

The Path from β -Carotene to Carlactone, a Strigolactone-Like Plant Hormone

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Strigolactones, phytohormones with diverse signaling activities, have a common structure consisting of two lactones connected by an enol-ether bridge. Strigolactones derive from carotenoids via a pathway involving the carotenoid cleavage dioxygenases 7 and 8 (CCD7 and CCD8) and the iron-binding protein D27. We show that D27 is a β -carotene isomerase that converts all-*trans*- β -carotene into 9-*cis*- β -carotene, which is cleaved by CCD7 into a 9-*cis*-configured aldehyde. CCD8 incorporates three oxygens into 9-*cis*- β -apo-10'-carotenal and performs molecular rearrangement, linking carotenoids with strigolactones and producing carlactone, a compound with strigolactone-like biological activities. Knowledge of the structure of carlactone will be crucial for understanding the biology of strigolactones and may have applications in combating parasitic weeds.

The biosynthetic carotenoid pathway provides precursors for smaller, physiologically important compounds such as the plant hormone abscisic acid (1), the vertebrate morphogen retinoic acid (2), and the fungal pheromone trisporic acid (3), which originate from oxidative cleavage of C-C double bonds in carotenoid backbones (4–7). The derivative compounds, named apocarotenoids (see fig. S1 for nomenclature and structures), may undergo structural modifications concealing their origin. Strigolactones, a prominent example of such compounds, are a group of metabolites with a common C₁₉ structure (e.g., 5-deoxystrigol, Fig. 1A) consisting of a tricyclic lactone (A, B, and C rings) connected via an enol ether bridge to a second lactone (D ring) (8). Several lines of evidence demonstrate that strigolactones derive from carotenoids. A putative biosynthetic pathway has been proposed, leading from a C₁₅ apocarotenal to the ABC C₁₄ skeleton, which is then coupled to a butenolide group (D ring) of unknown origin (9).

Released by plant roots, strigolactones trigger seed germination of root parasitic weeds such as witchweeds (*Striga* spp.) (10) and can induce hyphal branching in arbuscular mycorrhizal fungi, an essential step in establishing the symbiosis

(11). In plants, strigolactones inhibit tillering and shoot branching. High tillering (more branching) is observed in plant mutants disrupted in *Carotenoid Cleavage Dioxygenase 7* (CCD7) and *CCD8*, in *Dwarf27* (D27) encoding an iron-binding polypeptide with unknown catalytic properties, or in a cytochrome P450 gene represented by *More Axillary Growth 1* (MAX1) from

Arabidopsis thaliana (8, 12–17). The application of the synthetic strigolactone GR24 restored the wild type tillering/branching phenotype of these mutants, suggesting the involvement of CCD7, CCD8, D27, and MAX1 in strigolactone biosynthesis (8, 12–17).

CCDs are nonheme iron enzymes that cut C-C double bonds by incorporating a dioxygen, yielding carbonyl products (4–7). It was supposed that CCD7 from *Arabidopsis* (AtCCD7) cleaves all *trans* β carotene (C₄₀; Fig. 1A) into all *trans* β apo 10' carotenal (C₂₇) that is then further shortened by AtCCD8 to the C₁₈ ketone β apo 13 carotenone (Fig. 1B) (18), a biologically active compound affecting the growth of root hairs in plants (19). The sequential activity of the two cleavage enzymes was deduced from the coexpression of AtCCD7 and AtCCD8 in *Escherichia coli* strains accumulating all *trans* β carotene (18) and confirmed by in vitro studies showing the formation of β apo 13 carotenone from all *trans* β apo 10' carotenal by CCD8 enzymes from different plant species (20). However, the structure of β apo 13 carotenone has little in common with that of strigolactones, and treatment of rice *ccd8* mutant with this compound did not restore the wild type tillering phenotype (fig. S2).

We used an in vitro approach to investigate the activities of CCD7 and CCD8 from *Arabidopsis*,

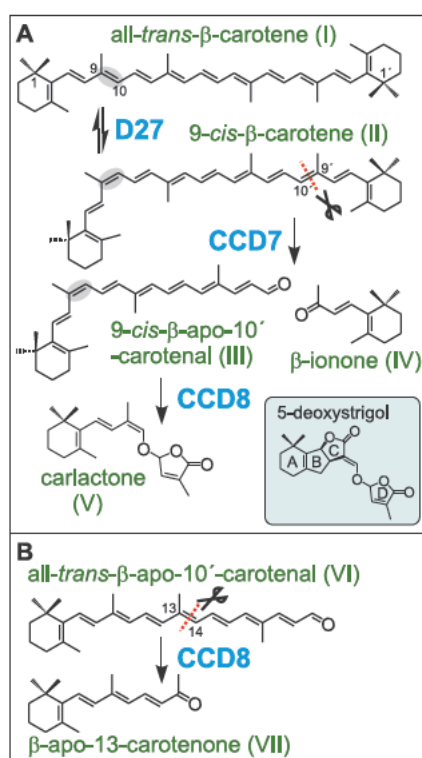


Fig. 1. The pathway to carlactone. **(A)** D27 catalyzes the isomerization of the C9-C10 double bond (shaded) in all-*trans*- β -carotene (I; C₄₀), leading to 9-*cis*- β -carotene (II) that is cleaved by CCD7 at the C9', C10' position into 9-*cis*- β -apo-10'-carotenal (C₂₇; III) and β -ionone (C₁₃; IV). A single enzyme, CCD8, converts III to carlactone (V) that already contains the D ring and the enol ether bridge of strigolactones such as 5-deoxystrigol (inset). **(B)** CCD8 also catalyzes a known CCD reaction by converting all-*trans*- β -apo-10'-carotenal into β -apo-13-carotenone. However, this reaction is slower than that with the *cis* isomer by about a factor of 10.

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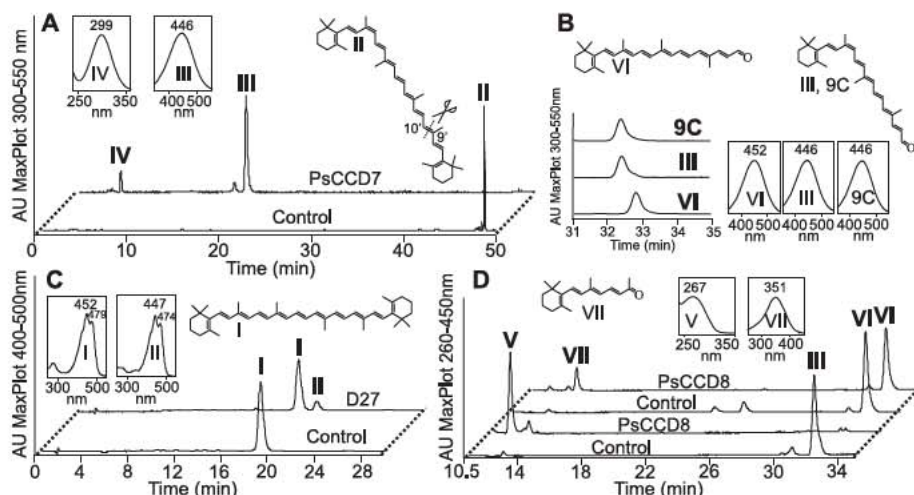
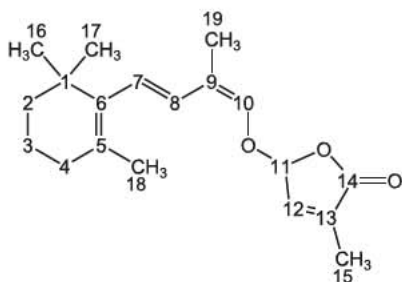


Fig. 2. HPLC analyses of in vitro incubations with PsCCD7, D27, and PsCCD8. (A) Incubation of PsCCD7 with 9-*cis*- β -carotene (II) yielded 9-*cis*- β -apo-10'-carotenal (III) and β -ionone (IV). (B) Confirming its 9-*cis* configuration, compound III coeluted with the reference compound 9-*cis*- β -apo-10'-carotenal (9C) 0.5 min earlier than with all-*trans*- β -apo-10'-carotenal (VI). (C) Incubation of purified D27 with all-*trans*- β -carotene (I) led to the formation of 9-*cis*- β -carotene (II). (D) PsCCD8 converted 9-*cis*- β -apo-10'-carotenal (III) into an apocarotenoid (V) different from β -apo-13-carotenone (VII). The samples were analyzed using separation systems A (A), B (B) and (D), and C (C) (see supporting online material). UV-Vis spectra are depicted in the insets. AU, arbitrary units.

Table 1. ^1H and ^{13}C NMR data of compound V: experimental ^1H and ^{13}C chemical shifts, ^1H - ^{13}C heteronuclear multiple bond correlations (HMBC), and relevant ^1H - ^1H nuclear Overhauser enhancements (NOE) data for compound V dissolved in and referenced to CD_2Cl_2 (^1H , 5.32 ppm; ^{13}C , 54.0 ppm).

	^1H	^{13}C	^1H - ^{13}C HMBC	1D ^1H -(^1H)-NOE
1	-	34.37		
2	1.46 (2 H, m)	39.86	C-1, C-3, C-4, C-6, C-16 and C-17	
3	1.61 (2 H, m)	19.59	C-1, C-2, C-4 and C-5	
4	2.00 (2 H, br t, 6.5 Hz)	33.24	C-2, C-3, C-5, C-6 and C-18	
5	-	129.38		
6	-	138.18		
7	6.05 (1 H, d, 16.2 Hz)	127.47	C-5 and C-9	H-16, H-17 and H-19
8	6.46 (1 H, d, 16.4 Hz)	127.47	C-6, C-9, C-10 and C-19	no NOE for H-10
9	-	117.62		
10	6.20 (1 H, s)	138.21	C-8, C-9, C-11 and C-19	H-11 and H-19 no NOE for H-8
11	5.97 (1 H, br p, 1.5 Hz)	100.65	C-10	H-10 and H-12
12	6.90 (1 H, br p, 1.5 Hz)	142.67		H-11 and H-15
13	-	135.03		
14	-	171.52		
15	1.95 (3 H, t, 1.5 Hz)	10.71	C-12, C-13 and C-14	H-12
16, 17	1.00 (6H, s)	29.02	C-1, C-2, C-6, C-7, C-16 and C-17	
18	1.69 (3H, s)	21.69	C-4, C-5, C-6, C-7, C-16 and C-17	
19	1.73 (3H, d, 1.2 Hz)	14.39	C-6, C-7, C-8 and C-9	



Pisum sativum, and *Oryza sativa*. Carotenoids occur in different *cis* configurations, a feature that may determine reaction specificities (1, 21). In deed, we show that the *cis* configuration of the C9 C10 double bond in β carotene is decisive for the course of the reactions in strigolactone biosynthesis (Fig. 1) and that this pathway is characterized by the function of a single enzyme, CCD8, in converting a 9 *cis* configured apocarotenal into a strigolactone like compound (Fig. 1A).

As shown by high performance liquid chromatography (HPLC) analysis, soluble fractions of *E. coli* cells expressing *P. sativum* CCD7 (PsCCD7) fused with thioredoxin (Fig. 2A), as well as purified PsCCD7 fused with glutathione *S* transferase (GST; fig. S3), cleaved 9 *cis* β carotene (II, C_{40}) into 9 *cis* β apo 10' carotenal (III, C_{27}) and β ionone (IV, C_{13}). All other β carotene isomers tested, including all *trans*, 13 *cis*, and 15 *cis* β carotene, were not converted (fig. S4, B to D). The products were identified by gas chromatography and liquid chromatography coupled to mass spectrometry (GC MS and LC MS) (fig. S5) and according to their ultraviolet/visible (UV/Vis) absorption spectra. The configuration of β apo 10' carotenal (III) was confirmed by HPLC comparison (Fig. 2B) with the reference compounds all *trans* β apo 10' carotenal (VI) and 9 *cis* β apo 10' carotenal (9C). *Arabidopsis* and *O. sativa* thioredoxin CCD7 crude preparations exhibited the same activity (fig. S4A). Thus, CCD7 enzymes are stereospecific for the 9 *cis* configuration and cleave the C9' C10' double bond in the *trans* moiety of 9 *cis* β carotene (Fig. 1A).

In plants, the carotenoid biosynthetic pathway provides β carotene in the all *trans* configuration (22), because the enzyme forming β carotene, lycopene β cyclase, acts as a selectivity filter for the all *trans* isomer (23). *Cis* isomers may arise nonspecifically from photo 60 isomerization (24) or thermo isomerization (25). Alternatively, a specific all *trans*/9 *cis* β carotene isomerase may be postulated to divert the carotenoid pathway into strigolactone biosynthesis by forming the authentic CCD7 substrate. We tested whether D27, a strigolactone biosynthetic enzyme with hitherto unknown catalytic properties (26), exerts this function. In vitro incubation of all *trans* and 9 *cis* β carotene with purified protein demonstrates that D27 is indeed a carotene isomerase that reversibly converts all *trans* β carotene into the CCD7 substrate 9 *cis* β carotene (Fig. 2C; see also fig. S6). These data suggest that D27 is the first all *trans*/9 *cis* β carotene isomerase reported so far and indicates that D27 is the enzyme providing the substrate for CCD7 in the course of strigolactone biosynthesis (Fig. 1A).

The configuration of the C9 C10 double bond of β apo 10' carotenal determines the nature of the product formed by CCD8. Incubation of thioredoxin PsCCD8 crude preparations with the CCD7 product 9 *cis* β apo 10' carotenal yielded an unknown compound (V, Fig. 2D) with an absorption maximum at 267 nm, lower than that of β apo 13 carotenone (VII, Fig. 2D) reported

to be formed by CCD8 from all *trans* β apo 10' carotenal (19, 20). Incubations of thioredoxin OsCCD8b and thioredoxin AtCCD8 led to the same results, demonstrating the generality of the finding that CCD8 enzymes use both β apo 10' carotenal isomers as substrates but can produce different products (Fig. 1 and fig. S7, A and B). Time courses of product formation (fig. S7C) show that the conversion of the *cis* substrate is faster than that of the *trans* substrate by a factor of ~10. We conclude that the conversion of the *cis* substrate into compound V is not a side reaction.

The structure of compound V was elucidated using electrospray ionization mass spectrometry (ESI MS) and 1D and 2D nuclear magnetic resonance (NMR) spectroscopy, exploiting NMR reference data (27) and ^1H , ^{13}C chemical shift prediction (27). The NMR data are summarized in Table 1. Both the ESI MS data ($[\text{M}+\text{H}]^+$: 303.19, $\text{C}_{19}\text{H}_{26}\text{O}_3$; fig. S8) and chemical shifts at C9, C10, and C11 pointed to oxygen as the linker between C10 and C11. This finding was

corroborated by taking advantage of 2D ^1H ^{13}C heteronuclear multiple bond correlation and 1D ^1H ^1H nuclear Overhauser enhancement NMR spectroscopy (figs. S9 to S15). ^1H and ^{13}C chemical shift prediction (28) confirmed the proposed structure. Thus, compound V [IUPAC nomenclature: 2',3',4',5'E 3 methyl 5 (3' methyl 1' oxa penta 2',4' dienyl 5' (2'',6'',6'' trimethylcyclohex 1'' en 1''yl)) 2(5H) furanone] is structurally similar to strigolactones (Fig. 1A), including the number of C atoms and the presence of a lactone. We have named compound V carlactone (Fig. 1A), reflecting its origin from β carotene and its relationship to strigolactones.

Carlactone was also produced when 9 *cis* β apo 10' carotenal was incubated with purified PsCCD8, instead of lysates of overexpressing *E. coli* cells, and in the absence of any added cofactor (fig. S16). Thus, the C skeleton of carlactone derives entirely from 9 *cis* β apo 10' carotenal, and CCD8 alone is capable of catalyzing all the reactions needed to form carlactone from this substrate, including the introduction of three oxygens and intramolecular rearrangement. A hypothetical mechanism for the conversion of 9 *cis* β apo 10' carotenal into carlactone is presented in fig. S17.

We first performed each assay with each enzyme individually. Strigolactone biosynthesis was initiated by the D27 mediated isomerization of all *trans* to 9 *cis* β carotene, followed by the CCD7 catalyzed formation of 9 *cis* β apo 10' carotenal that was converted by CCD8 into carlactone (Fig. 1A). To investigate the effect of interactions between enzymes, we then combined PsCCD7 and PsCCD8 *in vitro*. No products were detected with all *trans* β carotene as a substrate, but 9 *cis* β carotene was converted into carlactone (fig. S18A). Addition of the isomerase D27 to PsCCD7 and PsCCD8 allowed the formation of carlactone from all *trans* β carotene (fig. S18B).

The absence of carlactone in *d27*, *ccd7*, and *ccd8* mutants is the reason for the more branching, high tillering phenotype observed. We applied carlactone produced *in vitro* to the rice *d27*, *ccd7* (*htd 1*), and *ccd8* (*d10*) mutants and, as a control, to the wild type and to *d3*, a high tillering strigolactone signal perception mutant (17). After treatment for 28 days, tiller numbers of *d27*, *htd 1*, and *d10* mutants were reduced (*d27* from 5.3 to 3; *htd 1* from 6 to 2.3; *d10* from 5.7 to 1.3), whereas wild type and *d3* tiller numbers were not affected (wild type, 2; *d3*, 4.5) (Fig. 3, A and B). The activity of carlactone in restoring the wild type tillering phenotype in *d27*, *ccd7*, and *ccd8* mutants indicates that this compound is a likely precursor of strigolactones. Only a few reactions—dioxygenation followed by a dehydrogenation and simultaneous closure of the B and C rings (fig. S19B)—would be required to convert carlactone into 5 deoxystrigol. This appears feasible in view of a recent paper that shows the chemical synthesis of the strigolactone B/C ring system from a linear precursor in a single

acid catalyzed step (29). However, we have not been able to detect carlactone in plants.

Carlactone can induce seed germination of parasitic plants. We treated *Striga hermonthica* seeds with various concentrations of carlactone and with GR24 as a positive control. Carlactone, although not as effective as GR24, did induce seed germination in a concentration dependent manner (Fig. 3C). The germination promoting activity of carlactone shows that the enone moiety of the C ring of strigolactones (Fig. 1A) is not mandatory, contradicting previous suggestions (30, 31).

The comparatively simple structure of carlactone opens up new possibilities for designing strigolactone/carlactone analogs that could be applied to induce suicidal germination, a promising tool to combat root parasitic weeds that represent severe agricultural pests worldwide (32). Knowledge of the carlactone structure will greatly assist in identifying the bioactive compound(s) that regulate shoot branching and in elucidating further steps in strigolactone biosynthesis, including the activities of MAX1 (8, 14 17).

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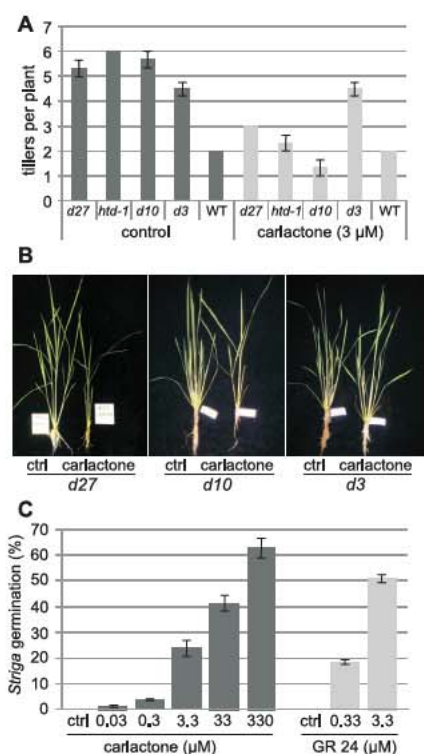


Fig. 3. Biological activity of carlactone. (A) Effect of carlactone on tillering in *d27*, *htd-1*, *d10*, *d3*, and the corresponding wild type (WT, Shiohari). Application of carlactone (3 μM daily for 2 weeks) rescued the high-tillering phenotype of the strigolactone-deficient rice mutants, whereas *d3* and WT rice remained unaffected. **(B)** Photographs were taken after 30 days. **(C)** Germination rate of *S. hermonthica* seeds 2 days after treatment with different concentrations of carlactone, GR24, or water (ctrl). Data are means \pm SD [A], $n = 4$; [C], $n = 4$ with 50 to 100 seeds per replicate.

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European patent application (number 11 003 470.9) on carlactone and its applications.