Planta (2010) 232:691–699
DOI 10.1007/s00425-010-1205-y

ORIGINAL ARTICLE

Overexpression of the *rice carotenoid cleavage dioxygenase 1* gene in Golden Rice endosperm suggests apocarotenoids as substrates *in planta*

Andrea Ilg · Qiuju Yu · Patrick Schaub · Peter Beyer · Salim Al-Babili

Received: 22 March 2010 / Accepted: 28 May 2010 / Published online: 13 June 2010

Abstract Carotenoids are converted by carotenoid cleavage dioxygenases that catalyze oxidative cleavage reactions leading to apocarotenoids. However, apocarotenoids can also be further truncated by some members of this enzyme family. The plant carotenoid cleavage dioxygenase 1 (CCD1) subfamily is known to degrade both carotenoids and apocarotenoids in vitro, leading to different volatile compounds. In this study, we investigated the impact of the rice CCD1 (OsCCD1) on the pigmentation of Golden Rice 2 (GR2), a genetically modified rice variety accumulating carotenoids in the endosperm. For this purpose, the corresponding cDNA was introduced into the rice genome under the control of an endosperm-specific promoter in sense and anti-sense orientations. Despite high expression levels of OsCCD1 in sense plants, pigment analysis revealed carotenoid levels and patterns comparable to those of GR2, pleading against carotenoids as substrates in rice endosperm. In support, similar carotenoid contents were determined in anti-sense plants. To check whether OsCCD1 overexpressed in GR2 endosperm is active, in vitro assays were performed with apocarotenoid substrates. HPLC analysis confirmed the cleavage activity of introduced OsCCD1. Our data indicate that apocarotenoids rather than carotenoids are the substrates of OsCCD1 *in planta*.

Electronic supplementary material The online version of this article (doi:10.1007/s00425-010-1205-y) contains supplementary material, which is available to authorized users.

A. Ilg · Q. Yu · P. Schaub · P. Beyer · S. Al-Babili (✉)
Faculty of Biology, Institute of Biology II,
Albert-Ludwigs University of Freiburg, Schaenzlestr. 1,
79104 Freiburg, Germany
e-mail: salim.albabili@biologie.uni-freiburg.de

Keywords Apocarotenoids · Carotenoids · Carotenoid cleavage dioxygenase · Golden Rice · Endosperm

Abbreviations

CCD1	Carotenoid cleavage dioxygenase 1
GR	Golden Rice
Gt1	Glutelin 1 promoter

Introduction

Carotenoids are lipophilic pigments synthesized by all photosynthetic organisms as well as by some fungi and heterotrophic bacteria. In plants, carotenoids exert a vital role in protecting the photosynthetic apparatus from photooxidation and represent essential constituents of the light harvesting and reaction center complexes. In addition, carotenoids confer their color to many flowers and fruits, contributing substantially to plant–animal communication (Cunningham and Gantt 1998; Hirschberg 2001; Fraser and Bramley 2004; DellaPenna and Pogson 2006). Besides these functions, carotenoids serve as precursors of physiologically important compounds termed apocarotenoids synthesized through oxidative cleavage (Moise et al. 2005; Bouvier et al. 2005; Auldridge et al. 2006a). Representative examples of apocarotenoids are the ubiquitous chromophore retinal, the chordate morphogen retinoic acid and the phytohormone abscisic acid (ABA). A further example is represented by the signalling molecules strigolactones responsible for attracting both symbiotic arbuscular mycorrhizal fungi and parasitic plants (Akiyama 2007; Bouwmeester et al. 2007). Strigolactones were also

recently shown to exert further functions as novel plant hormones regulating shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008). Roots infected with arbuscular mycorrhizal fungi accumulate cyclohexenone and mycorradicin derivatives (Schliemann et al. 2008), with the latter conferring a yellow pigmentation to infected roots (Walter et al. 2000). Other examples for apocarotenoids mediating pigmentation are bixin in *Bixa orellana* (Bouvier et al. 2003a) and saffron in *Crocus sativus* (Bouvier et al. 2003b). Some apocarotenoids, e.g., β-ionone, are released as volatiles supposed to contribute to plant–animal communication (Bouvier et al. 2005).

Animals lack the ability to synthesize carotenoids de novo. To satisfy their demands of retinoids, they rely on dietary vitamin A (retinol) or its precursors, e.g., β-carotene. Deprivation of these compounds in the human diet leads to vitamin A deficiency (VAD), which represents a severe health problem worldwide, particularly in developing countries (Mayer et al. 2008). VAD can result in permanent blindness and also impair the immune system, thereby exacerbating infectious diseases and leading to mortality during early childhood (Mayer et al. 2008). Therefore, the improvement of provitamin A (β-carotene) content of rice (Ye et al. 2000; Paine et al. 2005; Al-Babili et al. 2006), tomatoes (Romer et al. 2000; Rosati et al. 2000; Fraser et al. 2002; Dharmapuri et al. 2002), canola (Shewmaker et al. 1999; Ravanello et al. 2003), maize (Aluru et al. 2008), flaxseed (Fujisawa et al. 2008) and potatoes (Diretto et al. 2007; Ducreux et al. 2005) has been an important goal of plant biotechnology. In the case of rice, these efforts led to the generation of "Golden Rice 2" (GR2) that accumulates up to 37 μg/g carotenoids in the endosperm with β-carotene accounting for about 84% of these pigments (Paine et al. 2005; for review see Al-Babili and Beyer 2005).

Carotenoid cleavage dioxygenases constitute a widespread enzyme family mediating the cleavage of double bonds in the substrate backbone, thereby catalyzing the initial step in apocarotenoids formation. In plants, these enzymes are classified into **n**ine-***c*is*-**e**poxycarotenoid **c**leavage **d**ioxygenases (NCEDs) that cleave 9-*cis*-violaxanthin or 9′-*cis*-neoxanthin, leading to the ABA-precursor xanthoxin, and **c**arotenoid **c**leavage **d**ioxygenases (CCDs) that convert different carotenoids. There are four known subfamilies of plant CCD enzymes designated as CCD1, CCD4, CCD7 and CCD8. CCD4 enzymes from different species were shown to cleave β-carotene at the C9′–C10′position leading to β-ionone (C_{13}) (Rubio et al. 2008; Huang et al. 2009). Similar activities were reported for the Arabidopsis CCD7 enzyme (Booker et al. 2004; Schwartz et al. 2004) mediating the first cleavage step in the synthesis of strigolactones. The other Arabidopsis CCD enzyme (AtCCD8) acting on the same pathway was shown to convert the CCD7 product β-apo-10′-carotenal (C_{27}), which is formed besides β-ionone (C_{13}) by AtCCD7, into the β-apo-13-carotenone (C_{18}), upon coexpression of both enzymes in β-carotene accumulating *E. coli* cells (Schwartz et al. 2004). An in vitro study on CCD8 enzymes from Arabidopsis, rice and pea revealed a high specificity for β-apo-10′-carotenal(ol) and confirmed the formation of β-apo-13-carotenone (Alder et al. 2008). In contrast to CCD8 enzymes, CCD1 enzymes obtained through heterologous expression in *E. coli* exhibit a wide substrate specificity in converting various all-*trans* carotenes, e.g., β-carotene, zeaxanthin and lycopene (Giuliano et al. 2003; Bouvier et al. 2005; for review see Auldridge et al. 2006a), besides different apocarotenals, e.g., β-apo-8′-carotenal (Schmidt et al. 2006; Vogel et al. 2008), β-apo-10′-carotenal and apolycopenals (Ilg et al. 2009). In addition to this wide substrate specificity, CCD1 enzymes act on multiple sites in the backbone of monocyclic and acyclic carotenoids. The first CCD1 enzyme investigated (AtCCD1) was shown to mediate a double cleavage of carotenoids at the C9–C10 and C9′–C10′ double bonds, leading to two C_{13}-ketones, e.g., β-ionone, and one C_{14}-dialdehyde (Schwartz et al. 2001). Based on in *E. coli* expressed enzymes, this double cleavage was confirmed for CCD1s from other plant species including tomato (Simkin et al. 2004a; Vogel et al. 2008), maize (Sun et al. 2008; Vogel et al. 2008), strawberry (García-Limones et al. 2008), lemon (Kato et al. 2006), petunia (Simkin et al. 2004b), *Vitis vinifera* (Mathieu et al. 2005), melon (Ibdah et al. 2006) and rice (Ilg et al. 2009). Revisiting of CCD1 activities from *A. thaliana*, maize and tomato revealed a novel cleavage site in lycopene, the C5–C6 and/or C5′–C6′ position, leading to the flavor of volatile 6-methyl-5 hepten-2-one (MHO; Vogel et al. 2008). Recently, we have shown that heterologously expressed rice CCD1 (OsCCD1) can target an additional cleavage site, the C7–C8 double bond of apolycopenals and of monocyclic and acyclic carotenoids, yielding geranial (Ilg et al. 2009), a flavor released by tomatoes (Lewinsohn et al. 2005) and roses (Gang 2005).

While heterologously expressed CCD1s have been investigated extensively, there are only few reports on the activities of these enzymes *in planta*. Moreover, these reports pursued knockdown approaches, focusing on the impact of the release of C_{13}-volatiles in flowers (Simkin et al. 2004b) and fruits (Simkin et al. 2004a), or on apocarotenoid accumulation in mycorrhiza (Floss et al. 2008; Sun et al. 2008). In this study, we aimed at the elucidation of the impact of OsCCD1 activity on carotenoids accumulated in GR2 endosperm. For this purpose, the corresponding cDNA was introduced under the control of the endosperm-specific glutelin promoter 1 (Gt1). The absence of significant decrease in carotenoid levels of transgenic

tissues indicated that carotenoids were not the substrates *in planta*. This conclusion was supported by in vitro assays showing that the overexpressed enzyme was active on apocarotenoids and by similar pigmentation of endosperm tissues transformed with an anti-sense construct.

Materials and methods

Construction of pCA-RNCE and pCA-RNCE-as

The cDNA of *OsCCD1* was excised as an *Eco*RV/*Hin*dIII fragment from pCR-OsCCD1 (Ilg et al. 2009) and ligated into *Sma*I/*Hin*dIII- and alkaline phosphatase-treated pCA-Gt1, a pCAMBIA1305 derivative harboring the endosperm-specific glutelin1 (Gt1) promoter, to yield pCA-RNCE. To generate pCA-RNCE-as, the *OsCCD1* cDNA was excised as an *Pst*I/*Bam*HI fragment from pCR-OsCCD1 and ligated into accordingly digested and alkaline phosphatase-treated pCA-Gt1.

Rice transformation

Immature GR2 seeds (*Oryza sativa* var. japonica cv. Kaybonnet; obtained from the International Rice Research Institute, The Philippines) were collected at the milky stage (10–12 days after flowering), sterilized with 70% ethanol for 30–60 s and washed twice with sterile ddH_2O, followed by shaking for 70 min in 12% sodium hypochloride (w/v) solution containing 0.1% (v/v) Tween 20. Seeds were then washed six times with sterile ddH_2O, and embryos were isolated and cultivated for 8 days at 28°C in the dark on CIM medium (pH 5.8) containing 4.4 g L^{-1} MS basal salts, 2 mg L^{-1} 2,4-D, 1 mL L^{-1} Gamborg B5 vitamins (1,000×; Duchefa, Haarlem, The Netherlands), 0.5 g L^{-1} L-glutamine, 0.5 g L^{-1}L-proline, 0.3 g L^{-1}casein hydrosylate, 30 g L^{-1} sucrose and 3 g L^{-1} phytagel. After removing of starch and roots, calli were dried for 10–15 min on a filter paper and used immediately for transformation.

Agrobacterium tumefaciens (LBA 4404) transformed with pCA-RNCE or pCA-RNCE-as was prepared by inoculation of a single colony into 5 mL of YEB medium (pH 7.5), supplemented with kanamycin (50 μg mL^{-1}), rifampicin (25 μg mL^{-1}) and streptomycin (50 μg mL^{-1}), consisting of 5 g L^{-1} bacto-peptone, 1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g L^{-1} sucrose and 493 mg $L^{-1}MgSO_4$. After shaking at 28°C overnight, 50 μL of the culture was inoculated into 50 mL of YEB medium supplemented with the three antibiotics and grown at 28°C to an OD_{600} of approximately 0.6–1.0. Cells were harvested by centrifuging (10 min, 3,220*g*), resuspended in 50 mL PIM2 medium (Aldemita and Hodges 1996) containing 200 μM acetosyringone and cultured for 2–3 h at 28°C.

Agrobacterium suspension was transferred to a 50 mL tube, and the dried calli were added. After vacuum infiltration for 10 min, the mixture was incubated for 20 min at room temperature with occasional shaking. Calli were then transferred to a filter paper and dried for 10–15 min, transferred onto a filter placed on CC medium (pH 5.6) containing CC salts (Potrykus et al. 1979), 2 mg L^{-1} 2,4-D, 1 mL L^{-1} Gamborg B5 vitamins (1,000×; Duchefa), 0.05 g L^{-1} casein hydrosylate, 10 g L^{-1} glucose, 20 g L^{-1} sucrose, 36.4 g L^{-1} mannitol and 3 g L^{-1} phytagel, and supplemented with acetosyringone (200 μM). After 60 h incubation at 28°C in the dark, calli were transferred to CIM plates and incubated at 28°C in the dark and in the presence of antibiotics, as follows: 3 days on CIM plates containing 500 mg L^{-1} cefotaxime, 30 days on CIM plates containing 125 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime, 20 days on CIM plates containing 95 mg L^{-1} hygromycin and 200 mg L^{-1} cefotaxime, followed by 14 days on CIM plates containing 80 mg L^{-1} hygromycin and 100 mg L^{-1} cefotaxime. After repetition of the last step, calli were transferred to CIM medium supplemented with 30 g L^{-1}, sorbitol 0.5 mg L^{-1}, IAA, 1 mg L^{-1} zeatin, 25 mg L^{-1} hygromycin and containing 5.5 g L^{-1} (instead of 3 g L^{-1}) phytagel. After incubation for 20–30 days at 28°C in the light, plantlets were transferred to ROT medium (CIM with the following modifications: 2.2 g L^{-1} MS basal salts, 0.5 g L^{-1} casein hydrosylate, 15 g L^{-1} sucrose and 2.4 g L^{-1} phytagel) and grown for approximately 2 weeks at 28°C under 16-h/8-h light–dark cycle. Finally, plantlets were transferred to Yoshida solution (Yoshida et al. 1976), grown for 2 weeks at 28°C under 16-h/8-h light–dark cycle and moved to the greenhouse.

Southern-blot analysis

Genomic DNA was extracted from frozen leaves according to Soni and Murray (1994). As much as 12 μg of genomic DNA was digested with *Nco*I, allowing the determination of the orientation of introduced *OsCCD1* cDNA. Digested DNA was electrophoretically fractionated through a 0.8% (w/v) agarose gel before capillary transfer and immobilization on nylon membranes (Hybond N^+, Biosciences Europe, Freiburg, Germany). PCR amplified, digoxygenin-labeled (Roche Applied Science, Mannheim, Germany) fragment of *OsCCD1* (primers 5′ CTCTACTGGCTCTCCGGCAACT 3′ and 5′ TGCAGTATTAGCTGTCCCATGTCC 3′) was used as probe. Blotting, hybridization, washing and detection were performed following the procedure of Wünn et al. (1996).

Carotenoid analysis

Dehusked seeds harvested ca. 30 days after flowering were polished for 1 min using a commercial grain polisher (Kett,

Tokyo, Japan), and carotenoids were extracted and quantified spectrophotometrically according to Hoa et al. (2003).

For HPLC analyses, the extracts were dried and resuspended in 40 µL of chloroform, of which 10 µL was injected into the HPLC system as described in Hoa et al. (2003) with the following modifications: using a flow rate of 1 mL min^{-1}, the column was developed with a linear gradient from 57% A to 100% A within 10 min, then the conditions were maintained for 9 min. Subsequently, the flow rate was increased to 2 mL min^{-1} within 1 min, maintaining these final conditions for 3 min. Carotenoid peaks were integrated at their individual λ_{max}, and quantification was performed according to Hoa et al. (2003).

In vitro assays

In vitro assays were performed with milky-stage endosperm of four freshly harvested seeds in a final volume of 400 µL containing the substrate at a concentration of 40 µM in the incubation buffer described by Ilg et al. (2009). The apocarotenoid substrates were solubilized using octyl-β-glucoside at a final concentration of 1% (v/v). For this purpose, substrates were mixed with 100 µL of a 4% octyl-β-glucoside ethanolic solution, dried using a vacuum centrifuge and resuspended in 200 µL of water. Milky-stage endosperm of four freshly harvested seeds was added to 200 µL of 2× incubation buffer, homogenized and mixed with the solubilized substrate. Assays were incubated for 6 h at 28°C in the dark under gentle shaking. Extraction and HPLC analyses were performed according to Ilg et al. (2009).

Real-time RT-PCR assay

RNA from rice endosperm was isolated using the plant RNA purification reagent (Invitrogen, Paisley, UK) according to the manufacturer's protocol. RNA purification and on-column DNaseI digestion were performed using the Qiagen RNeasy mini kit (Quiagen, Hilden, Germany). First-strand cDNA synthesis was performed using the TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany), according to the manufacturer's protocol. Real-time RT-PCR assays were carried out with an ABIPrism 7000 (Applied Biosystems) using 18S rRNA levels for normalization (Human 18S rRNA PDAR; Applied Biosystems). For the detection of OsCCD1 transcript levels, SYBR Green (Eurogentech, Cologne, Germany) was used. Primers were designed using Primer Express software (Applied Biosystems; R-CCD.F: 5′ GAC GAAATGTTCGCTTTTGGA 3′; R-CCD.R: 5′ TGGTAA TGACCCGGTATGTACAGT 3′). The relative quantity of the transcripts was calculated using the comparative threshold cycle method (Livak 1997). Data were normalized first to the corresponding 18S rRNA levels and then expressed as relative to GR2 transcript levels.

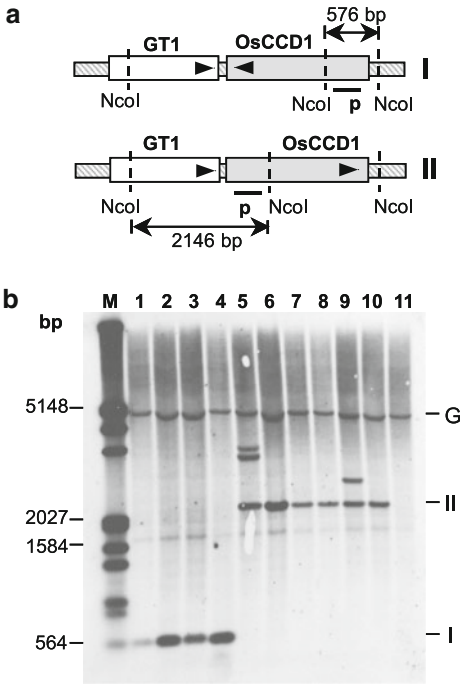

Fig. 1 Southern-blot analyses. **a** Scheme of the transformed expression cassettes. The DIG-labeled probe (p) was localized at the 5′-end of the *OsCCD1*-cDNA (shown in *gray*), allowing the detection of a 576-bp for the anti-sense (I, pCA-RNCE-as) and a 2146-bp *Nco*I-fragment for the sense construct (II, pCA-RNCE). Gt1, glutelin1 promoter. **b** Examples of Southern-blot analysis of transformed T1 anti-sense and sense plants. *M* marker, *lines 1–4* plants transformed with pCA-RNCE-as (anti-sense), *lines 5–10* plants transformed with pCA-RNCE (sense), *line 11* GR2. Hybridization with the DIG-labeled probe led to the detection of the expected anti-sense (I) and sense (II) fragments, respectively, which were absent in GR2. The band G corresponds to the wild-type copy of *OsCCD1*

Results

Generation of GR2 plants transformed with OsCCD1 cDNA

To transform GR2 plants, the two binary vectors pCA-RNCE-as (Fig. 1a, I) and pCA-RNCE (Fig. 1a, II) were constructed, containing the OsCCD1 cDNA under the control of the Gt1-promoter in anti-sense and sense orientation, respectively. The embryos of an experimental GR2 event in a cv. "Kaybonnet" background were isolated and transformed using *Agrobacterium tumefaciens*. Transgenicity of regenerated plants was confirmed by Southern-blot analyses, the examples of which are shown in Fig. 1b.

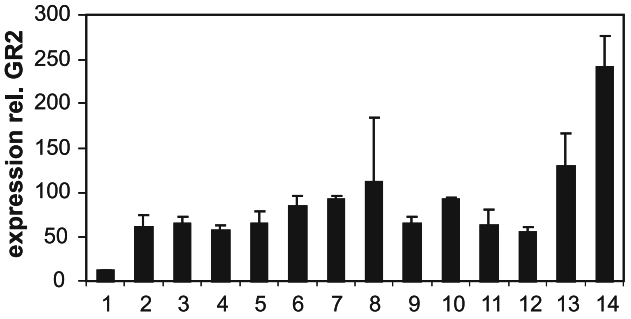

Fig. 2 Real-time RT-PCR analysis of *OsCCD1* mRNA levels in milky-stage endosperm. *OsCCD1*-mRNA levels in T2 milky-stage endosperm of different sense lines were determined and calculated relative to the *OsCCD1*-expression level of GR2. The depicted values represent the average of the levels of two siblings per line (±SE)

Eight anti-sense and 14 sense plants obtained from independent calli were selected for further investigation.

The introduced OsCCD1 is highly expressed in sense plants

To determine the expression levels of overexpressed OsCCD1, T1 seeds from sense plants were grown under hygromycin selection. Total RNA was then isolated from milky-stage T2 seeds, i.e., 10–12 days after flowering, and used for real-time RT-PCR analysis. The results obtained are shown in Fig. 2. Compared to the control (GR2), the OsCCD1 transcript levels showed a marked increase in all lines investigated, ranging from about 50-fold in line 2 to about 240-fold in line 14. Analysis of anti-sense plants revealed high levels of transcripts (Online Resource 1) corresponding to anti-sense mRNA that cannot be distinguished from sense transcripts by real-time RT-PCR.

Endosperms of sense and anti-sense plants exhibit similar variable carotenoid contents

To determine the impact of the transformation on carotenoid levels, endosperm of homozygous T2 seeds from sense and anti-sense plants were harvested 30 days after flowering, and carotenoids accumulated were extracted and quantified photometrically. Contrary to our expectation, overexpression of OsCCD1 did not lead to a pronounced decrease of carotenoid content. As shown in Fig. 3, the carotenoid levels in the endosperm of sense plants (black bars) exhibited a relatively high variability commonly found with this greenhouse-grown cultivar and were in a range of 11–27 μg g^{-1}, with the majority of the lines containing approximately 15–21 μg g^{-1}, a content similar to that of GR2 (hatched bar). These data indicated that endosperm carotenoid levels were not affected by OsCCD1 transcript levels. Consistently, endosperm samples of anti-sense plants (gray bars) did not contain higher carotenoid levels. Moreover, one of the anti-sense plants (line 15) even contained significantly reduced carotenoid level, compared to the GR2 control.

To shed light on a possible impact of the transformation on individual carotenoids, HPLC analysis was performed with extracts of homozygous T2 seeds from sense and anti-sense plants, and amounts of different carotenoids were calculated and compared with those of GR2. As shown in Table 1, the values determined were variable and did not allow the conclusion that overexpression of OsCCD1 leads to a decrease in a specific carotenoid. With the exception of lutein that showed slightly lower values in the sense and higher in the anti-sense plants, the contents of individual carotenoids did not correlate with the orientation of the introduced OsCCD1-cDNA.

Taken together, the transformation with *OsCCD1* in both, sense and anti-sense orientations, resulted in plants accumulating variable carotenoid amounts with an average comparable to the content of the GR2 control.

OsCCD1 overexpressed in endosperm is active in vitro

Since increased transcript levels do not necessarily result in functional enzymes, we checked the carotenoid cleavage activity of endosperm samples containing overexpressed OsCCD1 in vitro. For this purpose, incubations with

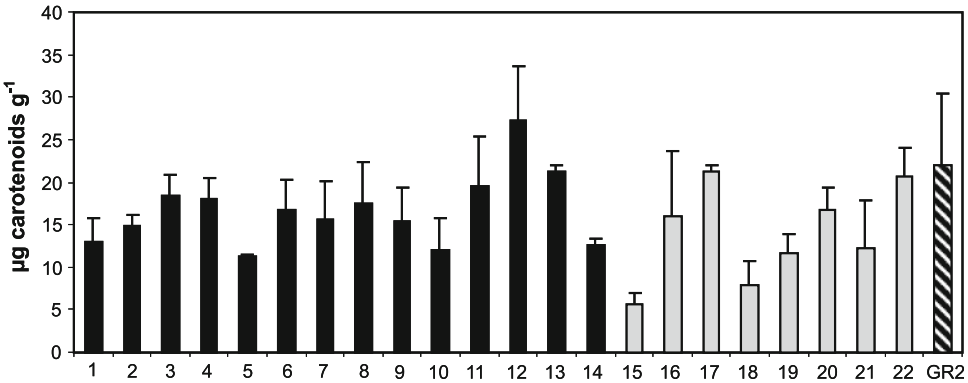

Fig. 3 Carotenoid content in T2 seeds of sense and anti-sense plants. The carotenoid levels in seeds of sense plants (*black*) and anti-sense plants (*gray*) were determined and compared to those of GR2 (*hatched*). Depicted values of sense and anti-sense plants represent the average of two biological replicas (±SE). The value for GR2 corresponds to the average of five replicas (±SD)

Table 1 Carotenoid composition in T2 seeds (30 days after flowering) of sense and anti-sense plants

Carotenoid	AS	S	GR2
Lutein	1.57 ± 0.63	0.93 ± 0.43	1.21 ± 0.23
Cryptoxanthin	3.88 ± 1.67	3.11 ± 0.89	2.06 ± 0.65
α-Carotene	10.57 ± 1.95	11.86 ± 3.86	16.89 ± 1.72
β-Carotene	70.04 ± 6.56	66.27 ± 10.77	63.96 ± 4.65
Phytoene	13.95 ± 6.23	17.84 ± 11.64	15.89 ± 3.70

Percentage of total carotenoids

The carotenoid compositions of seeds of anti-sense (AS) and sense (S) plants were compared to that of GR2. Carotenoids were quantified by HPLC analysis, according to Hoa et al. (2003). Depicted values represent the average of 8 anti-sense, 14 sense lines and 4 independent GR2 plants (±SD)

β-carotene, zeaxanthin, β-apo-10′-carotenal and apo-10′-lycopenal were performed. These two apocarotenoids (for structure see Fig. 4) were previously shown to be suitable substrates for OsCCD1 heterologously expressed in *E. coli* (Ilg et al. 2009). The assays were extracted and analyzed by HPLC. Incubation with β-carotene did not lead to detectable products. Similarly, only very weak activity was observed with zeaxanthin (data not shown). Much higher activities were detected with apocarotenoids. Incubation with β-apo-10′-carotenal (C_{27}, substrate I; Fig. 4b) led to the formation of β-ionone (C_{13}, product 3; Fig. 4) and the corresponding C_{14}-dialdehyde apo-10,10′-carotene-dial (product 2; Fig. 4). The nature of the two products was verified by comparison to authentic standards (data not shown), suggesting the cleavage of the C9–C10 double bond. In addition to these C_{13}- and C_{14}-compounds, two products were detected (products 1 and 4; Fig. 4), the nature of which could not be determined. Besides being cleaved, β-apo-10′-carotenal was also reduced to the corresponding alcohol, as indicated by peak E (Fig. 4), identical to β-apo-10′-carotenol in its UV–vis spectrum. However, the retention time of peak E was much longer than that of β-apo-10′-carotenol, pointing to esterification. The reduction of the aldehyde is corroborated by the presence of peak E in both GR2 and the OsCCD1 sense samples, in contrast to products 1–4 (Fig. 4) that were restricted to the latter. The incubation with apo-10′-lycopenal (C_{30}) led to four products (Fig. 4c) identical to those produced by heterologously expressed enzyme (Ilg et al. 2009), which were used as references. Products 2 and 5 (Fig. 4) were identified as apo-10,10′-carotene-dial (C_{14}) and pseudoionone (C_{13}), which were produced on cleavage of the C9–C10 double bond (A; Fig. 4d). Product 7 corresponded to the C_{19}-dialdehyde apo-6,10′-carotendial arising through the cleavage of the C5–C6 double bond (C; Fig. 4d), which also led to the volatile 6-methyl-5-hepten-2-one (C_8; MHO). Product 6 was identified as apo-8,10′-carotendial

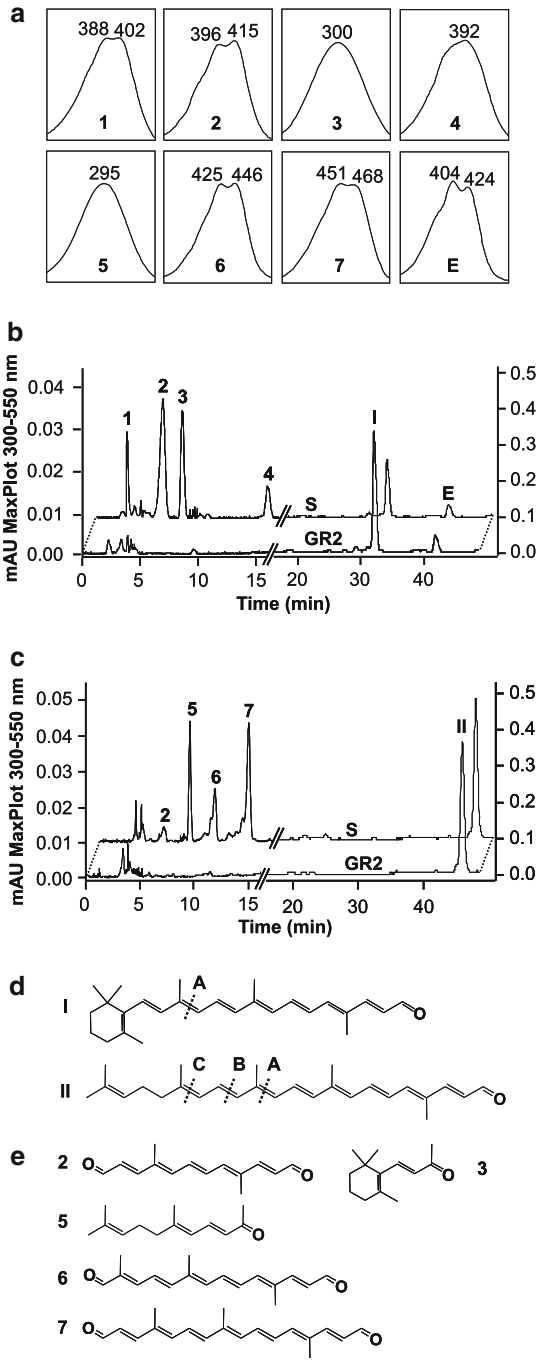

Fig. 4 HPLC analyses of incubations of apocarotenoids with milky-stage endosperm. Endosperm overexpressing *OsCCD1* (*line 5*, Fig. 2) was incubated with β-apo-10′-carotenal (I) and apo-10′-lycopenal (II). Incubation with I led to four products *1* unidentified compound, *2* apo-10,10′-carotendial (C_{14}), *3* β-ionone, and *4* unidentified compound. Incubation with II led to four products *2* apo-10,10′-carotendial (C_{14}), *5* pseudoionone, *6* apo-8,10′-carotendial (C_{17}), *7* apo-6,10′-carotendial (C_{19}) and *6* pseudoionone. **a** UV/vis-spectra of the detected compounds. **b** Chromatogram of the incubation with β-apo-10′-carotenal (I). **c** Chromatogram of the incubation with β-apo-10′-lycopenal (II). **d** Structures of the substrates with the deduced cleavage sites *A*, C9–C10; *B*, C7–C8; *C*, C5–C6. **e** Structures of the detected cleavage products. The peak E in **b** corresponds to esterified β-apo-10′-carotenol

(C_{17}), formed from geranial (C_{10}) through the cleavage of the C7–C8 double bond. As with β-apo-10′-carotenal, none of the cleavage products was detected in the control assay (GR2), suggesting their formation by overexpressed OsCCD1.

Discussion

Enzymatic studies with heterologously expressed plant CCD1 enzymes suggested their capability in cleaving β-carotene and other C_{40}-carotenoids, both in vitro and in carotenoid-accumulating *E. coli* cells, pointing to an involvement in carotenoid turnover *in planta*. In support of such a role, it was shown that loss of function of the *Arabidopsis CCD1* results in an increase of seed carotenoid content, but no other phenotype (Auldridge et al. 2006b). Consistent with this finding, it was reported that the maize *White cap* (*Wc*) mutant exhibits a dose-dependent carotenoid deficiency in kernels, due to enhanced CCD1 expression in the endosperm (Timmermans et al. 2004). Thus, these results indicate that downregulation of CCD1 activity may be a tool to enhance the carotenoid content in seeds of cereals, analogous to CCD4a in *Chrysantemum* petals (Ohmiya et al. 2006). To test this possibility and to investigate the carotenoid cleavage activity *in planta*, *OsCCD1* cDNA was expressed in GR2 endosperm in both sense and anti-sense orientations. Based on the observations with maize *White cap* (*Wc*) mutant, we expected pronounced changes in carotenoid content, especially on OsCCD1 overexpression. However, both sense and anti-sense transformations led to carotenoid contents that do not correlate with the orientation of the introduced cDNA or, in case of sense transformants, with the OsCCD1 expression levels. The absence of significant decrease in the latter transformants cannot be explained by expressed, but non-active, CCD1 enzyme. This conclusion was drawn from the cleavage of apocarotenoids by overexpressing endosperm samples in vitro, which was not detectable with the GR2 control. Our data indicate that apocarotenoids rather than carotenoids are the substrates of OsCCD1 *in planta*. This result can be explained by the absence of a transit peptide in CCD1 enzymes (Bouvier et al. 2005; Floss and Walter 2009), which is necessary to provide access to plastid-localized carotenoid substrates. The cytosolic localization of CCD1 enzymes distinguishes them from other plant carotenoid cleavage dioxygenases, e.g., CCD4 or CCD7, which occur together with their substrates, the carotenoids, in plastids.

The involvement of CCD1 enzymes in the formation of carotenoid-derived C_{13}-volatiles in plants was confirmed by knockdown experiments in tomato fruits and petunia flowers, leading to decreased emission of β-ionone (Simkin et al. 2004a, b). In addition, recent studies on the CCD1 from maize suggested its involvement in the formation of cyclohexenone and mycorradicin in mycorrhizal roots (Sun et al. 2008). New insights into the nature of the substrates cleaved by CCD1 in vivo were provided by analysis of mycorrhizal hairy roots of *Medicago truncatula*. Using RNA interference (RNAi), it was shown that repression of *CCD1* gene in this tissue leads to reduction of mycorrhizal apocarotenoids and accumulation of C_{27}-apocarotenoids, pointing to apo-10′-carotenal as the substrate *in planta* (Floss et al. 2008) and revealing CCD1 as an apocarotenoid oxygenase similar to the cyanobacterial enzymes SynACO (previously designated as Diox1; Ruch et al. 2005) and NosACO (Scherzinger et al. 2006). Therefore, it is supposed that MtCCD1 acts as a second cleavage enzyme converting C_{27}-apocarotenals released from plastids after formation by other carotenoid cleavage dioxygenases, i.e., CCD7 or CCD4, which utilize C_{40} carotenoids (for review, see Floss and Walter 2009). Supporting the scenario of sequential cleavage reactions as route for formation of mycorrhizal apocarotenoids, it was recently shown that downregulation of CCD7 in tomato decreases strigolactones content as well as the levels of cyclohexenone and mycorradicin (Vogel et al. 2010). The necessity of an initial cleavage enzyme, e.g., CCD4 or CCD7, for CCD1 activity in mycorrhiza is in agreement with our results showing that overexpression of OsCCD1 alone does not lead to degradation of carotenoids in the GR2 endosperm. However, in contrast to mycorrhizal hairy roots of *Medicago truncatula*, downregulation of CCD1 activity in GR2 endosperm did not lead to accumulation of apocarotenoids, as suggested by HPLC analysis (data not shown). Hence, it could be assumed that CCD4 or CCD7 activities are low or absent in GR2 endosperm. Accordingly, expression analyses of rice CCD4a (accession no AY838897), CCD4b (accession no. AY838898) and CCD7 (accession no. XP_473418) enzymes revealed low expression levels, compared to seedlings (data not shown). Low transcript level was also determined for OsCCD1 in the endosperm. These low expression levels are consistent with the absence of cleavage activities of rice endosperm when incubated with apocarotenoids or carotenoids in vitro. As shown in Fig. 4, the cleavage of β-apo-10′-carotenal and apo-10′-lycopenal was only detected with endosperm samples overexpressing OsCCD1. Similarly, incubation with C_{40} carotenoids, i.e., zeaxanthin, revealed weak activity that was observed only on overexpression of OsCCD1 (data not shown).

The CCD1 subfamily is widely represented in plants. Moreover, homologous enzymes occur even in cyanobacteria. The *Nostoc* sp. PCC 7120 enzyme NosCCD (previously designated as NSC1; Marasco et al. 2006) can be considered as an ortholog of plant CCD1s enzymes, since it

shows similar substrate specificities and cleavage pattern (Scherzinger and Al-Babili 2008). Due to its cytosolic localization and induction by high light, it was supposed that NosCCD was responsible for scavenging cytosolic apocarotenoids that may arise due to this stress condition (Scherzinger and Al-Babili 2008). It could be speculated that plant CCD1 fulfill a similar function, converting plastid-released apocarotenoids arising through destructive, non-enzymatic oxidative cleavage processes, into volatiles. Such a scenario might explain the wide substrate specificity and the multiple cleavage sites, which distinguish CCD1 enzymes from other plant carotenoid cleavage dioxygenases.

Acknowledgments This work was funded by The HarvestPlus research consortium (http://www.harvestplus.org) and by the Deutsche Forschungsgemeinschaft (DFG) Grant AL 892/1-4. We are indebted to Dr. Rhodora Aldemita for valuable discussions.

References

Akiyama K (2007) Chemical identification and functional analysis of apocarotenoids involved in the development of arbuscular mycorrhizal symbiosis. Biosci Biotechnol Biochem 71:1405–1414

Al-Babili S, Beyer P (2005) Golden Rice—five years on the road—five years to go? Trends Plant Sci 10:565–573

Al-Babili S, Hoa TT, Schaub P (2006) Exploring the potential of the bacterial carotene desaturase CrtI to increase the beta-carotene content in Golden Rice. J Exp Bot 57:1007–1014

Aldemita RR, Hodges TK (1996) *Agrobacterium tumefaciens*-mediated transformation of *japonica* and *indica* rice varieties. Planta 199:612–617

Alder A, Holdermann I, Beyer P, Al-Babili S (2008) Carotenoid oxygenases involved in plant branching catalyse a highly specific conserved apocarotenoid cleavage reaction. Biochem J 416:289–296

Aluru M, Xu Y, Guo R, Wang Z, Li S, White W, Wang K, Rodermel S (2008) Generation of transgenic maize with enhanced provitamin A content. J Exp Bot 59:3551–3562

Auldridge ME, McCarty DR, Klee HJ (2006a) Plant carotenoid cleavage oxygenases and their apocarotenoid products. Curr Opin Plant Biol 9:315–321

Auldridge ME, Block A, Vogel JT, Dabney-Smith C, Mila I, Bouzayen M, Magallanes-Lundback M, DellaPenna D, McCarty DR, Klee HJ (2006b) Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family. Plant J 45:982–993

Booker J, Auldridge M, Wills S, McCarty D, Klee H, Leyser O (2004) MAX3/CCD7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel signaling molecule. Curr Biol 14:1232–1238

Bouvier F, Dogbo O, Camara B (2003a) Biosynthesis of the food and cosmetic plant pigment bixin (annatto). Science 30:2089–2091

Bouvier F, Suire C, Mutterer J, Camara B (2003b) Oxidative remodeling of chromoplast carotenoids: identification of the carotenoid dioxygenase CsCCD and CsZCD genes involved in Crocus secondary metabolite biogenesis. Plant Cell 15:47–62

Bouvier F, Isner JC, Dogbo O, Camara B (2005) Oxidative tailoring of carotenoids: a prospect towards novel functions in plants. Trends Plant Sci 10:187–194

Bouwmeester HJ, Roux C, Lopez-Raez JA, Bécard G (2007) Rhizosphere communication of plants, parasitic plants and AM fungi. Trends Plant Sci 12:224–230

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 49:557–583

DellaPenna D, Pogson BJ (2006) Vitamin synthesis in plants: tocopherols and carotenoids. Annu Rev Plant Biol 57:711–738

Dharmapuri S, Rosati C, Pallara P, Aquilani R, Bouvier F, Camara B, Giuliano G (2002) Metabolic engineering of xanthophyll content in tomato fruits. FEBS Lett 519:30–34

Diretto G, Al-Babili S, Tavazza R, Papacchioli V, Beyer P, Giuliano G (2007) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. PLoS One 4 B(4):e350

Ducreux LJ, Morris WL, Hedley PE, Shepherd T, Davies HV, Millam S, Taylor MA (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of beta-carotene and lutein. J Exp Bot 56:81–89

Floss DS, Walter MH (2009) Role of carotenoid cleavage dioxygenase 1 (CCD1) in apocarotenoid biogenesis revisited. Plant Signal Behav 4:172–175

Floss DS, Schliemann W, Schmidt J, Strack D, Walter MH (2008) RNA interference-mediated repression of MtCCD1 in mycorrhizal roots of *Medicago truncatula* causes accumulation of C27 apocarotenoids, shedding light on the functional role of CCD1. Plant Physiol 148:1267–1282

Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res 43:228–265

Fraser PD, Romer S, Shipton CA, Mills PB, Kiano JW, Misawa N, Drake RG, Schuch W, Bramley PM (2002) Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. Proc Natl Acad Sci USA 99:1092–1097

Fujisawa M, Watanabe M, Choi SK, Teramoto M, Ohyama K, Misawa N (2008) Enrichment of carotenoids in flaxseed (*Linum usitatissimum*) by metabolic engineering with introduction of bacterial phytoene synthase gene *crtB*. J Biosci Bioeng 105:636–641

Gang DR (2005) Evolution of flavors and scents. Annu Rev Plant Biol 56:301–325

García-Limones C, Schnäbele K, Blanco-Portales R, Luz Bellido M, Caballero JL, Schwab W, Muñoz-Blanco J (2008) Functional characterization of FaCCD1: a carotenoid cleavage dioxygenase from strawberry involved in lutein degradation during fruit ripening. J Agric Food Chem 56:9277–9285

Giuliano G, Al-Babili S, von Lintig J (2003) Carotenoid oxygenases: cleave it or leave it. Trends Plant Sci 8:145–148

Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagès V, Dun EA, Pillot JP, Letisse F, Matusova R, Danoun S, Portais JC, Bouwmeester H, Bécard G, Beveridge CA, Rameau C, Rochange SF (2008) Strigolactone inhibition of shoot branching. Nature 455:189–194

Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol 4:210–218

Hoa TT, Al-Babili S, Schaub P, Potrykus I, Beyer P (2003) Golden Indica and Japonica rice lines amenable to deregulation. Plant Physiol 133:161–169

Huang FC, Molnár P, Schwab W (2009) Cloning and functional characterization of carotenoid cleavage dioxygenase 4 genes. J Exp Bot 60:3011–3022

Ibdah M, Azulay Y, Portnoy V, Wasserman B, Bar E, Meir A et al (2006) Functional characterization of CmCCD1, a carotenoid

cleavage dioxygenase from melon. Phytochemistry 67:1579–1589
Ilg A, Beyer P, Al-Babili S (2009) Characterization of the rice carotenoid cleavage dioxygenase 1 reveals a novel route for geranial biosynthesis. FEBS J 276:736–747
Kato M, Matsumoto H, Ikoma Y, Okuda H, Yano M (2006) The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. J Exp Bot 57:2153–2164
Lewinsohn E, Sitrit Y, Bar E, Azulay Y, Meir A, Zamir D, Tadmor Y (2005) Carotenoid pigmentation affects the volatile composition of tomato and watermelon fruits, as revealed by comparative genetic analyses. J Agric Food Chem 20:3142–3148
Livak KJ (1997) User Bulletin No. 2: ABI PRISM 7700 Sequence Detection System. PE Applied Biosystems, Foster City, CA, USA, pp 11–15
Marasco EK, Vay K, Schmidt-Dannert C (2006) Identification of carotenoid cleavage dioxygenases from Nostoc sp. PCC 7120 with different cleavage activities. J Biol Chem 281:31583–31593
Mathieu S, Terrier N, Procureur J, Bigey F, Gunata Z (2005) A carotenoid cleavage dioxygenase from *Vitis vinifera* L.: functional characterization and expression during grape berry development in relation to C13-norisoprenoid accumulation. J Exp Bot 56:2721–2731
Mayer JE, Pfeiffer WH, Beyer P (2008) Biofortified crops to alleviate micronutrient malnutrition. Curr Opin Plant Biol 11:166–170
Moise AR, von Lintig J, Palczewski K (2005) Related enzymes solve evolutionarily recurrent problems in the metabolism of carotenoids. Trends Plant Sci 10:178–186
Ohmiya A, Kishimoto S, Aida R, Yoshioka S, Sumitomo K (2006) Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in chrysanthemum petals. Plant Physiol 142:1193–1201
Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchliffe E, Adams JL, Silverstone AL, Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat Biotechnol 23:429–430
Potrykus I, Harms CT, Lörz H (1979) Callus formation from cell culture protoplasts of corn (*Zea mays* L.). Theor Appl Genet 54:209–214
Ravanello MP, Ke D, Alvarez J, Huang B, Shewmaker CK (2003) Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. Metab Eng 5:255–263
Romer S, Fraser PD, Kiano JW, Shipton CA, Misawa N, Schuch W, Bramley PM (2000) Elevation of the provitamin A content of transgenic tomato plants. Nat Biotechnol 18:666–669
Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. Plant J 24:413–419
Rubio A, Rambla JL, Santaella M, Gómez MD, Orzaez D, Granell A, Gómez-Gómez L (2008) Cytosolic and plastoglobule-targeted carotenoid dioxygenases from *Crocus sativus* are both involved in beta-ionone release. J Biol Chem 283:24816–24825
Ruch S, Beyer P, Ernst H, Al-Babili S (2005) Retinal biosynthesis in eubacteria: in vitro characterization of a novel carotenoid oxygenase from *Synechocystis* sp. PCC 6803. Mol Microbiol 55:1015–1024
Scherzinger D, Al-Babili S (2008) In vitro characterization of a carotenoid cleavage dioxygenase from Nostoc sp. PCC 7120 reveals a novel cleavage pattern, cytosolic localization and induction by highlight. Mol Microbiol 69:231–244
Scherzinger D, Ruch S, Kloer DP, Wilde A, Al-Babili S (2006) Retinal is formed from apo-carotenoids in *Nostoc* sp. PCC7120: in vitro characterization of an apo-carotenoid oxygenase. Biochem J 398:361–369
Schliemann W, Ammer C, Strack D (2008) Metabolite profiling of mycorrhizal roots of *Medicago truncatula*. Phytochemistry 69:112–146
Schmidt H, Kurtzer R, Eisenreich W, Schwab W (2006) The carotenase AtCCD1 from *Arabidopsis thaliana* is a dioxygenase. J Biol Chem 281:9845–9851
Schwartz SH, Qin X, Zeevaart JAD (2001) Characterization of a novel carotenoid cleavage dioxygenase from plants. J Biol Chem 276:25208–25211
Schwartz SH, Qin X, Loewen MC (2004) The biochemical characterization of two carotenoid cleavage enzymes from *Arabidopsis* indicates that a carotenoid-derived compound inhibits lateral branching. J Biol Chem 279:46940–46945
Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DY (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J 20:401–412
Simkin AJ, Schwartz SH, Auldridge M, Taylor MG, Klee HJ (2004a) The tomato *carotenoid cleavage dioxygenase 1* genes contribute to the formation of the flavor volatiles β-ionone, pseudoionone, and geranylaceton. Plant J 40:882–892
Simkin AJ, Underwood BA, Auldridge M, Loucas HM, Shibuya K, Schmelz E et al (2004b) Circadian regulation of the PhCCD1 carotenoid cleavage dioxygenase controls emission of beta-ionone, a fragrance volatile of petunia flowers. Plant Physiol 136:3504–3514
Soni R, Murray JA (1994) Isolation of intact DNA and RNA from plant tissues. Anal Biochem 218:474–476
Sun Z, Hans J, Walter MH, Matusova R, Beekwilder J, Verstappen FW, Ming Z, van Echtelt E, Strack D, Bisseling T, Bouwmeester HJ (2008) Cloning and characterisation of a maize carotenoid cleavage dioxygenase (ZmCCD1) and its involvement in the biosynthesis of apocarotenoids with various roles in mutualistic and parasitic interactions. Planta 228:789–801
Timmermans MC, Brutnell TP, Becraft PW (2004) The 46th Annual Maize Genetics Conference. Unlocking the secrets of the maize genome. Plant Physiol 136:2633–2640
Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K, Kyozuka J, Yamaguchi S (2008) Inhibition of shoot branching by new terpenoid plant hormones. Nature 455:195–200
Vogel JT, Tan BC, McCarty DR, Klee HJ (2008) The carotenoid cleavage dioxygenase 1 enzyme has broad substrate specificity, cleaving multiple carotenoids at two different bond positions. J Biol Chem 283:11364–11373
Vogel JT, Walter MH, Giavalisco P, Lytovchenko A, Kohlen W, Charnikhova T, Simkin AJ, Goulet C, Strack D, Bouwmeester HJ, Fernie AR, Klee HJ (2010) SlCCD7 controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. Plant J 61:300–311
Walter MH, Fester T, Strack D (2000) Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the 'yellow pigment' and other apocarotenoids. Plant J 21:571–578
Wünn J, Klöti A, Burkhardt PK, Ghosh Biswas GC, Launis K, Iglesias VA, Potrykus I (1996) Transgenic indica rice breeding line IR58 expressing a synthetic *cryIA*(*b*) gene from *Bacillus thuringiensis* provides effective insect pest control. Biotechnology 14:171–176
Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303–305
Yoshida S, Forno DA, Cook JH, Gomez KA (1976) Routine procedures for growing rice plants in culture solution. In: Yoshida S, Forno DA, Cook JH, Gomez KA (eds) Laboratory manual for physiological studies of rice. International Rice Research Institute, Los Banos, Philippines, pp 61–66