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Reduced levels of cadinane sesquiterpenoids in cotton plants expressing antisense (+)-δ-cadinene synthase

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Abstract

Cotton plants were transformed with an antisense construct of *cdn*1-Cl, a member of a complex gene family of δ-(+)cadinene (CDN) synthase. This synthase catalyzes the cyclization of (*E*,*E*)-farnesyl diphosphate to form CDN, and in cotton, it occupies the committed step in the biosynthesis of cadinane sesquiterpenoids and heliocides (sesterterpenoids). Southern analyses of the digestion of leaf DNA from R_o, T_o, and T_1 plants with *Hind* III, *Pst* I and *Kpn* I restriction enzymes show the integration of antisense *cdn*1-C1 cDNA driven by the CaMV 35S promoter into the cotton genome. Northern blots demonstrate the appearance of *cdn* synthase mRNA preceding CDN synthase activity and the formation of gossypol in developing cottonseed. T_2 cottonseed show a reduced CDN synthase activity and up to a 70% reduction in gossypol. In T_1 leaves the accumulated amounts of gossypol, hemigossypolone and heliocides are reduced 92.4, 83.3 and 68.4%, respectively. These data demonstrate that the integration of antisense *cdn*1-C1 cDNA into the cotton genome leads to a reduction of CDN synthase activity and negatively impacts on the biosynthesis of cadinane sesquiterpenoids and heliocides in cotton plants.

Keywords: *Gossypium arboreum*; Cotton; Malvaceae; Antisense cDNA; δ-Cadinene synthase activity; Gossypol

1. Introduction

There are several cadinane sesquiterpenoids and heliocides (sesterterpenoids) deposited in pigment glands in cotton plants that function in pathogen and insect resistance (Stipanovic et al., 1999). A proposed pathway for the biosynthesis of these compounds is shown in Fig. 1. Infection of cotton stele tissue with conidia of *Verticillium dahliae* that induced the formation of sesquiterpenoid phytoalexins also induced 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) mRNA and HMGR activity, demonstrating the important role of HMGR in the biosynthesis of the sesquiterpenoids (Bianchini et al., 1994). Studies with specifically labeled mevalonic acid (MVA) or acetate demonstrated the folding pattern of farnesyl diphosphate (**1**, FDP) required for gossypol (**2**) formation (Masciadri et al., 1985; Stipanovic et al., 1986). Subsequently, the enzymatic product of the cyclization of *E*,*E*-FDP (**1**) in cotton extracts was identified as (+)-δ-cadinene (**3**, CDN) (Benedict et al., 1995; Davis and Essenberg, 1995; Chen et al., 1995). The enzymatic mechanism of CDN synthase for the formation of the cadinane structure of cotton sesquiterpenoids was shown to involve the isomerization of **1** to a nerolidyl intermediate, cyclization to a *cis*-germacradienyl cation, a 1,3-hydride shift, cyclization to a cadinanyl cation and deprotonation to form **3** (Benedict et al., 2001). CDN synthase catalyses the committed step in the formation

Abbreviations: MVA, mevalonic acid; FDP, farnesyl diphosphate; CDN, (+)-δ-cadinene; CaMV, cauliflower mosaic virus; G, gossypol; dHG, desoxyhemigossypol; HG, hemigossypol; HGQ, hemigossypolone; DOXP, 1-deoxy-D-xylulose-5-phosphate; $H_{1\text{-}4}$, heliocides$_{1\text{-}4}$; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; dpa, days past anthesis

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of the cadinane sesquiterpenoids from **1** at a branch point in the MVA pathway. **2** is the sesquiterpenoid formed in cottonseed with only traces of desoxyhemigossypol (**4**, dHG) and hemigossypol (**5**). In cotton foliage hemigossypolone (**6**) is formed from dHG **4** (Stipanovic et al., 1999). A Diels–Alder reaction accounts for the cycloaddition of myrcene or β-ocimene to **6** to form heliocides$_{1–4}$ (**7**–**10**) (Stipanovic, 1992). The monoterpenes or its precursors are synthesized by the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway in the plastids (Arigoni et al., 1997; Lichenthaler et al., 1997; Adam et al., 1998).

There is an interest in controlling the biosynthetic pathways for the formation of natural products in plants by altering the activity of key enzymes with antisense constructs (Robbins et al., 1998; Tanaka et al., 1999; Mahoud and Croteau, 2001). Four cDNAs of *cdn* synthase (*cdn*1-C1, *cdn*1-C14, *cdn*1-A and *cdn*1-C2) have been isolated from *Gossypium arboreum* (Chen et al., 1995, 1996) and in this paper we show that antisense *cdn*1-C1 cDNA driven by a Cauliflower Mosaic Virus (CaMV) 35S promoter had a negative impact on the level of cadinane sesquiterpenoids (**2**, **6**) and heliocides (**7**–**10**) in cotton plants.

2. Results and discussion

Southern analyses of *Hind* III digestion of the pCGN 1578 plasmid containing the antisense *cdn*1-C1 cDNA construct and leaf DNA from T_0 and T_1 plants probed with radiolabeled *cdn*1-C1 cDNA probe showed a 1.22 kb and a 0.66 kb fragment resulting from *Hind* III digestion of 2 internal sites in the antisense *cdn*1-C1

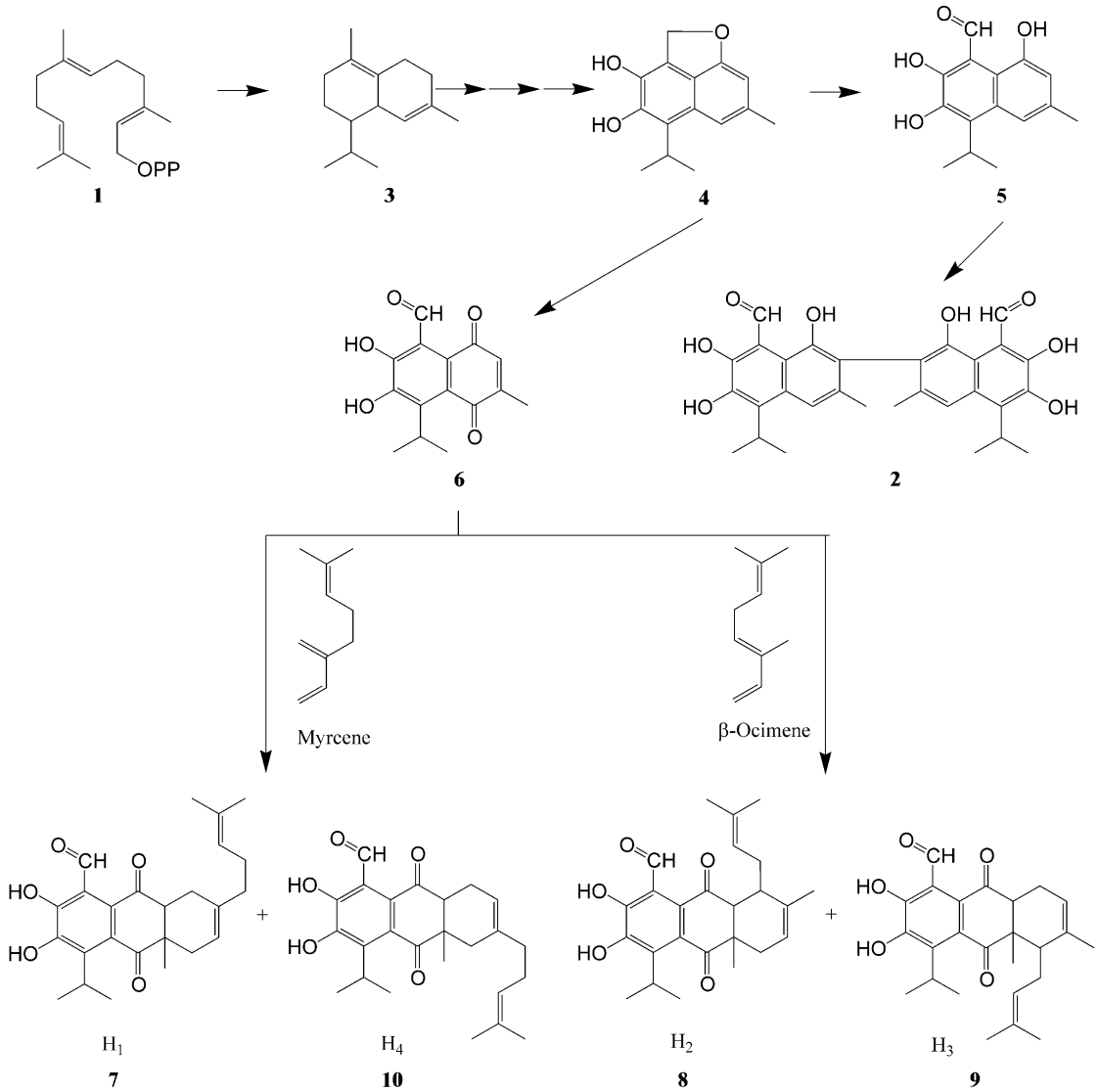

Fig. 1. Proposed reaction sequence for the biosynthesis of gossypol (**2**) in cottonseed and gossypol (**2**), hemigossypolene (**6**) and heliocides$_{1–4}$ (**7**–**10**) in cotton leaves.

cDNA and a site external to the 3′ end of the cDNA. No comparable fragments were produced from the *Hind* III digestion of the R_0 leaf DNA (data not shown).

Southern blots of *Pst* I digestion of the pCGN 1578 plasmid containing the antisense construct and leaf DNA from R_0, T_0 and T_1 plants probed with radiolabeled *cdn*1-C1 cDNA show that *Pst* I digestion excises the entire cassette and produces a slightly smaller than 3 kb fragment from the pCGN 1578 plasmid (data not shown). A similar fragment is produced from the *Pst* I digestion of the T_0 and T_1 leaf DNA, whereas comparable size DNA fragment is produced from the *Pst* I digestion of the R_0 leaf DNA (data not shown).

The plasmid pCGN 1578 with the antisense construct, leaf DNA from R_0, T_0, and T_1 plants were amplified by PCR with VirD primers. A 560 bp fragment was produced from the PCR of the pCGN 1578 plasmid but no fragments were produced from the PCR amplification of the R_0, T_0 and T_1 DNA indicating no *Agrobacterium* DNA contamination of the R_0, T_0 and T_1 leaf DNA.

Southern blots were made of the *Kpn* I digestion of the pCGN 1578 plasmid containing the antisense construct and DNA from leaves of R_0, T_0 and 2CD1,2CB1,2CG3,2CH2 and 2CA3 T_1 plants probed with radiolabeled *Npt* II DNA. Only one *Kpn* I restriction site lies within the T-DNA borders containing the antisense cassette. The *Kpn* I digestion of the pCGN 1578 plasmid produces a single fragment from the linearization of the plasmid DNA (data not shown). There is no fragment detected from the *Kpn* I digestion of the non-transformed R_0 control plants. The digestion of the T_0 and T_1 leaf DNA produces a different size fragment than is produced from the linearized plasmid DNA. These results and the Southern analyses of the *Hind* I and *Pst* I digestions of leaf DNA demonstrate the integration of antisense *cdn*1-C1 cDNA construct into a unique location in the cotton genome of the T_0 and T_1 plants. Twelve of the 15 T_1 plants produced from a single T_0 plant had inherited the transgene.

No attempt was made to treat the T_1 plants with kanamycin to determine the ratio for the segregation of the antisense insert. Many times in examining the Southerns of the T_1 leaf DNA the *Npt* II was absent after integration of the T-DNA into the cotton genome leaving the CaMV 35S promoter—antisense *cdn*1-C1 cDNA—CaMV 35S terminator insert without the *Npt* II gene.

The kinetic analyses of the formation of *cdn* synthase mRNA, CDN synthase activity and **2** formation in developing cottonseed of untransformed plants are shown in Fig. 2. The Northern blots show that *cdn* synthase mRNA was detected at 23 days past anthesis (dpa) and reached steady-state levels from 30 to 50 dpa. CDN synthase activity first appeared between 26 and 33 dpa, reached a maximum at 38 dpa then sharply declined. The accumulation of **2** began between 25 and 33 dpa and reached a maximum level at 45 dpa. These results demonstrate that the formation of *cdn* synthase mRNA preceded the formation of CDN synthase activity and **2**. The alteration of the *cdn* synthase mRNA with antisense *cdn*1-C1 cDNA should negatively

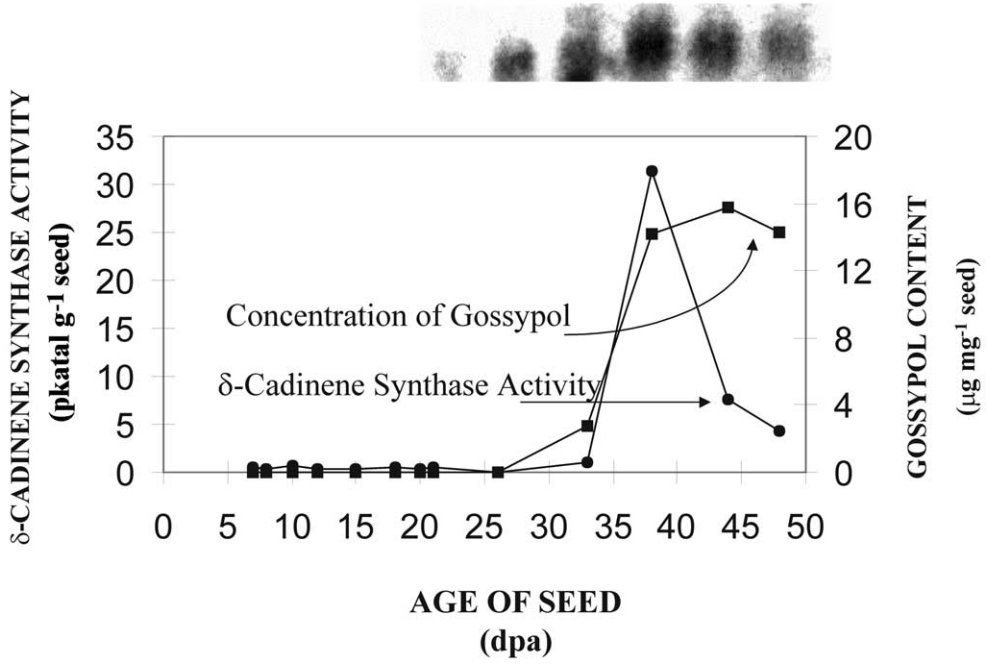

Fig. 2. The developmental charger in *cdn* synthase mRNA, CDN synthase activity and **2** in maturing cottonseed.

impact on **2** formation in cottonseed. These results are similar to the timing of gene expression of *cdn* synthase, appearance of CDN synthase activity and the formation of **4** and **2** in cotton stele tissue (Alchanati et al., 1998) and developing seed (Meng et al., 1999).

The CDN synthase activity and **2** content of T_2 cottonseed from selected T_1 plants is shown in Table 1. The synthase activity varies from 30.5 to 10.7 pkat/g seed for a 10.9–68.7% reduction compared to the activity in the non-transformed control seed. There is a 17.4–39.2% reduction of **2** in these T_2 cottonseed compared to levels in control seed. Although other reactions together with the synthase may limit **2** formation in cottonseed the results demonstrate that an alteration of CDN synthase activity with antisense *cdn*1-C1 cDNA leads to a lowering of the level of **2** in the seed.

The frequency distribution of **2** in 113 individual seed was determined from randomly selected T_2 seed produced on T_1 plants that were generated from a single T_0 plant (Fig. 3). Twelve of these plants were transformed and three were non-transformed. Compared to the distribution of **2** in the control seed from R_1 plants, there is a marked shift of **2** in the T_2 cottonseed to lower values. The range of **2** in these seed was from 15–16 to 4–5 μg/mg seed (0–70% reduction). There was no attempt to demonstrate the distribution of the antisense *cdn*1-C1 cDNA insert segregating in the T_2 population. The aim of these analyses was to analyze a greater population of

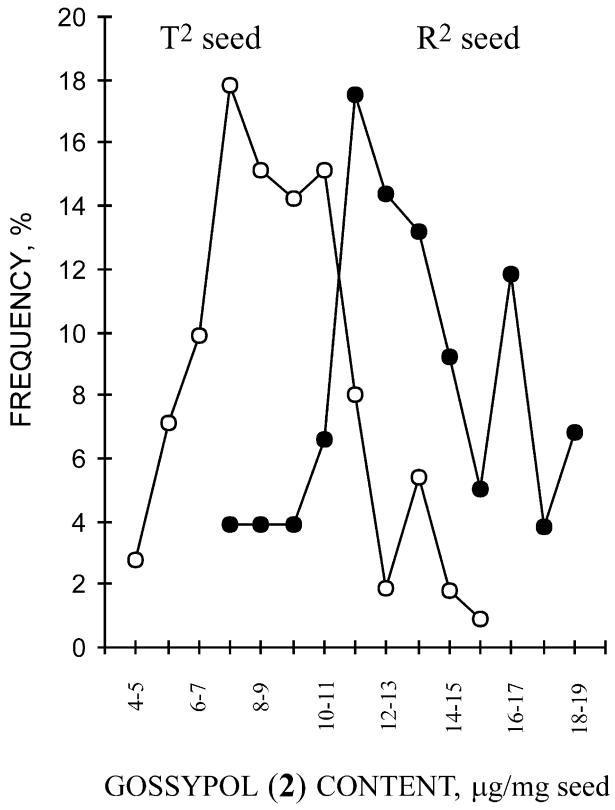

Fig. 3. The frequency distribution of **2** in control R_2 (76) seed and randomly selected T_2 (113) seed.

Table 1
The effect of antisense *cdn*1-C1 cDNA on CDN synthase activity and **2** levels in T_2 cottonseed from selected T_1 cotton plants

R_1 controlplants	CDN synthase[a] activity in R_2 seed (pkatal/g seed)	T_1 Plants	CDN synthase activity in T_2 seed (pkatal/g seed)	Gossypol (2) content[b] in T_2 seed (μg/mg seed)
1	36.9	2CH2	11.7±0.9[c] (65.1%)[d]	8.0±1.2[4] (39.2%)[d]
2	31.9	2CA3	10.7±0.9 (68.7%)	9.6±0.6 (27.3%)
3	34.3	2CB1	25.6±1.9 (25.0%)	10.8±3.0 (18.3%)
4	31.1	2CD1	8.8±0.7 (74.2%)	9.5±1.9 (28.1%)
5	35.8	2CA4	30.5±2.2 (10.9%)	10.9±2.0 (17.4%)

[a] Mean of CDN synthase activity in R_2 control seed was 34.0 pkatal/g seed with a standard deviation of 2.5 pkatal/g seed.
[b] Mean of gossypol concentration in 76 individual mature R_2 seed was 13.3 μg/mg with a standard deviation of 2.9 μg/mg.
[c] Standard deviation values.
[d] The values in parenthesis are the percent reduction compared to R_2 control seed.

Table 2
The affect of antisense *cdn*1-C1 cDNA on the levels of gossypol, hemigossypolone and heliocides$_{1-4}$ in cotton leaves from T_1 plants

Plant[a]	Hemigossypolone (μg/mg leaf)	Gossypol (μg/mg leaf)	Heliocide$_{1-4}$ (μg/mg leaf)
2CH2	0.18±0.03[b] (78.5%)[c]	0.023±0.008 (85.3%)	0.62±0.12 (66.8%)
2CA3	0.066±0.015 (92.4%)	0.023±0.009 (85.3%)	0.58±0.10 (68.4%)
2CB1	0.73±0.23 (17.4%)	0.38±0.09 (0%)	5.09±0.98 (0%)
2CD1	0.18±0.04 (79.7%)	0.013±0.006 (91.7%)	1.08±0.27 (41.9%)
2CA4	0.86±0.17 (0%)	0.20±0.04 (0%)	3.08±0.58 (0%)

[a] Level of HGQ, G and total H_{1-4} in 15 individual leaves of R_1 control plants was 0.86±0.16 (standard deviation) μg/mg, 0.16±0.05 μg/mg and 1.86±0.42 μg/mg, respectively.
[b] Standard deviation values.
[c] The values in parenthesis are the percent reduction compared to the concentration in R_1 control leaves.

T_2 cottonseed than in Table 1 to further demonstrate that the lowering of CDN synthase activity in the T_2 cottonseed correlated with the modulation of **2**.

HPLC separation of the cadinane sesquiterpenoids (**2** and **6**) and heliocides (**7**–**10**) extracted from the leaves of an R_1 non-transformed control plant and a T_1 plant show that the level of these terpenoids in the control leaves is comparable to the level in other cotton plants. The level of **2** in the control leaves was about 1/100 the level deposited in cottonseed (Table 1). Measurements of CDN synthase activity in the cotton leaves were not consistent, but compared to control leaves, the range of reduction of the terpenoids in the T_1 leaves was **2**, 0–91.7%; **6**, 0–92.4% and **7**–**10**, 0–66.8% (Table 2). The biosynthesis of the cadinane sesquiterpenoids in the cotton leaves is dependent on the MVA pathway for the formation of **1** and on the activity of CDN synthase for the formation of the cadinane ring structure. **2** is formed from **3** through **4** and **5**. CDN synthase furnishes 15 carbons of the **4** required for the formation of (**7**–**10**) by the Diels–Alder reaction. The non-mevalonate plastid pathway that is known to account for the biosynthesis of monoterpenes may provide the precursors for the 10 carbon monoterpenes used in the Diels–Alder reaction for the synthesis of H_{1-4}. The above data demonstrate that the integration of the antisense *cdn*1-C1 cDNA into the cotton genome negatively impacts on the formation of the sesquiterpenoids and heliocides in the leaf tissue.

3. Conclusions

The integration of the antisense *cdn*1-C1 cDNA into the cotton genome altered the activity of CDN synthase and suppressed the biosynthesis of cadinane sesquiterpenoids in cottonseed and cadinane sesquiterpenoids and heliocides in cotton foliage. These results demonstrate that the activity of CDN synthase at the first committed step in sesquiterpenoid formation, at a branch point in the MVA pathway, controls the flux of carbon into the sesquiterpenoids. Results of fusing a cottonseed specific promoter (Song et al., 2000) with antisense *cdn*1-C1 cDNA led only to a 20% reduction of G in seed of transgenic cotton plants (Benedict, unpublished). The success of the antisense *cdn*1-C1 cDNA in our experiments in regulating sesquiterpenoid formation was in part due to the fusion with the CaMV 35S promoter. The expression of the antisense construct was sufficient to negate the transcripts from the expression of multiple constitutive *cdn* synthase genes. Use of the general plant promoter has allowed an assessment of the role of CDN synthase activity in controlling the level of sesquiterpenoids in several tissues of the cotton plant. These data are important to the cotton industry in replacing expensive procedures to remove toxic gossypol (**2**) from cottonseed and cottonseed oil and to regulating sesquiterpenoids and heliocides in cotton foliage for pathogen and insect resistance.

4. Experimental

4.1. Chemicals

[1-^{3}H]-(*E*,*E*)-Farnesyl Diphosphate (832.5 GBq mmol^{-1}) was purchased from New England Nuclear. The ammonium salt of farnesyl diphosphate (**1**) was purchased from Sigma. [α-^{32}*P*]-d-Cytidine triphosphate (220 TBq mmol^{-1}) was purchased from Amersham.

4.2. Plants

The regenerated R_0, R_1 cotton plants and the transformed T_0, T_1 plants (*Gossypium hirsutum* cv. Coker 312) were grown in a Biohazard level I and II Plant Growth Facility. The plants were grown in 5-gal pots containing Metro-Mix 700 soil, fertilized weekly with Peters 20–20–20 fertilizer and watered daily. The day temperature ranged between 85 and 90 °F and the night temperature ranged between 80 and 85 °F. The photoperiod was 14 h day – 10 h night. Light irradiance was 1000–2000 μmol m^{-2} s^{-1}.

4.3. Construction of the binary vector

The cloning plasmid pRTL 2 (obtained from Dr. James Carrington formerly of Texas A&M) was used to build the antisense construct. The TEV leader sequence was excised with *Xho* I and *Bam* HI leaving the Cauliflower Mosaic Virus (CaMV) 35S promoter and the CaMV 35S terminator. The *G. arboreum cdn* 1-C1 cDNA obtained from Dr. Peter Heinstein, Purdue University was ligated into the pRTL 2 vector in the reverse direction between the CaMV 35S promoter and terminator. This cassette was excised with *Pst* I and ligated into the binary vector pCGN1578 (Calgene) which contains the kanamycin resistant cassette CaMV 35S promoter *Npt* II—CaMV 35S terminator. The freeze-thaw method was used to introduce the antisense construct into disarmed *Agrobacterium tumefaciens* strain EHA 101 (Hood et al., 1986). The methods for hypocotyl-transformation and regeneration of transgenic plants (Firoozabady et al., 1987) were used to integrate the antisense constructs into the cotton genome.

4.4. Southern analyses

Genomic DNA was extracted from the leaves of the R_0, R_1, T_0, T_1 cotton plants (Paterson et al., 1993). Ten μg of DNA from each sample digested with restriction

enzymes *Hind* III, *Pst* I or *Kpn* I was used for electrophoresis in 1% Agarose gel. DNA was blotted onto a Zeta-Probe GT genomic nylon membrane. The membrane was hybridized with radiolabeled *cdn*1-C1 cDNA or *Npt* II probes prepared using Random Primer DNA labeling system (GIBCOBRL) and [α-^{32}P]-d-CTP.

4.5. PCR analysis

Vir D4 primers 5′-GAAGAAAGCCGAAATAAAGAGG-3′ and 5′-TTGAACGTATAGTCGCCGATAG-3′ were used for PCR analysis for detection of *Agrobacterium* DNA contamination in the DNA isolated from the regenerated or transformed plants. The amplification reaction contained 1 μg leaf DNA, 1.3 μM each primer, 0.2 mM of each dNTP, appropriate amounts of Taq polymerase and buffer. The PCR profile was 94 °C, 10 s; 55 °C, 30 s and 72 °C, 1 min for a total of 30 cycles on the DNA thermal cycler.

4.6. Isolation and assay of CDN synthase

To determine the development of CDN synthase activity in maturing cottonseed the flowers on the R_1 and T_1 plants were tagged to indicate the day of anthesis. Bolls were collected at different dpa for analysis of CDN synthase activity in the developing seed. The seed were excised from the different age bolls, delinted and the seed coat removed. The seed were weighed and ground to a powder in liquid N_2 in a mortar. (Several seed from the same boll were collected for dry weight determinations.) The powder was further ground in 8 ml/g seed in 0.1M Tris–HCl buffer pH 7.5 containing 5 mM GSH and 5% (w/v) of insoluble PVP. The homogenate was centrifuged at 13,000 *g* in a Sorvall RC-3B centrifuge for 20 min and the soluble supernatant fraction removed. The pellet was washed three times with the homogenizing buffer. The original supernatant fraction and washings were combined and centrifuged at 100,000 *g* for 90 min in a Beckman L8–55M ultracentrifuge. The soluble supernatant fraction was used for the enzyme analyses.

The CDN synthase activity was determined by a radiolabeled assay (Alchanati et al., 1998). The reaction mixture for the enzymatic cyclization of [1-^{3}H]-(E,E)-FDP to [^{3}H]-CDN contained: 200 μl of soluble enzyme extract, 15 μl 0.1 M GSH (pH 7.5), 40 μl 0.2 M KF, 3 μl 0.1 M $MgCl_2$, 4 μl of [1-^{3}H]-(E,E)-FDP containing 8.04 nmol and 5.79×10^5 dpm of radioactivity in a total volume of 300 μl. The reaction mixture was incubated at 30 °C for 20 min and the reaction stopped by extracting the aqueous fraction with 2.0 ml hexane:EtOAc (3:1 v/v). The aqueous fraction was extracted two more times and the extracts combined. A 600 μl aliquot was assayed for radioactivity in Cocktail D (New England Nuclear) with a Beckman Liquid Scintillation Spectrometer. As shown by HPLC analysis, the [^{3}H]-CDN accounted for the radioactivity in the hexane-EtoAc extract. The CDN synthase activity was expressed in pkatal/g seed. The CDN synthase activity in the T_2 cottonseed from different T_1 plants was assayed on the day of peak activity (Fig. 2).

4.7. Northern blots

The amount of *cdn* synthase mRNA in different age maturing cottonseed was determined over a period of 5–48 dpa. The seed were excised from the bolls collected at different ages and immediately frozen in liquid N_2. The remainder of the seed were collected from the bolls for enzyme and gossypol analyses. The seed RNA was isolated from 1 g seed using an RNeasy® total plant RNA kit developed by Qiagen (catalogue #74904). Absorbance at 260 and 280 nm was used to determine purity and concentration of total RNA. Aliquots containing 4 μg RNA were loaded onto a 1.2% denaturing gel and electrophoresed at 22 V. Gels were blotted onto Zeta-Probe GT (Bio-Rad) membranes following the alkaline Northern blotting protocol recommended by Bio-Rad. The *cdn*1-C1 cDNA probe was cut from the Bluescript SK-plasmid vector with restriction enzymes *Bam* HI and *Xho* I and radiolabeled by the random primer method. Hybridization was by the standard procedure recommended by Bio-Rad. After the final post hybridization wash, the blots were exposed to Kodak XAR5 film at −80 °C. Based on sequence similarity the *cdn* 1-C1 cDNA probe was also likely to detect the mRNA of *cdn*1-A, *cdn* 1-C14 as well as mRNA of *cdn*1-C1 (Meng et al., 1999).

4.8. Analysis of gossypol (**2**) content in cottonseed

The gossypol (**2**) content of cottonseed was determined by HPLC (Stipanovic et al., 1988). Individual seed were ground in liquid N_2 to a powder and transferred to a 125 ml Erlenmeyer flask. EtOH:H_2O:ether:HOAC (59:24:17:02, v/v/v/v, 20 ml) was added and the flask capped. The flask was placed on a New Brunswick Scientific G-10 Gyrorotory Shaker at 350 rpm for 30 min at room temp. The flask contents were quantitatively filtered through a Buchner filter using a Whatman #1 filter paper into a 50 ml flask. One ml of this filtrate was filtered through a fritted glass funnel (0.45 μm) into a crimp-top vial for HPLC analysis.

The HPLC was performed on a Hewlett-Packard 1090 Liquid Chromatograph equipped with a diode array detector, multi-channel integrator and auto injector using an analytical Scientific Glass Engineering 250 GL4-ODS-H12/5 column at 55 °C with an isocratic solvent system of H_2O: acetonitrile: EtoH: isopropyl alcohol: DMF: MeOH: EtOAc: phosphoric acid (37.4: 20.2: 16.7: 12.1: 5.1: 4.6: 3.8: 0.1, v/v/v/v/v/v/v/v) with a

flow rate of 1.25 ml/min and a run time of 30 min. The injection volume was 50 μl. The chromatogram signal was monitored at 272 nm and the spectra over 200–400 nm were stored. The amount of G in the cottonseed was calculated using a calibration curve established with an authentic sample of **2** at quantities of 25–6000 ng. The calibration curve for **2** (retention time of 10.37 min) is described by $Y=1238X-8.11$ where X is the amount of **2** in μg and Y is the area of the **2** peak in mAU s.

4.9. Analysis of 2, 6 and 7–10 in cotton leaves

Fully expanded leaves from the third to the fifth node down from the apex to the main stem were collected, frozen in liquid N_2 and lyophilized. Individual dry leaves were ground to a powder in a mortar and 100 mg of leaf tissue was transferred to a 125 ml Erlenmeyer flask. Hexane:EtOAc (3:1 v/v, 10 ml), 10% HCl (200 ml) and glass beads (15 ml) were added and the flask capped. The flask was placed on a Gyrorotary Shaker at 350 rpm for 30 min at room temp. The solution was filtered through a fritted glass funnel into a 50 ml pear-shaped flask. The residue was washed hexane:EtOAc (3:1 v/v, 3 × 5 ml) and the original extract and washings were combined and added to a 50 ml flask. The solvent was evaporated under reduced pressure on a rotary evaporator. The residue was dissolved in hexane:EtOAc (3:1 v/v, 150 μl) and the flask washed with solvent (3 × 150 ml). The original solution and the washes were combined and loaded onto a silica Sep-Pak. The Sep-Pak was dried with N_2 and eluted with isopropyl alcohol: acetonitrile:H_2O:EtOAc (37:22:41:6 v/v/v/v 5 ml). The eluant (1 ml) was transferred to a crimp-top vial for HPLC analysis as described previously for the analysis of **2** in cottonseed.

Amounts of **2**, **6** and **7**–**10** in the leaves were quantified using standard curves obtained for authentic samples of **2**, **6**, **7** and **8** and H_2 with concentrations in the range of 25–6000 ng. Standard curves for **7** and **8** were used for **9** and **10**.

For HGQ (retention time 3:09 min) the standard curve is given by $Y=1810X-33.54$ where X is the amount of terpenoid in μg and Y the area of the HGQ peak in mAU-s. For G (retention time 10.37 min) $Y=1238\ X\ -8.11$. For H_1, H_2, H_3, and H_4 (retention times 11.27, 11.68, 12.84 and 13.59 min, respectively) $Y=1652X-89.70$.

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