

Carotenoid cleavage dioxygenases and their apocarotenoid products in plants

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Abstract Carotenoid cleavage dioxygenases (CCDs) catalyze the cleavage of carotenoids in various organisms. In *Arabidopsis*, the CCD enzyme family consists of nine members, which are further divided into two subfamilies: five 9-*cis* epoxy-carotenoid dioxygenases (NCEDs) and four CCDs. The NCEDs are involved in the biosynthesis of the plant hormone abscisic acid (ABA). CCDs are only distantly related to the NCEDs, and their substrate specificities and activities differ from those of the NCEDs. Recent evidence indicates that apocarotenoids produced by the action of CCDs play various roles in the growth and development of plants; these apocarotenoids include a novel plant hormone that controls shoot branching and pollinator attractants produced in reproductive organs. This review focuses on enzymes belonging to the CCD subfamily; their enzymatic activities and the functions of their apocarotenoid products are discussed.

Key words: Apocarotenoid, carotenoid, carotenoid cleavage dioxygenase, strigolactone.

Carotenoids are widely distributed C₄₀ isoprenoid pigments with polyene chains containing up to 15 conjugated double bonds. More than 700 naturally occurring carotenoids have been identified (Britton et al. 2004). They furnish flowers and fruits with distinct colors (e.g., yellow, orange, and red), which attract pollinators, and play important roles in photosynthesis, light harvesting, and prevention of photooxidative damage (reviewed in Niyogi 2000).

Specific enzymatic cleavage of carotenoids produces various types of apocarotenoids, some of which play important biological functions in the growth and development of both plants and animals. The most well-known apocarotenoids are vitamin A and abscisic acid (ABA). Vitamin A is an important visual pigment and signaling molecule in animals (DellaPenna and Pogson 2006), and ABA plays a key role in seed development and in responses to environmental stresses related to loss of water (reviewed in Nambara and Marion-Poll 2004). In addition to these bioactive compounds, carotenoid catabolism produces diverse apocarotenoid compounds that are also essential for plant growth and development. One of these compounds is involved in apical dominance and has been categorized as a novel plant hormone, and other compounds provide fruits and flowers with unique aroma and color for attracting pollinators. This review

focuses on the recent progress in the study of the functions of apocarotenoids and carotenoid cleavage dioxygenases (CCDs), a major class of carotenoid-cleaving enzymes involved in the production of apocarotenoids.

The CCD family

The first protein found to specifically cleave carotenoids, viviparous14 (VP14), was identified by analysis of a viviparous ABA-deficient mutant of maize (*Zea mays*; Schwartz et al. 1997; Tan et al. 1997). Analysis of the enzymatic activity of VP14 showed that it cleaves the 11,12(11',12') double bonds of the 9-*cis* isomers of neoxanthin and violaxanthin to yield xanthoxin, the precursor of ABA (Figure 1). The pioneering work on VP14 facilitated the discovery of related enzymes in different plant species and other organisms. Analysis of the genome sequence of *Arabidopsis thaliana* led to the definition of nine clades of dioxygenases (Tan et al. 2003). Five of these, the 9-*cis* epoxy-carotenoid dioxygenases (NCEDs; NCED2, NCED3, NCED5, NCED6, and NCED9), are closely related to and have the same activity as VP14. The remaining four (CCD1, CCD4, CCD7, and CCD8) have low sequence homologies to the NCEDs, and their enzyme activity and

Abbreviations: ABA, abscisic acid; *bps1*, *bypass1*; *BRC1*, *BRANCHED1*; CCD, carotenoid cleavage dioxygenase; *dad*, *decreased apical dominance*; *htd*, *high-tillering dwarf*; *max*, *more axillary growth*; NCED, 9-*cis* epoxy-carotenoid dioxygenase; *rms*, *ramosus*; *TB1*, *TEOSINTE BRANCHED1*; VP14, viviparous14.

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substrate specificity also differ from those of the NCEDs. Orthologs belonging to the NCED and CCD subfamilies have been identified in many plant species, and they are named on the basis of their homology to those of *Arabidopsis*.

Apocarotenoids involved in vegetative growth

Control of apical bud outgrowth is a key determinant of plant architecture. The number of branches is controlled largely by the action of the plant hormone auxin (Booker et al. 2003). The axillary buds located in the axil of each leaf do not grow to form branches by the action of auxin, which is supplied from the growing shoot apex, and removing the auxin source by decapitation induces branching. The phenomenon is termed “apical dominance”. Auxin prevents branching by downregulating cytokinin synthesis in buds, where cytokinin enhances bud outgrowth (Tanaka et al. 2006). Although auxin is known to play a role in apical dominance, the molecular mechanism by which the hormone exerts its effect on branching is largely unknown. Recently, analyses of branching mutants have shown that carotenoid-derived signaling molecules are required for auxin-mediated apical dominance (reviewed in Mouchel and Leyser 2007).

CCD7 and CCD8 are involved in branching-signal production

By genetic analysis, researchers have identified mutants showing increased numbers of branches in *Arabidopsis more axillary growth* (*max*) mutants, petunia (*Petunia hybrida*) *decreased apical dominance* (*dad*) mutants, and pea (*Pisum sativum* L.) *ramosus* (*rms*) mutants (Napoli 1996; Beveridge 2000; McSteen and Leyser 2005). Axillary buds of these mutants are resistant to the inhibitory effect of exogenously applied auxin for bud outgrowth, indicating that the *MAX*, *DAD*, and *RMS* genes act downstream of auxin and are involved in apical dominance (reviewed in Ongaro and Leyser 2007). Grafting experiments with the branching mutants have provided evidence that a novel signaling molecule transported from root to shoot inhibits branching. The existence of this novel branching signal was first demonstrated in the *dad-1* mutant. The branching phenotype of the *dad-1* mutant is restored to wild type when it is grafted onto wild-type root stock, indicating that a long-range graft-transmissible signal that inhibits lateral branching is produced in roots (Napoli 1996). When the *max* and *rms* mutants are grafted to wild-type root stock, the branching phenotype of these mutants is restored, which indicates that their branching is also controlled by long-distance signaling from the root (Beveridge 2000; Morris et al. 2001; Booker et al. 2005;

Foo et al. 2005).

In *Arabidopsis*, four nonallelic mutants (*max1–max4*) were isolated, and the *MAX* loci were characterized. The *Arabidopsis MAX3* and *MAX4* genes were identified as *CCD7* and *CCD8*, respectively (Booker et al. 2004; Schwartz et al. 2004). Both *CCD7* and *CCD8* are expressed predominantly in the root. Reciprocal grafting between *max3* and *max4* showed that *CCD7* and *CCD8* function when they are present in the same tissue and that the signal produced by the successive action of *CCD7* and *CCD8* is acropetally transported via the xylem to the shoot, where the signal affects the outgrowth of buds. The recombinant AtCCD7 protein catalyzes cleavage of the 9,10 double bond of β -carotene to yield a C₂₇ aldehyde (10'-apo- β -carotenal) and a C₁₃ ketone (β -ionone). *CCD8* subsequently catalyzes the cleavage of the C₂₇ aldehyde at the 13,14 double bond to produce a C₁₈ ketone (13-apo- β -carotenone). A double-mutant analysis together with a reciprocal-grafting analysis showed that *MAX1* acts downstream of *MAX3* and *MAX4* and modifies the signal in the shoot (Booker et al. 2005). *MAX1* encodes a class III cytochrome P450, whose substrate is often produced by the action of dioxygenase. Discovery of the *MAX* genes allowed the identification of orthologs of the *DAD* and *RMS* genes. *RMS1* (Sorefan et al. 2003) and *DAD1* (Snowden et al. 2005) are *MAX4* orthologs; *RMS5* (Johnson et al. 2006) and possibly *DAD3* (Simons et al. 2007) are *MAX3* orthologs. In rice (*Oryza sativa*), *dwarf* (*d*) and *high-tillering dwarf* (*htd*) mutants showing an increased tillering phenotype were identified (Ishikawa et al. 2005; Zou et al. 2005). The *D10* and *HTD1* genes were cloned and found to encode orthologs of *MAX4* and *MAX3*, respectively (Zou et al. 2006; Arite et al. 2007). These findings suggest that the *MAX* pathway is conserved widely across monocots and eudicots.

Although the possible involvement of *MAX3* and *MAX4* in the production of the signaling molecule was demonstrated and the enzymatic activities of these proteins were characterized, the molecular nature of the long-distance signal was unknown until recently, when the carotenoid-derived signal was identified as strigolactone in both pea and rice (Gomez-Roldan et al. 2008; Umehara et al. 2008; Figure 1). Strigolactones are a group of compounds that trigger the germination of parasitic plant seeds and stimulate colonization of roots by symbiotic arbuscular mycorrhizal fungi (Akiyama et al. 2005; Bouwmeester et al. 2007). Because the *rms* mutants are defective in interacting with arbuscular mycorrhizal fungi, the *rms* gene was thought to be involved in strigolactone biosynthesis. Gomez-Roldan et al. (2008) found that *rms* mutants are deficient in strigolactone and that application of strigolactone restores the branching phenotype to the wild type. A very small amount of strigolactone is sufficient to inhibit

bud outgrowth in both *ccd8* and *ccd7* pea plants. In *Arabidopsis* and rice, strigolactone also inhibits bud outgrowth (Umehara et al. 2008). Strigolactone is now recognized as a novel plant hormone because it meets the criteria for categorization as a plant hormone: (1) it is a small molecule active at low concentration; (2) it is produced in a specific organ distant from the target tissue; and (3) it moves throughout the plant via the phloem.

Perception of branching signals

Although the long-distance signaling molecule was identified as strigolactone, information about the genes responding to the signal inside the buds to inhibit growth is limited. Reciprocal-grafting analysis showed that MAX2, RMS4, and D3 are required to locate at each node possibly for perceiving the signal (Stirnberg et al. 2002; Johnson et al. 2006; Arite et al. 2007). MAX2, RMS4, and D3 are members of the F-box leucine-rich repeat family and may function in an Skp1p-cullin-F-box (SCF) protein complex that is involved in the poly-ubiquitination and targeting of proteins for degradation (Stirnberg et al. 2007). TRANSPORT INHIBITOR RESISTANT1 (TIR1; Kepinski and Leyser 2005) and FLAVIN-BINDING, KELCH-REPEATS F-BOX1 (FKF1; Imaizumi et al. 2005) are members of the SCF protein family and act as auxin and photoperiodic receptors, respectively. The putative biochemical functions of MAX2, RMS4, and D3 are consistent with the predicted action of these proteins in the signal transduction of strigolactones.

Aguliar-Martínez et al. (2007) have demonstrated that *BRANCHED1 (BRC1)* is involved in the MAX signaling pathway in *Arabidopsis*. *BRC1* expression is restricted to axillary buds and downregulated in *max* mutants. Loss-of-function mutants show increased branching. The MAX pathway is therefore considered to suppress axillary bud outgrowth through transcriptional control of *BRC1*. *BRC1* was isolated as the gene closest to maize *TEOSINTE BRANCHED1 (TB1)*, a TCP transcriptional factor, (Kosugi and Ohashi 2002). *TB1* expression in axillary buds of maize is higher than that of teosinte, which results in suppression of bud growth. Not only the sequences but also the functions of the two genes in controlling shoot branching are related, which suggests that a common mechanism of signal transduction, and signaling molecule production, is conserved across monocots and eudicots.

Leaf growth and carotenoid-derived signals

Another component of the carotenoid-derived signaling mechanism was demonstrated by the analysis of a *bypass1 (bps1)* mutant of *Arabidopsis*. The *bps1* mutant shows defective growth and development in the shoot and root (van Norman et al. 2004). Grafting and root-

excision experiments showed that a graft-transmissible signal capable of inhibiting shoot development is produced in the root. In addition, the signal is produced in mutants where ABA- and MAX-dependent signals are not produced, indicating that the signal is neither ABA nor strigolactone (van Norman and Sieburth 2007). The *bps1* phenotype is partly restored by the application of carotenoid biosynthesis inhibitors such as norflurazon and CPTA, indicating that the signal produced in the roots of the *bps1* mutant is derived from carotenoids. *BPS1* encodes a protein that belongs to a small family whose function is unknown. Further study of the biological activity of BPS1 is required to understand the nature of the signaling molecule.

Carotenoid-derived color, flavor, and aroma

A variety of apocarotenoids derived from specific cleavage of carotenoids provide unique color, flavor, and aroma to fruits and flowers of many plant species. Two classes of CCDs (CCD1 and CCD4) are involved in the formation of the apocarotenoids.

CCD1 is involved in flavor and aroma formation

Volatile terpenoid compounds derived from carotenoids, such as β -ionone, β -cyclocitral, geranylacetone, and pseudoionone, are important components of flavor and aroma in many fruits, vegetables, and ornamental plants (Figure 1). These apocarotenoids play a role in attracting pollinators. For example, α - and β -ionol attract *Batrocera latifrons* (Diptera: Tephritidae; Flath et al. 1994; McQuate and Peck 2001), and β -ionone attracts beetles (Donaldson et al. 1990). However, little is known about the enzymes responsible for the synthesis of these volatile compounds.

CCD1 contributes to the formation of apocarotenoid volatiles in the fruits and flowers of several plant species. Orthologs of *AtCCD1* have been found in a variety of species, including petunia (Simkin et al. 2004b), tomato (*Solanum lycopersicum*; Simkin et al. 2004a), crocus (*Crocus sativus*; Bouvier et al. 2003b, Rubio et al. 2008), grape (*Vitis vinifera*; Mathieu et al. 2005), melon (*Cucumis melo*; Ibdah 2006), citrus (Kato et al. 2006), rice (Ilg et al. 2008), nectarine (*Prunus persica*; Baldermann et al. 2005), rose (*Rosa damascena*; Huang et al. 2009a), and *Medicago truncatula* (Floss et al. 2008). The emission of apocarotenoid volatiles is temporally associated with *CCD1* expression in petunia corolla (Simkin et al. 2004b), grape fruits (Mathieu et al. 2005), and tomato fruits (Simkin et al. 2004a). In addition, *CCD1* loss-of-function mutants show decreased levels of β -ionone emission in tomato fruit and petunia flowers. These results indicate that CCD1 plays an important role in the formation of apocarotenoid volatiles in fruits and flowers. However, suppression of

CCD1 expression does not affect the carotenoid content in the tissues. One reason for this result is considered to be the limited access of *CCD1* to its substrate. In *Arabidopsis*, members of the *CCD* subfamily, except *AtCCD1*, contain a plastid-targeting signal and are located in plastids (Tan et al. 2003). In contrast, the *CCD1*s reported so far lack a plastid-targeting signal and are located in the cytoplasm (Bouvier et al. 2003b; McCarty and Klee 2006). *CCD1* access to its substrates is most likely limited to carotenoids located in the outer envelope of the plastids, because substantial amounts of β -carotene are present in the outer envelope of pea (Markwell et al. 1992) and spinach (*Spinach oleracea*; Douce et al. 1973).

Enzymatic activity of *CCD1*

The enzymatic activity of *CCD1* is still a matter of debate. Schmidt et al. (2006) demonstrated that recombinant *AtCCD1* obtained symmetrically cleaves the 9,10(9',10') double bonds of multiple carotenoid substrates to produce a C_{14} dialdehyde and two C_{13} cyclohexone derivatives. Additional *CCD1* cleavage sites at the 5,6(5',6') and 7,8(7',8') double bonds of lycopene have also been reported (Ilg et al. 2008; Vogel et al. 2008; Huang et al. 2009a). Floss et al. (2008) demonstrated the *in planta* *CCD1* activity using a symbiotic plant–microbe interaction system: when the expression of *M. truncatula* *CCD1* (*MtCCD1*) is strongly suppressed in the mycorrhizal root, reduction in the amount of C_{14} cleavage products and accumulation of C_{27} apocarotenoids (not C_{40} carotenoids) is observed, whereas the amount of C_{13} cleavage products is reduced to 30–50% of that produced by an empty-vector control. The differential reduction of C_{13} and C_{14} apocarotenoids led Floss et al. to propose that, *in planta*, *MtCCD1* catalyzes the cleavage of C_{27} to C_{13} and C_{14} but not the cleavage of C_{40} to C_{27} and C_{13} . On the basis of the results obtained by the *MtCCD1* analysis, the following mechanism of carotenoid degradation by *CCD1* was postulated: conversion of C_{40} to C_{27} and C_{13} likely occurs primarily inside the plastid, and then, these two apocarotenoids are exported from the plastid to the cytoplasm, where the C_{27} apocarotenoid is further cleaved by *CCD1* to C_{13} and C_{14} . This mechanism provides a clear explanation of how cytoplasmic *CCD1* accesses its substrates and why severe *CCD1* suppression does not affect carotenoid content in the tissue. It seems, therefore, that the mechanism of carotenoid cleavage found in mycorrhizal roots is common to many plant species.

CCD4 is involved in unique pigment formation

Some plants accumulate apocarotenoids that are economically valuable. For example, crocus flowers accumulate apocarotenoids such as crocin, crocetin,

microcrocin, and safranal, which are responsible for the orange-yellow color, flavor, and aroma of saffron (Figures 1 and 2). Bouvier et al. (2003b) showed that these compounds are synthesized through the cleavage of zeaxanthin at the 7,8(7',8') double bonds by zeaxanthin cleavage dioxygenase *CsZCD*. Bouvier et al. (2003a) also identified the genes involved in the synthesis of bixin (Figure 1), which is extracted from the seeds of the tropical tree *Bixa orellana* and is one of the most important colorants in the food and cosmetic industries worldwide. Synthesis of bixin starts with the cleavage of lycopene at the 5,6(5',6') double bonds by lycopene cleavage enzyme *BoLCD*. The gene encoding *BoLCD* was isolated using degenerate primers based on the *CsZCD* sequence, and sequence of *BoLCD* is closest to that of *CsZCD*. Synthesis of bixin requires two additional enzymes: bixin aldehyde dehydrogenase (*BoBADH*) and norbixin methyltransferase (*BonBMT*). Bixin accumulation results from coexpression of *LCD*, *ZCD*, and *BADH* in lycopene-producing *Escherichia coli* (Bouvier et al. 2003a). Both *CsZCD* and *BoLCD* fall into the same clade as *CCD4* (Bouvier et al. 2005). Recent work has shown that *CsZCD* represents an incomplete *CsCCD4* sequence and that full-length *CsCCD4* catalyzes the conversion of β -carotene to β -ionone in *E. coli*, implying the 9,10(9',10') cleavage activity of *CsCCD4* (Rubio et al. 2008). These results indicate that the *in planta* activity of both *CsZCD* and *BoLCD* should be reconsidered.

CCD4 is involved in white petal color formation

In chrysanthemums, the white petal color is dominant over the yellow and is postulated to arise from a single dominant gene that inhibits carotenoid formation (Hattori 1991). By differential screening, a gene that is expressed specifically in white petals was identified (Ohmiya et al. 2006). The gene (designated *CmCCD4a*) is highly homologous to *AtCCD4*. Suppression of *CmCCD4a* expression by RNAi turns the white petal color to yellow, suggesting that the white color results from degradation of carotenoids by *CmCCD4a*. Analysis of carotenogenic gene expression supports this suggestion: in white chrysanthemum petals, all the genes encoding carotenoid biosynthesis enzymes are expressed, indicating that carotenoids are biosynthesized in white petals (Kishimoto and Ohmiya 2006). Whether the mechanism of white color formation found in chrysanthemum petals is applicable to other plant species is a question of great interest. Little is known, however, about the *CCD* expressed in petals of other plants. In petals of rose (*Rosa damascena*), 15 apocarotenoids have been identified, and the existence of a *CCD* that cleaves diverse carotenoid substrates has been postulated (Eugster and Märki-Fischer 1991). Recently, orthologs of *CCD1* and *CCD4* were identified

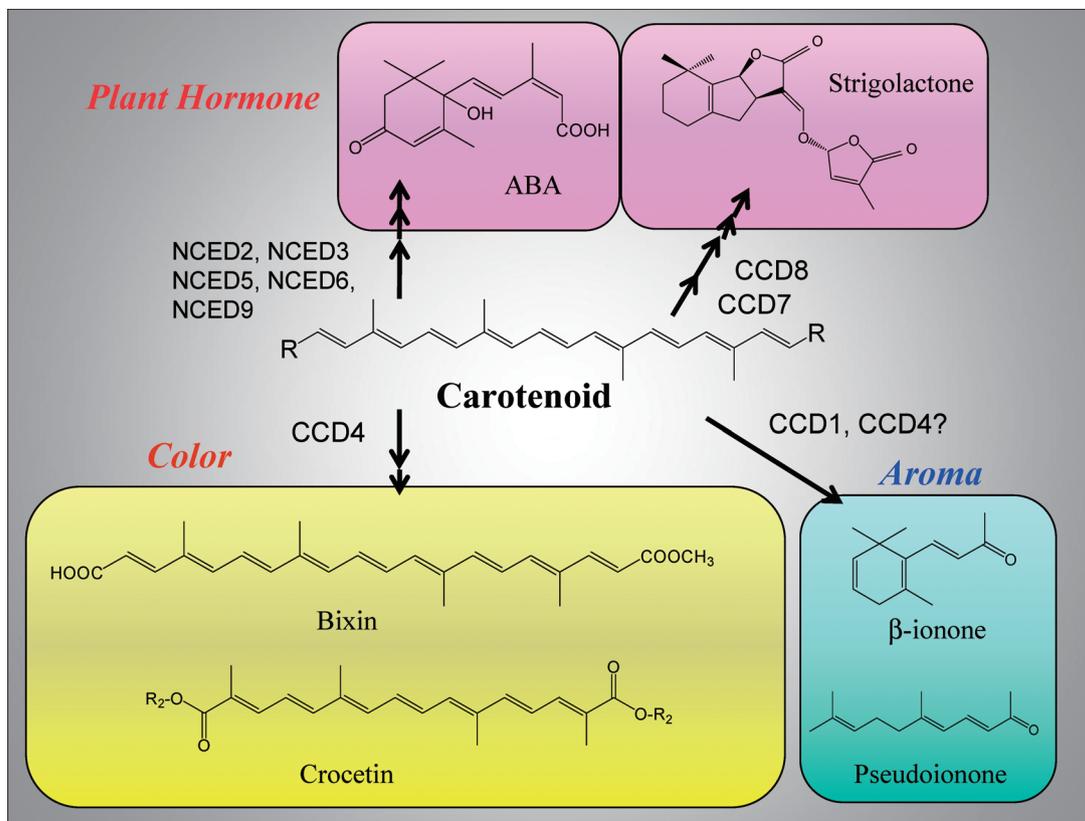


Figure 1. Apocarotenoids and the CCDs or NCEDs responsible for their production.



Figure 2. The crocus flower has stigmas with blood-red color derived from crocin, crocetin, and picrocrocin.

in rose, and the possible involvement in carotenoid degradation in flowers was demonstrated (Huang et al. 2009a; Huang et al. 2009b).

In chrysanthemums, mutation of petal color from white to yellow sometimes occurs by means of the loss of the *CmCCD4a* gene (Boase et al. 1997; Ohmiya et al. 2006). However, 'Jimba', the most popular white-flowered cultivar in Japan, rarely mutates to yellow either

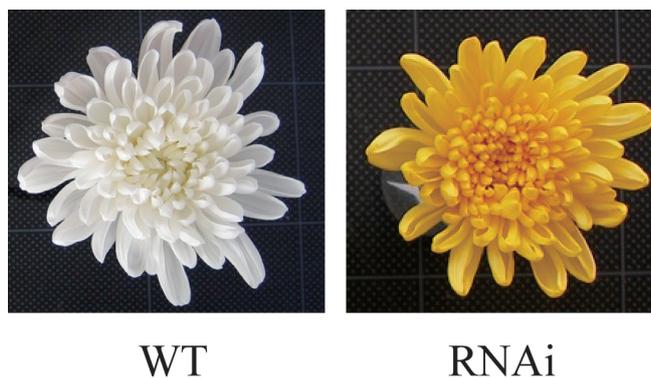


Figure 3. Chrysanthemum flowers of wild-type 'Jimba' (WT) and a transgenic plant harboring *CmCCD4a* RNAi (RNAi).

by spontaneous mutation or by induction. My co-workers and I recently succeeded in manipulating the petal color of 'Jimba' from white to yellow by *CmCCD4a* RNAi (Figure 3; Ohmiya et al. 2009). It is interesting to note that the growth properties of the RNAi plant differ from those of the wild-type plant. The number of apical bud outgrowths is substantially reduced, and vegetative tissue tends to be small in the RNAi plants. It is possible that apocarotenoids produced by the action of *CmCCD4a* in petals are basipetally transported and affect vegetative growth. If so, apocarotenoids produced in petals must have different functions from the apocarotenoid

produced in roots (i.e., strigolactone). As mentioned earlier in this review, strigolactone suppresses branching in petunia and *Arabidopsis* (Gomez-Roldan et al. 2008). On the other hand, apical bud outgrowth is suppressed in RNAi 'Jimba' plants in which apocarotenoid production in petals is suppressed. Although the mechanism that controls branching in chrysanthemum is not known and the compounds produced by the action of CmCCD4a *in planta* have not been determined yet, it is possible that apocarotenoids structurally related to strigolactone are produced *in petals* and transported down the stem, where they antagonistically affect branching. Further study is needed to identify the compounds produced by CmCCD4a and other CCD homologs in chrysanthemums.

Perspective

The green tissues of most plants have similar carotenoid profiles, containing carotenoids essential for photosynthesis. In contrast, the carotenoid profiles of fruits and flowers are distinctive and depend on the plant species (Tanaka et al. 2008). It is interesting to note that the same is true of carotenoid catabolism. Carotenoid-derived bioactive compounds, ABA and possibly strigolactones, are widely distributed in vegetative tissues of seed plants and play fundamental roles in growth and development. In contrast, the modes of carotenoid degradation in fruits and flowers are diverse and vary according to plant species. The reproductive organ-specific distribution of both carotenoids and apocarotenoids evolved to attract pollinators. How organ-specific distribution is regulated remains largely unknown. Therefore, studies of transcriptional and posttranscriptional regulation of carotenoid metabolic pathways are essential to gain further understanding of the function and organ-specific distribution of carotenoids and apocarotenoids.

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