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Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family

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Summary

Arabidopsis thaliana **has nine genes that constitute a family of putative carotenoid cleavage dioxygenases (*CCDs*). While five members of the family are believed to be involved in synthesis of the phytohormone abscisic acid, the functions of the other four enzymes are less clear. Recently two of the enzymes, CCD7/MAX3 and CCD8/MAX4, have been implicated in synthesis of a novel apocarotenoid hormone that controls lateral shoot growth. Here, we report on the molecular and genetic interactions between *CCD1*, *CCD7/MAX3* and *CCD8/MAX4*. CCD1 distinguishes itself from other reported CCDs as being the only member not targeted to the plastid. Unlike *ccd7/max3* and *ccd8/max4*, both characterized as having highly branched phenotypes, *ccd1* loss-of-function mutants are indistinguishable from wild-type plants. Thus, even though CCD1 has similar enzymatic activity to CCD7/MAX3, it does not have a role in synthesis of the lateral shoot growth inhibitor. Rather, it may have a role in synthesis of apocarotenoid flavor and aroma volatiles, especially in maturing seeds where loss of function leads to significantly higher carotenoid levels.**

Keywords: branching, hormones, seeds.

Introduction

Apocarotenoids are a class of terpenoid compounds generated by oxidative cleavage of carotenoids. They perform essential biological functions in plants, animals and photosynthetic bacteria. Examples of biologically active apocarotenoids include the phytohormone abscisic acid (ABA) and retinol (vitamin A), an essential component of the visual cycle in animals. Economically, apocarotenoids are valued as colorants and spices. Examples are bixin, an apocarotenoid used as a colorant in foods and cosmetics, and saffron, a spice extracted from the styles of crocus flowers (Winterhalter and Rouseff, 2002). The biosynthesis of apocarotenoids begins with cleavage of a carotenoid molecule at one of its double bonds. In plants, this cleavage can occur non-enzymatically but the predominant mechanism is enzymatically via the activity of a family of carotenoid cleavage dioxygenases (Booker *et al.*, 2004; Bouvier *et al.*, 2003; Iuchi *et al.*, 2001; Schwartz *et al.*, 1997, 2001; Sorefan *et al.*, 2003; Tan *et al.*, 1997, 2003).

The first gene encoding a carotenoid cleavage dioxygenase was isolated from the maize (*Zea mays*) ABA-deficient viviparous mutant, *vp14*. VP14 catalyzes the first step in ABA biosynthesis, the cleavage of either of two 9-*cis*-epoxycarotenoids, violoxanthin or neoxanthin at the 11,12 double bond. Because of its preferred substrate, VP14 is called a 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Schwartz *et al.*, 1997). In the Arabidopsis (*A. thaliana*) genome, nine putative carotenoid cleavage dioxygenases have been identified based on sequence homology to VP14 (Table 1). Four of the Arabidopsis dioxygenases (NCED2, NCED3, NCED6 and NCED9) have the same activity as VP14 and are designated

Table 1 Comparison of the carotenoid cleavage dioxygenases (CCD) and *NCED* gene structures and identities to VP14

Family member	Gene ID	Intron no.	% Identity to VP14
AtCCD1	*At3g63520*	13	37
AtNCED2	*At4g18350*	0	64
AtNCED3	*At3g14440*	0	67
AtCCD4	*At4g19170*	0	41
AtNCED5	*At1g30100*	0	66
AtNCED6	*At3g24220*	0	57
AtCCD7	*At2g44990*	5	21
AtCCD8	*At4g32810*	5	26
AtNCED9	*At1g78390*	0	67

NCEDs. NCED5 has high homology to VP14, though its activity has not been determined (Iuchi *et al.*, 2001; Tan *et al.*, 2003). The remaining four proteins diverge from the NCEDs and have been given the generic designation carotenoid cleavage dioxygenases (CCDs). These include CCD1, CCD4, CCD7 and CCD8.

CCD1 (Schwartz *et al.*, 2001) and CCD7 (Booker *et al.*, 2004; Schwartz *et al.*, 2004) have previously been shown to have activity on multiple carotenoid substrates. They are specific in their site of cleavage, both having activity at the 9,10 double bond of their substrates. Although CCD1 cleaves symmetrically at both the 9,10 and 9′,10′ bonds, CCD7 has been reported to cleave only asymmetrically. Thus, CCD1 cleaves β-carotene to produce two C_{13} products (both β-ionone) and one central C_{14} dialdehyde (Schwartz *et al.*, 2001), while CCD7 produces one β-ionone and the C_{27} 10′-apo-β-carotenal (Schwartz *et al.*, 2004). AtCCD8 has been shown to further catabolize the C_{27} apocarotenoid derived from CCD7 cleavage of β-carotene (Schwartz *et al.*, 2004). This result suggests that CCD7 and CCD8 may act sequentially in a metabolic pathway. Consistent with this interpretation, loss-of-function mutants of either CCD7 or CCD8 have a developmentally altered phenotype. The *ccd7* and *ccd8* mutants are allelic to *max3* and *max4*, respectively. These mutants, named for more axillary growth, exhibit proliferation of both leaves and inflorescences emerging from typically dormant axillary buds (Booker *et al.*, 2004; Sorefan *et al.*, 2003). Thus, the available data are consistent with a model in which they act sequentially to produce an inhibitor of lateral shoot growth. Because the apocarotenoid generated by CCD7/MAX3 and CCD8/MAX4 action is graft transmissible (Booker *et al.*, 2004; Sorefan *et al.*, 2003), it probably represents a novel class of apocarotenoid phytohormone.

Results

Phenotypes of loss-of-function CCD *mutants*

In order to address the relative roles of CCD1, CCD7 and CCD8 in the growth and development of Arabidopsis plants, we identified loss-of-function mutations in each gene (Figure 1). For CCD7/MAX3 we used either a previously described ethylmethane sulfonate (EMS)-induced allele, *max3-9* (Booker *et al.*, 2004), or a T-DNA knockout obtained from the Salk population, *max3-11*. This line has an insertion in the fifth intron of *CCD7* and effectively removes the sixth (and final) exon. Loss-of-function mutants for *CCD8* were isolated from the Wisconsin Knockout Facility (Krysan *et al.*, 1999) and the SAIL populations (Sessions *et al.*, 2002) and designated *max4-5* and *max4-6* to conform to the already established nomenclature of mutations at the *CCD8/MAX4* locus. The T-DNA insertions in the fifth and sixth exons are present in *max4-5* and *max4-6*, respectively. A single loss-of-function mutant for *CCD1* was obtained from the SAIL population of insertional mutants. This line contains a T-DNA within the sixth intron and was designated *ccd1-1*. Because only a single insertional mutant was available for *CCD1*, the level of *CCD1* transcript was validated by real-time TaqMan reverse transcriptase (RT)-PCR. The mutant had no detectable *CCD1* transcript.

Both *max4-5* and *max4-6* exhibited a significant decrease in petiole length and were highly branched. The axillary buds, which are typically delayed in growth in wild-type plants, grew out to produce leaves and inflorescences. The *max4-5* and *max4-6* plants had smaller rosette diameters

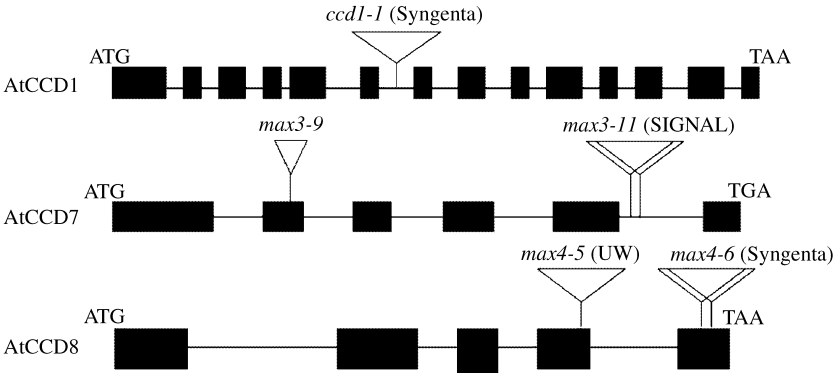

Figure 1. Carotenoid cleavage dioxygenases (*CCD*) mutant alleles.
Exons are represented by thick lines, introns by thin lines and insertions by inverted triangles. The start (ATG) and stop points for translation are indicated. Insertion events were identified from the Syngenta (SAIL), the Salk Institute (SIGNAL) and the University of Wisconsin (UW) T-DNA insertion collections. All of the insertion points were verified by sequencing of the junction fragments. The *max3-11* and *max4-6* insertions are tandem T-DNA repeats. The EMS-induced *max3-9* allele was previously reported in Booker *et al.* (2004).

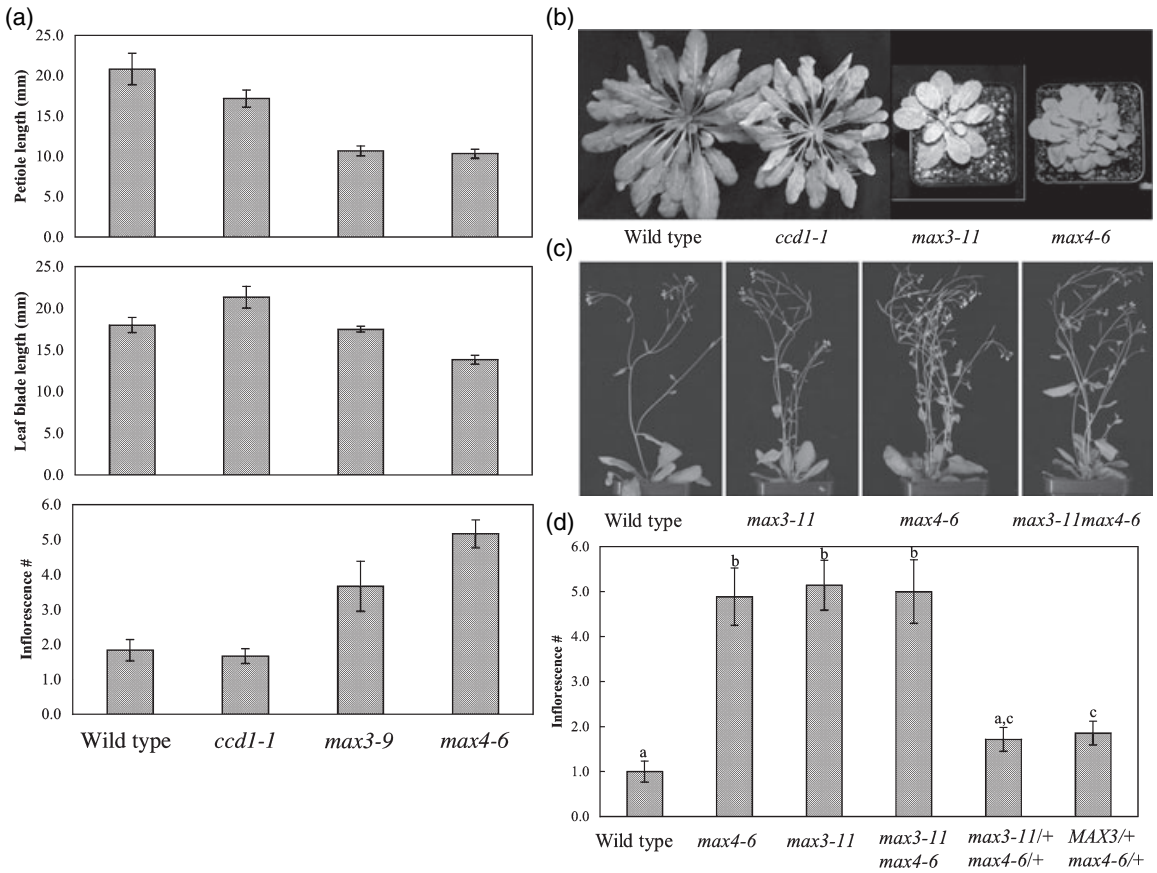

Figure 2. Carotenoid cleavage dioxygenases (*CCD*) mutant phenotypes.
(a) Petiole length, leaf blade length and inflorescence number of *ccd1-1*, *max3-9* and *max4-6* plants compared with wild type (±SE).
(b) Top view of CCD mutant and wild-type rosettes.
(c) Lateral shoot formation in the *max3-11 max4-6* double mutant compared with each single mutant and wild type.
(d) Inflorescence number of the *max3* and *max4* single mutants compared with the *max3 max4* double mutant and to the *CCD8/MAX4* heterozygotes.

due to a decrease in the lengths of petioles and leaf blades compared with wild-type plants. Together, the above phenotypes contributed to the bushy appearance of the *max4* mutants (Figure 2a,b). The branching phenotype was more pronounced when plants were grown on short days due to the nearly tenfold increase in inflorescence number in the mutant versus wild-type plants (data not shown). The branching phenotype is consistent with the previous observations of Sorefan *et al.* (2003). Introduction of a wild-type copy of the *CCD8*/*MAX4* cDNA under the control of the constitutive Figwort Mosaic Virus 35S promoter (Richins *et al.*, 1987) rescued the mutant phenotypes. The complementation establishes that the phenotypes are associated with the loss of *CCD8* function.

CCD7/MAX3 has been classified as a carotenoid cleavage dioxygenase based on its demonstrated ability to cleave multiple carotenoids including lycopene, β-carotene and zeaxanthin (Booker *et al.*, 2004). Similarly, CCD1 is a non-specific carotenoid cleavage dioxygenase with activity at the 9,10 double bond of multiple carotenoid substrates (Schwartz *et al.*, 2001). Despite the similarities in CCD1 and CCD7/MAX3 activity, the *ccd1* and *max3* plants do not exhibit similar phenotypes (Figure 2). No significant phenotypic alterations were observed for *ccd1-1* plants under the growth conditions used. The plants were indistinguishable from wild type in petiole and leaf blade length as well as in inflorescence number.

ccd1 *loss of function affects seed carotenoid content*

As CCD1 was shown *in vitro* to cleave several carotenoid molecules (Schwartz *et al.*, 2001), the consequence of the *ccd1* loss of function on seed carotenoid composition was determined. Leaf carotenoids were also measured but no significant differences were observed (data not shown). Carotenoids from dry seeds of wild type and mutant were extracted and quantified by HPLC (Table 2). In general, the major carotenoid of wild-type Arabidopsis seed is lutein, while antheraxanthin and zeaxanthin are present in levels significantly higher than in leaf tissue and accumulation of

Table 2 Wild type and *ccd1-1* seed carotenoid compositions

	Lutein	β-Carotene	Neoxanthin	Violaxanthin	Antheraxanthin	Zeaxanthin	β-xanthophylls	Total carotenoids
Wild type (Col)	29.9 ± 1.8 (79)	0.15 ± 0.01 (0.4)	0.9 ± 0.05 (2.4)	3.3 ± 0.24 (8.7)	2.0 ± 0.13 (5.4)	1.6 ± 0.1 (4.1)	8.0 ± 0.6 (21)	37.8 ± 2.3
ccd1-1	36.3 ± 1.2* (70)	0.28 ± 0.02** (0.5)	3.7 ± 0.05** (7.2)	7.6 ± 0.23** (14.5)	2.4 ± 0.12 (4.6)	1.8 ± 0.05 (3.4)	15.8 ± 0.5** (30)	52.1 ± 1.2*

The amount of carotenoid is expressed as nmol pigment g^{-1} seed. Each value is the mean of three experiments ±SD, with the relative molar percentage of each carotenoid given in parentheses. *ccd1-1* values marked with a single or double asterisk are significantly different from wild type (Student's *t*-test, $P < 0.05$ or $P < 0.01$, respectively).

β-carotene is extremely low. *ccd1-1* seed had a 37% increase in total carotenoids relative to wild type, due mainly to a greater than twofold and fourfold increase in violaxanthin and neoxanthin, respectively. This is reflected in a near doubling of the absolute levels of β-carotene-derived xanthophylls in *ccd1-1*. The lutein level in *ccd1-1* was increased to the greatest extent in absolute terms compared with other carotenoids. To validate that the increased carotenoid content was due to the loss of *CCD1* function, the *ccd1-1* mutant was complemented with a 35S-CCD1 cDNA construct. We identified three independent lines with transgene expression at or above the level of the endogenous gene. In each case, the transgene significantly reduced seed carotenoid content relative to the *ccd1-1* parent ($P < 0.01$; data not shown). These data indicate that CCD1 metabolizes carotenoids in seeds and its loss leads to higher seed carotenoid content.

Double-mutant analysis

The identical phenotypes of the *max3* and *max4* mutants, together with the *in vitro* CCD7/MAX3 and CCD8/MAX4 enzyme data (Schwartz *et al.*, 2004) support a model in which CCD7/MAX3 and CCD8/MAX4 act in a single pathway to synthesize an inhibitor of bud outgrowth in Arabidopsis. It is also possible that CCD7/MAX3 and CCD8/MAX4 act in independent pathways both of which contribute to the production of branch-inhibiting compounds. If the latter were true, then a double *max3 max4* mutant may be predicted to have an additive phenotype compared with either single mutant. Therefore, a cross between *max3-11* and *max4-6* was made. The *max3-11 max4-6* double mutant was phenotypically indistinguishable from either single mutant (Figure 2c,d) indicating an interaction consistent with both genes functioning in the same pathway. Interestingly, both classes of plants genotyped as heterozygous for *CCD8*/*MAX4* (*max3*/+, *max4*/+ and *MAX3*/*MAX3*, *max4*/+) showed a slight increase in inflorescence number compared with wild type ($P = 0.076$ and $P = 0.029$, respectively). This evidence of a quantitative dosage effect of *CCD8*/*MAX4* on inflorescence number suggests that CCD8/MAX4 activity is a limiting step in the pathway.

Hormone levels in the max4 *mutant*

Auxin originating from the apex of the plant promotes apical dominance (Ward and Leyser, 2004). We were therefore interested in determining whether auxin levels are altered in mutants lacking CCD8/MAX4 activity. Free IAA (indole-3-acetic acid) was measured in *max4-6* rosettes to determine if altered auxin content was the cause of the branching phenotype. Because of the relatedness of CCD8/MAX4 to the ABA biosynthetic proteins, ABA was also measured. While ABA has been implicated in bud inhibition (Chatfield *et al.*, 2000), none of the NCED loss-of-function mutants display a shoot branching phenotype (B.C. Tan and W.T. Deng, University of Florida, Gainesville, FL, USA, personal communication). Similar to *max3* plants (Booker *et al.*, 2004), no significant change in either hormone was seen in *max4-6*. Levels of IAA were 13.65 ± 2.04 ng g^{-1} fresh weight (FW) in wild type versus 13.12 ± 0.92 ng g^{-1} FW in *max4-6*. Levels of ABA were 5.69 ± 0.30 ng g^{-1} FW in wild type versus 6.60 ± 0.96 ng g^{-1} FW in *max4-6*. Therefore, the branching phenotype seen in the *max4* mutants is not a consequence of altered IAA or ABA levels within Arabidopsis.

CCD *expression*

The loss-of-function phenotypes are consistent with different roles for CCD1 and CCD7/MAX3 in growth and development, in contrast to the seemingly identical roles of CCD7/MAX3 and CCD8/MAX4. In order to further understand CCD function, expression of *CCD1* and *CCD8/MAX4* in wild-type Arabidopsis (ecotype Columbia) was determined. We previously showed highest expression of *CCD7/MAX3* in root tissue (Booker *et al.*, 2004). Northern blot analysis was not sensitive enough to detect the low levels of *CCD8*/*MAX4* mRNA. Therefore, transcript abundance was measured by quantitative real-time RT-PCR, using TaqMan primers and probes. Figure 3 shows transcript abundance as a percentage of mRNA calculated by comparison with a standard curve. *CCD1* was present in all tissue types tested, with its highest expression in flower and silique tissue. On average, *CCD1* expression was 50-fold higher than the previously reported *CCD7*/*MAX3* expression. *CCD8*/*MAX4* transcripts

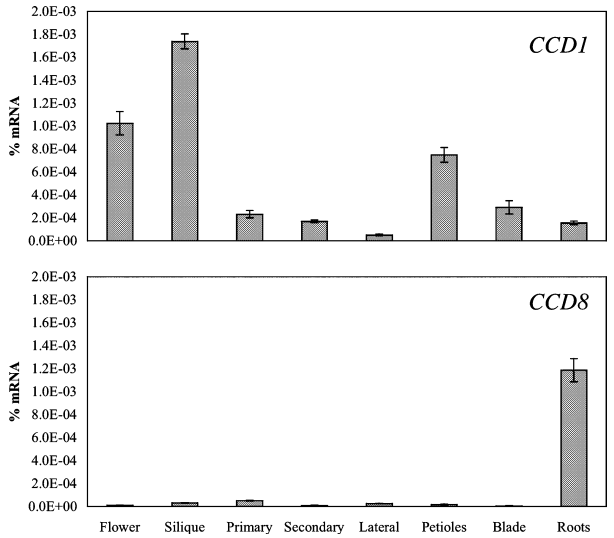

Figure 3. Expression patterns of CCD1 and CCD8.
Ribonucleic acid was extracted from petioles, leaf blades and roots before bolting. Primary, secondary and lateral stem tissue was used after the inflorescences and cauline leaves were removed. The flowers and siliques were collected from both primary and secondary inflorescences. The RNA was quantified by real-time RT-PCR (±SD). CCD1 and CCD8 expression is shown in the top and bottom panels, respectively.

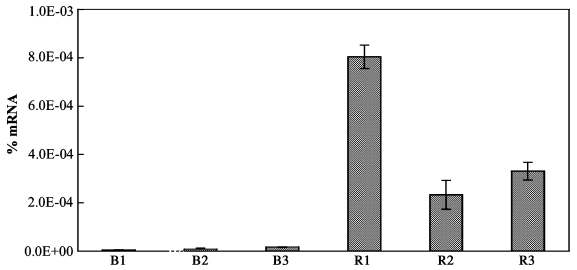

Figure 4. *CCD8* expression in leaves and roots.
Levels of CCD8 were determined by real-time RT-PCR in leaf blade and root tissue at three developmental time points: B1, leaf blade before bolting; B2, leaf blade after emergence of primary inflorescence; B3, leaf blade after emergence of secondary inflorescences; R1, root before bolting; R2, root after emergence of primary inflorescence; R3, root after emergence of secondary inflorescences.

were detected in all tissues tested at levels comparable to *CCD7*/*MAX3*, with the exception of root tissue, where expression was much higher. *CCD8*/*MAX4* transcript was highest in roots prior to bolting. Previously it was shown that wild-type roots grafted onto *max4-1* shoots rescued the *max4-1* phenotype (Sorefan *et al.*, 2003). Thus, the increased expression seen in roots relative to other tissue was intriguing. We therefore compared root expression before and after emergence of the primary inflorescence, and after emergence of secondary inflorescences (Figure 4). Transcript abundance in root tissue decreased by an average of 65% after the emergence of primary and secondary inflorescences. In contrast, the low level of transcript in leaf blade was not altered after emergence of axillary shoots.

Subcellular localization

The enzymes responsible for carotenoid biosynthesis are located within plastids (Cunningham and Gantt, 1998). Due to their hydrophobic nature carotenoids for the most part remain in the plastid. Because carotenoids are the putative substrates for CCDs, the subcellular localization of the CCDs was determined. Using *in vitro* pea chloroplast uptake studies, CCD7/MAX3 was previously shown to be targeted to the plastid stroma (Booker *et al.*, 2004). Using the same *in vitro* assay, CCD1 was not taken up by isolated chloroplasts (Tan *et al.*, 2001). The chloroplast prediction program TARGETP (v. 1.0) (Emanuelsson *et al.*, 2000) predicts CCD8/MAX4 to be localized to the chloroplast and assigns a transit peptide of 56 amino acids. In order to determine the subcellular localization of CCD1 and CCD8/MAX4, green fluorescent protein (GFP) fusions were constructed and transiently expressed in tobacco protoplasts (Figure 5a). Under the conditions used, the BY2 cells do not contain differentiated chloroplasts and a co-expressed marker was used to mark the plastids. The results of the localization experiments indicated that CCD1 is cytoplasmic while CCD7 and CCD8 are located inside the plastids.

To validate the *in vivo* uptake results and determine the suborganellar localization, *in vitro* chloroplast import assays were performed following the procedure of Cline *et al.* (1993) (Figure 5b). VP14, the maize NCED, is chloroplast localized and served as a positive control for plastid import. Previously, VP14 was shown to be targeted to the stroma and, to a lesser extent, associated with the thylakoid membrane (Tan *et al.*, 2001). Following *in vitro* transcription and translation, the CCD precursor proteins were incubated with isolated pea chloroplasts. After import reactions, intact chloroplasts were either treated with the protease, thermolysin, or fractionated into envelope, stroma and thylakoid compartments. Results show that CCD1 was sensitive to thermolysin treatment whereas CCD8/MAX4 was resistant. Fractionation of the chloroplast revealed that CCD8/MAX4 is localized to the stroma (Figure 5b). In addition, the reduced size of the imported mature protein indicated the existence of a cleaved transit peptide. These results, together with the previous results obtained for CCD7/MAX3, indicate that CCD7/MAX3 and CCD8/MAX4 are localized within the plastid while CCD1 is cytoplasmic.

CCD8/MAX4 activity

CCD7/MAX3 has been shown to cleave multiple carotenoid substrates (Booker *et al.*, 2004). We tested the activity of CCD8/MAX4 on carotenoid substrates by expressing the full-

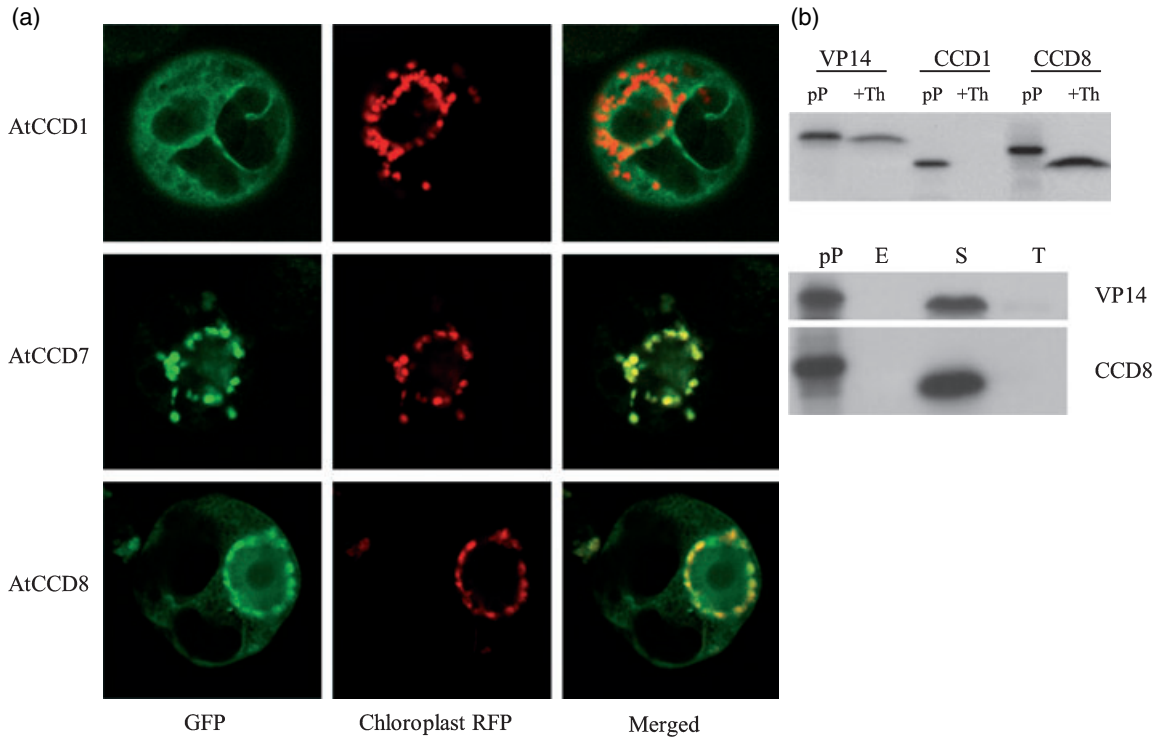

Figure 5. Carotenoid cleavage dioxygenases subcellular localization.
(a) CCD–GFP fusions transiently expressed in tobacco BY2 protoplasts (left) compared with a chloroplast marker (center). The merged images are shown on the right.
(b) Import of *in vitro* transcribed and translated CCD precursor proteins (pP) into pea chloroplasts were determined and compared with the previously studied chloroplast imported proteins VP14. Following import, chloroplasts were treated with thermolysin (+Th). Chloroplasts were further fractionated to determine suborganellar localization of CCD8 to the envelope (E), stroma (S) or thylakoid (T).

length CCD8/MAX4 with an N-terminal GST (glutathione-S-transferase) fusion in *Escherichia coli* strains engineered to accumulate β-carotene, lycopene or zeaxanthin (Cunningham *et al.*, 1996; Sun *et al.*, 1996). When expression of CCD8/MAX4 was induced, accumulation of each of these carotenoids was significantly reduced (Figure 6). Despite this reduction in carotenoid accumulation we were unable to identify the cleavage product, either by HPLC analysis of cell or growth media extracts or by gas chromatography analysis of collected volatiles. It is likely that the products were further catabolized in *E. coli*, as has been previously observed by others (von Lintig and Vogt, 2000). Therefore we were unable to determine the substrate specificities of CCD8/MAX4. The lack of an accumulating product is likely due to further metabolism by *E. coli*. The catabolism of carotenoids in each of the three *E. coli* strains does indicate that CCD8/MAX4 enzyme is capable of acting upon carotenoid substrate(s) in the absence of CCD7/MAX3. Because lycopene and β-carotene are both intermediates in the synthesis of zeaxanthin, we cannot rule out that CCD8/MAX4 is only active on a single carotenoid including, or between, phytoene and lycopene in the biosynthetic pathway. However, the catabolism of lycopene clearly indicates that the enzyme can cleave carotenoid substrates other than 10′-apo-β-carotenal.

Discussion

Carotenoids have critical roles in plant growth, acting as light-harvesting pigments and preventing oxidative stress (van den Berg *et al.*, 2000). They are also important substrates for a class of carotenoid cleavage dioxygenases that synthesize phytohormone apocarotenoids such as ABA. Recent compelling data support the existence of a novel, so far unidentified apocarotenoid hormone controlling leaf and lateral shoot growth (Booker *et al.*, 2004; Sorefan *et al.*, 2003). This apocarotenoid is generated at least in part by CCD7/MAX3 and/or CCD8/MAX4. Previous experiments indicated that CCD1 and CCD7/MAX3 have similar activities, the former oxidatively cleaving multiple carotenoids symmetrically at the 9,10 and 9′,10′ positions and the latter only at the 9,10 position (Booker *et al.*, 2004; Schwartz *et al.*, 2001, 2004). Here we have shown that while loss of *CCD7*/*MAX3* has a major impact on growth and development, loss of *CCD1* has no obvious morphological effect. While *CCD1* is highly expressed in multiple tissues, the protein is cytoplasmic. In contrast, *CCD7*/*MAX3* is expressed at much lower levels and the encoded protein is localized to the inside of plastids, the predominant sites of carotenoid accumulation.

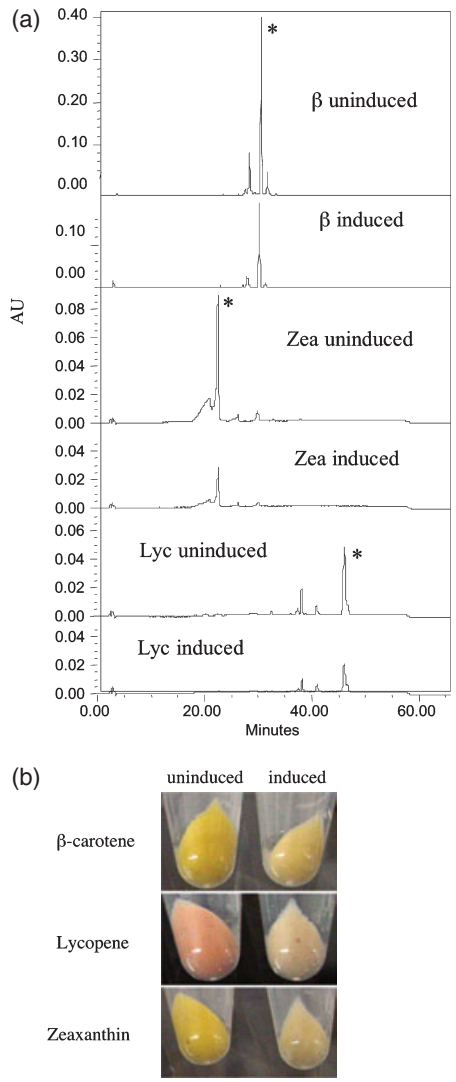

Figure 6. Expression of CCD8/MAX4 in carotenoid-producing strains of *E. coli*.
Escherichia coli engineered to accumulate the carotenoids β-carotene (βC), lycopene (LYC) or zeaxanthin (Zea) were transformed with an arabinose-inducible CCD8-GST.
(a) Carotenoid levels with and without induction of CCD8 expression were determined by HPLC analysis.
(b) Visual comparison of pelleted cells 3 h post-induction.

What is the function of CCD1 in Arabidopsis? One hint about its function is the high level of expression in reproductive tissues. CCD1 homologs have been identified in *Phaseolus vulgaris* (Schwartz *et al.*, 2001), *Crocus sativus* (Bouvier *et al.*, 2003), tomato (*Lycopersicon esulentum*) (Simkin *et al.*, 2004a) and petunia (*Petunia hybrida*) (Simkin *et al.*, 2004b). Experiments in both tomato and petunia verified the activity of CCD1 *in vivo* (Simkin *et al.*, 2004a,b). Tomato contains two closely related genes encoding CCD1 homologs. When the *LeCCD1* genes are co-suppressed, decreases in β-ionone and geranylacetone emission from fruits occurs (Simkin *et al.*, 2004a). β-Ionone and geranylacetone are important contributors to tomato flavor (Baldwin *et al.*, 2000). In petunia *PhCCD1* expression is diurnally regulated (Simkin *et al.*, 2004b), indicating a likely role in the synthesis of diurnally regulated floral volatiles for attracting pollinating moths. Pollination by insects is most probably not a typical means of fertilization for a self-pollinating plant like Arabidopsis. However, it may be beneficial to maintain a means by which diversity in genetic makeup can be obtained (Aharoni *et al.*, 2003).

Some of the CCD1 apocarotenoid products have been reported to have antimicrobial activities (Fester *et al.*, 1999). Thus, expression of *CCD1* in all tissues, and particularly in reproductive tissues and seeds, may be important for plant defense. Although the carotenoid substrates for CCD1 are largely inaccessible to a cytoplasmic enzyme, significant quantities of carotenoids are found in the outer plastid envelope (Douce *et al.*, 1973; Markwell *et al.*, 1992) and would likely be accessible to the enzyme.

The observation that seeds in the *ccd1* loss-of-function mutant contain significantly higher levels of carotenoids is particularly interesting. Catabolism of carotenoids in seeds may serve a protective function associated with apocarotenoid synthesis. Since many of these apocarotenoid volatiles are also important flavor and aroma volatiles, their production in seeds and fruits may be an aid to seed dispersal by animals. Since CCD1 is cytoplasmic and the carotenoid substrates are principally localized in the plastid, CCD1-mediated carotenoid catabolism is probably limited largely to seed maturation when the embryo degreens as light-harvesting complexes are degraded. We have observed a similar CCD1-associated loss of carotenoids upon maize seed maturation as well (B.C. Tan and D.R. McCarty, University of Florida, Gainesville, FL, USA, unpublished). It has not escaped our attention that this CCD1-associated carotenoid catabolism has negative consequences for nutritional quality of the seed.

The lack of an effect on lateral shoot growth in the *ccd1-1* knockout line is consistent with the observation that CCD7/MAX3 and CCD8/MAX4 are localized within the plastid. Thus, the 9,10 cleavage activity must be located within the plastid, most likely coordinately with CCD8/MAX4. A biosynthetic pathway leading to production of the novel signaling compound is evidenced by the identical phenotypes seen in the *max3* and *max4* mutants as well as the *max3-11 max4-6* double mutant. Loss of a single copy of *CCD8*/*MAX4* resulted in an increase in inflorescence number indicating an exquisite sensitivity of the phenotype to dosage of *CCD8*/*MAX4*. This observation suggests that CCD8/MAX4 activity may limit production of a branching signal. Furthermore, the *CCD8*/*MAX4* heterozygotes irrespective of *CCD7*/*MAX3* genotype (*max3*/+, *max4*/+ and *MAX3*/*MAX3*, *max4*/+) were indistinguishable from each other, indicating that a single dose of *CCD7*/*MAX3* is sufficient to maintain wild-type branching. For

this reason, we suggest that CCD8/MAX4 activity is likely to be a key regulated step in the pathway.

We do not yet know the identity of the apocarotenoid phytohormone. Our previous results indicated that CCD7/MAX3 has broad substrate specificity, recognizing both linear and cyclic carotenoid substrates, cleaving them at the 9,10 position to generate two aldehydes (Booker *et al.*, 2004). Schwartz *et al.* (2004) determined that for β-carotene, CCD7/MAX3 makes an asymmetric cleavage that generates a C_{13} (β-ionone) and a C_{27} aldehyde (10′-apo-β-carotenal). They further showed that CCD8/MAX4 cleaves the C_{27} aldehyde to generate a C_{18} aldehyde and a C_9 dialdehyde. They did not observe any activity with CCD8/MAX4 alone, suggesting that CCD8/MAX4 acts sequentially on the CCD7/MAX3 product(s). Although we have not yet determined the products, our data indicate that CCD8/MAX4 does act directly upon carotenoid substrates. Expression of the enzyme in *E. coli* led to depletion of carotenoids in strains synthesizing lycopene, β-carotene or zeaxanthin. Because β-carotene is an intermediate in zeaxanthin synthesis, we cannot rule out catabolism of β-carotene in the zeaxanthin-producing strain. However, lack of accumulation of lycopene clearly indicates a broader substrate specificity than previously believed. Indeed, in HPLC assays we have observed depletion of phytoene from a phytoene-accumulating *E. coli* strain (data not shown). This suggests that CCD8/MAX4 has a less stringent substrate requirement than previously suspected. However, no one has precisely determined substrate affinities for CCD7 and CCD8 and these enzymes may have stringent specificities *in vivo*. Since CCD8/MAX4 is active in the absence of CCD7/MAX3, the question of order of action between the two enzymes is still an open one. CCD7/MAX3 and CCD8/MAX4 may form heterodimers *in vivo*, although Schwartz *et al.* (2004) showed that CCD8/MAX4 cleaved the product of the mouse β-*Dioxi*I enzyme, which also catalyzes 9,10 cleavage of β-carotene. These observations also raise interesting questions concerning the identity of the translocated apocarotenoid. If CCD8/MAX4 can act upon carotenoid substrates in the absence of CCD7/MAX3, that product may be inaccessible to or unrecognized by CCD1 since the latter enzyme cannot complement a *ccd7*/*max3* mutant. Alternatively, CCD1, because it symmetrically cleaves a wide range of carotenoids, may simply produce an inactive apocarotenoid. Clearly further experiments addressing the activities of all these enzymes are needed.

Historically, apical dominance was thought to be controlled by the ratio of auxin to cytokinin found within the plant. It has been postulated that auxins, produced in the apex of the plant, travel down the stem and inhibit the growth of axillary meristems. While this is largely true, recent studies in Arabidopsis (Booker *et al.*, 2004; Sorefan *et al.*, 2003; Stirnberg *et al.*, 2002), pea (*Pisum sativum*) (Beveridge, 2000; Foo *et al.*, 2005; Morris *et al.*, 2001; Rameau *et al.*, 2002) and petunia (Napoli, 1996; Snowden *et al.*, 2005) point to a more complex mechanism controlling branching in plants. The *ramosus* (*rms*) mutants in pea exhibit increased branching but have normal to high auxin levels and normal auxin transport (Beveridge, 2000; Beveridge *et al.*, 1996; Morris *et al.*, 2001; Rameau *et al.*, 2002). PsRMS1 is orthologous to AtCCD8/MAX4 (Sorefan *et al.*, 2003). As reported here, the *max4-6* mutant, like *rms1*, also contains wild-type levels of auxin. These observations lead to the hypothesis that this branching phenotype is independent of auxin signaling. However, in the Arabidopsis *max4-1* mutant auxin sensitivity was decreased (Sorefan *et al.*, 2003) indicating a potential link to auxin signaling. Reduced auxin sensitivity was also seen in *rms1* and *rms2*. Normal sensitivity was regained when *rms1* and *rms2* shoots were grafted onto wild-type roots, suggesting that a graft-transmissible signal provided by wild-type roots restored auxin sensitivity to mutant shoots. How the apocarotenoid signaling molecule interacts with auxin to influence branching is clearly complex and remains to be determined.

Two additional mutants, *max1* and *max2* (Stirnberg *et al.*, 2002), have identical phenotypes to *max3* and *max4* and are probably involved in the production and perception of the graft transmissible signal produced by CCD7/MAX3 and CCD8/MAX4. MAX2, originally identified as ORE9 and later isolated from an EMS screen for branching mutants, is a member of the F-box LRR family of proteins (Stirnberg *et al.*, 2002; Woo *et al.*, 2001). These proteins function in the ubiquitin-mediated degradation of proteins targeted for destruction by the proteosome. MAX1 has recently been identified as a cytochrome P450 (Booker *et al.*, 2005). Reciprocal grafting experiments among all *max* plants indicate that CCD7/MAX3, CCD8/MAX4 and MAX1 are active in both roots and shoots while MAX2 functions only in the shoots (Turnbull *et al.*, 2002). The model hypothesizes that CCD7/MAX3 and CCD8/MAX4 cleave a carotenoid molecule inside the plastid to make a mobile intermediate that is subsequently acted on by MAX1 to produce a signal perceived by MAX2, leading to the inhibition of branching (Booker *et al.*, 2005). The demonstrated roles for carotenoid cleavage dioxygenases in Arabidopsis, pea and petunia as well as the existence of homologous sequences in the monocots maize and rice (B.C. Tan and D.R. McCarty, unpublished results) indicate a broadly conserved mechanism for controlling lateral branching in plants.

Experimental procedures

Isolation of loss-of-function mutants

Three publicly available T-DNA populations were used to obtain mutants in this study: the Wisconsin knockout population (Krysan *et al.*, 1999; Weigel *et al.*, 2000), the Syngenta Arabidopsis Insertion Library (SAIL) (Sessions *et al.*, 2002) and the Salk Institute Genomic Analysis Laboratory (Signal) population (Alonso *et al.*, 2003). *ccd1-1* and *max4-6* were obtained from the SAIL population. *max3-11* was

obtained from the Signal population and *max4-5* was obtained from the Wisconsin knockout facility. For each population, PCR was used to identify a plant homozygous for the T-DNA insert. Gene-specific primers used in these reactions were the following: CCD1, F 5′-cagagtgttggatcgttgctggaagaaag-3′/R 5′-tcctggagttgttcctgtgaataccagac-3′, CCD7 F 5′-gctcatgtcttccacaaaatcactcaact-3′/R 5′-aaccatgaaaacccatcggaaacgtcaaa-3′, and CCD8 F 5′-aaaaccgcatcaaaacttaccgtcaaact-3′/R 5′-ttgcgaattgataggtggaaccagtgaac-3′. The junctions between the T-DNA inserts and *CCD*s were sequenced for verification. Southern analysis using the selectable marker as a probe showed that *ccd1-1* has multiple inserts and *max3-11* and *max4-6* have two tandem inserts (data not shown).

Auxin and abscisic acid measurements

Wild-type and *max4-6* plants were grown on short days (8-h day/16-h night) until their rosettes contained 15–20 leaves, at which time rosettes were frozen individually. Abscisic acid and IAA were quantified following the procedure of Schmelz *et al.* (2004). Tissue from six rosettes was analyzed individually and the measurements were averaged.

Plant growth conditions and measurements

Plants were grown under Cool White and Gro-lux (Sylvania, Danvers, MA, USA) bulbs at 50 μmol m^{-2} sec^{-1}. Temperatures ranged from 19 to 22°C. Short days consisted of 8 h of light and 16 h of dark, while long days consisted of 16 h of light and 8 h of dark. Measurements of petiole length and leaf blade length were taken from the sixth leaf on the rosette. A combined inflorescence number was obtained by counting every inflorescence, emerging from either the primary meristem or axillary meristems, 1 week in long days and 2 weeks in short days after observation of emergence of the primary inflorescence. For all measurements, data from at least six plants were averaged.

Cloning of CCD8

In order to demonstrate complementation, a wild-type copy of *CCD8* was introduced into the *max4-6* allele. The *CCD8* cDNA in pBlueScript (KS) was a gift from Steve Schwartz. A single-nucleotide mutation was found in the cDNA clone and corrected using a Clonetech (Palo Alto, CA, USA) mutagenesis kit. The sequence matched that of the annotated gene in GenBank (*At4g32810*). *CCD8* cDNA was amplified using the following primers F 5′-caccatggcttctttgatcacaaccaaagc-3′/R 5′-ttaatctttggggatccagcaaccatg-3′, put into the Gateway pENTR2B vector (Invitrogen, Carlsbad, CA, USA) and sequenced. *CCD8* cDNA was recombined from pENTR2B into pDEST15 (Invitrogen) for expression with a GST tag. *CCD8* cDNA was transferred from CCD8pENTR2B to pDESTOE (Booker *et al.*, 2004) by recombination for expression in plants. pDESTOE contains the constitutive Figwort Mosaic Virus 35S promoter and the nitric oxide synthase (NOS) terminator as well as the plant selection gene *NPTII*. The plasmid was introduced into *Agrobacterium tumefaciens* and transformed into *max4-6* plants via the floral dip method (Clough and Bent, 1998). One line found to show expression of *CCD8* by real-time RT-PCR analysis was taken to homozygosity.

Real-time RT-PCR

To determine the major sites of *CCD* expression, tissues for RNA were harvested from Columbia plants grown in soil on short days for 2.5 months. Plants were then switched to long days in order to promote flowering. Once plants bolted, the primary inflorescence stem (primary stem minus flowers and cauline leaves), flower and green silique tissues were collected. Secondary inflorescence stems (secondary stem minus flowers and cauline leaves) were collected once they reached 8 cm in height. For analysis of *CCD8* expression in roots at three different stages of development, roots were collected from soil-grown plants prior to bolting, following emergence of the primary inflorescence, and following emergence of secondary inflorescences. Root tissue was carefully rinsed with water to remove soil and frozen in liquid nitrogen. Total RNA was isolated as described (Chang *et al.*, 1993).

All RNA was DNaseI (Ambion, Austin, TX, USA) treated at 37°C for 30 min. Deoxyribonuclease was removed using the RNeasy kit from Qiagen (Valencia, CA, USA). Ribonucleic acid was visualized on agarose gels and quantified by spectrophotometry. An Applied Biosystems GeneAmp 5700 Real-Time RT-PCR machine was used with TaqMan One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA) and reaction conditions were as per the manufacturer's specifications using 250 ng RNA per reaction in a 25 μl reaction volume. The primer/probe pairs were as follows: CCD1 F primer 5′-acaagagattgacccactccttca-3′, probe 5′-FAM-tgctcacccaaaagttgacccggt-TAMRA-3′, R primer 5′-tgtttacattcggctattcgca-3′; and CCD8 F primer 5′-tgataccatctgaaccattcttcgt-3′, probe 5′-FAM-cctcgacccggtgcaacccat-TAMRA-3′, R primer 5′-cgatatcaccactccatcatcct-3′. Transcript quantities were determined by comparison with a standard curve. Transcript for use in the production of standard curves was synthesized with T7 polymerase *in vitro* in the presence of ^{3}H-UTP from *CCD1*pBK-CMV (linearized with *Not*I) and *CCD8*pENTR2B (linearized with *Xba*I). Quantities were then normalized to ribosomal RNA, which was detected using the TaqMan Ribosomal RNA Control Reagents kit by Applied Biosystems. Tissue expression patterns were determined for three biological replicates. Figures 3 and 4 show one replicate with error bars corresponding to the duplicate reactions performed for each RNA sample.

Real-time RT-PCR was used to determine whether any *CCD1* transcript was detectable in the *ccd1-1* mutant using 10-day-old plate-grown Arabidopsis. Seeds were stratified for 4 days at 4°C and then grown at 22°C under cool white fluorescent lights with a 16-h light/8-h dark photoperiod. The plants were harvested at the same time on the 10th day in each biological replicate. Total RNA was isolated using the Qiagen RNeasy plant kit and on column DNase treatment was performed with the Qiagen RNase free DNase kit as specified by the manufacturer. The RNA was then quantified and analyzed by real-time RT-PCR as described above. The primers and probe used were designed to span the sixth intron of *CCD1*, the site of the T-DNA insertion, which were CCD1 forward primer 5′-atcaccatggaaaacttctagcatt-3′, probe 5′-FAM-caggaggcagataagccgtacgtcatca-black hole quencher-3′, CCD1 reverse primer 5′-gcaggtctccatcttccaaaac-3′.

Subcellular localization by in vitro *import assays*

Transcription of *CCD*s and *Vp14* were controlled by the SP6 promoter in the pSP64-PolyA vector (Promega, Madison, WI, USA) and pSMS64 vector (Anderson and Smith, 1986), respectively. The *CCD1* cDNA in pBK-CMV (Stratagene, La Jolla, CA, USA) was a gift from B.C. Tan. *CCD1*pBK-CMV was digested with *Pst*I/*Sma*I and ligated into pSP6-PolyA (Promega). Cloning of *CCD8* into the pSP64-PolyA vector was performed by digestion (*Sal*I partial/*Xba*I) of CCD8pENTR2B, followed by ligation into pSP6-PolyA. *In vitro* transcription and translation were performed using the coupled transcription/translation (TNT) wheat germ extract system by Promega

as specified by the manufacturer. A total of 6 μg plasmid DNA was used in a 100 μl reaction volume and SP6 polymerase was used for transcription. Translation products were labeled with ^{3}H-leucine. Reactions were incubated for 30 min at 25°C. A 2 μl aliquot of TNT reaction products was set aside and the remaining reaction mix was used for import into the chloroplast.

Pea chloroplasts were isolated and import assays were performed as described (Cline *et al.*, 1993). Chloroplasts were divided into two fractions, one for protease treatment with thermolysin and the other for subfractionation. Chloroplasts were subfractionated into a membrane fraction, stromal fraction and thylakoid fraction by differential centrifugation. Thermolysin-treated whole chloroplasts and chloroplast subfractions were electrophoresed on 12.5% SDS-polyacrylamide gels. The TNT products were run beside import samples for size comparisons. Gels were incubated in DMSO followed by 2,5-diphenyloxazole (PPO) in DMSO, rinsed with water, dried and exposed to film.

Subcellular localization by in vivo *transient assays*

The *CCDs* were put into the Gateway-ready pDESTOE-GFP vector (Moussatche, 2004) by recombination with CCD1pENTRD, CCD7pENTRD or CCD8pENTR2B for C-terminal fusion to GFP. Protoplasts used for transfection were isolated from BY2 tobacco cells. BY2 cells were cultured in a modified Murashige–Skoog medium (Duchefa, Roubaix, France). Other additives were as described by Nagata *et al.* (1992), except the further inclusion of 100 mg l^{-1} myo-inositol. Cell cultures (50 ml in 250 ml Erlenmeyer flasks) were kept in the dark at 25°C under agitation. The cells (2 g) were digested in 20 ml solution containing 1.0% (w/v) cellulase 345 (Cayla, Toulouse, France), 0.2% (w/v) pectolyase Y-23 (Seishin Pharmaceutical, Tokyo), 0.6 M mannitol and Tris MES [2-(4-morpholino)-ethane sulfonic acid] 25 mM, pH 5.5, at 37°C for 90 min. Protoplasts were transfected by a modified polyethylene glycol method as described by Abel and Theologis (1994) and Negrutiu *et al.* (1987). Typically, 0.2 ml of protoplasts suspension (0.5×10^6 ml^{-1}) were transfected with 20 μg sheared salmon sperm carrier DNA and 20 μg of the DNA plasmid carrying the appropriate constructs. Transfected protoplasts were then incubated for 16 h at 25°C. Co-transfections were carried out with different *CCD* constructs fused to GFP and a *cRecA* gene encoding chloroplast targeted protein fused to modified Red Fluorescent Protein (Cao *et al.*, 1997).

Confocal images of transfected protoplasts were acquired with a confocal laser scanning system (Leica TCS SP2, Leica DM IRBE; Leica Microsystems, Wetzlar, Germany) equipped with an inverted microscope (Leica) and a 40× water immersion objective (numerical aperture 0.75). For GFP, the samples were illuminated with a 488 nm ray line of an argon laser and the emission light collected in the 500–525 nm spectral range. The mRFP fluorescence was observed with the 543 nm ray line of a helium neon laser for excitation and emitted light was collected in the 560–660 nm spectral range.

Seed carotenoid determinations

The carotenoid contents of seeds of wild-type and *ccd1* mutant were determined as previously described (Tian *et al.*, 2003).

Complementation of the ccd1-1 *mutation*

The full-length *CCD1* cDNA was cloned into a Gateway entry vector (Invitrogen) and was recombined into a plant transformation destination vector under the control of the Figwort Mosaic Virus (FMV) promoter. The DNA was transferred to *ccd1-1* plants via the whole plant dipping method similar to that described by Clough and Bent (1998). Seed germination on medium containing kanamycin (50 μg ml^{-1}) (Fischer Scientific, Fair Lawn, NJ, USA) was used to identify plants containing the transferred DNA. Real-time PCR was used to monitor the expression of *CCD1* in the transgenic plants and those plants with increased *CCD1* accumulation were selected. Seed from T_3 plants containing the transferred DNA was then measured for carotenoid content.

CCD8 activity in carotenoid accumulating E. coli strains

BL21AI (Invitrogen) cells were transformed with expression plasmids for β-carotene, lycopene or zeaxanthin (Cunningham *et al.*, 1996; Sun *et al.*, 1996). Cells with visible expression of these carotenoids were made chemically competent with calcium chloride and then transformed with either CCD8pDEST15 or the vector control pDEST17. Pre-cultures were grown overnight in Luria–Bertani (LB) medium in a rotary shaker at 37°C with 25 μg ml^{-1} chloramphenicol (carotenoid gene-containing plasmid) and 100 μg ml^{-1} carbenicillin (pDEST15) or 100 μg ml^{-1} spectinomycin (pDEST17). Two ml of pre-culture was used to inoculate 100 ml of LB medium and the cultures were grown at 37°C in the absence of antibiotics with 0.1% (w/v) glucose, shaking at 300 r.p.m. in the dark until an OD_{600} 1.5 was reached. The cells were then pelleted by centrifugation at room temperature and resuspended in 100 ml fresh LB. The cultures were grown for 30 min at 25°C in the dark shaking at 300 r.p.m. The expression of CCD8-GST was then induced by the addition of 0.1% arabinose. The cultures were grown for an additional 3 h at 25°C and then harvested by centrifugation. Carotenoids were extracted from the pelleted cells by vortexing in 2 ml formaldehyde, followed by extraction in 2 ml methanol and 8 ml ethyl ether. The ethyl ether phase was collected and washed twice with an equal volume of water. The organic phase was dried down to 0.5 ml and approximately 100 μl was injected on the HPLC for analysis (Fraser *et al.*, 2000). The injection volume was adjusted for cell density of the cultures at the time of extraction.

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