen, Germany). β -carotene and lycopene were obtained from Roth (Karlsruhe, Germany). Zeaxanthin was isolated from *Synechocystis* sp. PCC6803; neoxanthin, and lutein from spinach. Rosafluene-dialdehyde was prepared enzymatically using the AtCCD1 from *A. thaliana* and β -apo-8'-carotenal according to [44]. β -apo-10'-carotenol was prepared by reducing the corresponding aldehyde with NaBH₄ in an EtOH solution. β -apo-10'-carotenoic acid was obtained enzymatically from the corresponding aldehyde using a NAD-dependent dehydrogenase from *Neurospora crassa* [45].

In vitro assays were performed in a total volume of 200 μ l. 50 μ l of substrates (160 μ M) in EtOH were mixed with 50 μ l of ethanolic Triton X-100 (0.4%, v/v; Sigma, Deisenhofen, Germany), dried using a vacuum centrifuge and then resuspended in 50 μ l water. The prepared substrates were then mixed with 100 μ l of 2 X incubation buffer containing 2 mM TCEP, 0.4 mM FeSO₄ and 2 mg/ml catalase (Sigma, Deisenhofen, Germany) in 100 μ l of 200 mM Hepes-NaOH, pH 8. Purified OsCCD8b was then added to a final concentration of 400 ng/ μ l. Crude assays were performed using 50 μ l of the soluble fractions of overexpressing cells. The assays were incubated for 2 h at 28 °C. Extraction was done by adding two volumes of each acetone and light petroleum/diethylether (1:4, v/v). After centrifugation, the epiphase was collected.

For *in vivo* assays, carotenoid-accumulating strains were generated by transforming XL-1 Blue *E. coli* cells with the plasmids pLYC, p- β and pZEA, pACYC (Fermentas GmbH; St. Leon-Rot, Germany) derivatives encoding the *Erwinia herbicola* genes required for lycopene, β -carotene and zeaxanthin biosynthesis, respectively. The obtained strains were then transformed with pThio-At8, pThio-Ps8, pThio-Os8b and the control plasmid pThio. Overnight cultures of transformed cells were inoculated into 50 ml of LB-medium, grown at 28 °C to an OD₆₀₀ of 0.5 and induced with arabinose (0.2%, w/v)

for 16 h. Cells were then harvested, extracted with chloroform/MeOH (2:1, v/v) and dried extracts were subjected to HPLC analyses.

Analytical methods

Substrates were quantified spectrophotometrically at their individual λ_{\max} using extinction coefficients calculated from E1% as given by Barua and Olson [46]. The concentrations of the rosafluene-dialdehyde, β -apo-10'-carotenol and -carotenoic acid were estimated based on the concentrations of the precursor apocarotenals. Protein concentration was determined using the BioRad protein assay kit (BioRad, Hercules, CA). For HPLC, a Waters system (Eschborn, Germany) equipped with a photodiode array detector (model 996) was used. The separation was performed using a YMC-Pack C₃₀-reversed phase column (250 x 4.6 mm i.d., 5 μ m; YMC Europe, Schermbek, Germany) with the solvent systems B: MeOH/ *tert*-butylmethyl ether/water (120:4:40, v/v/v) and A: MeOH/ *tert*-butylmethyl ether (500:500, v/v). The column was developed at a flow-rate of 1 ml/min with a gradient from 100% B to 43% B within 45 min, then to 0% B within 1 min, maintaining the final conditions for another 14 or 29 min at a flow-rate of 2 ml/min. For the analyses of assays containing rosafluene-dialdehyde, the separation was performed using the solvent systems B: MeOH/water (35:65, v/v) and A: MeOH/*tert*-butylmethyl ether/water (500:500:100, v/v/v). The column was developed for 10 min with solvent B at a flow rate of 1.4 ml/min, followed by a linear gradient from 100% B to 50% B within 10 min at a flow rate of 1.4 ml/min, then to 100% A and a flow rate of 2 ml/min within 1 min, maintaining these final conditions for another 4 min.

LC-MS analyses of HPLC-purified products were performed using a Thermo Finnigan LTQ mass spectrometer coupled to a Surveyor HPLC system consisting of a Surveyor Pump Plus, Surveyor PDA Plus and Surveyor Autosampler Plus (Thermo Electron, Waltham, MA, USA). Separations were carried out using a Thermo Hypersil GOLD C₁₈-reversed phase column (150 x 4.6 mm i.d., 3 μ m) with the solvent system A: MeOH/water/*t*-butylmethyl ether (50/45/5, v/v/v) and B: MeOH/water/*t*-butylmethyl ether (27/3/70, v/v/v) with the water containing 0.1 g/l ammonium acetate. The column was developed isocratically at a flow rate of 450 μ l min⁻¹ with 90% A and 10 % B for 5 min followed by a linear gradient to 5% A and 95% B in 10 min. The final conditions were maintained for 5 min at a flow-rate of 900 μ l min⁻¹. The identification of β -apo-13-carotenone was carried out using APCI in positive mode. Nitrogen was used as sheath and auxiliary gas, which were set to 20 and 5 arbitrary units, respectively. The vaporizer temperature was 225 °C and capillary temperature 175 °C. The source current was set to 5 μ A and the capillary voltage was 49 V.

RESULTS

In vivo and *in vitro* assays with recombinant OsCCD8b

To investigate the carotenoid cleavage activity of OsCCD8b, the corresponding cDNA, encoded in the plasmid pThio-Os8b, was expressed in β -carotene-, zeaxanthin- and lycopene-accumulating *E. coli* strains, a frequently used *in vivo* system to determine the enzymatic activity of carotenoid synthesizing and cleaving enzymes [47, 8]. Subsequent HPLC analyses revealed a pronounced reduction in the carotenoid levels in lycopene and zeaxanthin strains. However, no cleavage products were detected (Supplemental Fig. 1). Due to the limited variety of substrates that can be offered *in vivo*, we further explored the activity of the enzyme *in vitro* using supernatants of *OsCCD8b*-expressing BL21(DE3) *E. coli* cells. Based on the assumption that OsCCD8b may cleave apocarotenoids rather than their carotenoid precursors, we applied synthetic apocarotenals as substrates. Among the apocarotenoids presented in Fig. 1, we could only detect a weak cleavage activity with β -apo-10'-carotenal, indicated by the appearance of small amounts of a novel product (data not shown). To improve the enzymatic activity, the *thioredoxin-OsCCD8b* fusion was expressed in BL21(DE3) *E. coli* cells harboring the vector pGro7, which encodes the chaperones groES-groEL. This resulted in a striking improvement of the enzymatic activity, as indicated by the strong bleaching (not shown) of the β -apo-10'-carotenal assays. HPLC analyses revealed the conversion of β -apo-10'-carotenal into a more polar compound (Fig. 2A). Based on the UV-Vis-spectrum and the elution pattern, we assumed that the product detected was β -apo-13-carotenone. To prove identity, the product formed was purified and analyzed using LC-MS. As shown in Fig. 3B, the OsCCD8b product exhibited the expected $[M+H]^+$ molecular ion of 259.27 and a MS² spectrum identical to the authentic reference compound (Fig. 3A). This suggests that OsCCD8b cleaves β -apo-10'-carotenal (C₂₇) at the C13-C14-double bond yielding the ketone β -apo-13-carotenone (C₁₈). This activity must also lead to the formation of a C₉-dialdehyde (Fig. 4), which was not detected with the extraction and separation protocol used.

Apocarotenals are frequently reduced to alcohols or oxidized to form acids *in vivo*. To investigate the impact of these variants on the cleavage activity of OsCCD8b, we produced the corresponding alcohol using NaBH₄ and acid with an aldehyde dehydrogenase from *Neurospora crassa* [45]. The HPLC analyses showed that the incubation of β -apo-10'-carotenol resulted in the formation of β -apo-13-carotenone (C₁₈) as above (Fig. 2B), while β -apo-10'-carotenoic acid was not converted (Supplemental Fig. 2C).

In contrast to β -apo-10'-carotenal/ol, there was no conversion with any of the other apocarotenals differing in chain lengths (Supplemental Fig. 3), i.e., β -apo-12'- (C₂₅), β -apo-8'-carotenal (C₃₀) and *rosafuene-dialdehyde* (C₁₄). Other C₂₇-compounds with different end-groups, the acyclic apo-10'-lycopenal and the ring-hydroxylated, (3R)-3-OH- β -apo-10'-carotenal, were not cleaved either (Supplemental Fig. 2). To further explore the substrate specificity, we applied several C₄₀-carotenoids, including lycopene, β -carotene, zeaxanthin, lutein, and neoxanthin *in vitro*. However, no cleavage

activity was detected in the subsequent HPLC analyses (Supplemental Fig. 4). It has been reported that the expression of AtCCD7 in β -carotene-accumulating *E. coli* cells led to the formation of β -ionone [23]. This implies the formation of β -apo-10'-carotenal and/or rosafluene-dialdehyde as further products. In consideration of the orthology of AtCCD7 and OsCCD7, and accounting for the possibility that the latter may also produce rosafluene-diladehyde, we incubated OsCCD8b with this compound. However, we did not detect any significant conversion (Supplemental Fig. 3C).

***In vitro* and *in vivo* assays with recombinant AtCCD8 and PsCCD8**

The rice enzyme OsCCD8b shows about 74% similarity to the dicot enzymes AtCCD8 (MAX4) and PsCCD8 (RMS1). To compare the substrate specificities of AtCCD8 and PsCCD8 with the rice enzyme, the corresponding cDNAs were cloned into the pBAD/Thio expression-system. Based on the experience with OsCCD8b, the two cDNAs were co-expressed with the chaperones groES-groEL in *BL21(DE3) E. coli* cells. *In vitro* assays were then performed with the synthetic substrates depicted in Fig. 1 using the supernatants of expressing cells. Subsequent HPLC analyses (Fig. 2) revealed a clear-cut conversion of β -apo-10'-carotenal and β -apo-10'-carotenol into β -apo-13-carotenone (C_{18}). The identity of the produced ketone was verified using LC-MS analyses (as above; not shown). In contrast, none of the other synthetic substrates was cleaved, as determined by HPLC (Supplemental Fig. 2, 3). Similarly, we did not detect any cleavage activity with rosafluene-diladehyde (Supplemental Fig. 3), β -apo-10'-carotenoic acid (Supplemental Fig. 2) and with the C_{40} -carotenoids lycopene, β -carotene, zeaxanthin, lutein and neoxanthin (Supplemental Fig. 4). In line with these *in vitro* results, the expression of the two cDNAs, embedded in the corresponding pThio-plasmids, in *E. coli* cells accumulating lycopene, β -carotene and zeaxanthin did not result in any HPLC-detectable products, albeit a reduction in the carotenoid contents occurred (Supplemental Fig. 1, see Discussion).

***In vitro* assays with purified OsCCD8**

The *in vitro* results presented in this study were all obtained with supernatants of CCD8s-expressing *E. coli* cells. To confirm these results, we performed *in vitro* assays using purified thioredoxin-OsCCD8 fusion (Supplemental Fig. 5). The substrate specificity was identical, however, the overall activity was significantly lower (data not shown).

DISCUSSION

Tillering is an important agronomic trait for grain production in rice and other monocot crops; however, the molecular factors that control this branching process have just begun to emerge. Recent mutant analyses of high-tillering rice mutants suggest the involvement of the two carotenoid oxygenases OsCCD7 and OsCCD8b in determining the number of tillers, by inhibiting the outgrowth

of axillary buds [42, 43]. This indicated the presence of a novel, unidentified signalling molecule, which, by orthology and analogy with the situation in *Arabidopsis*, may be a carotenoid derivative. In *A. thaliana*, lateral branching is supposed to be under the control of an apocarotenoid derivative acting in concert with auxin. This derivative is initially synthesized by the successive actions of the carotenoid oxygenases AtCCD7 (MAX3) and AtCCD8 (MAX4) [21, 36], further modified by MAX1 [48], a class III cytochrome P450, and perceived by MAX2 [49], a F-box leucine-rich repeat protein. The carotenoid origin of this signal molecule was deduced from the enzymatic activities of AtCCD7 and AtCCD8. However, conflicting data have been reported on the corresponding substrate specificities. It was shown that AtCCD7 exhibited wide substrate specificity in carotenoid-accumulating *E. coli* cells, converting the linear C₄₀ carotenoid ζ -carotene and the cyclic β -carotene and potentially also lycopene, δ -carotene and zeaxanthin [23]. Regional specificity was determined at the C9'-C10' double bond; no information could be given whether cleavage also occurred at the C9-C10 position. This resulted in the formation of the respective C₁₃-compounds and implied the formation of a C₂₇-aldehyde and/or a C₁₄-dialdehyde (rostafluene-dialdehyde). Another report confirmed lycopene and β -carotene cleavage but excluded zeaxanthin as a substrate. However, the second cleavage product was identified as a C₂₇-apocarotenoid, and hence asymmetric cleavage was proposed, confirmed by *in vitro* assays showing the conversion of β -carotene into β -apo-10'-carotenal (C₂₇) [36]. The second enzyme involved, AtCCD8, was not investigated *in vitro* so far. However, it showed wide substrate specificity in *E. coli* as well [37], where β -carotene-, lycopene- and zeaxanthin-accumulating cells reduced their carotenoid content upon its expression. This contradicts results showing that this enzyme did not produce apocarotenoids when expressed alone in carotenoid-accumulating *E. coli* cells. Only upon co-expression with AtCCD7, the C₁₈ ketone β -apo-13-carotenone was detected as a new compound together with the AtCCD7 products. By sequence homology and the similarities of the phenotypes obtained, it was assumed that PsCCD7/PsCCD8 (RMS5/RMS1) from pea and OsCCD7/OsCCD8b from rice are functionally equivalent to AtCCD7/AtCCD8 from *Arabidopsis*.

Given the conflicting data, it is unclear whether firstly, AtCCD8 can cleave carotenoids, and secondly whether it can convert apocarotenoids other than β -apo-10'-carotenal (C₂₇). The cleavage of several substrates could result in many different products, and thus may imply the occurrence of a family of branching inhibitors in *A. thaliana*, instead of only one. Third, the question arises whether the CCD8 family members PsCCD8, OsCCD8b and AtCCD8 catalyze the identical reaction to produce one precursor of the branching inhibitor that is identical across plant species. To clarify these points, we have undertaken the outlined approach with CCD8 enzymes, considering plants, for which respective mutants have been characterized, i.e., rice as a monocot and the dicots pea and *Arabidopsis*.

To address these questions, we first expressed the three cDNAs in carotenoid-accumulating *E. coli* cells. However, we could not detect any cleavage product, albeit decreases in the substrate contents were observed. Our results are consistent with a former *in vivo* study on AtCCD8 in which no

cleavage products were detected in decolorized *E. coli* cultures [37]. To explain these contradictory data, we checked the cleavage of several carotenoids, including the ones accumulated in the *E. coli* system, *in vitro*, using enzyme preparations readily active on β -apo-10'-carotenal/ol. As shown in Supplemental data, Fig. 4, we could neither observe significant reductions in the substrate levels, nor detect any cleavage product. Hence, we concluded that the decreased carotenoid contents in the *E. coli* cells are the result of interference with carotenoid biosynthesis through pleiotropic effects caused by the overexpression. In fact, we occasionally observe bleaching of such cells due to the formation of inclusion bodies even when the expressed oxygenase gene is inactivated by mutation.

OsCCD8b was insoluble in *E. coli*; however, the problem could be solved satisfactorily by expressing a thioredoxin-fusion together with the groES-groEL chaperone system. All carotenoids tested were not converted, pointing to apocarotenoids as the authentic substrates. The use of synthetic apocarotenoids showed that this was in fact the case. However, in marked contrast to other apocarotenoid-cleaving oxygenases investigated so far (see below), the enzyme showed a very narrow specificity, being active exclusively with β -apo-10'-carotenal/ol. Only these substrates were converted into the C₁₈-compound β -apo-13-carotenone. The elucidation of the substrate specificity of AtCCD8 and PsCCD8 revealed the identical selectivity *in vitro*. Consequently, we did not detect any conversion of the C₁₄-compound rosafluene-dialdehyde, which may arise through the cleavage of carotenoids at the C9-C10 and C9'-C10' double bonds, catalyzed by AtCCD7 [23].

The high selectivity reported here seems unique. Several carotenoid oxygenases, such as CCD1 from *A. thaliana* [14, 44] and the enzymes CarX [10] and CarT [13] from *Fusarium*, were shown to convert C₄₀-carotenoids and apocarotenoids. In contrast, the cyanobacterial enzymes SynACO [16] and NosACO [17] cleaved only apocarotenoids, but of varying chain lengths and functional groups. However, these inhomogenous apocarotenoid substrates were all targeted at the same site, the C15-C15' double bond, to produce the C₂₀-compounds retinal and its derivatives. The structure of SynACO provides some clues on the parameters determining substrate specificities and, in case of relaxed specificities, on the maintenance of high regional specificity of cleavage [50]. SynACO contains a hydrophobic substrate binding tunnel leading to the Fe²⁺-4-His arrangement in the reaction center. The geometry of this tunnel accounts for the wide substrate specificity where the β -ionone ring is arrested at the entrance while its depth allows to accommodate C₂₅- up to C₃₅-apocarotenoids. The cleavage site is then determined by the constant distance between the β -ionone ring and the reaction center, leading to cleavage at the C15'-C15 double bond in all cases. The high substrate and regional specificity of the CCD8 family members investigated here may be the result of a similar structure in which the distance between the β -ionone ring and the reaction center governs the C13-C14 cleavage site. However, it can be speculated that the substrate tunnels of these enzymes are less deep and therefore restrictive with respect to chain length. In addition, the geometry of the tunnel-entrance could exclude hydroxylated β -ionone rings by an unknown mechanism.

The absence of a more branching phenotype in the *Arabidopsis* mutant *lut2*, which lacks α -carotene and its derivatives [51], suggests that the branching inhibitor is synthesized either from acyclic or from β -cyclic carotenoids. Similarly, the normal branching of mutants impaired in violaxanthin and neoxanthin synthesis [52] indicates that these epoxy-carotenoids play no role as carotenoid precursors. Hence, it can be concluded that the branching inhibitor is derived from β -zeacarotene, γ -carotene, β -carotene or β -cryptoxanthin, since the cleavage of these carotenoids by CCD7 at the C9'-C10' double bond results in the formation of the CCD8 substrate β -apo-10'-carotenal (Fig. 4). The high substrate specificity of the CCD8 enzymes implies an equivalently high product specificity of the CCD7 family members. In support of this, it was shown that the *max3-Arabidopsis* phenotype, caused by a lesion in *AtCCD7*, can be rescued by introducing the *OsCCD7* cDNA under the control of the constitutive 35S promoter [42]. It is therefore surprising to note the wide substrate specificity of *AtCCD7* and its ability to cleave several carotenoids *in vivo* [23, 36]. This may indicate that the biological function of CCD7s is not restricted to the formation of the branching inhibitor.

Taken together, the concordance of the reactions mediated by the CCD8s investigated here suggests an ancient pathway for the synthesis of the branching inhibitor, which arose prior to the monocot-dicot divide. Moreover, it can be assumed that β -apo-13-carotenone represents the common apocarotenoid precursor of the branching inhibitor. Adding on the evolutionary perspectives, β -apo-13-carotenone is supposed to be the precursor of the well-known fungal pheromone trisporic acid [53]. During the preparation of this manuscript, a report appeared describing a novel function of β -apo-13-carotenone, designated as d'orenone, in blocking the growth of root hairs by interfering with PIN2-mediated auxin transport [54], thus supporting the biological relevance of our findings.

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FIGURE LEGENDS

Figure 1 Structure of the apocarotenoid substrates used for *in vitro* assays with OsCCD8b, AtCCD8 and PsCCD8

I: rosafluene-dialdehyde (C₁₄), II: β -apo-12'-carotenal (C₂₅), III: β -apo-10'-carotenal (C₂₇), IV: β -apo-10'-carotenol (C₂₇), V: β -apo-10'-carotenoic acid (C₂₇), VI: (3R)-3-OH- β -apo-10'-carotenal (C₂₇), VII: apo-10'-lycopenal (C₂₇) and VIII: β -apo-8'-carotenal (C₃₀). The three enzymes converted only compound III and its corresponding alcohol (IV).

Figure 2 HPLC analysis of *in vitro* incubations with β -apo-10'-carotenol /ol

A: The incubations (2 h) of β -apo-10'-carotenal (2) with the enzymes OsCCD8b, PsCCD8 and AtCCD8 led to the formation of product (1) which resembled β -apo-13-carotenone in its UV/Vis spectrum and elution characteristics. **B:** The substrate β -apo-10'-carotenol (3) yielded the same product (1). The UV/Vis spectra of the substrates and the product are shown in the insets. For structures, see Figs. 1 and 4.

Figure 3 LC-MS analysis of the authentic β -apo-13-carotenone standard (A) and the purified OsCCD8b product (B)

The purified product of OsCCD8b (compound 1, Fig. 1) showed the expected [M+H]⁺ molecular ion of 259.27. The MS² spectrum was identical with the authentic reference (A) including the 217, 201, 185, 175, 159, 119 and 85 [M+H]⁺ fragments. These data suggest the OsCCD8b-product to be β -apo-13-carotenone; its structure is given in A.

Figure 4 Proposed pathway for the synthesis of the plant branching inhibitor

The first step, catalyzed by CCD7s, must lead to β -apo-10'-carotenal, the CCD8-substrate determined in this work. In the second, very specific step, the CCD8s (OsCCD8b, AtCCD8 and PsCCD8) mediate the cleavage of only β -apo-10'-carotenal/ol at the C13-C14 double bond leading to the C₁₈-ketone β -apo-13-carotenone and a C₉-product. Further modifications of one of the two products, likely β -apo-13-carotenone, then lead to the branching inhibitor(s) active in monocots and dicots.

Figure 1

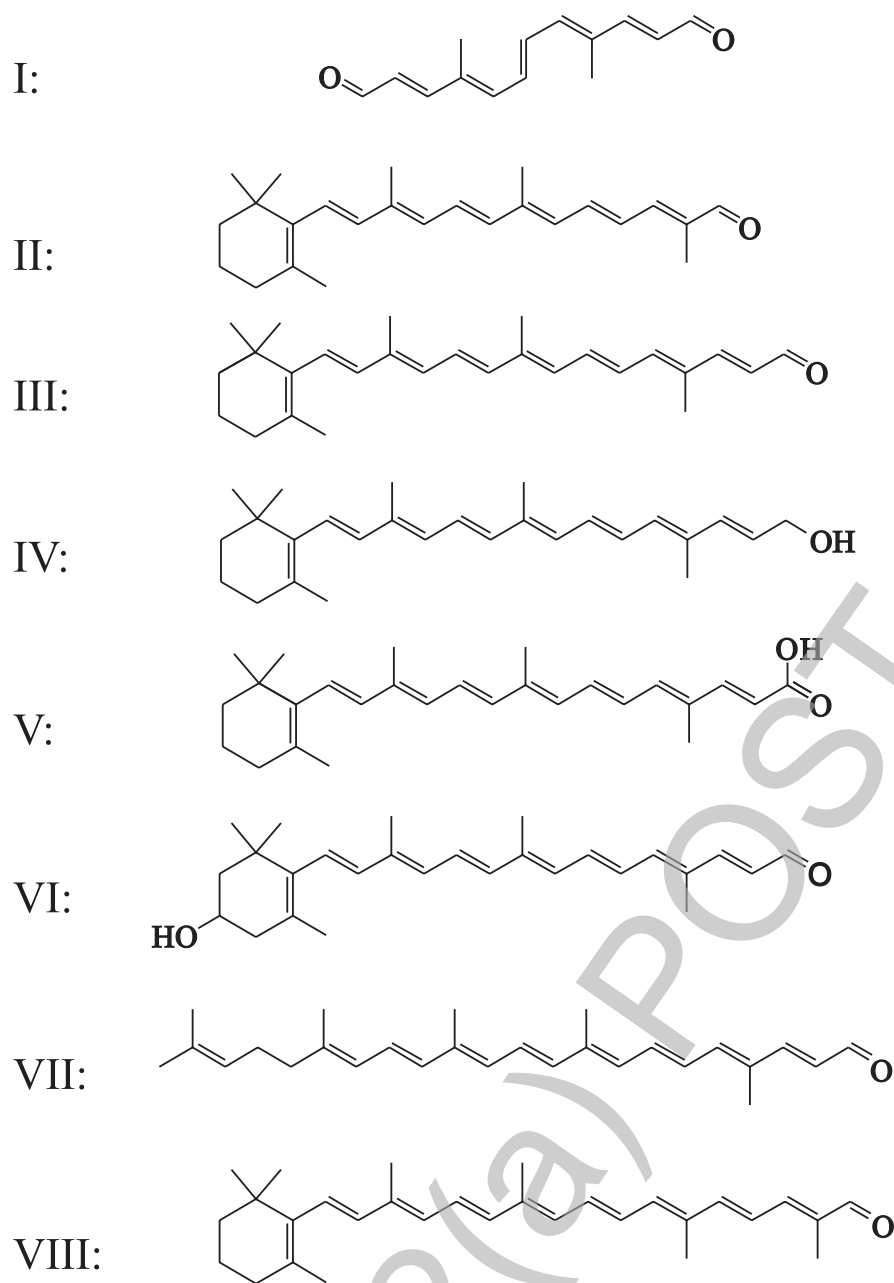


Figure 2

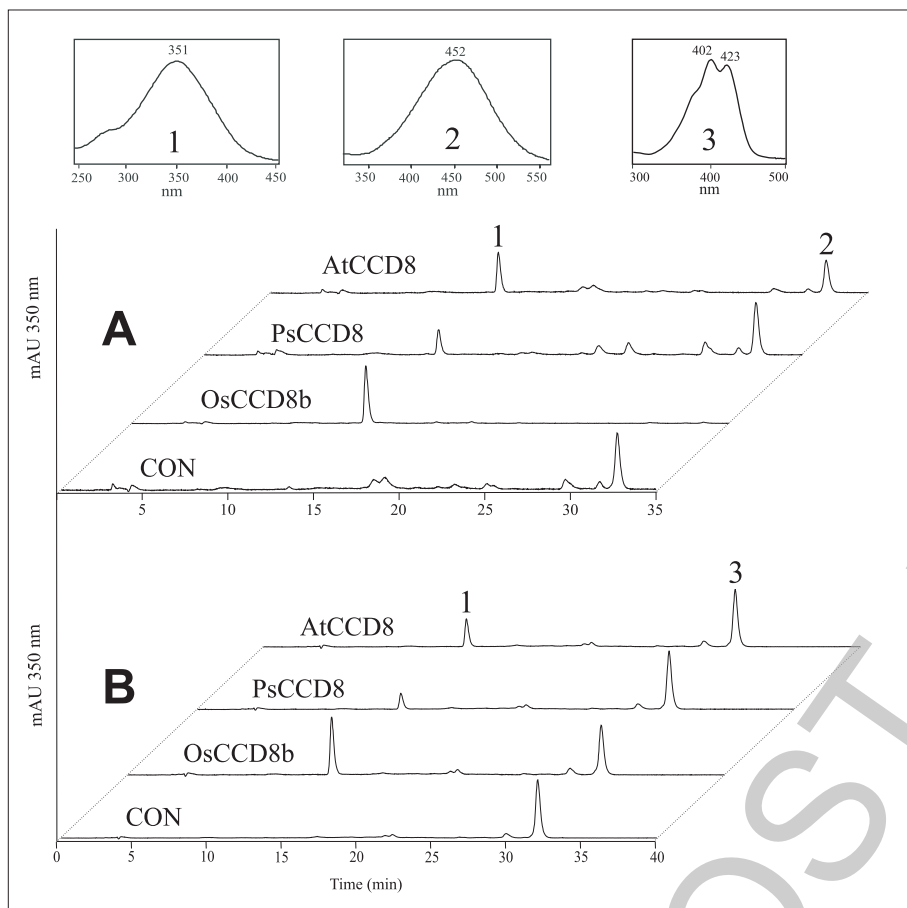


Figure 3

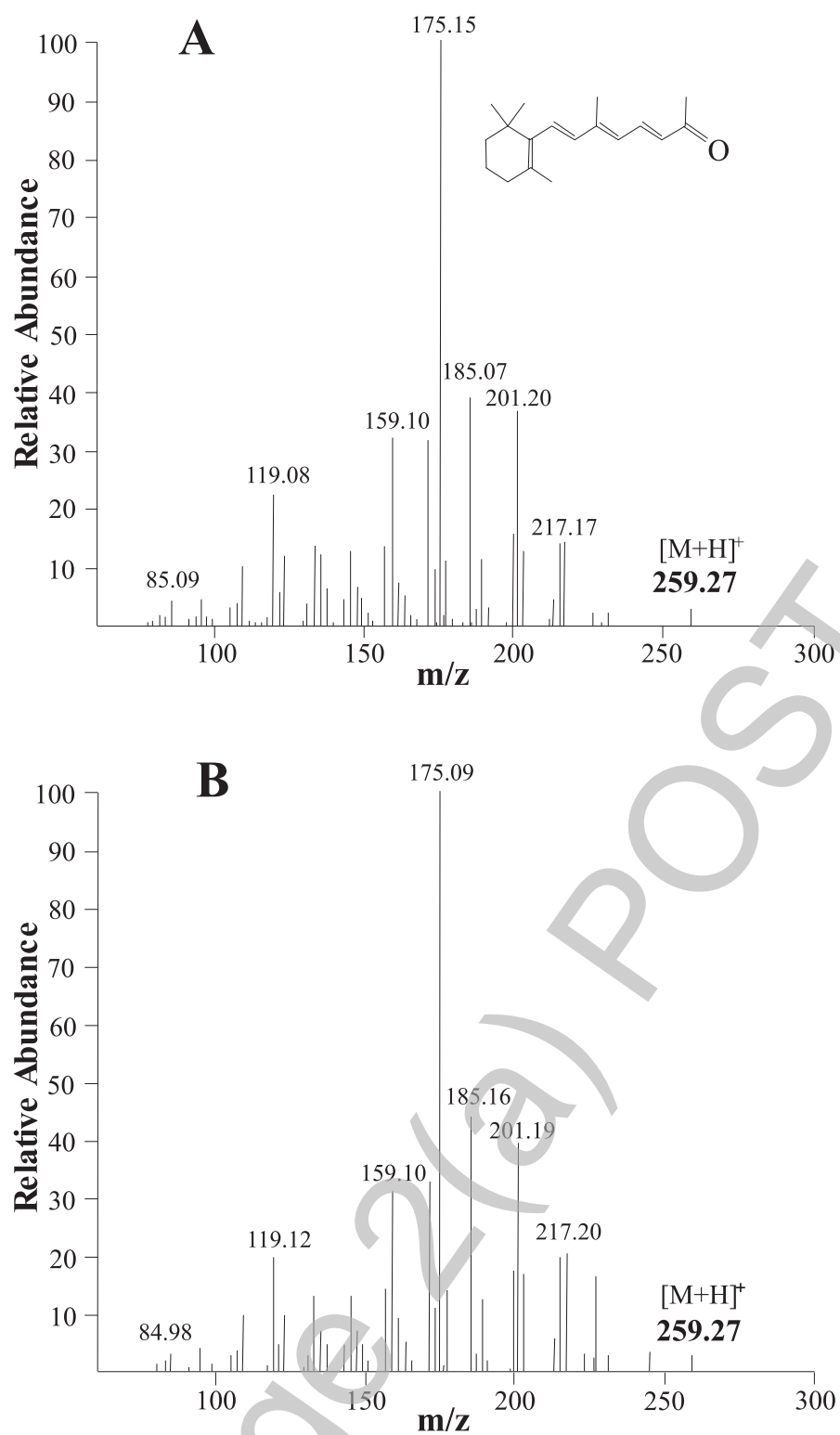


Figure 4

