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Cloning and Heterologous Expression of a Second (+)-δ-Cadinene Synthase from *Gossypium arboreum*

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Screening of a *Gossypium arboreum* L. cv. Nanking cDNA library resulted in the identification and cloning of a second (+)-δ-cadinene synthase. A probe for the screens was prepared by PCR using primers based on conserved sequences in farnesyl diphosphate cyclases and genomic DNA as a template. This second cDNA clone encodes a protein that is 80% identical to the recently described (+)-δ-cadinene synthases CAD1-C1 and C14 from *G. arboreum* and maintains a significant degree of homology to the other known mono-, sesqui-, and diterpene synthases. As in the case of CAD1-C1 (+)-δ-cadinene synthase from cultured cotton cells, the synthesis of the second CAD1-A mRNA was induced by treatment of cotton cell suspension cultures with a partially purified elicitor preparation from the phytopathogenic fungus *Verticillium dahliae*. Expression of CAD1-A mRNA was quantitated with reverse transcription PCR and showed that CAD1-A mRNA was maximally increased 8-fold, 6 h after addition of elicitor. Heterologous expression of this second cDNA produced a 64 kD protein that catalyzed the cyclization of farnesyl diphosphate to (+)-δ-cadinene, the identical product produced by CAD1-C1. The steady-state kinetic parameters of CAD1-A were similar to CAD1-C, showing a K_m of 7 mM farnesyl diphosphate and k_{cat} of 0.039 s^{-1} at 30 °C. However, the optimal pH and Mg^{2+} concentration for CAD1-A activity were significantly higher than those observed for CAD1-C.

The elicitation of stress conditions in plants upon interaction with biotic elicitors, like phytopathogenic microbes, results in a number of discrete metabolic changes.[1] These inducible defense responses in plant cells include biosynthesis of secondary metabolites,[2] synthesis of reactive oxygen species,[3] hydroxyproline-rich proteins,[4] protease inhibitors,[5] lignin-like material,[6] callose,[7] and hydrolytic enzymes,[8] and the hypersensitive response,[9] etc. The resulting metabolic changes are designed to facilitate resistance of the plant tissue to colonization by the pathogen. Thus, the induction of the biosynthesis of secondary metabolites with antimicrobial activity, collectively called phytoalexins, serves to inhibit further growth of the phytopathogen in plant tissue.

Most of the metabolic alterations are initiated by the induction of gene expression. For example, the synthesis of all the biosynthetic enzymes catalyzing reactions in the pathways leading from phenylalanine to flavonols, isoflavonoids, anthocyanins, and lignin precursors are induced in a variety of plants such as parsley, pea, chickpea, alfalfa, and soybean upon stimulation with fungal-derived elicitor molecules.[11] In tobacco,[12] the biosynthesis of the sesquiterpenoid antimicrobial metabolite capsidiol is induced by fungal cell wall hydrolysates or cellulase.[13] Specifically, the elicitor-induced regulation of capsidiol formation in tobacco is initiated by the induction of the synthesis of the sesquiterpene cyclase, 5-*epi*-aristolochene synthase, with the concomitant supression of the primary metabolic enzyme squalene synthase required for the formation of sterols. This elicitor-induced metabolic shift and expression of 5-*epi*-aristolochine synthase results in the preferential utilization of farnesyl diphosphate for the synthesis of the phytoalexin capsidiol.[14] A variety of terpenoids in other plant families are synthesized upon interaction of a phytopathogen with plant cells in culture and with intact plantlets.[15]

In cotton, a similar induction of the esquiterpenoid pathway is observed. The interaction of the wilt-producing fungus *Verticillium dahliae* with *Gossypium arboreum* cells in culture initiated the *de novo* synthesis of the cyclic sesquiterpene aldehydes: hemigossypol, gossypol, and derivatives.[10] Phytopathogenic bacterial elicitation of the synthesis of cadinane-type sesquiterpenes from farnesyl diphosphate can be observed in foliar tissue of resistant cotton varieties.[16] Subsequently, a farnesyl diphosphate cyclase was cloned from fungal elicitor-treated *G. arboreum* cell suspension cultures and expressed in *E. coli*.[17] The enzyme catalyzed the formation of (+)-δ-cadinene.[16,17] During the isolation of this clone, additional probing of the *G. arboreum* cDNA library resulted in the identification of a second farnesyl diphosphate cyclase cDNA, encoding a protein with 80% identity to the (+)-δ-cadinene synthase from *G. arboreum*.[17] The cloning, heterologous expression, and characterization of the second farnesyl diphosphate cyclase are reported herein.

Results and Discussion

As described previously,[17] the original PCR was performed on genomic DNA isolated from cell suspension cultures of *G. arboreum* L. cv. Nanking, using primers 3876 and 3819, obtained from Dr. Joe Chappell, University of Kentucky. Both primer sequences were based on the conserved regions in the 5-*epi*-aristolochene synthase (EAS) gene isolated from tobacco.[11] From the PCR's, a 503 bp genomic DNA fragment was identified. Alignment of the PCR product sequence with

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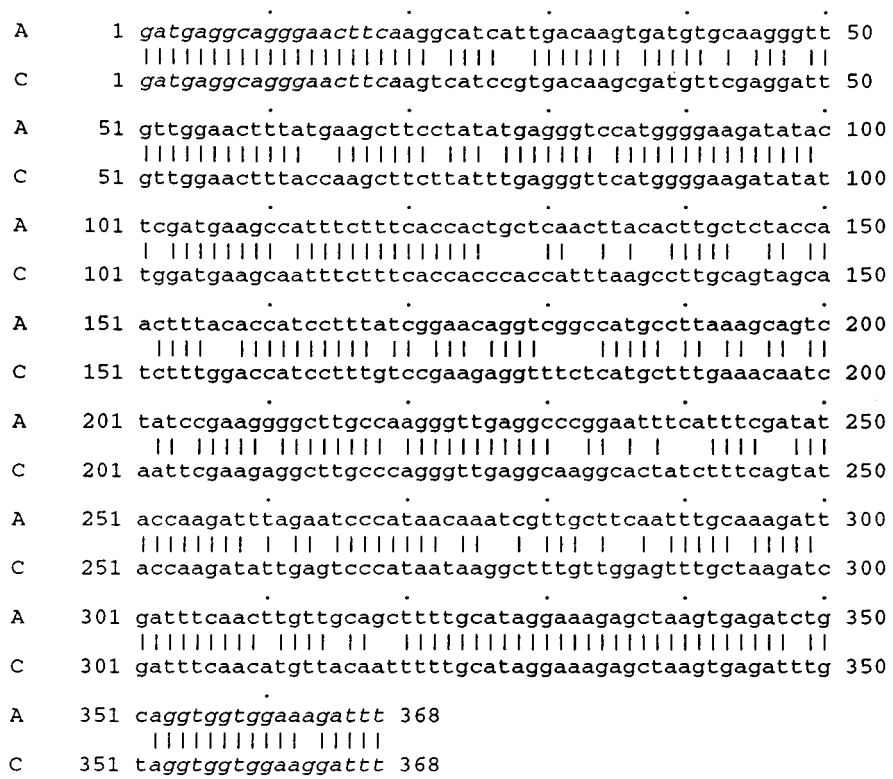

Figure 1. Alignment of two reverse transcriptase-polymerase chain reaction products, A and C.1 or C.14. Primers used in the amplification are 9310 (sense) and 9315 (antisense). Their complimentary sequences are indicated by italic letters. Both fragments, labeled with [α-^{32}P]dCTP by random priming, were used as probes to screen a *G. arboreum* cDNA library.

Table 1. Primers Used in PCR and RT-PCR

primer	DNA sequence	position in *cad* 1-A
sense		
3876	GAAATTGATGAGATTTTGT	+128 − 146[a]
9310	GATGAGGCAGGGAACTTCA	+415 − +433
93160	CCACTGGCAACTTACA	+536 − +552
93126	ATAAGGATGAAATGCGTCC	+50 − +68[b]
94E02	GCAGCTGTGGATCC CATGGCTTCACAAGTT	−15 − +15 (*Bam*HI)
antisense		
9315	ARRTCYTTCCACCACCT	+766 − +782
3819	CCATATGCTAGAGATCGAG	+802 − +820*
93T1600	TTGGACTGAATCAATGAAG	+225 − +243**
93T550	AGGAAGCTTCATAAAGTTC	+566 − +584 (*Hin*d III)

[a] Positions in tobacco 5-epi-aristolochene synthase,[11] obtained from J. Chappell, University of Kentucky. [b] Positions in *cad*1-C and *cad*1-C14.[17]

the predicted region in the EAS gene indicated a 60% DNA sequence identity, including a 97 bp intron. Internal primers 9310 and 9315 (Table 1) were then synthesized according to the sequence of the cotton genomic DNA fragment and used in subsequent RT-PCR's. Templates for the RT-PCR's were total RNA isolated from *G. arboreum* cells 10 h after elicitation with an acetone-precipitated preparation of *V. dahliae*. A UniZap cDNA library was constructed from poly(A+) RNA prepared from *V. dahliae*-treated *G. arboreum* cell cultures.[17] A hybridization probe prepared previously, consisting of a mixture of fragments A and C (Figure 1), was used to isolate two closely related cDNA clones (97%, amino acid identity). These DNA sequences matched those of *cad*1-C and encoded 554 amino acid proteins that showed cadinene synthase activity.[17]

The cDNA library was screened a second time with only fragment A. Ten individual cDNA clones were identified after two rounds of selection, each containing cDNA inserts of 1.9 kb. DNA sequence analysis of four isolates indicated that they represented full-length clones containing fragment A. The cDNA was distinct from those previously reported that encoded (+)-δ-cadinene synthase[17] and was named *cad*1-A.

Table 2. Identities and Similarities between Predicted Peptides of Plant Terpene Cyclases[a]

	A	C1	C14	EAS	CAS	LIM
A		79.60	79.42	50.00	40.22	31.17
C1	90.25		96.94	47.99	38.09	32.13
C14	89.53	98.56		48.72	38.81	33.39
EAS	66.18	65.93	65.93		42.23	33.09
CAS	63.77	62.46	62.46	64.72		30.80
LIM	53.15	53.25	53.25	53.68	52.62	

[a] Identities (%) are represented in the upper right half of the table and similarities (%) in the lower left half of the table. A, C1, and C14 represent three cDNA clones of sesquiterpene cyclases from *G. arboreum*;[17] EAS, 5-*epi*-aristolochene synthase from *N. tabacum*;[11] CAS, casbene synthase from *R. communis*;[32] LIM, 4*S*-limonene synthase from *M. spicata*.[33]

An open reading frame in cDNA clone A codes for a 555 amino-acid protein (Figure 2) with a predicted molecular weight of 64 062. The predicted protein sequence is consistent with a plant terpene cyclase function since amino acid alignments show a high degree of homology with related enzymes. As shown in Table 2, the highest identity (80%) was found with the recently discovered (+)-δ-cadinene synthase from cotton encoded by *cad*1-C1 and *cad*1-C14. The cDNA *cad*1-A reported here is, however, distinct on the basis of these comparisons. As expected, there are additional close relationships between the protein encoded by cDNA *cad*1-A with other plant terpene cyclases[13,18−27] (Table 2). Of particular significance is the tobacco FPP cyclase, 5-*epi*-aristolochene synthase (EAS), which main-

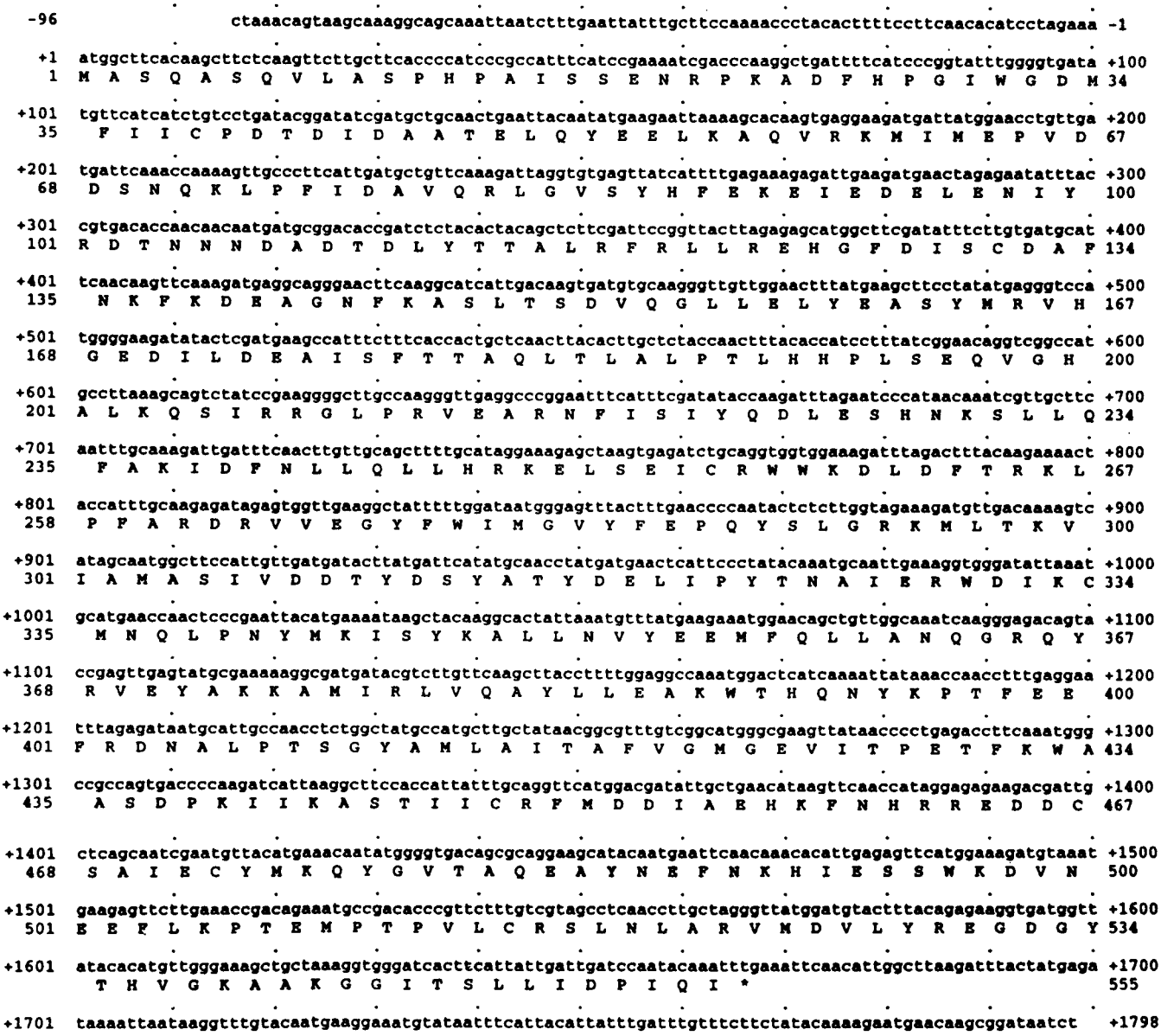

Figure 2. Nucleotide and predicted amino acid sequences of the pXA-T7 (+)-*δ*-cadinene synthase cDNA clone from *G. arboreum*.

Table 3. Segments of Conceived Amino Acid Homology in Plant Sesquiterpene Cyclases[a]

A	119FRLLREHGF	256RWWKDLDF	267LPFARDR	306IVDDTYD	448CRFMDDIA	472IECYM	519LNLAR
C1	118FRLLREHGY	255RWWKDLDF	266LPYARDR	305IVDDTYD	447CRFMDDIA	471IECYM	518LNLAR
C14	118FRLLREHGY	255RWWKDLDF	266LPYARDR	305IVDDTYD	447CRFMDDIA	471IECYM	518LNLAR
EAS	113FRLLRQHGF	251RWWKD	262LPYARDR	301IVDDTFD	443CRVIDDTA	471IECCM	514LNLAR

[a] Numbers indicate amino acid positions in each protein. C1 and C14 are cotton (+)-*δ*-cadinene synthases previously reported.[17] EAS is 5-*epi*-aristolochene synthase from *N. tabacum*.[11]

tains a 50% identity.[13] This protein bears a more distant relationship with the diterpene cyclase casbene synthase (CAS)[26] and the spearmint monoterpene cyclase 4*S*-limonene synthase (LS).[27] A further search of the protein databases (Genbank, EMBL, and Swisspro) did not identify related sequences of significant homologies. These data clearly implicated the protein encoded by the cotton cDNA *cad*1-A to be a FPP cyclase related to cadinene synthase.

Short regions of significant similarity were previously identified in *cad*1-C[17] and were conserved in the protein encoded by *cad*1-A. Table 3 lists seven segments that have particularly high similarity in the known plant FPP cyclases. The aspartate-rich motif (I, L, V)DDXXD, present at positions 306−312 of the protein (Table 3, Figure 2), has been considered a common consensus sequence for the broader classes of terpenoid cyclases and isoprenoid diphosphate synthases.[27−34] A related aspartate rich sequence (positions 452−453) is also highly conserved in the plant FPP cyclases. Two histidine residues at amino acids 88 and 125, and the cysteine residue at amino acid 471 (Figure 2), are conserved in all plant terpene cyclases. These two histidine residues have been postulated to participate in the active sites of (4*S*)-limonene synthase, according to amino acid-directed[35] and mechanism-based inhibitors.[36]

The 5′-terminal region flanking the coding DNA sequence in *cad*1-A was modified by PCR to incorporate a unique *Bam*HI site and to remove noncoding DNA.

Table 4. Purification of (+)-δ-Cadinene Synthase Cloned from *G. arboreum* and Expressed in *E. coli*

purification steps	total protein (mg)	specific activity (nmol mg^{-1} h^{-1})	total activity (nmol h^{-1})	recovery (%)	purification factor
crude extract	132	64	8434	100	1
streptomycin treated	90	93	8370	99	1.5
affinity column	6	1205	7291	86	19

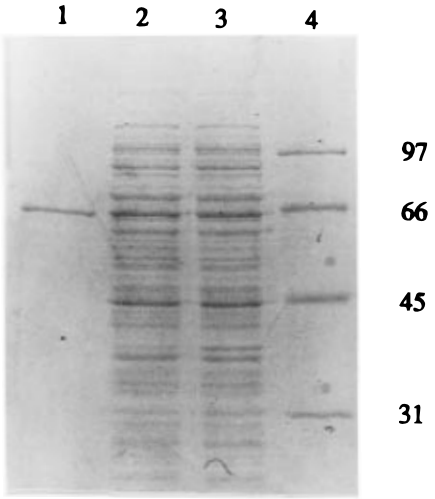

Figure 3. SDS−PAGE (10%) of protein extract from *E. coli* BL21(DE3)p*LysS* transformed by pXA-T7. Lane 1: 15 μg of purified protein from induced cultures. Lanes 2 and 3: 15 μg of protein from *E. coli* cells induced with IPTG. Lane 4: molecular mass markers (97.4, 66.2, 45, and 31 kD, top to bottom). The gel was stained with Coomassie brilliant blue.

The major portion of the noncoding region at the 3′-end was removed by restriction modification and facilitated direct subcloning of the coding region into the *E. coli* expression vector pET28b (Novagen). This vector encodes a heptahistidyl leader sequence under the control of the T7 RNA polymerase promoter. *E. coli* strain BL-(DE3)p*Lys*S was transformed with pXA-T7. Protein production was induced in cultures grown at 25 °C with 0.01 mM IPTG. Cultures grown at higher temperatures and induced with 1 mM IPTG resulted in 90% of the induced fusion protein being in the form of insoluble inclusion bodies.

Fusion proteins of the predicted molecular mass were detected on SDS−PAGE of cell-free extracts from the induced *E. coli* cultures. The target protein produced was purified by a two-step process (Table 4). Following elution from a 1 × 5 cm His-Bind column and electrophoresis on 10% SDS−PAGE, a single major band was apparent (Figure 3). The molecular mass of the fusion protein CAD1-A was estimated by SDS−PAGE as 66 kD. This observed mass is consistent with a heptahistidyl fused to the plant FPP cyclase encoded in *cad*1-A.

The characteristics of the proteins were also analyzed by immunoblotting at each step of purification with a monoclonal antibody 5G9 raised against tobacco EAS.[13] In each blotting experiment the heptahistidyl fusion protein from cotton, (+)-δ-cadinene synthase (CAD1-C1), was included as a positive control. Despite the functional homology and close structural identity, the plant FPP cyclase from *cad*1-A did not cross-react with the antibody 5G9,[17] suggesting that this antibody recognized unique epitopes in CAD1-C and EAS.

The specific activity of the purified fusion protein CAD1-A was found to be 1.2 mmol of products formed mg $protein^{-1}$ h^{-1}. The K_m of the CAD1-A protein was found to be 7 μM FPP, when assayed at 30 °C for 10 min. Removal of the N-terminal leader sequence (15 amino acids) by thrombin cleavage, followed by elution of the protein on an His-Bind column, provided an enzyme with 1.8-fold higher specific activity (2.2 mmole of product mg $protein^{-1}h^{-1}$) that displayed a similar K_m. The specific activity of CAD1-A is similar to that of protein CAD1-C (1.1 μmol of products mg $protein^{-1}$ h^{-1} at 30 °C). In comparison, the thrombin-cleaved CAD1-C protein displayed a K_m of 10 μM. These parameters are consistent with other plant terpene cyclases.[13,18−23,25,35]

Pentane extractions of the CAD1-A-catalyzed reaction were used for product characterization. Capillary GC analysis showed that the product of the CAD1-A-catalyzed reaction was identical to the product of the CAD1-C-catalyzed reaction. MS analysis revealed a molecular ion at *m*/*z* 204 (42% RA) and a fragmentation pattern as previously found for the product extracted from the CAD1-C-catalyzed reactions.[17] These data are consistent with a cadinene structure. ^{1}H NMR analysis supported a δ-cadinene structure, and stereochemical analyses on a chiral capillary GC of the CAD1-A product verified the compound as the (+)-enantiomer. Elution of the product was observed with the product from CAD1-C and the material isolated from cade oil.[17,48]

Despite their identical catalytic activities, CAD1-A and CAD1-C required different assay conditions for optimal activities. The pH optimum of protein CAD1-A was 8.7 (20 mM Tris/HCl), dropped to 80% at pH 7 (20 mM HEPES) and to 62% at pH 6.75 (20 mM MES). Highest activity of protein CAD1-C was observed in the range of pH 7 (20 mM HEPES) to 7.5 (20 mM Tris/HCL). At pH 8.7 (20 mM Tris/HCl) the catalytic activity of Cad1-C was reduced to 60%. Another distintive feature of the (+)-δ-cadinene synthases CAD1-A and CAD1-C were their metal dependence for optimal activities. Enzymatic activities of CAD1-A was dependent on the divalent metal Mg^{2+} with optimal activity obtained at 2.5 mM $MgCl_2$, whereas CAD1-C required 15 mM $MgCl_2$ for optimal activity.[17]

Previously, northern analysis showed that elicitation of *G. arboreum* tissue cultured cells with *V. dahliae* elicitor increased CAD1-C1 mRNA 8-fold after 10 h when compared to control cultures.[17] A similar analysis was carried out for CAD1-A mRNA production. However, to specifically measure mRNA levels for CAD1-A and CAD1-C1 in the same sample, quantitative RT-PCR was used.[37−39] This procedure uses a genomic DNA sequence as an internal standard to quantitate an unknown cDNA. The standard DNA fragment selected was identical to the unknown *cad*1-A except for the presence of a 97 bp intron. Two genomic DNA fragments were prepared from *G. arboreum* DNA using PCR, after TA-cloning and amplification in *E. coli* (Figure 4). Competitive RT-PCR using primers specific for *cad*1-A and *cad*1-C1 allowed for the quantitative analyses of mRNA for the two similar sequences.

+536	CCACT	GCTCAACTTA	CACTTGCTCT	ACCAACTTA	CACCATCCTT	
	TATCGGAACA	GGTCGGCCAT	GCCTTAAAGC	AGTCTATCCG	AAGGGCTTG	
	CCAAGGGTTG	AGGCCCGGAA	TTTCATTTCG	ATATACCAAG	ATTTAGAATC	
	CCATAACAAA	TCGTTGCTTC	AATTTGCAAA	GATTGATTTC	AACTTGTTGE	
	AGCTTTTGCA	TAGGAAAGAG	CTAAGTGAGA	TCTGCAGGTA	AGTGTTTGGA	
	GATCTTTAAA	GCTATGAAGT	CTAATACTAT	TTCAATTGAT	CACACGACTG	
	TTGCTGACAT	TTTATGATGC	TTTTTTTAGG	TGGTGGAAAG	ATTT	+782
+50	A	TAAGGATGAA	ATGCGTCCCA	AAGCCGATTT	TCAGCCTAGC	
	ATTTGGGAG	ATCTCTTCCT	CAACTGTCCC	GACAAGGTAT	ACATACATAT	
	GACCATATTG	GTATATAATC	TTAACAGGTT	ATTTGTTTTA	TTTAGTGATG	
	CAATGTGAAA	AAAAATTTCA	TTATTTTGAG	CAGAATATTG	ATGCTGAAAC	
	TGAAAAGCGC	CACCAACAAT	TGAAAGAAGA	AGTGAGGAAG	ATGATTGTGG	
	CACCAATGGC	TAATTCAACC	CAAAAGTTAG	CCTTCATTGA	TTCAGTCCAA	+243

Figure 4. Genomic DNA fragments used as internal standards in the quantitative analysis of mRNA coding for CAD1-A (top) and CAD1-C1 (bottom). Both fragments were obtained by PCR using genomic DNA from *G. arboreum* as templates and the specific primers 93160/9315 for *cad*1-A and 93126/93T1600 for *cad*1-C1 and C14 (Table 1). The introns are underlined.

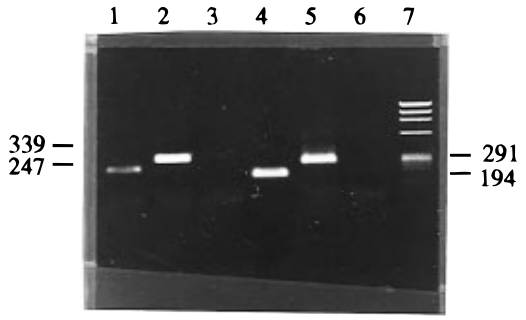

Figure 5. Agarose gel electrophoretic analysis of PCR products from the amplification of *G. arboreum* genomic DNA internal standards and *cad*1-A or *cad1*1-C1−C14. Primers for the products shown in lanes 1−3 were 93160/9315, specific for *cad*1-A nucleotide sequences; for the products in lanes 4−6 the primers were 93126/93T1600, specific for *cad*1-C1 or C14 nucleotide sequences. The following templates were used: lanes 1 and 4, reverse transcripts of 20 ng total RNA isolated 6 h after elicitation of a *G. arboreum* cell suspension culture with partially purified *V. dahliae* elicitor; lanes 2 and 5, amplification of 50 ng genomic DNA; lane 3, 0.1 ng of pXC-T7 cDNA; and lane 6, 0.1 ng of pXA-T7. In lane 7, DNA markers (1353−194 bp) were separated.

Selection of the correct primers was based on specificity for the *cad*1-A and *cad*1-C1 mRNA. Thus, sense primer 93160 and antisense primer 9315 were used to measure *cad*1-A mRNA and sense primer 93126 together with antisense primer 93T1600 for *cad*1-C1 and *cad*1-C14. By using these specific primers, it was possible to obtain two distinct fragments on agarose gel electrophoresis (Figure 5). Cross priming events were excluded, since control reactions with the specific primers and incorrect *cad*1 templates did not show any detectable products on agarose gels. Control samples from nonelicited cell suspension cultures contained 2.3×10^{-5} pmol of *cad*1-C1 mRNA in 1 μg of total RNA but only 12.6×10^{-7} pmol of c*cad*1-A mRNA (Figure 6). The actual number of target mRNA molecules present in each initial sample could be higher, as the efficiency of cDNA synthesis using oligo (dT) was reported to be 40−50%.[40] Despite the 100-fold differences in the basal mRNA levels, elicitor action induced both (+)-δ-cadinene synthase transcripts in *G. arboreum* Nanking cell suspension cultures. A 13-fold induction of *cad*1-C1 was observed after 6 h of elicitation, and *cad*1-A mRNA was 8-fold higher under similar conditions.

A reason for the presence of two enzymes catalyzing the same reaction in secondary metabolism in cotton cells is unclear at this time. The existence of multiple closely related isozymes in phytoalexin biosynthesis has been observed for the rate-limiting enzymes in flavonoid formation, phenylalanine ammonia lyase (PAL),[41,42] and chalcone synthase (CHS).[11] In terpenoid biosynthesis, induced by defense related signals, multiple isozymes of 5-*epi*-aristolochene synthase (EAS) in tobacco[12] and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in potato[44] are expressed. In all plants investigated, the genes for these enzymes are transiently activated by different elicitor signals. For example, CHS isozymes are differentially induced by UV and visible light and by fungal elicitation.[11] Similarly, HMGR isozymes are differentially induced by wounding or fungal elicitors. In the potato, this differential elicitation results in the wound-induced elevation of steroid biosynthesis and fungal pathogen-induced biosynthesis of sesquiterpene phytoalexins.[44] These mechanisms allow plants to respond to different environmental stimuli in a programmed manner by activating specific metabolic responses. Clearly, both (+)-δ-cadinene synthase isozymes, CAD1-A and CAD1-C1, are induced in *G. arboreum* cell suspension cultures elicited with *V. dahliae*. However, plant cell cultures exhibit undifferentiated growth, and the expression of the two isozymes in intact cotton plants analogous to differential expression of rate-limiting isozymes in other plants upon differential elicitation, or tissue specific elicitation, cannot be excluded.

Experimental Section

Materials. Chemicals and biochemicals were obtained from Sigma Chemical Co. except as indicated. [1(*n*)-^{3}H]farnesyl diphosphate, triammonium salt, 19.6 Ci/mmol, was obtained from Amersham, Arlington Heights, IL. The plasmids pBluescript SK(+) were from Stratagene and pET28b from Novagen; ZAP cDNA synthesis kit and Gigapack II packaging extract were from Stratagene; Wizard PCR preps DNA purification system was from Promega; RadPrime DNA labeling system was from Gibco BRL; sequence version 2.0 DNA

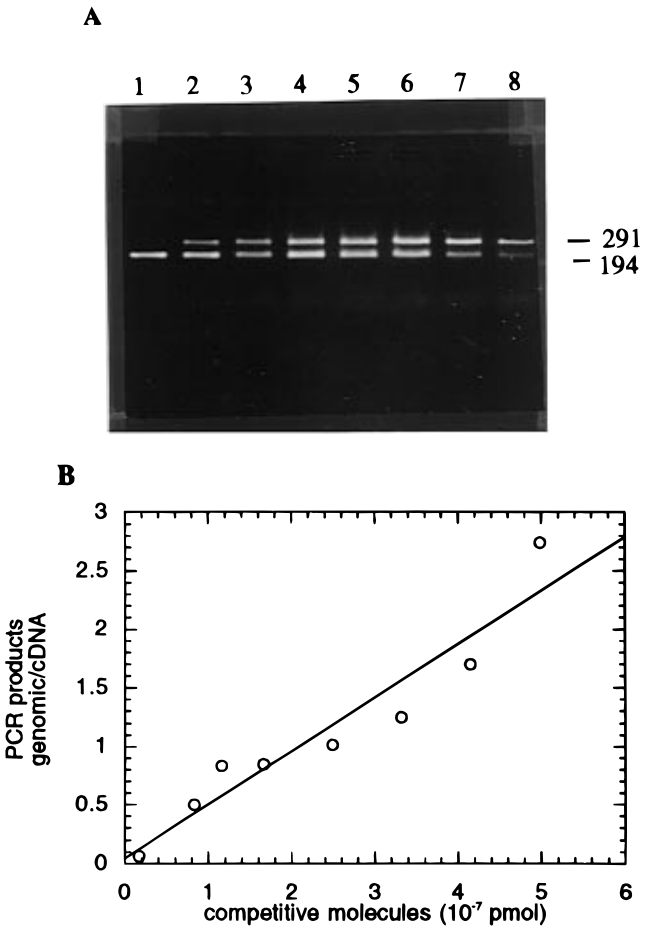

Figure 6. (A) Competitive PCR of *cad*1-C1 or C14 (lower band, 194 bp) prepared from total RNA-extracted *G. arboreum* cells 6 h after elicitation with partially purified elicitor from *V. dahliae* and an internal standard genomic DNA (pXgC-SK, upper band, 291 bp) prepared from *G. arboreum* cells. All reactions contained primers 93126/93T1600, specific for *cad*1-C1 or C14. The internal standard genomic DNA, pXgC-SK, concentration was 0.33/0.83/132;1.66,2.49; 3.32, 4.35, and 4.98 $\times 10^{-6}$pmol in tube 1 through 8. (B) The amounts of the PCR products were determined by scanning the agarose electrophoretic gels and plotting the ratios of the PCR products obtained with the genomic DNA to the cDNA template, against the number of molecules of internal standard genomic DNA, pXgC-SK, added to each PCR.

sequencing kit was from USB; His-Bind resin was from Novagen; and nitrocellulose filters BA85 and NAS85 were purchased from Schleicher & Schuell. *E. coli* strains XL1-Blue, XL1-Blue-MRF, and SOLR were purchased from Stratagene; strains DH5a and BL21-(DE3) were obtained from Novagen.

Plant Cell Cultures. Cells of *Gossypium arboreum* L. cv. Nanking were maintained in liquid LS medium[45] and transferred every 7 days. For elicitation experiments, cells in the exponential growth phase were treated with acetone precipitated elicitor from *Verticillium dahliae* strain 277 or TA, at a final concentration of 1 μg protein mL^{-1} culture, as previously described.[10,46] In time-course experiments for the quantitation of mRNA by the competitive reverse transcription polymerase chain reaction (RT-PCR), 2 ccm of cells (ca. 0.15 g dry wt) were transferred to 50 mL of fresh LS medium. After a 48 h incubation, 0.5 mL of elicitor (50 μg of elicitor protein) was added to each flask. The treated cells were collected at desired time intervals, and the nucleic acids were extracted. While a 100-fold increase of gossypol equivalents can be detected after a 72 h elicitation period, cell growth rates decreased by about 50−70% in these elicited cultures.[10,19] No elicitor was added to control flasks.

Isolation of Nucleic Acids and Polymerase Chain Creation. The isolation of genomic DNA and total RNA as well as the purification of poly(A$^+$) RNA from 3-day old cell suspension cultures of *G. arboreum* cv. Nanking were as described.[17] PCR with genomic DNA or total RNA for RT-PCR were performed with the primers listed in Table 1 and executed as previously described.[17] PCR products were purified using the Wizard PCR Preps DNA purification kit (Promega). DNA fragments were amplified in *E. coli* XL1-Blue, after TA-ligation with pBluescript (SK+). Recombinant plasmids were isolated and analyzed for DNA inserts by restriction digests and the resulting products sequenced.[17]

cDNA Library Construction. Cell suspension cultures of *G. arboreum* treated with elicitor from *V. dahliae* for 10 h were used as a source of total RNA and the subsequent isolation of mRNA. The synthesis of cDNA, ligation to lUniZAP XR, packaging (Gigapack II), and infection of *E. coli* strain XL1-Blue-MRF' was as described.[17]

cDNA Probes and Screening of cDNA Library. For the initial PCR, *G. arboreum* genomic DNA, as template, and primers 3876 (sense, Table 1) together with antisense primer 3819 (Table 1) were used. The primers 3876 and 3819 were a gift from Dr. Joe Chappell, University of Kentucky. PCR's were performed with 5−50 ng of template DNA in 10 mM Tris/HCl, pH 8.8, containing 2.5 U of AmpliTaq DNA polymerase, 200 μM dTNP, 1.5 mM $MgCl_2$, and 0.8 μM of each primer in a total volume of 100 μL. The initial denaturation stage was performed at 94 °C for 3 min. Each thermal cycle was composed of 1 min at 94 °C, annealing for 1 min at 40 °C, and extension for 1 min at 72 °C for a total of 30 cycles. The reaction mixture was diluted 80-fold, and a 1 μL aliquot was used as template in a second PCR amplification using the identical conditions, yielding a 503 bp DNA fragment. From the nucleotide sequence of this fragment internal primers 9310 (sense) and 9315 (antisense) were synthesized (Table 1). These specific primers were then used in a RT-PCR with total RNA, isolated from *G. arboreum* cells treated with elicitor for 10 h, as template. A 368 bp DNA fragment was detectable by agarose gel electrophoresis of a 5 mL aliquot of the RT-PCR mixture.

The RT-PCR products were purified using Wizard PCR Preps DNA purification system (Promega). The fragments, which were A-tailed by an incubation at 72 °C for 10 min following the PCR thermal cycles, were ligated with pBluescript SK(+) that was *Eco*RV digested and T-tailed by incubation with 1 nmol dTTP and 2.5 U of Taq DNA polymerase in a 100 μL reaction.[20] Recombinant plasmids were isolated after amplification in *E. coli* XL1-Blue (Stratagene) and analyzed for DNA inserts by restriction digests of the purified plasmids. Sequence analysis of the 368 bp segment showed that the product contained two DNA fragments, C and A, exhibiting a 79% nucleotide homology. Screening of the cDNA library with a mixture of fragments C and A as probe identified several hybridizing clones, including

*cad*1-C1 and *cad*1-C14, [17] but clones with sequences corresponding to fragment A were not found. However, some plasmids contained only fragment A. This DNA fragment was isolated and labeled with [α-^{32}P]dCTP by random labeling and used as a probe to screen a *G. arboreum* cDNA library a second time, as described.[17] The positive rescued plasmid clone was named pXA.

Nucleotide Sequencing and Analysis of Nucleic Acids. Single- and double-stranded forms of template DNA were used for sequencing reactions by the dideoxynucleotide chain-termination method. Both sense and antisense strands of the isolated cDNA clones or the TA-cloned PCR products were completely sequenced with vector specific and synthetic internal primers. DNA sequences and the deduced peptide sequences were analyzed and aligned with related genes by Genetics Computer Group programs, Sequence Analysis Software Package.

Subcloning into pET28b. The expression vector pET28b harboring the *cad*1-A was constructed in two steps. First, the 5' region of *cad*1-A was modified to remove the nontranslated sequence. A sense primer 94EO2 (Table 1) was designed to introduce a *Bam*HI site via PCR with the antisense primer 93TA550 (Table 1) using pXA1-SK, containing the *cad*1-A insert, as the DNA template. Digestion of the PCR product with *Bam*HI and *Eco*RV resulted in a fragment encompassing bases +1 to +126. The original pXA1-SK was digested with *Bam*HI (from vector) and *Eco*RV (from insert, at base 126), and the large fragment was purified by agarose gel electrophoresis. The digested PCR product was ligated with the recovered large fragment to create the intermediate plasmid construct pXA2-SK. The 3'-noncoding region of pXA2-SK was deleted by digestion with *Afl*II (from insert at base + 1681, Figure 2) and *Kpn*I (from vector) and the large fragment was purified by agarose gel electrophoresis. The rcovered DNA fragment (5 μg) was treated with 4 U of Klenow fragment and 0.5 mM dNTPs followed by ligation of the resultant blunt ends to arrive at pXA3-SK. The entire coding region for CAD1-A was recovered by *Bam*HI and *Xho*I digestion, which was cloned into the identical sites in pET28b (Novagen) to make the final product pXA-T7. The fidelity of the PCR amplification steps was verified in the coding sequence of *cad*1-A by dideoxy sequencing of pXA-T7.

Protein Production. *E. coli* BL21(DE3)p*Lys*S transformed with pXA-T7 was used for the *in vivo* protein expression. Bacteria were grown, induced with IPTG, protein extracted, and (+)-δ-cadinene synthase purified as described.[17]

Sesquiterpene Synthase Assay. The radiochemical assay for FPP transformations was performed in a total volume of 100 μL containing 20 mM Tris/HCl, pH 8.7, 0.5 mM $MgSO_4$, 5 mM dTT, 2 μg of purified protein, and 30 μM [1-^{3}H]-FPP (14.3 μCi/μmol, Amersham). For kinetic investigations, the FPP was varied over the range from 1 to 70 μM. Reactions were initiated by addition of the substrate and incubated at 30 °C in 1.5 mL capped Eppendorf tubes. After 10 min or a designated time, the tubes were placed on ice and 10 μL of 0.5 M EDTA was added to terminate the reaction. The mixture was extracted with 200 μL of hexane. Quantitation of the radioactivity in the extracts was accomplished by liquid scintillation counting (Beckman, LS 1801). In cases where crude extracts were assayed, 50 mg of silica gel, activated at 100 °C for 1 h prior to use, was added to remove [1-^{3}H]farnesol produced by phosphatase contamination.

The product of the reaction was verified by capillary GC and GC−MS as described.[17] The stereochemistry of the reaction product was determined by chiral column chromatography[48] in the laboratory of Dr. Margaret Essenberg (Oklahoma State University).

Quantitative Analysis of mRNA by Competitive RT-PCR. Competitive RT-PCR was performed essentially as described by Gilliland *et al.*[37,38] and Vanden Heuvel *et al.*[23] Competitive DNA fragments were prepared by PCR from genomic DNA templates followed by TA-cloning. For gene *cad*1-A the sense primer 93160 (+536 to +552, Table 1) and antisense primer 9315 (+766 to +782) were used, and the template was genomic DNA isolated from cultured cells of *G. arboreum*. The PCRs were performed in 10 mM Tris/HCl pH 8.8 containing 2.5 U of AmpliTaq DNA polymerase, 200 μM dNTP, 1.5 mM $MgCl_2$, 0.8 μM of each primer, and 50 ng of genomic DNA in a total volume of 100 μL. The reaction was heated at 94 °C for 3 min and immediately cycled 35 times at 94, 50, and 72 °C (30 s for each step) followed by the final cycle containing a 10 min elongation at 72 °C. The PCR product was checked by agarose gel electrophoresis and purified by a Wizard PCR Preps DNA purification system (Promega). After TA-cloning to pBluescript SK(+), the plasmid, pXgA-SK, was amplified in *E. coli* strain XL1-Blue. DNA sequencing revealed a 339 bp genomic DNA fragment with a 92 bp intron located in a position between +767 and +768 (Figures 4 and 5). The plasmids, pXgA-SK and pXgC-SK, were diluted and used as internal standards in subsequent competitive PCR's.

Total RNA was prepared from cell suspension cultures of *G arboreum* at 0, 2, 4, 6, 10, and 24 h post elicitor treatment. For each sample, 2 μg of total RNA was added to a 20 μL solution, and the reverse transcription was performed as described above. An aliquot of the reverse transcription incubation, equivalent to 20 ng of total RNA, was added to each 50 μL of PCR mixture containing 0.005−10 attomol of competitive DNA templates of either of the two genomic fragments, pXgA-SK or pXgC-SK. The PCR was performed as described for competitive DNA fragment preparation. After thermocycling, aliquots of 5 μL of each PCR mixture were electrophoresed on 1.8% agarose gel, and the DNA fragments were visualized with ethidium bromide staining and photography (Figure 6A). Densitometry was carried out using a pdi laser gel scan densitometer (Huntington Station, NY). Quantitation of target mRNA was determined as described.[38,39] Briefly, the ratio of the genomic DNA PCR product to the cDNA PCR product was plotted against the amount of each of the internal standard added to the PCR (Figure 6B). The number of competitive DNA molecules, where the ratio is equal to 1, represents the amount of target cDNA present in the sample. The number was then corrected by a fragment size factor (339/247 for *cad*1-A and 291/194 for *cad*1-C) and by a factor of 2, since the competitive DNA templates are double stranded.

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Abbreviations used: FPP, farnesyldiphosphate; EAS, 5-*epi*-aristolochene synthase; CAS, casbene synthase; LIM, limonene synthase, CHS, chalcone synthase; PAL, phenylalanine ammonia lyase; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; *cad*, cadinene synthase cDNA; CAD, protein product of *cad*; pX, plasmid; LS, Linsmaier−Skoog; IPTG, isopropyl β-D-thiogalactoside; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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