Coordinated Accumulation of (+)-δ-Cadinene Synthase mRNAs and Gossypol in Developing Seeds of *Gossypium hirsutum* and a New Member of the *cad*1 Family from *G. arboreum*

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A new member of the (+)-δ-cadinene synthase (CAD1) family was isolated from a *Gossypium arboreum* cDNA library. This cDNA encodes a protein that showed 97.3%, 96.9%, and 79.2% sequence identities with the proteins encoded by previously isolated cDNAs of *cad*1-C1, *cad*1-C14, and *cad*1-A, respectively. It may be grouped into the *cad*1-C subfamily as *cad*1-C2. Seeds of a glanded cotton cultivar, *G. hirsutum* cv. Sumian-6, were collected at different intervals during maturation, and the *cad*1 mRNA levels were analyzed by quantitative RT-PCR. The transcripts could be detected in seeds of 27 DPA (days postanthesis) and increased dramatically along with the seed maturation, which coordinated with an increase in sesquiterpene cyclase activities and subsequently the accumulation of gossypol. The transcription level detected with primers specific to *cad*1-C (including at least C1, C14, and *cdn*1) was higher than that detected with primers specific to *cad*1-A, and mRNA was detected also with *cad*1-C2-specific primers. This investigation indicates that, in developing seeds of the glanded cotton cultivar, genes of both the CAD1-C and CAD1-A subfamilies are expressed and there is an active biosynthesis of cadinene-type sesquiterpenes.

Plant defense mechanisms involve the induced expression of a complex assortment of genes, including those coding for enzymes catalyzing the formation of antimicrobial secondary metabolites (phytoalexins). In cotton [*Gossypium hirsutum* L. and *Gossypium arboreum* L. (Malvaceae)], the production of cadinene-type sesquiterpenes was induced clearly in plants inoculated with the phytopathogenic bacterium *Xanthomonas campestris*[1,2] and in cell suspension cultures treated with elicitors prepared from the wilt disease-causing fungus *Verticillium dahliae*.[3] However, cotton sesquiterpenes, including the phenolic aldehyde gossypol, also are constitutive components present in subepidermal pigment glands.[4,5] The occurrence of pigment glands is a distinctive character in the tribe Gossypieae of the family Malvaceae.[4] The glands are found throughout the plant, and they are particularly dense in cotyledons of mature seeds of most cotton cultivars, and high levels of sesquiterpenes (mainly gossypol) are accumulated and deposited in seed glands.[6,7]

Sesquiterpene cyclase catalyzes the cyclization of linear farnesyl diphosphate (FPP), the first step committed to the formation of various cyclic sesquiterpenes.[8−10] Previous investigations have characterized three cDNAs of the cotton sesquiterpene cyclase, (+)-δ-cadinene synthase (CAD1), from *G. arboreum*. On the basis of sequence similarities, they were grouped into two subfamilies, *cad*1-C (including C1 and C14) and *cad*1-A.[11,12] A cDNA from *G. hirsutum*, *cdn*1, which is over 95% identical to *G. arboreum cad*1-C1 and *cad*1-C14, has been isolated recently.[13] In addition, the enzyme was purified from bacterial-inoculated *G. hirsutum* folia tissues.[14] In *G. arboreum* cell suspension cultures, mRNA levels of both *cad*1-C and *cad*1-A subfamilies were increased by 10 times at 6−10 h posttreatment with *V. dahliae* cellular extracts, followed by increased levels of cyclase proteins.[11,12] Similarly, the expression of genes of a number of other plant terpene cyclases, such as tobacco 5-*epi*-aristolochene synthase (EAS),[15,16] *Hyoscyamus muticus* vetispiradiene synthase,[17] and *Ricinus communis* casbene synthase,[18] was induced in cell cultures or plant tissues by elicitation.

Table 1. Primers Used in PCR Amplifications[a]

forward	reverse	specificity
93126	93T1450	*cad*1-C1,[b] *cad*1-C14;[b] *cdn*1[c]
ataaggatgaaatgcgtcc	gaagcttggtaaagttcca	
93160	T1098	*cad*1-A[d]
ccactgctcaacttacat	attcgggagttggttcatg	
93900	TM2	*cad*1-C2
tttgcataggaaagagcta	ctctattgctgagcaatcat	
H3F	H3R	histone 3[e]
gaagcctcatcgataccgtc	ctaccactaccatcatggc	

[a] Primer sequences are listed from 5′ to 3′. Specificity refers to hybridization (perfect matching) with specific *cad*1 or histone 3 cDNAs. [b] Reference 11. [c] Reference 13. [d] Reference 12. [e] Reference 27.

Formation of pigment glands and their sesquiterpene content is genetically and developmentally controlled in cotton species but appears not to be related to environmental stimuli.[19] In developing cottonseeds, the young cotyledons are well protected by the seed-coat. It has been reported that, when cottonseeds deteriorate through the action of *Aspergillus niger*, no induced synthesis of terpenoid aldehydes could be detected.[6] Therefore, induced expression of the cotton CAD1 may not account for the formation of sesquiterpenes in glanded seeds, and expression of the cyclase genes may be regulated developmentally as well. Here we report the time-course of CAD1 gene transcription and, concomitantly, the sesquiterpene cyclase

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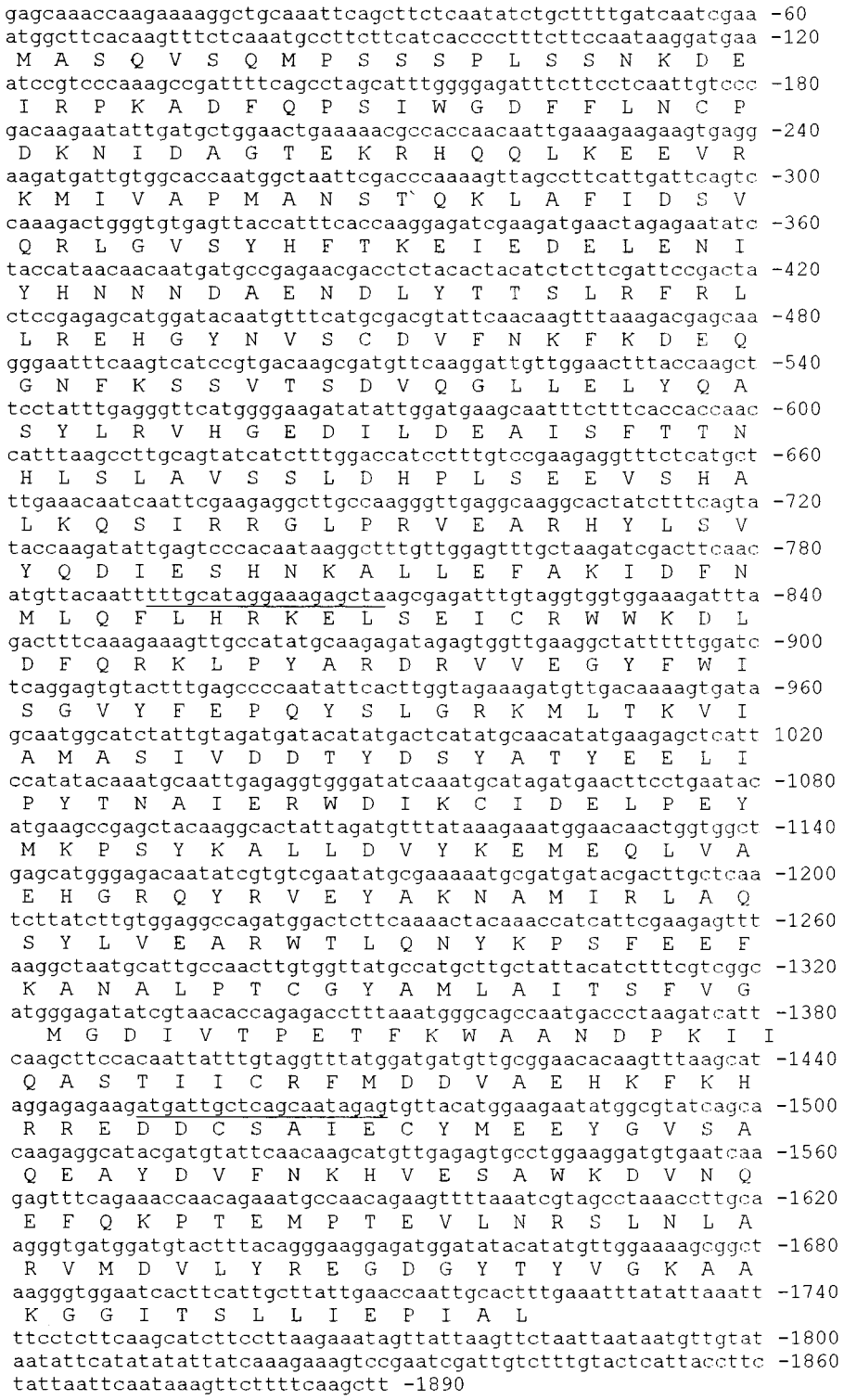

Figure 1. Nucleotide and deduced amino acid sequences of (+)-δ-cadinene synthase cDNA *cad*1-C2 from *G. arboreum* (EMBL accession number Y16432). Primers used in cDNA isolation and RT-PCR analysis are underlined.

activities and gossypol accumulation in developing seeds of a glanded upland cotton cultivar, *G. hirsutum* cv. Sumian-6. During this investigation, a new member of the *cad*1-C subfamily was identified, and it was found also expressed in seeds of this cultivar.

Results and Discussion

To isolate new members of the cotton CAD1 family, PCRs were performed on a cDNA library prepared from elicitor-treated cells of *G. arboreum*,[11] with various primers hybridizing to *cad*1. Nucleotide sequences of one of the PCR fragments showed high homology to *cad*1-C1 and *cad*1-C14. On the basis of sequence information of this fragment, a specific reverse primer TM2 (Table 1) was applied, together with a forward primer 93900, to screen the cDNA library using a PCR-96-well plate method.[20] This isolated a cDNA clone that contained an insert of 1890 bp, with an open reading frame encoding a protein of 554 residues (Figure 1), exactly the same size as the proteins encoded by *cad*1-C1 and *cad*1-C14.[11] Alignment of its nucleotide and de-

duced amino acid sequences with members of the *G. arboreum cad*1 family revealed the highest identity with *cad*1-C1, 95.6% at nucleotide sequence level and 97.3% at amino acid sequence level, comparable to the identities between *cad*1-C1 and *cad*1-C14 (96.9% at amino acid sequence level). In the 3' noncoding region, its sequence identities with other members of the *cad*1-C subfamily were comparatively lower (86.4% with *cad*1-C1 and 85.8% with *cad*1-C14). The identity between this cDNA and *cad*1-A was significantly lower (79.2% at amino acid sequence level), again comparable to the homology between *cad*1-A and other members of *cad*1-C.[12] Apparently, it is a new member of the *cad*1-C subfamily of the *G. arboreum* (+)-*δ*-cadinene synthase family and is herein referred to as *cad*1-C2.

The cotton CAD1 is encoded by a multimember gene family. Until now from the diploid species *G. arboreum*, three members of the *cad*1-C subfamily have been isolated and sequenced at the mRNA level, including *cad*1-C1, *cad*1-C14,[11] and *cad*1-C2 (reported herein), whereas only one member of *cad*1-A has been isolated.[12] All three members of the *cad*1-C subfamily of *G. arboreum*, as well as *cdn*1 from the upland cotton *G. hirsutum*,[13] show a high nucleotide sequence identity among each other in noncoding regions, particularly in the 5'-end region upstream to the translation start codon. Possibly these individual genes were derived from gene duplication. In tobacco, formation of sesquiterpenoid phytoalexins is also catalyzed by multiple isozymes of EAS, and the tobacco genome is believed to contain 12−15 copies EAS genes.[15,16]

Total RNAs were isolated from seeds of a glanded cotton cultivar *G. hirsutum* cv. Sumian-6, at four intervals ranging from 20, 27, 35, to 40 days postanthesis (DPA), approximately corresponding to stages of embryogenesis of the later cotyledon, early maturation, mid-maturation, and late maturation.[21] Tiny pigment glands were visible in developing cotyledons at 30 DPA, the mid-maturation stage, and the glands were clearly distinguishable in cotyledons of 40 DPA, the late maturation stage. Seeds were completely mature when desiccation took place at ~56−60 DPA.

The CAD1 gene transcription was analyzed by RT-PCR using primers specific to *cad*1-A, *cad*1-C2, or all other known members of the *cad*1-C subfamily (including *cdn1* from *G. hirsutum*), respectively (Table 1). Sequences of primers 93126 and 93T1450 would otherwise match all members of the *cad*1-C subfamily reported so far, but for primer 93126 a single nucleotide at the 14th position does not match *cad*1-C2. Using this pair of primers, transcripts of *cad*1-C could be detected in seeds of the early maturation stage (27 DPA), and stronger bands on agarose gel were found with seeds of the mid-maturation and late maturation stages (35 and 40 DPA, Figure 2A). A similar pattern of RT-PCR products was obtained with *cad*1-A specific primers, though after 30 cycles of amplification the band obtained from seeds of 27 DPA was too weak to be observed on the agarose gel (Figure 2B). By using primers specific to *cad*1-C2, RT-PCR products amplified from the samples of 40 DPA were observed (data not shown).

The relative transcription levels of these genes were further analyzed by quantitative PCR, in which ^{32}P-dATP was applied in 25 cycles of amplification, and the endogenous histone 3 mRNA was analyzed as an internal standard (Table 2). No above-background signals were obtained from seeds of 20 DPA. The *cad*1 transcripts were detectable in seeds of 27 DPA and increased along with seed maturation, reaching a high level in the late maturation stage (40 DPA). The mRNA level of *cad*1-C (possibly including at least *cad*1-C1, *cad*1-C14, and *cdn*1) was four to five times higher than that of *cad*1-A, but the time-course pattern was similar. For *cad*1-C2, a certain level of transcript was detectable only in the seeds of 40 DPA. These results indicated that, in seeds of the glanded cultivar *G. hirsutum* cv. Sumian-6, transcripts of *cad*1 sometimes appeared close to the early maturation stage (27 DPA) and dramatically increased along with seed

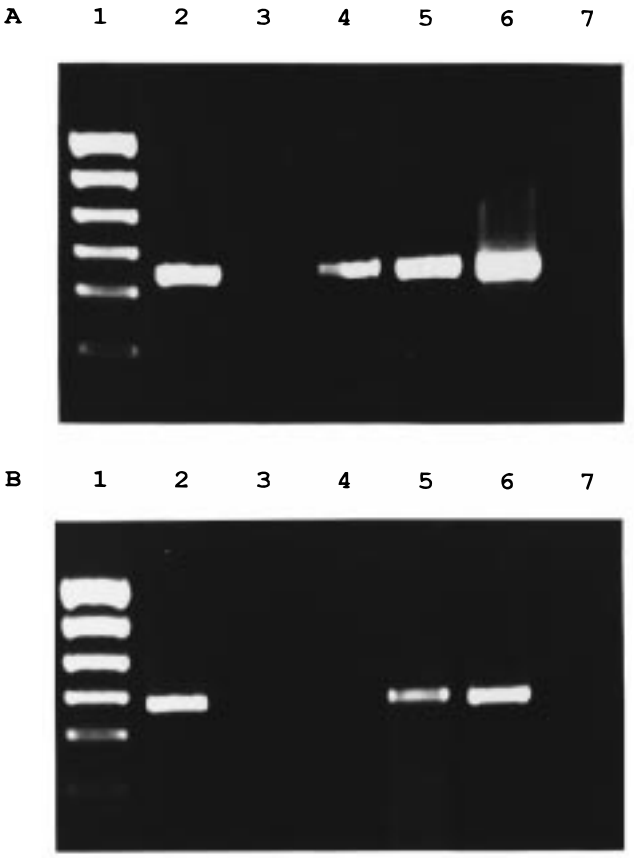

Figure 2. RT-PCR products amplified from total RNAs from seeds of *G. hirsutum* cv. Sumian-6. Molecular markers were 1543, 994, 695, 515, 377, and 237 bp (lane 1). A: Amplification of *cad*1-C (including at least *cad*1-C1, *cad*1-C14, and *cdn*1) with primers 93126 and 93T1450. PCRs were performed on the templates of *cad*1-C1 in pBluescript(−) as a positive control (lane 2) and on reverse transcripts of the RNA isolated from seeds of 20 DPA (lane 3), 27 DPA (lane 4), 35 DPA (lane 5), and 40 DPA (lane 6). As a negative control, *cad*1-A in pBluescript(−) was applied as a template (lane 7). B: Amplification of *cad*1-A with primers 93160 and T1098. PCRs were performed on the templates of *cad*1-A in pBluescript(−) as a positive control (lane 2) and of *cad*1-C1 in pBluescript(−) as a negative control (lane 7). For templates in lane 3 through lane 6, see part A.

Table 2. Relative mRNA Levels of (+)-*δ*-Cadinene Synthase in Developing Seeds of a Glanded Cotton Cultivar *Gossypium hirsutum* cv. Sumian-6[a]

postanthesis (days)	internal standard (histone 3) (dpm)	*cad*1-C1, C14, *cnd*1, etc. (dpm)	*cad*1-C2 (dpm)	*cad*1-A (dpm)
20	631	101	85	82
27	672	682	118	281
35	747	1046	102	434
40	621	2189	370	646

[a] Relative mRNA levels were estimated by RT-PCR using primers specific to *cad*1-A, *cad*1-C2, and other members of *cad*1-C (see Table 1). Each reaction mixture contained ^{32}P-dATP, and the RT-PCR product was quantitated by counting the radioactivity incorporated into the product. As an internal standard, *G. hirsutum* histone 3 mRNA[27] was analyzed with primers H3F and H3R (see Table 1). The background counts were 60−115 dpm.

Table 3. Sesquiterpene Cyclase Activity and Gossypol Content in Developing Cottonseeds[a]

days postanthesis	sesquiterpene cyclase activity (nmol FPP/h/mg)	pigment glands	sesquiterpene aldehydes (mg/g dry wt)	gossypol equivalents (mg/g dry wt)
Gossypium hirsutum cv. Sumian-6 (glanded cultivar)				
20	UD	invisible	UD	UD
27	24.3	invisible	trace	UD
35	74.6	visible	3.37	0.6
40	98.8	visible	11.86	4.7
60	50.0	visible	15.50	12.4
G. hirsutum cv. GL-5 (glandless cultivar)				
60	UD	no	UD	UD

[a] Sesquiterpene aldehydes were quantitated with a phloroglucinol/HCl reagent, and gossypol content was determined with the same reagent after TLC separation. UD: undetectable.

maturation. These kinetics of *cad*1 transcripts are similar to the accumulation pattern of cotton Class I *Lea* (late embryogenesis-abundant) mRNAs reported by Galau et al.[21]

It has to be mentioned that, although the primers used in RT-PCR analysis were based on all known members of the cotton CAD1 family reported so far (Table 1), sequence information of only one member of the *G. hirsutum* CAD1 is available; thus, bias could be introduced during RT-PCR due to sequence differences between the two cotton species. However, the kinetics of CAD1 mRNA accumulation detected here are in good agreement with the result of cyclase assay. Specific activities of the sesquiterpene cyclase increased concomitantly with the *cad*1 transcripts in developing cottonseeds of this glanded cultivar (Table 3). No activities were detectable in seeds of 20 DPA. The highest specific activities were found in seed kernel protein extracts at the late maturation stage (40 DPA), and the activities dropped to half in seeds at desiccation (60 DPA).

When reacted with phloroglucinol/HCl reagent to examine the presence of sesquiterpene aldehydes,[22] acetone extracts of seeds of 20 DPA did not show any above background absorbency at OD555, whereas only trace amounts of sesquiterpene aldehydes were found in seeds of 27 DPA. Afterward, there was a rapid accumulation of sesquiterpene aldehydes. As gossypol is reported to be the main (often the only) component of sesquiterpene aldehydes accumulating in seeds of various glanded *G. hirsutum* cultivars and *G. arboreum*,[6,7] it was isolated from the acetone extract after TLC separation, in order for more accurate estimation. Quantitative analysis showed a rapid accumulation of gossypol in seeds after 35 DPA, and, at desiccation (60 DPA), gossypol accounted for 1.2% of the seed kernel dry weight (Table 3).

Neither detectable sesquiterpene cyclase activities nor sesquiterpene aldehydes were found in seeds of a glandless cultivar, *G. hirsutum* cv. GL-5 (Table 3). RT-PCR analysis did not detect any *cad*1 transcripts either (data not shown). This is in agreement with an earlier report that glandless cottonseeds contain little or no gossypol equivalents.[23]

Cotton is mainly cultivated for its fiber. However, the seeds of cotton plant are potentially useful as a foodstuff additive. The seed kernel contains on the average 30% oil and over 30% protein. However, the nutritional value of cottonseeds is limited because of the presence of sesquiterpene aldehydes, mainly gossypol, which are toxic to monogastric animals. The present investigation has indicated that cotton CAD1 genes are expressed in developing seeds and that there is a close correlation of kinetics between the levels of *cad*1 transcripts, sesquiterpene cyclase activity, and the formation of sesquiterpene aldehyde gossypol. Thus, it may be concluded that, in glanded cottonseeds, there is an active biosynthesis of cadinene-type sesquiterpenoids, resulting in the formation and accumulation of gossypol. In addition, mRNAs of both the *cad*1-C and *cad*1-A subfamilies were detected in glanded *G. hirsutum* seeds during maturation, although transcription levels of the former were much higher than the latter. These results are of interest in designing a strategy to regulate sesquiterpene aldehyde formation in seeds by means of plant genetic engineering. It has been found that, in *G. arboreum* cell suspension cultures elicited with *V. dahliae*, both the CAD1-A and CAD1-C isozymes are induced.[12] Further investigation of the intact cotton plant will reveal if various members of the CAD1 family are differentially regulated upon differential elicitation or at different developmental stages.

In tobacco, the sesquiterpene cyclase EAS regulates the formation of the phytoalexin, capsidiol. The tobacco EAS gene shows localized inducible expression only, as determined by promoter-GUS fusion analysis.[16] The cotton sesquiterpene cyclase genes seem to have a different expression pattern. While induced CAD1 gene expression and sesquiterpene aldehyde formation have been found in fungal elicitor-treated cell suspension cultures[11] and bacterial-inoculated tissues,[1] at least in glanded seeds, expression of CAD1 genes is developmentally regulated, in coordination with the formation of glands in which gossypol accumulates.

Experimental Section

Plant Material. A glanded upland cotton cultivar, *G. hirsutum* L. cv. Sumian-6, and a glandless upland cotton cultivar *G. hirsutum* L. cv. GL-5, both from Jiangsu Agriculture Academy, Nanjing, were grown in a greenhouse. For each flower, the flowering date was recorded. Seeds were collected at 20, 27, 35, and 40 DPA and when mature (60 DPA). For each collection, seeds were sampled from six different flowers. Experiments were carried out in triplicate, and mean values were taken for quantitative estimation.

Screening of the cDNA Library. The λ-UniZAP cDNA library was constructed from mRNAs prepared from *V. dahliae* elicitor-treated cell suspension cultures of *G. arboreum* L. cv. Nanking, as previously described.[11] The library was screened using a PCR-96-well plate method as described.[20] Briefly, the library of about 10^7 pfu was spread into 96 wells and mixed with *E. coli* strain XL1-Blue. After overnight incubation at 37 °C, aliquots from each well were used as templates in PCR amplification. A forward primer 93900 and a reverse primer TM2 (Table 1) were used, and PCR was carried out at 94 °C for 15 s, 52 °C for 30 s, and 72 °C for 1 min, for 30 cycles altogether. Positive wells were then selected for the next round. After four rounds of screening, a positive clone was isolated and the plasmid excised according to Stratagene's protocol. DNA sequences were determined with a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE-ABI, Warrington, UK).

RNA Analysis. Total RNA was isolated from the seed kernel by the cold phenol method,[24] followed by further

purification with the RNeasy Plant Total RNA kit (Qiagen, Chatsworth, CA). The first strand cDNA was synthesized with 1 μg total RNA in a 20 μL reaction, using a reverse transcription system (Promega, Madison, WI), and then 1 μL of the reaction mixture was used in PCR amplification carried out as described above. Primers specific to different members of *cad*1 (Table 1) were applied. Quantitative PCR analysis was carried out as described.[24,25] Briefly, the ^{32}P-dATP of 0.1 μCi was included in each PCR mixture (30 μL) containing the four dNTPs (200 μM each), and the primers (0.2 μM each) listed in Table 1 were applied for 25 cycles of amplification. The labeled products were then electrophoresed on a 1.5% agarose gel, together with cold products that were amplified from the corresponding cDNA. The band was cut out and rinsed twice with double-distilled H_2O, and the radioactivity was determined in a scintillation counter. As an internal standard, histone 3 mRNAs were analyzed in a separate tube with the same method. A forward primer H3F and a reverse primer H3R (Table 1) were synthesized according to published *G. hirsutum* histone 3 cDNA sequences.[27]

Sesquiterpene Cyclase Assay. Proteins were isolated from seed kernels with a buffer containing 50 mM Tris/Cl, 10 mM EDTA (pH 8.0), 1% β-mercaptoethanol, and 20% glycerol. Protein concentrations were determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Specific activities of sesquiterpene cyclase were assayed as described.[11,28] Briefly, seed proteins of 15 μg were added to 200 μL of buffer containing 0.15 μCi [^{3}H] FPP (Sigma, St. Louis, MO), 15 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 3.5 nmol FPP. After incubation at 37 °C for 10 min, the reaction mixture was extracted twice with 150 μL hexane, and the extract was treated with activated silica gel powder. Radioactivity was determined in a scintillation counter.

Sesquiterpene Aldehyde Analysis. Seed kernels were immersed in liquid nitrogen and ground into a fine powder. An aliquot (100 mg) was extracted with acetone at room temperature for 30 min. Total sesquiterpene aldehydes were then quantitated using the phloroglucinol assay.[22] A standard curve was made for gossypol (Sigma, St. Louis, MO). The acetone extract was further separated by TLC (silica gel G_{254}), and gossypol was included as a standard. After developing with ethyl acetate−hexane (1:1) or with *n*-butanol−acetic acid−water (4:1:5), the plates were sprayed with 5% ethanolic phloroglucinol/HCl, and the color was developed at 55 °C, as described.[29] For quantitation, the plates were visualized under UV light, the spot identical to gossypol was scraped and extracted with the reagent (1% phloroglucinol, 2 N HCl in 95% EtOH), and gossypol was then quantitated as described above.

Abbreviations used: CAD, (+)-δ-cadinene synthase; *cad*, cDNA or mRNA of CAD; DPA, days postanthesis; EAS, 5-*epi*-aristolochene synthase; FPP, farnesyl diphosphate; PCR, polymerase chain reaction; OD, optical density; RT, reverse transcription.

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