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Selective Inhibition of Carotenoid Cleavage Dioxygenases

PHENOTYPIC EFFECTS ON SHOOT BRANCHING[*S]

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Members of the carotenoid cleavage dioxygenase family catalyze the oxidative cleavage of carotenoids at various chain positions, leading to the formation of a wide range of apocarotenoid signaling molecules. To explore the functions of this diverse enzyme family, we have used a chemical genetic approach to design selective inhibitors for different classes of carotenoid cleavage dioxygenase. A set of 18 arylalkyl-hydroxamic acids was synthesized in which the distance between an iron-chelating hydroxamic acid and an aromatic ring was varied; these compounds were screened as inhibitors of four different enzyme classes, either *in vitro* or *in vivo*. Potent inhibitors were found that selectively inhibited enzymes that cleave carotenoids at the 9,10 position; 50% inhibition was achieved at submicromolar concentrations. Application of certain inhibitors at 100 μM to *Arabidopsis* node explants or whole plants led to increased shoot branching, consistent with inhibition of 9,10-cleavage.

Carotenoids are synthesized in plants and micro-organisms as photoprotective molecules and are key components in animal diets, an example being β-carotene (pro-vitamin A). The oxidative cleavage of carotenoids occurs in plants, animals, and micro-organisms and leads to the release of a range of apocarotenoids that function as signaling molecules with a diverse range of functions (1). The first gene identified as encoding a carotenoid cleavage dioxygenase (CCD)[2] was the maize *Vp14* gene that is required for the formation of abscisic acid (ABA), an important hormone that mediates responses to drought stress and aspects of plant development such as seed and bud dormancy (2). The VP14 enzyme cleaves at the 11,12 position (Fig. 1) of the epoxycarotenoids 9′-*cis*-neoxanthin and/or 9-*cis*-violaxanthin and is now classified as a 9-*cis*-epoxycarotenoid dioxygenase (NCED) (3), a subclass of the larger CCD family.

Since the discovery of *Vp14,* many other CCDs have been shown to be involved in the production of a variety of apocarotenoids (Fig. 1). In insects, the visual pigment retinal is formed by oxidative cleavage of β-carotene by β-carotene-15,15′-dioxygenase (4). Retinal is produced by an orthologous enzyme in vertebrates, where it is also converted to retinoic acid, a regulator of differentiation during embryogenesis (5). A distinct mammalian CCD is believed to cleave carotenoids asymmetrically at the 9,10 position (6) and, although its function is unclear, recent evidence suggests a role in the metabolism of dietary lycopene (7). The plant volatiles β-ionone and geranylacetone are produced from an enzyme that cleaves at the 9,10 position (8) and the pigment α-crocin found in the spice saffron results from an 7,8-cleavage enzyme (9).

Other CCDs have been identified where biological function is unknown, for example, in cyanobacteria where a variety of cleavage specificities have been described (10–12). In other cases, there are apocarotenoids with known functions, but the identity or involvement of CCDs have not yet been described: grasshopper ketone is a defensive secretion of the flightless grasshopper *Romalea microptera* (13), mycorradicin is produced by plant roots during symbiosis with arbuscular mycorrhyza (14), and strigolactones (15) are plant metabolites that act as germination signals to parasitic weeds such as *Striga* and *Orobanche* (16).

Recently it was discovered that strigolactones also function as a branching hormone in plants (17, 18). The existence of such a branching hormone has been known for some time, but its identity proved elusive. However, it was known that the hormone was derived from the action of at least two CCDs, max3 and max4 (*m*ore *ax*illary growth) (19), because deletion of either of these genes in *Arabidopsis thaliana*, leads to a bushy phenotype (20, 21). In *Escherichia coli* assays, AtCCD7 (max3) cleaves β-carotene at the 9,10 position and the apocarotenoid product (10-apo-β-carotene) is reported to be further cleaved at 13,14 by AtCCD8 (max4) to produce 13-apo-β-carotene (22). Also recent evidence suggests that AtCCD8 is highly specific, cleaving only 10-apo-β-carotene (23). How the production of 13-apo-β-carotene leads to the synthesis of the complex strigolactone is unknown. The possibility remains that the enzymes may have different specificities and cleavage activities *in planta.* In addition, a cytochrome P450 enzyme (24) is believed to be involved in strigolactone synthesis and acts in the pathway downstream of the CCD genes. Strigolactone is

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental materials, Table S1, and Figs. S1 and S2.

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[2] The abbreviations used are: CCD, carotenoid cleavage dioxygenase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; *max*, more axillary growth; WT, wild type; GST, glutathione *S*-transferase; HPLC, high performance liquid chromatography; NAA, α-naphthalene acetic acid; ACO, apocarotenoid-15,15′-oxygenase.

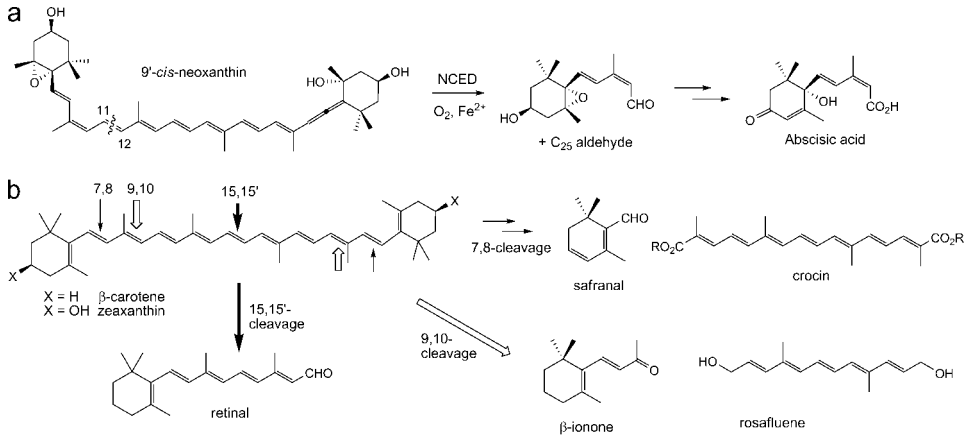


FIGURE 1. **Reactions catalyzed by the carotenoid cleavage dioxygenases.** *a*, 11,12-oxidative cleavage of 9′-*cis*-neoxanthin by NCED; *b*, oxidative cleavage reactions on β-carotene and zeaxanthin.

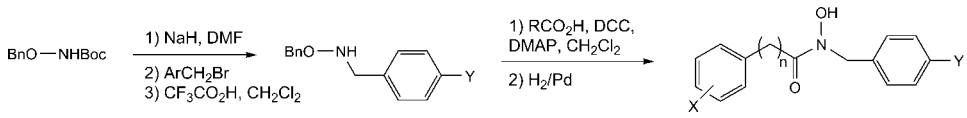


FIGURE 2. **Synthetic route for preparation of hydroxamic acid inhibitors.**

thought to effect branching by regulating auxin transport (25). Because of the involvement of CCDs in strigolactone synthesis, the possibility arises that plant architecture and interaction with parasitic weeds and mycorrhyza could be controlled by the manipulation of CCD activity.

Although considerable success has been obtained using genetic approaches to probe function and substrate specificity of CCDs in their native biological contexts, particularly in plant species with simple genetic systems or that are amenable to transgenesis, there are many systems where genetic approaches are difficult or impossible. Also, when recombinant CCDs are studied either *in vitro* or in heterologous *in vivo* assays, such as in *E. coli* strains engineered to accumulate carotenoids (26), they are often active against a broad range of substrates (5, 21, 27), and in many cases the true *in vivo* substrate of a particular CCD remains unknown. Therefore additional experimental tools are needed to investigate both apocarotenoid and CCD functions in their native cellular environments.

In the reverse chemical genetics approach, small molecules are identified that are active against known target proteins; they are then applied to a biological system to investigate protein function *in vivo* (28, 29). This approach is complementary to conventional genetics since the small molecules can be applied easily to a broad range of species, their application can be controlled in dose, time, and space to provide detailed studies of biological functions, and individual proteins or whole protein classes may be targeted by varying the specificity of the small molecules. Notably, functions of the plant hormones gibberellin, brassinosteroid, and abscisic acid have been successfully probed using this approach by adapting triazoles to inhibit specific cytochrome P450 monooxygenases involved in the metabolism of these hormones (30).

In the case of the CCD family, the tertiary amines abamine (31) and the more active abamineSG (32) were reported as specific inhibitors of NCED, and abamine was used to show new functions of abscisic acid in legume nodulation (33). However, no selective inhibitors for other types of CCD are known. Here we have designed a novel class of CCD inhibitor based on hydroxamic acids, where variable chain length was used to direct inhibition of CCD enzymes that cleave carotenoids at specific positions. We demonstrate the use of such novel inhibitors to control shoot branching in a model plant.

EXPERIMENTAL PROCEDURES

Synthesis of Tertiary Amine Inhibitors—Abamine was synthesized according to published procedures (31, 34).

Synthesis of Hydroxamic Acid Inhibitors—Synthesis is shown in Fig. 2 and structures are given in Table 1. *N*-Boc,*O*-benzyl-hydroxylamine was treated with NaH in DMF, followed by the appropriate benzyl or alkyl bromide (35). Deprotection was carried out by treatment with 1% trifluoroacetic acid in dichloromethane, to give the *N*-substituted hydroxylamine. Hydroxamic acid formation was carried out using DCC (1.1 equiv.) and 4-dimethylaminopyridine (0.2 equiv.) and the appropriate carboxylic acid, in dichloromethane. The hydroxamic acid products were purified by silica gel column chromatography. B1, D12, and D13 were prepared by activation of the appropriate acid with methyl chloroformate and triethylamine in THF, and reaction with hydroxylamine hydrochloride. The intermediate for synthesis of B1 was synthesized from β-ionone (36); B1 was isolated as a 2:1 mixture of E/Z isomers. Spectroscopic data and yields for analogues D1–D13, F1–4, and B1 are available as supplemental materials.

In Vitro NCED Enzyme Assay—We overexpressed LeNCED1 in *E. coli*, as an N-terminal His_6 fusion protein (supplemental materials). Cell-free extract containing recombinant LeNCED1 was prepared in 100 mM bis-Tris buffer (pH 6.7). 15-μl extract was pre-activated by addition of iron (II) sulfate (20 mM, 1 μl) and ascorbic acid (20 mM, 1 μl) on ice for 2 min, prior to use. This aliquot of enzyme was then added to an assay (150 μl total volume) containing 100 mM bis-Tris buffer (pH 6.7), 0.05% v/v Triton X-100, 1.0 mg ml^{-1} catalase, and 3 μg of 9′-*cis*-neoxanthin. The 9′-*cis*-neoxanthin substrate was prepared as described in supplemental materials. The enzyme assay was incubated for 15 min in the dark at 20 °C. Water (700 μl) was then added, and the products extracted with ethyl acetate (3 × 1 ml). The organic solvent was removed at reduced pressure, the residue was dissolved in methanol (200 μl), and then 100 μl was injected onto a Phenomenex C_{18} reverse phase HPLC column, and a gradient of 5–10% methanol in acetonitrile/0.05% triethylamine was applied at 0.5 ml min^{-1} over 20 min, detecting at 440 nm. NCED inhibition assays contained 1–100 μM inhibitor; inhibition was calculated from the product formation after 15 min, compared with a control assay with no inhibitor

present. Retention times: 9′-*cis*-neoxanthin, 10.2 min; C_{25} product, 6.5 min.

In Vitro LeCCD1a Enzyme Assay—We overexpressed LeCCD1a in *E. coli*, as a GST fusion protein (supplemental materials). The *in vitro* assay of LeCCD1a was based on reported methods (37), and was carried out in a 200-μl total volume in a 96-well microtiter plate, with the signal detected at 485 nm. To prepare substrate solution for each assay, 5 μl of 4% (w/v in ethanol) apo-8′-carotenal (Sigma) was mixed with 25 μl of 4% (w/v in ethanol) β-octylglucoside (Sigma), the ethanol was then evaporated under nitrogen, and the residue dissolved in 150 μl of phosphate-buffered saline buffer containing 10 mM sodium ascorbate by incubation at 20 °C for 30 min. 50 μl of cell-free extract containing recombinant LeCCD1a was added, and the reactions monitored over 30 min at 20 °C.

In Vivo Enzyme Assays in E. coli—Genes of interest (supplemental Table S1) were cloned into the vector pET30c (Novagen) fused directly to the initial ATG codon with no tag, or into pGEX-4T such that the gene was fused to an N-terminal GST tag. All genes were full-length except AtCCD7, which had the chloroplast signal sequence removed (supplemental materials). This gene, when fused to GST in the pGEX-4t vector (GE Healthcare), showed greater CCD activity than when expressed in a pET vector without a tag. Therefore, this construct was used in subsequent assays. The plasmids were transferred to the *E. coli* expression strain BL21(DE3), harboring pAC-BETA (38), and therefore producing β-carotene.

For each inhibitor assay, 2.5 ml of LB medium with the appropriate antibiotics (25 μg ml^{-1} chloramphenicol and 50 μg ml^{-1} kanamycin or 100 μg ml^{-1} ampicillin) and 2.5 μM isopropyl-1-thio-β-D-galactopyranoside, was prepared. Inhibitors (0.1 M in 100% ethanol) were added to the media to a final concentration of 100 μM. The media was then inoculated with 0.25 ml of overnight culture (grown at 37 °C with the appropriate antibiotics) and incubated with shaking (200 rpm) at 28 °C for 16 h. 1 ml of culture was harvested by microcentrifugation and resuspended thoroughly in 1 ml of ethanol containing 0.2% Triton X-100. After vortexing, the extract was incubated at room temperature in the dark for 3 h, again vortexed and then spun in a microcentrifuge for 5 min at 13,000 rpm. The supernatant was removed, and the $A_{453} - A_{550}$ was measured. The amount of β-carotene was calculated using a standard curve generated from a dilution series of β-carotene (Sigma) in ethanol with 0.2% Triton X-100 (supplemental Fig. S1). The relative inhibition was calculated by the equation: $(C_i - C_c)/(C_l - C_c) \times 100$, where C_i is the carotenoid level with inhibitor and CCD present, C_c is the level with CCD but without the inhibitor and C_l is the level in a strain where lacZ is expressed instead of the CCD, and no inhibitor is present. Thus the increase in β-carotene due to inhibition of the CCD ($C_i - C_c$) was expressed relative to the maximum possible β-carotene content when CCD is absent ($C_l - C_c$).

Growth of Arabidopsis—Wild-type (ecotype Col-0) and the *max3-9* mutant in the Col-0 background (21) were grown in double Magenta pots (Sigma) on 30 ml of ATS media (39) containing 1% sucrose and 0.8% agar, supplemented, where stated, with 100 μM inhibitor from a 100 mM stock in ethanol. Seeds were sterilized by immersion for 1 min in 70% ethanol and 4 min in 50% household bleach before being washed five times in distilled water. Six seeds were placed in each Magenta pot, they were vernalized in the dark at 4 °C for 2 days, and then placed in a growth room (24 °C, 16-h light period, 150 μmol m^2 s^{-1} photosynthetically active radiation) for 45 days before being photographed. Side shoots from rosette nodes were counted for each plant.

Axillary Bud Outgrowth Assay in Arabidopsis Stem Sections—Assays were performed essentially as described (40) with the following modifications. Small Petri dishes (50-mm diameter, 20-mm depth) were filled with 10 ml of ATS (39) supplemented with 1% agar and 1% sucrose. Inhibitors and α-naphthalene acetic acid (NAA) were added to the agar before pouring to give 100 μM and 1 μM, respectively. Thus, when the central strip was cut out of the agar, both the apical and basal media contained both NAA and inhibitor. Any nodes in which the apical end had curled out of the media or in which bud length was less than 2 mm at the end of the experiment were discounted. Measurement of the shoot length was performed every 24 h. For each assay a logistic curve was fitted using Genstat (10th edition, VSN international) with the fitcurve directive and the lag phase was calculated by extrapolating the linear part of the curve and the initial plateau (see supplemental materials). The x value of where these two lines intersected represented the lag phase.

Statistical Analysis—For data in Figs. 4 and 5, analysis was by Residual Maximum Likelihood (REML) in Genstat10. In both cases, an F-test showed that overall the treatment effects were highly significant ($p < 0.001$). The maximum value of the least significant difference (LSD) was calculated by multiplying the maximum standard error of differences by a *t*-value ($p = 0.05$), and is presented on the graphs. There were 105 and 157 degrees of freedom for the LSDs shown in Figs. 4 and 5, respectively. The maximum LSD was used because individual LSDs varied but if differences between means were significant using the maximum values then they were also significantly different at the individual value for any two selected means.

RESULTS

Inhibitor Design and Synthesis—NCED was proposed to be a dioxygenase (3), with a reaction mechanism involving a carbocation intermediate, followed by formation of a dioxetane ring or a Criegee rearrangement prior to cleavage (41); such a mechanism was supported by ^{18}O labeling experiments with AtCCD1 (37), and was the most likely mechanism based on computational studies of the ACO crystal structure (42).

It was reported that the tertiary amine abamine (see Fig. 3*a* for structure) is a reversible competitive inhibitor ($K_i = 39$ μM) of recombinant NCED and that it inhibited abscisic acid production *in planta* at 50–100 μM concentration (31). AbamineSG, with an extended 3 carbon linker between the methyl ester and the nitrogen atom, was subsequently developed with an improved activity ($K_i = 18.5$ μM) (32). The precise mechanism of action of abamine is uncertain, but our hypothesis was that the protonated amine mimics a carbocation intermediate in the catalytic mechanism, with the oxygenated aromatic ring bound in place of the hydroxy-cyclohexyl terminus of the carotenoid substrates (41), as shown in Fig. 3. Inhibition may be due in part to chelation of the essential metal ion cofactor by the

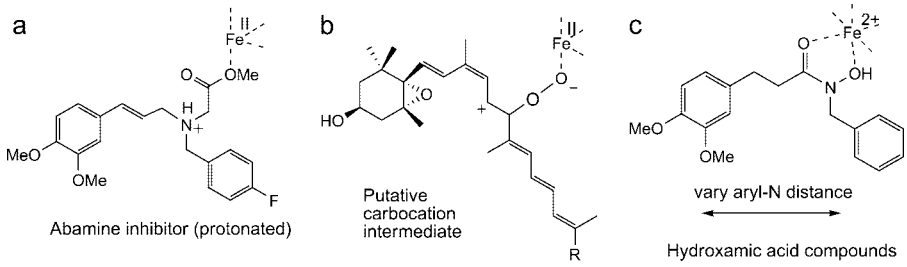


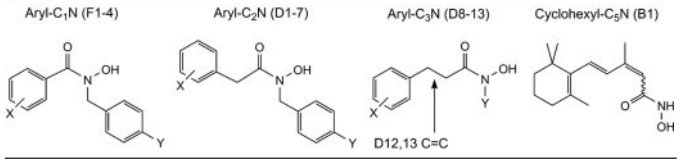
FIGURE 3. **Inhibitor design.** Protonated abamine (*a*), a carotenoid carbocation intermediate (*b*), and a hydroxamic acid inhibitor (*c*) are shown bound to the iron(II) cofactor of a CCD.

TABLE 1

Inhibition of recombinant LeCCD1a and LeNCED1 enzymes using *in vitro* assays

Enzyme assays, using *E. coli* cell extracts containing the recombinant CCD, were initially carried out at 100 μM inhibitor concentration; for compounds showing ≥95% inhibition of LeCCD1a at this concentration, IC_{50} values were also determined. NT, not tested. Chemical structures of hydroxamic acid inhibitors are shown below, with X and Y given in the table. The structure of abamine is given in Fig. 3.

Inhibitor				*LeCCD1a* (9,10/9′,10′)		*LeNCED1* (11,12)
Class	Name	X	Y	Inhibition @ 100 μM (%)	IC_{50} (μM)	Inhibition @ 100 μM (%)
Abamine				35 ± 15[a]	210	20
Aryl-C_1N	F1	4-OMe	H	>95	2.0	0
	F2	4-OMe	F	>95	2.5	0
	F3	3,4-$(OMe)_2$	H	50		2
	F4	3,4-$(OMe)_2$	F	0		0
Aryl-C_2N	D1	4-OH	H	>95	0.9	27
	D2	4-OH	F	>95	0.8	29
	D3	3,4-$(OH)_2$	F	>95	0.8	4
	D4	4-OMe	F	>95	2.5	33
	D5	3,4-$(OMe)_2$	H	>95	8.0	8
	D6	3,4-$(OMe)_2$	F	>95	9.0	18
	D7	3,4-OCH_2O	F	>95	3.0	33
Aryl-C_3N	D8	3,4-$(OMe)_2$	CH_2Ph	61		40
	D9	4-OMe	CH_2Ph	>95	10	27
	D10	3,4-$(OMe)_2$	n-octyl	65		14
	D11	4-OMe	n-octyl	53		15
	D12	3,4-$(OMe)_2$	H	26		11
	D13	4-OMe	H	46		13
Ring-C_5N	B1			90	20	5



[a] Mean and range of two independent experiments.

methyl ester of abamine. However, a derivative of abamine, containing an acid group (COOH) in place of the methyl ester, was not active (32), even though in theory this should be more effective at binding the iron cofactor.

Hydroxamic acids are known to act as inhibitors of several different classes of metalloenzymes, such as the matrix metalloproteases, by chelation of the essential metal ion cofactor (43). Therefore, hydroxamic acid analogues were synthesized, in which the hydroxy-cyclohexyl terminus of the carotenoid substrate was mimicked as above by an oxygenated aromatic ring, and the hydroxamic acid functional group was positioned at variable distance from the aromatic ring. Thus, a collection of aryl-C_3N analogues (D8–D13), aryl-C_2N analogues (D1–D7), and aryl-C_1N analogues (F1–F4) was also synthesized (Table 1). The 4-fluorobenzyl substituent, found to promote activity in the abamine series (31), was included in the collection of hydroxamic acids. The synthetic route, shown in Fig. 2, involves coupling of the appropriate acid with a substituted *O*-benzyl hydroxylamine, followed by deprotection. One hydroxamic acid containing a longer C_5 spacer from a cyclohexyl moiety (B1) was also synthesized from β-ionone. A set of 18 hydroxamic acids was then used for inhibitor screening; numbering of chemical compounds is given in Table 1.

Specificity of Inhibition in Vitro for Tomato Genes LeNCED1 and LeCCD1a—To screen the inhibitors against enzymes which cleave carotenoids at the 9,10 position, we used the recombinant tomato LeCCD1a protein (44), because this type of enzyme can be studied using an *in vitro* colorimetric assay with β-apo-8′-carotenal as substrate (37). To establish the specificity of the inhibitors, they were also tested against the tomato LeNCED1 recombinant protein which cleaves 9-*cis* carotenoids at the 11,12 position (45). For this enzyme, the cleavage reaction was monitored by C_{18} reverse phase HPLC, using 9′-*cis*-neoxanthin as substrate. As reported by others (46), each enzyme activity was found to be unstable (lifetime < 24 h) toward storage or purification; therefore enzyme assays were carried out using recombinant cell-free extract (no cleavage activity was observed using *E. coli* extract lacking the recombinant CCD gene).

Against LeNCED1, several hydroxamic acids (notably D8, D7, and D4) showed 1.5–2-fold higher inhibitory activity than the designated NCED inhibitor, abamine (31), which in our hands showed only 20% inhibition at 100 μM concentration (see Table 1). Against LeCCD1a, potent inhibition was observed by all the aryl-C_2N hydroxamic acids, and certain other hydroxamic acids. 4-methoxyaryl hydroxamic acids were effective inhibitors in each series, but the most potent inhibition was observed with the 4-hydroxyaryl hydroxamic acids D1, D2, and D3, which gave IC_{50} values of 0.8 – 0.9 μM. In contrast, abamine gave an IC_{50} value of 210 μM against LeCCD1a (Table 1).

Comparison of inhibition data for LeNCED1 and LeCCD1a shows that all the active compounds show some selectivity toward LeCCD1a, with compounds D3, F1, and F2 showing high levels of inhibition of LeCCD1a, and little or no inhibition of LeNCED1 (Table 1).

In Vivo Activity of Inhibitors Applied to E. coli Strains Expressing CCDs—Colored *E. coli* strains that produce various carotenoids can be constructed by expression of enzymes for carotenoid synthesis (26). Upon co-expression of the appropriate CCD, the bacteria lose their color due to cleavage of the carotenoids to colorless products (4, 6, 27). This technique was employed here to further explore the specificity of inhibitors, and to test their activity *in vivo*. The level of carotenoid in each CCD-expressing strain was compared with the level of the carotenoid in a control strain producing β-carotene but lacking any CCD gene. The difference in the carotenoid levels gave a measure of CCD activity, and inhibition of this activity was measured by addition of inhibitors to the growing medium.

The inhibitors were tested against four β-carotene-producing *E. coli* strains (supplemental Table S1). Three of the strains expressed highly divergent CCDs that cleave at the 9,10 posi-

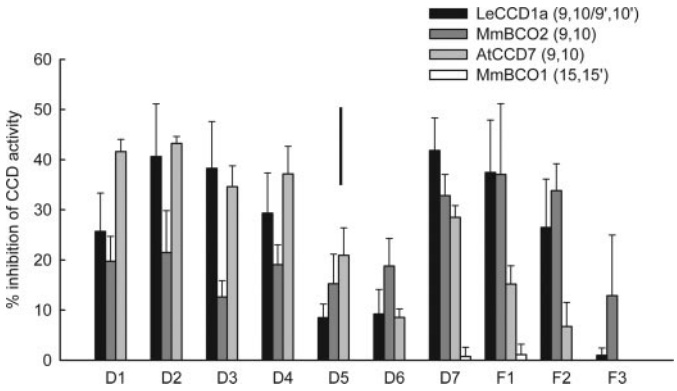


FIGURE 4. **The relative inhibition of four CCDs in *E. coli*.** CCD genes were expressed in *E. coli* strains that produce β-carotene. The strains were grown in the presence or absence of inhibitors (100 μM) for 16 h. This concentration of inhibitor was within the linear range of the *E. coli* response (see supplemental Fig. S2). The relative inhibition of each class of CCD was determined by the increase in β-carotene accumulation in the presence of the inhibitor, a value of 0 would indicate β-carotene levels equal to when no inhibitor was present, and a value of 100 would equal the maximum level of β-carotene as observed in strains lacking a CCD (see "Experimental Procedures" for equation). Error bars represent the S.E., $n = 4$. The floating *black bar* represents the least significant difference ($p < 0.05$) for comparison of any two means.

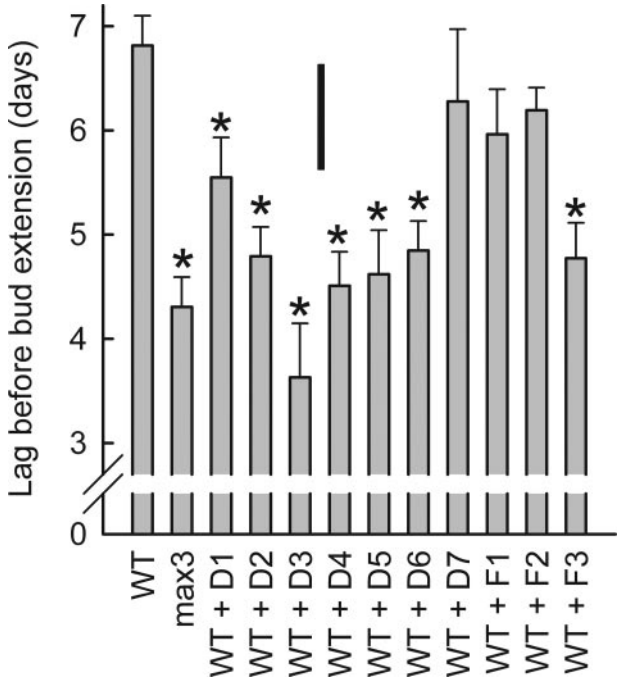


FIGURE 5. **The effect of inhibitors on the outgrowth of buds from excised *Arabidopsis* nodes in the presence of 1 μM NAA.** The graph shows lag time before the commencement of bud outgrowth for Col-0 (WT) in the presence or absence of 100 μM inhibitor. A null mutant of *AtCCD7* (*max3*) was included without inhibitor as control. Values represent means from five independent experiments; $n = 35$ (WT), $n = 18$ (*max3*), $n = 14–16$ (WT plus inhibitors). The floating *black bar* represents the least significant difference for comparison of any two means, and *asterisks* indicate values significantly different from the WT ($p < 0.05$).

tion: AtCCD7 from *Arabidopsis* (21) and MmBCO2 from mouse (6) which both cleave at a single site (9,10 or 9′,10′ but not both), and the tomato enzyme LeCCD1a (44) which cleaves at both sites in the same substrate molecule (9,10/9′,10′ activity). The fourth strain expressed another mouse CCD, MmBCO1 (47), which cleaves centrally at 15,15′. Ourselves and other researchers (48) have found that expressing CCDs (and presumably other proteins) can lead to loss of carotenoids by nonspecific means. However, detection of cleavage products by HPLC in the *E. coli* cells and media confirmed that in all four strains used here, CCD cleavage was the cause of carotenoid loss. NCED activity could not be studied in *E. coli* cells because the enzyme required for production of the 9-*cis* carotenoid substrates of NCED has not yet been identified. We synthesized the genes *CsZCD* (9) and *BoLCD* (49), expressed them in *E. coli*, and looked for the reported 5,6 and 7,8 cleavage activities both *in vitro* and in *E. coli* cells. However, we were not able to detect activity, and so it was not possible to test inhibitors against the 5,6 and 7,8 cleavage specificities.

The compounds showed different patterns of inhibition against the three 9,10 enzymes (Fig. 4). The activity of the compounds against LeCCD1a *in vivo* (Fig. 4) mirrored the activity observed *in vitro* (Table 1): D5 and D6 exhibited relatively weaker inhibition activity than the other D compounds and F3 exhibited virtually none. A different pattern was obtained with AtCCD7 in the *E. coli* system (Fig. 4), with the compounds F1 and F2, which exhibited good activity against LeCCD1a, showing poor inhibition. In contrast F1 and F2 were the most effective compounds at inhibiting MmBCO2 (Fig. 4). The 15,15′ cleavage enzyme MmBCO1 was not inhibited to any significant extent by any of the compounds tested (Fig. 4).

Stimulation of Shoot Branching in Arabidopsis Stem Sections by Application of Inhibitor—Auxin inhibits the outgrowth of axillary buds in wild-type *Arabidopsis* plants. In the *AtCCD7* and *AtCCD8* null mutants (*max3* and *max4*, respectively) the response to auxin is reduced, presumably due to a block in formation of an apocarotenoid hormone (recently shown (17, 18) to be strigolactone or a related compound) that suppresses branching (19), and axillary buds extend earlier, leading to formation of side branches. An *in vitro* assay was previously developed in which the growth of axillary buds from isolated sections of *Arabidopsis* stem was used to assess *max* mutants (40). In such assays, it was reported that bud outgrowth of the *max4-1* mutant (*AtCCD8*) was 2 days earlier than for wild-type (20) and a similar phenotype is expected of the highly branched *max3-9* mutant (21). We tested hydroxamic acid inhibitors at 100 μM in this assay and found that D1 to D6, and F3 all significantly ($p < 0.05$) advanced the timing of bud outgrowth in wild-type, with the advancement ranging from 1 day (D1) to 3 days (D3) (Fig. 5). This earlier bud outgrowth was equivalent to that observed in the AtCCD7-null mutant *max3-9* (Fig. 5), and indicates an inhibition of AtCCD7 and/or possibly AtCCD8 in this tissue. The effect of the inhibitors in this assay only partially mirrored the activities in the *E. coli* assay, with compounds F1 and F2 having a relatively small activity in both the bud outgrowth assay (Fig. 5) and the *E. coli* AtCCD7 assay (Fig. 4). However, in the case of F3 there was disagreement because it was inactive in the *E. coli* assay for AtCCD7, but it was active in stimulating bud outgrowth. One possibility in this case is that F3 stimulated branching by inhibiting AtCCD8 (not tested *in vitro* or in *E. coli*) rather than AtCCD7.

Stimulation of Shoot Branching in Whole Arabidopsis Plants—Inhibitors were also applied to *Arabidopsis* whole plants grown under sterile conditions in agar. The *max3-9* plants (Fig. 6*b*)

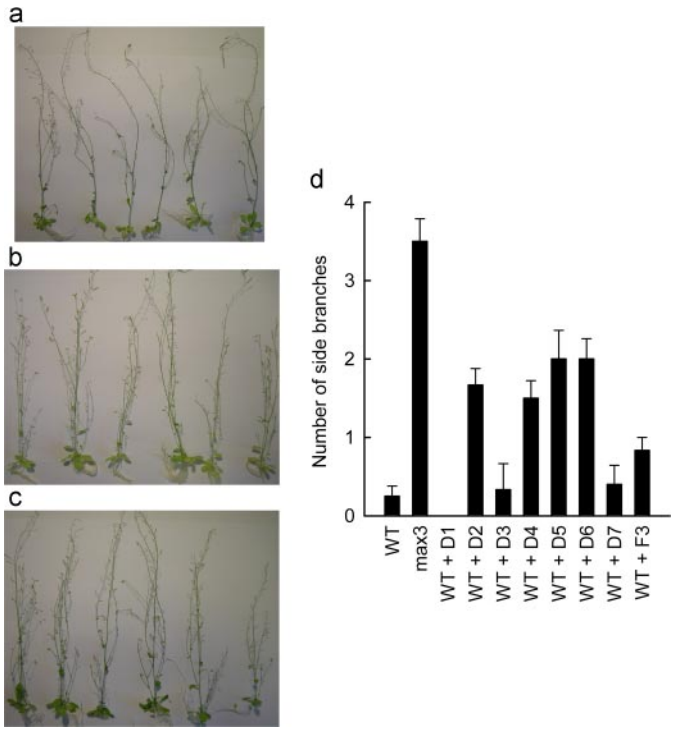


FIGURE 6. **Branching phenotypes of *Arabidopsis* plants grown in agar media for 45 days supplemented with inhibitor D6.** Images are shown: (*a*) Col-0 (WT) without inhibitor; (*b*) *max3-9* mutant without inhibitor; (*c*) Col-0 with 100 μM D6. The numbers of rosette branches were also quantified (*d*). Error bars represent S.E., $n = 6$ to 12.

and those treated with D2, D4, D5, and D6 (Fig. 6*c* shows D6-treated plants) exhibited a bushy appearance compared with the untreated wild-type controls (Fig. 6*a*). This bushy appearance was due to the increased number of side branches from the rosette nodes, with *max3-9* plants exhibiting 3 to 4 side branches compared with a mean of 0.25 for wild type. Inhibitor treated plants were intermediate (mean of ~2 branches) and so partially mimicked *max3-9* (Fig. 6*d*).

F1 and F2 were toxic to whole plants at 100 μM (data not shown). D3 was active in the stem section assay (Fig. 5), but in whole plants D3 had a negative effect on *Arabidopsis* growth when added to the agar media, and a toxicity effect was suggested by the observation that roots grew across the agar surface rather than penetrating. This general growth effect may have masked any possible stimulatory effects of D3 on side branching.

DISCUSSION

We have designed and tested a new class of inhibitor of the carotenoid cleavage dioxygenase family that is based on a structural mimic of the substrate that positions an iron-chelating hydroxamic acid group within the active site. Positioning was achieved by varying the distance between the hydroxamic acid and an aromatic ring so that it matched the distance within the carotenoid substrate between the proximal cyclic end-group and the cleavage site. Crystal structure of ACO, a cyanobacterial CCD, indicates that cleavage position is likely to be determined by the distance between the Fe(II) catalytic center and the opening of the long non-polar tunnel that allows access to carotenoid substrates (11). This idea is supported by the observation that for NosCCD (from *Nostoc* sp. PCC 7120) cleavage of the monocyclic γ-carotene occurs at the 7′,8′ position where the proximal terminus is linear, but at the 9,10 position when the proximal terminus has a more compact cyclic end group (48); indeed it was suggested that the cyclic end group may be arrested at the entrance of the tunnel (48).

We predicted from this crystal structure, and our model for the cleavage mechanism (Fig. 3), that aryl-C_1N, aryl-C_2N and aryl-C_3N compounds would be selective for 7,8, 9,10 and 11,12 cleavage reactions, respectively; we tested these classes against enzymes with 9,10, 11,12, and 15,15′ specificities. Certain aryl-C_1N compounds (F1, F2) were effective inhibitors of 9,10 but not 11,12 or 15,15′ cleavages. The aryl-C_2N compounds were potent inhibitors of 9,10 enzymes, but also had a moderate 11,12 inhibition activity. The aryl-C_3N compounds were much less potent against 9,10 enzymes, and although this group contained the best 11,12 inhibitor (D8), they all still maintained a somewhat greater selectivity toward the 9,10 cleavage. In comparison, a further analogue, abamineSG, was reported to be more active against the 11,12 cleavage than the 9,10 cleavage; at 100 μM it inhibited AtNCED3 by 78% and AtCCD1 by ≤20% (32). None of the compounds tested inhibited the 15,15′ enzyme, presumably because the spacing was too small. Thus we conclude that the strategy of varying the positioning of the hydroxamic acid group was only moderately successful, since some overlap existed between the classes. Nevertheless, individual compounds were identified with very high specificity to the 9,10 cleavage *in vitro*, *e.g.* IC_{50} for F1 was 2.0 μM but no inhibition of LeNCED1 was detected.

The inhibitors also exhibited different patterns of activity in *E. coli* against the three different enzymes with 9,10 cleavage activity. For example, F1 and F2 had high inhibitory activity against LeCCD1a and MmBCO2 but were relatively ineffective against AtCCD7. Such differences are not surprising since MmBCO2 shares only 17–23% amino acid identity with the two plant 9,10 enzymes (LeCCD1a and AtCCD7), which are themselves highly divergent, with only 19% identity to each other. This indicates that the variants of the hydroxamic acid inhibitors are able to distinguish between enzymes that have similar activities but highly divergent primary structure.

The *E. coli* system proved useful in measuring the efficacy of the inhibitors *in vivo*. For example, the *E. coli* assays showed F1 and F2 were poor inhibitors against AtCCD7 and this was confirmed in the *Arabidopsis* bud outgrowth assay (Fig. 5), which measures AtCCD7 and/or AtCCD8 activity. However, although D5 and D6 were poor in the *E. coli a*ssays they showed the largest effect on whole *Arabidopsis* plants, giving the greatest number of side branches. Also D1 and D3 appeared to be good inhibitors of the 9,10 enzymes *in vitro* (Table 1), in *E. coli* (Fig. 4) and in the bud outgrowth assay (Fig. 5), but D3 had negative effects on growth, which confounded the branching assay in whole plants, whereas plants treated with D1 grew normally and without an increase in branching. D1 and D3 both contained a more polar hydroxyl group on the aryl ring, therefore it is possible that these compounds are more actively transported or metabolized in the plant.

Overall, the different activities observed in different assays suggest that factors such as uptake, metabolism, and effects on

non-target processes may play a role in determining the suitability and effectiveness of the inhibitors *in planta*. Our results underline the importance of performing secondary screens in the biological systems where the compounds are to be used. Here we have been able to demonstrate that D2, D4, D5, and D6 appear to inhibit CCDs in all the assays tested, including *in planta*, without negative unintended effects on whole plants. These compounds represent useful chemical genetic agents to explore the function of CCDs in plants, animals, and micro-organisms.

Using the inhibitors described here, it will now be possible to inhibit the CCD(s) involved in branching in a wide range of plant species and then look for changes in carotenoids and apocarotenoids; this could provide a powerful approach for the identification of the precursors of strigolactone, the identity of other active strigolactone-related compounds, and to the further elucidation of the biosynthetic pathway. The inhibitors could also be used to probe for functional variation in the role of strigolactone between species. Branching-promoting chemicals may have applications in horticulture where compact plant architecture is often highly desirable, *e.g.* in orchard crops (50).

Other biological systems where genetic manipulations are not practical include the production of saffron (9) and bixin (49) in *Crocus* and *Bixa* plants respectively, where the *in vivo* substrates of the CCDs involved are not clear, and also in the study of the functions of mycorradicin and strigolactone in plant interactions with mycorrhyza (51) and parasitic weeds (15), respectively. Finally, there may be pharmaceutical applications for inhibitors of BCO2 in humans because products from 9,10 carotenoid cleavage have been implicated in DNA damage and carcinogenesis (52, 53).

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