Antisense Suppression of a (+)-δ-Cadinene Synthase Gene in Cotton Prevents the Induction of This Defense Response Gene during Bacterial Blight Infection But Not Its Constitutive Expression[1][w]

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In cotton (*Gossypium hirsutum*) the enzyme (+)-δ-cadinene synthase (CDNS) catalyzes the first committed step in the biosynthesis of cadinane-type sesquiterpenes, such as gossypol, that provide constitutive and inducible protection against pests and diseases. A cotton cDNA clone encoding CDNS (*cdn*1-C4) was isolated from developing embryos and functionally characterized. Southern analysis showed that CDNS genes belong to a large multigene family, of which five genomic clones were studied, including three pseudogenes and one gene that may represent another subfamily of CDNS. CDNS expression was shown to be induced in cotton infected with either the bacterial blight or verticillium wilt pathogens. Constructs for the constitutive or seed-specific antisense suppression of *cdn*1-C4 were introduced into cotton by Agrobacterium-mediated transformation. Gossypol levels were not reduced in the seeds of transformants with either construct, nor was the induction of CDNS expression affected in stems of the constitutive antisense plants infected with *Verticillium dahliae* Kleb. However, the induction of CDNS mRNA and protein in response to bacterial blight infection of cotyledons was completely blocked in the constitutive antisense plants. These results suggest that *cdn*1-C4 may be involved specifically in the bacterial blight response and that the CDNS multigene family comprises a complex set of genes differing in their temporal and spatial regulation and responsible for different branches of the cotton sesquiterpene pathway.

Plants enlist a complex array of physical and chemical defenses to protect themselves against herbivory and diseases. Cotton plants (*Gossypium* spp.) have numerous inducible defense mechanisms that are important for their ability to respond to changing biotic threats, including the synthesis of volatile terpenes (Pare and Tumlinson, 1997), phytoalexins, tannins, tyloses, pathogenesis-related proteins, as well as lignification, and the release of active oxygen species (Bell, 1981). The rapidity and intensity of an induced defense response is often a critical factor for resisting disease (Bell, 1981). Two economically important diseases of cultivated cotton (*Gossypium hirsutum*) that have been studied in detail with respect to the host defense responses they elicit are verticillium wilt, caused by the fungal pathogen *Verticillium dahliae* Kleb., and bacterial blight, caused by *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye (*Xcm*).

Phytoalexins are low-M_r antimicrobial compounds that accumulate in the plant in response to infection or abiotic elicitors (Kuć, 1995). The dominant phytoalexins important for defense against *V. dahliae*, for example, are the cotton terpenoid aldehydes (TAs; 8-hydroxylated cadinanes), including the dimeric sesquiterpene, gossypol, and its precursors desoxyhemigossypol (desHG) and hemigossypol (HG; Fig. 1), and their methyl ethers (Stipanovic et al., 1975, 1977; Veech et al., 1976; Heinstein et al., 1979). The cotton TAs are both inducible and developmentally regulated. The developmentally regulated forms are generally stored as a mixture with other pigments and oils within the lysigenous subepidermal gossypol glands that are characteristic of all Gossypium species (Fryxell, 1968). Cotton TAs are also present in the epidermis and hairs of developing roots, providing an additional defense against root-born pathogens. The root tips lack gossypol, and this explains the preferred route for infection by *V. dahliae* through the root tip (Mace et al., 1974; Bell, 1994). Induced TAs are exuded into the xylem vessels in response to vascular infection with *V. dahliae*, desHG being the most toxic (Mace et al., 1976, 1985, 1990; Mace, 1978).

The cotton cadinane-type sesquiterpenes also include the cadalene derivatives (7-hydroxylated cadinanes) that are produced mostly as phytoalexins and in response to stress (Bell, 1986). Green tissues infected

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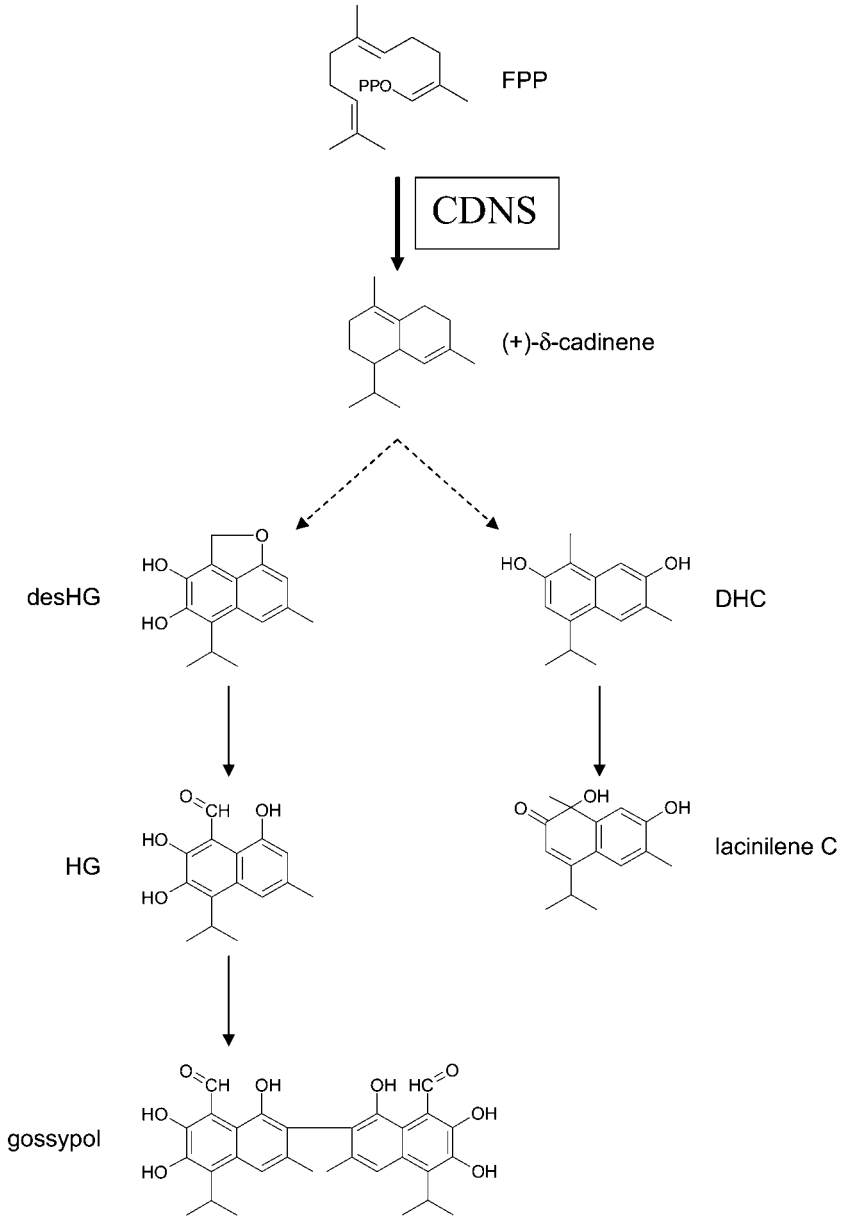

Figure 1. The proposed biosynthetic pathway for the cotton TAs (left branch) and cadalene derivatives (right branch). DHC, 2,7-dihydroxycadalene. Dashed lines indicate several reactions are involved.

with incompatible races of *Xcm* produce the cadalene derivatives as their major phytoalexins as part of the hypersensitive response. These are an important component of resistance to bacterial blight (Essenberg et al., 1982; Pierce and Essenberg, 1987; Pierce et al., 1996) in addition to the induced formation of desHG and HG (Abraham et al., 1999). 2,7-Dihydroxycadalene is the biosynthetic precursor of the cadalene derivatives, spontaneously oxidizing to form lacinilene C (Stipanovic et al., 1981; Fig. 1).

The cotton TAs and cadalene derivatives are sesquiterpenes (C_{15}) derived from a cytosolic branch of terpenoid metabolism, via the mevalonate pathway (Heinstein et al., 1962, 1970). Farnesyl diphosphate (FPP) is generated as the linear carbon skeleton of the sesquiterpenes in cotton (Essenberg et al., 1985; Stipanovic et al., 1986; Liu et al., 1999a). The various sesquiterpene synthases cyclize FPP to form the molecular frameworks of the different sesquiterpene types (Gershenzon and Croteau, 1993); in cotton, (+)-δ-cadinene synthase (CDNS; EC 4.6.1.11) catalyzes the cyclization of cis-trans-FPP to (+)-δ-cadinene via a nerolidyl diphosphate intermediate and is the first committed step in cadinane-type sesquiterpene biosynthesis and a likely control point (Essenberg et al., 1985; Bell, 1986; Stipanovic et al., 1986; Benedict et al., 1995; Davis and Essenberg, 1995; Alchanati et al., 1998).

The CDNS enzyme was first purified from a glandless cotton mutant by Davis et al. (1996) as a soluble hydrophobic monomer with a molecular mass of 64 to 65 kD. Chen et al. (1995) first cloned and functionally characterized a CDNS from the A-genome diploid

cotton *Gossypium arboreum*. Southern hybridization, enzyme purification, and genomic-library screening have since revealed that CDNS genes comprise a large multigene family in cotton (Chen et al., 1995, 1996; Davis et al., 1996; Meng et al., 1999; Tan et al., 2000; this study) similar to the terpene cyclase genes found in other plants (Facchini and Chappell, 1992; Back and Chappell, 1995). Several allelic and gene family variants of the cotton CDNS gene have since been isolated from both *G. arboreum* (Chen et al., 1996; Liu et al., 1999a; Meng et al., 1999; Tan et al., 2000) and the allotetraploid (A + D genomes) species *G. hirsutum* (Davis et al., 1998; this study). CDNS enzyme and transcripts are induced in cotton stems infected with *V. dahliae* (Benedict et al., 1995; Alchanati et al., 1998; Bianchini et al., 1999; Tan et al., 2000; this study), in cotton suspension cultures treated with *V. dahliae* elicitors (Chen et al., 1995, 1996; Liu et al., 1999a), and cotton cotyledons infected with *Xcm* (Davis and Essenberg, 1995; Davis et al., 1996; this study). CDNS is also developmentally regulated and CDNS transcripts increase during seed development in association with the biosynthesis and deposition of gossypol in the lysigenous storage glands of the embryo (Meng et al., 1999; Martin et al., 2003).

Two major subfamilies of the Gossypium CDNS multigene family, cdn1-A and cdn1-C, have been proposed based on sequence relatedness and appear to be differentially regulated at the transcriptional level (Meng et al., 1999; Tan et al., 2000). A third subfamily, cdn1-B, is represented by a single genomic clone, but no details have been published concerning the expression pattern or functional attributes of this gene. The nomenclature of the subfamilies and genes representing cotton CDNS genes has not been consistent in the literature, and therefore this study follows the nomenclature used in a recent publication by Martin et al. (2003). The individual genes representing CDNS will be given in italics and the families in plain font (e.g. *cdn*1-C1 and cdn1-C, respectively).

Besides its presence in roots and foliar tissues of the plant, gossypol is the dominant TA in the storage glands of the cotyledons in the developing and mature cottonseed. Gossypol is toxic to nonruminant animals, and so it reduces the commercial value of seed meal used for animal feeds and must be removed from cottonseed oil prior to human consumption. Naturally occurring glandless mutants of cotton are available that lack significant constitutive production of gossypol and other TAs, but these are highly prone to insect damage in the field and have had limited commercial use. Some Australian native diploid species such as *Gossypium sturtianum* also have a glandless seed phenotype but still possess foliar gossypol glands that afford them a high degree of protection against herbivory. Introgression of this desirable glandless seed/glanded plant trait from *G. sturtianum* into the cultivated cottons has had limited success because of the genome incompatibility between these species. Genetic engineering may provide another means of generating the trait of gossypol-free seed in cultivated cotton species through the disruption of terpenoid biosynthesis specifically in seeds, but this will require a greater understanding of the complex set of genes regulating the synthesis of the cotton sesquiterpenes. Reported herein is the isolation and characterization of CDNS genes from *G. hirsutum* and the use of one seed-expressed gene in antisense transformation constructs designed to reduce CDNS expression in transgenic cotton plants. Differential silencing of multigene families has been achieved for, as an example, rice (*Oryza sativa*) glutelin genes (Kusaba et al., 2003), and was found to occur via a posttranscriptional gene silencing (PTGS) mechanism (Wang and Waterhouse, 2000). Antisense expression of CDNS genes was envisaged as a way to activate this silencing mechanism in cotton, thereby blocking the cadinane-type sesquiterpene pathway and abolishing gossypol production in the transformants. The particular CDNS gene used in this study, however, did not affect gossypol levels in transformants with either a constitutive or seed-specific antisense construct, but appeared to be specific for the defense response to *Xcm*, implicating a complex system of differential regulation of the CDNS multigene family.

RESULTS

Isolation of a CDNS cDNA Clone

Degenerate PCR primers were designed to the conserved amino acid sequences of a number of terpene cyclases and from a region conserved in the two then-known CDNS genes from *G. arboreum*, *cdn*1-C1 (accession no. U23206) and *cdn*1-C14 (accession no. U23205; Chen et al., 1995). These primers were used to amplify a specific gene region of CDNS sequences from *G. hirsutum* genomic DNA. Two cloned PCR products displayed high homology to the *G. arboreum* CDNS genes. The CSZ-7 PCR product was 698 bp in length and the coding region was identical to the *cdn*1-C14 cDNA from *G. arboreum*, a member of the cdn1-C subfamily of CDNS genes in Gossypium as defined by Meng et al. (1999). The CSZ-2 product was 459 bp long and only 73% identical to the DNA sequence of CSZ-7, but 97% identical to the *cdn*1-A genomic clone from *G. arboreum* (accession no. Y18484; Chen et al., 1996).

The CSZ-7 clone was used as a probe to screen a *G. hirsutum* cDNA library (Liu et al., 1999b) made from the RNA of developing embryos, at a time during which gossypol biosynthesis is actively occurring (Loguercio et al., 1999; Meng et al., 1999). Clone C6 was the longest clone obtained from the screen, at a length of 1,837 bp including the poly(A)$^+$ tail, and was renamed *cdn*1-C4 (accession no. AF270425). The *cdn*1-C4 cDNA clone is 99.3% identical at the DNA sequence level to *cdn*1-C from *G. hirsutum* (accession no. U88318; Davis et al., 1998), which corresponds to five amino acid differences between them. Both

G. hirsutum CDNS genes belong to the cdn1-C subfamily, which also includes *cdn*1-C1; *cdn*1-C2 (accession no. Y16432), *cdn*1-C3 (accession no. AF174294), and *cdn*1-C14, all from *G. arboreum*.

Alignment of *cdn*1-C4 with other CDNS genes revealed it was a near full-length cDNA clone, lacking the 5′-untranslated region and 9 bp that encode the first three amino acids of the protein product. Conceptual translation indicates a *cdn*1-C4 protein product of 551 amino acids and 63.8 kD, slightly less than the endogenous CDNS enzyme due to the truncated nature of the clone. Amino acid residues conserved among terpene cyclases (Bohlmann et al., 1998) are also present in the predicted *cdn*1-C4 protein sequence, most notably the three Asp residues in the highly conserved DDXXD motif thought to be involved in metal ion-diphosphate binding (McGarvey and Croteau, 1995; Yamaguchi et al., 1996; Lesburg et al., 1997; Starks et al., 1997).

Genomic Representatives of CDNS in Cotton

The full-length *cdn*1-C4 clone was used as a probe for high-stringency Southern hybridization of restriction enzyme-digested genomic DNA from various cotton species and varieties. A complex banding pattern was evident (Fig. 2) and was consistent with previous data suggesting that terpene cyclases involved in secondary metabolism are usually members of large multigene families, including cotton CDNS genes (Chen et al., 1995, 1996; Meng et al., 1999; Tan et al., 2000). The same banding pattern was observed across all *G. hirsutum* cultivars owing to the low level of DNA polymorphism within current cultivars (Liu et al., 2001); however, the two diploid species had fewer bands that were mostly polymorphic with respect to the allotetraploids and each other. Due to the relatively high level of identity of different terpene cyclase genes, the bands hybridizing weakly with the *cdn*1-C4 probe may not be CDNS genes but perhaps different closely related terpene cyclase genes, but this would have to be verified by cloning and functional analysis of each.

The screening of a *G. hirsutum* genomic library with a *cdn*1-C4 probe resulted in the isolation of 21 genomic clones. Seven clones have been fully sequenced so far, representing five different sequences. Three clones appear nonfunctional and are predicted to be pseudogenes, as is common in large multigene families (Force et al., 1999; Aubourg et al., 2002; Schuler and Werck-Reichhart, 2003). A single-base deletion in the coding region of *cdn*1-C7 (GenBank accession no. AY800007) results in a frame shift and premature termination, which leads to a protein of only 201 amino acids. Similarly, *cdn*1-C8 (GenBank accession no. AY800008) has a single-base substitution that gives rise to a stop codon and a protein product of only 121 amino acids. In *cdn*1-C6 (GenBank accession no. AY800006) there are two mutations, the first single-base substitution resulting in a premature stop codon leading to a predicted protein product of only 49 amino acids, and a deletion further downstream would also lead to

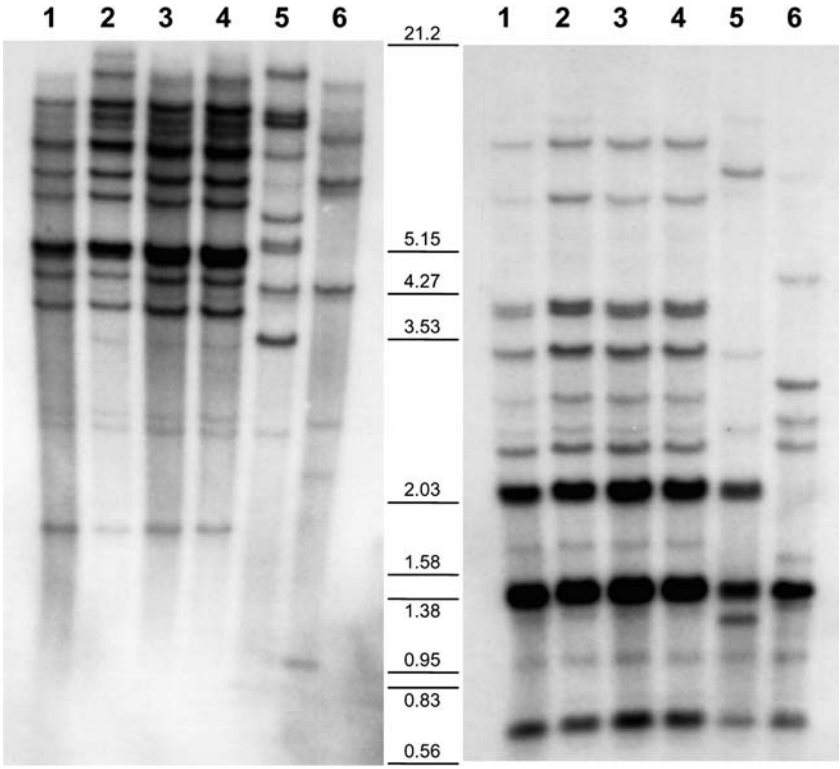

Figure 2. *cdn*1-C4 is part of a large multigene family in Gossypium species. Southern hybridization of *Eco*RI-digested (left section) and *Hin*dIII-digested (right section) cotton genomic DNA probed with the *Eco*RI insert of the *cdn*1-C4 clone labeled with [α-^{32}P]dCTP (10 μCi μL^{-1}) employing the New Megaprime Random Labeling kit (Amersham) and purified by standard methods (Sambrook et al., 1989). DNA was electrophoresed in an agarose gel and capillary blotted onto Hybond-N^{+} membrane (Amersham). The membranes were prehybridized and hybridized at 42°C with formamide and washed at high stringency as described in Mitchell et al. (1989). Lane 1, *G. hirsutum* cv Coker 315; lane 2, *G. hirsutum* cv Sicala V2; lane 3, *G. hirsutum* cv DP16 glanded; lane 4, *G. hirsutum* cv DP16 glandless; lane 5, *G. arboreum*; lane 6, *G. sturtianum*. Molecular weight markers are shown in kbs. There are no internal *Eco*RI sites in the *cdn*1-C4 gene; however, there are three internal *Hin*dIII sites.

premature translation termination due to a frame shift (data not shown). These single-nucleotide changes were rigorously confirmed by visual assessment of the chromatogram for sequence quality and repeated sequencing over the region where the mutation was located and, in the case of *cdn*1-C7, by sequencing an independent lambda isolate.

Genomic clones *cdn*1-C5 (accession no. AY800106) and *cdn*1-D1 (accession no. AY800107) are predicted to encode functional CDNS proteins. The coding region of *cdn*1-C5 is identical to the sequence of *cdn*1-C4 and is likely to represent the genomic counterpart of this cDNA. *cdn*1-C5 and *cdn*1-D1 encode full-length transcripts of 1,918 bp and 1,917 bp, respectively, and protein products of 554 amino acids. *cdn*1-D1 is only 87% and 85% identical to *cdn*1-C5 at the nucleotide and amino acid sequence levels, respectively, and almost equidistantly related to the *cdn*1-B gene from *G. arboreum*. A phylogenetic tree of the cotton CDNS genes highlights the relatedness of members within the CDNS subfamilies and the distance between the different subfamilies (Fig. 3). *cdn*1-D1 is different enough from the other CDNS genes of cotton to suggest that it belongs to another subfamily designated as cdn1-D. BLAST searches of the GenBank expressed sequence tag (EST) collection has identified two *Gossypium raimondii* seedling ESTs (CO080572.1 and CO098975.1) and a *G. hirsutum* seedling hypocotyl and root EST (CD486420.1) that were greater than 99% identical to *cdn*1-C5, suggesting that this is a D-genome gene. No direct equivalents of *cdn*1-D1 (or *cdn*1-B) were found in the EST collections for Gossypium species, although most ESTs other than the 65,000 from *G. raimondii* are from fibers that may not make terpenes.

The genetic structure of CDNS genes is well conserved, including the number, positions, and sizes of exons and introns, and also corresponds well to the genomic clones of other terpene cyclase genes such as tobacco (*Nicotiana tabacum*) 5-*epi*-aristolochene synthase (see supplemental material; Back and Chappell, 1995). Approximately 2 kb of promoter regions from each CDNS genomic clone was sequenced and a very low level of sequence conservation was found between all CDNS promoter regions. There were discrete regions of similarity mostly in the vicinity of the predicted TATA box and transcription start site but the similarity did not extend significantly upstream (data not shown).

Characterization of the *cdn*1-C4 Protein Product

The protein encoded by *cdn*1-C4 was expressed in *Escherichia coli* with an N-terminal 10× His tag to facilitate protein purification. The recombinant protein had a predicted length of 591 amino acids and a molecular mass of 68.5 kD. The recombinant protein was purified by affinity chromatography under denaturing conditions for the generation of polyclonal antibodies in rabbits (CDNS-Ab) and under native conditions for functional enzyme analysis and characterization of the

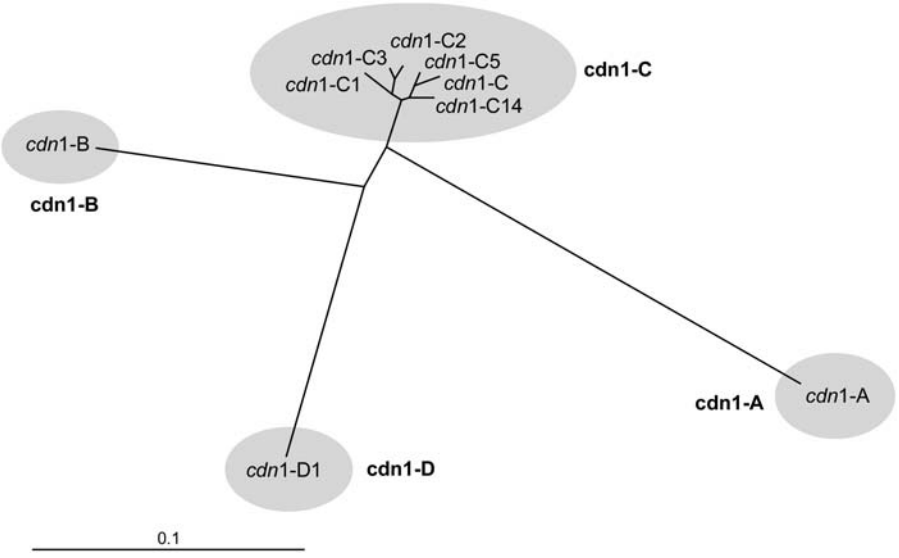

Figure 3. Phylogenetic tree of CDNS protein sequences showing the subfamilies cdn1-A, cdn1-B, cdn1-C, and cdn1-D. Conceptually translated amino acid sequences were aligned using PILEUP (Genetics Computer Group, 1999). The best model of molecular evolution was determined using PUZZLE. A matrix was obtained with PROTDIST using the Kimura 2-parameter model and gamma distribution. Trees were generated by the neighbor-joining algorithm using NEIGHBOR (all the above in PHYLIP 3.5) and graphical output made in DRAWTREE (PHYLIP 3.6). Branch lengths are indicative of genetic distance (number of substitutions per position) with the scale bar shown. *cdn*1-C5 has been used here as the full-length equivalent of *cdn*1-C4. GenBank accession numbers: *cdn*1-A, Y18484; *cdn*1-B, X95323; *cdn*1-C, U88318; *cdn*1-C1, U23206; *cdn*1-C2, Y16432; *cdn*1-C3, AF174294; *cdn*1-C5, AY800106; *cdn*1-C14, U23205; and *cdn*1-D1, AY800107.

reaction product. Enzyme assays utilized tritium-labeled FPP as a substrate, and activity was measured by the amount of hexane extractable radioactivity produced, revealing a specific activity of 2,922 nmol [^{3}H]FPP consumed mg^{-1} h^{-1}. The enzyme exhibited typical Michaelis-Menton kinetics with a K_m value of 10.6 μM FPP and a k_{cat} of 0.027 s^{-1}. These values are of a similar magnitude to those reported for other cotton CDNS genes expressed in bacteria (Chen et al., 1995, 1996), although the specific activity is not as high as reported for the protein purified from cotton by Davis et al. (1996).

Comparative capillary gas chromatography-mass spectrometry (GC-MS) was employed to confirm that the hexane extractable product was (+)-δ-cadinene. A significant peak at 7.96 min (GC program 1) in the *cdn*1-C4 enzyme assay extract coincided with the retention time of the authentic (+)-δ-cadinene standard. Additionally, the mass spectra of the 7.96-min peak from the enzyme assay had the following characteristic ions (with relative abundances as percent of mass-to-charge ratio 161 base peak shown in parentheses): 205(10), 204(68), 189(23), 162(23), 161(100), 145(7), 134(52), 133(13), 129(6), 128(7), 121(2), 120(7), 119(54), 115(9), and 105(48). The (+)-δ-cadinene standard characteristic ions and relative abundances were nearly identical. Stereochemical analyses by chiral-capillary GC verified the compound as the (+) enantiomer using the essential oil from *Angelica archangelica* root as a source of the (−) and (+) enantiomers (37.9 min and 38.66 min, respectively, using GC program 2).

Comparative nuclear magnetic resonance (NMR) analysis revealed that the one-dimensional and two-dimensional spectra of the (+)-δ-cadinene standard and the product of the recombinant enzyme assay were identical and corresponded with that described for (+)-δ-cadinene (Davis et al., 1996). These results are consistent with the *cdn*1-C4 gene encoding a functional CDNS enzyme.

Generation of Antisense CDNS Plants

The 35SC6as transformation construct was designed for the constitutive antisense suppression of the *cdn*1-C4 gene in cotton, and the LeC6as construct for seed-specific suppression. Agrobacterium-mediated transformation of the regenerable cultivar *G. hirsutum* cv Coker 315 with the LeC6as construct resulted in the generation of eight independent fertile primary transformants. The 35SC6as transformation generated five independent fertile transformants. This was a low efficiency compared to other cotton transformations using the same binary vector without the *cdn*1-C4 cDNA. It was noted that numerous plantlets died soon after transfer from tissue culture media to soil (D. Llewellyn and B. Townsend, unpublished data), suggesting they may have had a heightened sensitivity to pathogen infection or were altered in vigor.

Southern hybridization and NPTII enzyme assays were employed to determine the transgene copy number and segregation in subsequent generations. The T_3 transgenic lines analyzed in this study (LeC6as 3, LeC6as 4, LeC6as 50, 35SC6as 6, 35SC6as 23, and 35SC6as 51) were all independent lines homozygous for a single copy of the transgene (examples shown in Fig. 4).

Seed Gossypol Levels in Antisense *cdn*1-C4 Transgenics

HPLC was employed to quantify gossypol levels in mature cotton seed. Coker 315 mature seed contained gossypol levels of 1.301% ± 0.026% free gossypol as a percentage of total dry weight, a value similar to that observed by other researchers (Lukefahr and Fryxell, 1966; Dilday, 1986; Brubaker et al., 1996; Percy et al., 1996). Analysis of the gossypol content of seed from LeC6as and 35SC6as lines revealed no detectable

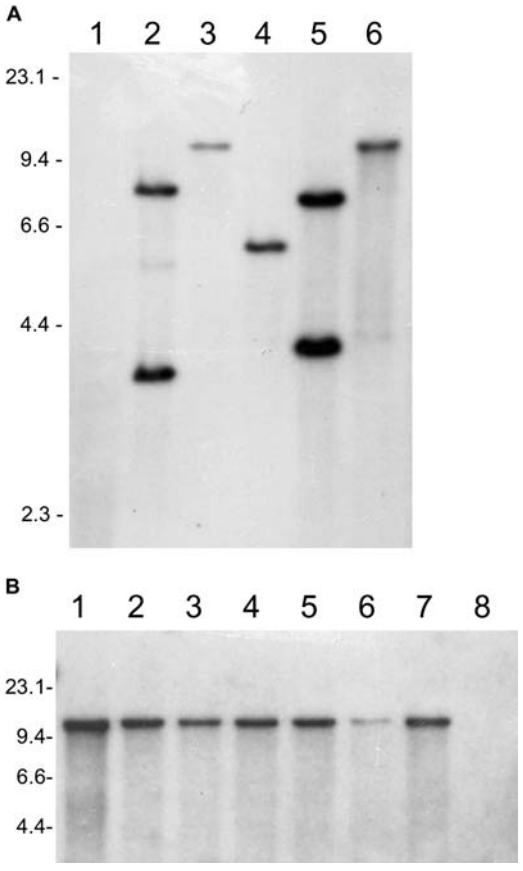

Figure 4. Examples of transgene copy number and segregation testing of cotton transformants by Southern hybridization. Genomic DNA (15 μg) was digested with *Eco*RI and hybridized at high stringency with a radiolabeled 1.0-kb *Bam*HI/*Sma*I fragment of the NPTII gene from p35SKN (Huang and Dennis, 1989) as described for Figure 2. A, Independent T_0 generation transformants. Lane 1, Coker 315 untransformed; lane 2, 35SC6as 14; lane 3, 35SC6as 22; lane 4, 35SC6as 23; lane 5, 35SC6as 42; and lane 6, 35SC6as 51. From this blot, only lines 35SC6as 23 and 51 are presented in this study. B, Testing uniformity segregation in the T_2 generation of the insertion in 35SC6as 51. Lane 1, T_0 plant; lanes 2 to 7, six T_2 plants, which tested positive for the transgene by NPTII assays; and lane 8, Coker 315 untransformed. Lambda *Hin*dIII size markers are indicated in kbs.

reduction in gossypol levels when compared to the untransformed control line. Gossypol levels in 35SC6as 6, LeC6as 4, and LeC6as 50 seed were not significantly higher than the control, ranging between 1.329% and 1.447% of dry weight. However, three of the six lines tested had significantly higher seed gossypol levels than Coker 315, as determined using a *t* test with 95% confidence intervals to compare the means of each data set. Most significantly different was the 35SC6as 23 seeds, which had 2.133% ± 0.063% gossypol, 64% higher than Coker 315. 35SC6as 51 and LeC6as 3 seeds had 1.561% ± 0.122% and 1.575% ± 0.058% gossypol, respectively, which was also significantly higher than the control. The significant increase in gossypol levels of some of these lines may be explained by either genetic variation arising from the tissue culture process, or the plants may have been suffering from physiological stresses. Untransformed Coker 315 was used as the control in this experiment as opposed to empty-vector transformed lines because the high level of variability introduced by the cotton tissue culture and regeneration process was likely to have given a broad range of values for gossypol content in any transformed control lines. As we were looking for complete suppression of gossypol synthesis it was of more interest to compare gossypol levels to an untransformed control. Detailed analysis of segregation of any interesting gossypol phenotype with the introduced transgene after backcrossing into the parental line would be required to confirm that it was indeed caused by the transgene and not somaclonal variation. As we did not observe any agronomically useful phenotype, this is beyond the scope of this study.

Induction of CDNS in Infected 35SC6as Transgenics

An *Xcm* isolate BT07 belonging to Race 1 (as characterized using a standard set of differential cotton host lines [data not shown]) was used to evaluate the defense responses of the transgenic antisense plants. Coker 315 is susceptible to infection by Race 1 and other Races of *Xcm* found in Australia, as well as the Australian *V. dahliae* isolate used in this study. The cotyledons of untransformed Coker 315 and 35SC6as lines 6, 23, and 51, were infected with *Xcm* BT07 suspension or a mock suspension and harvested at various times after inoculation to evaluate changes in CDNS expression. Similarly, the stems of transformed and untransformed plants were infected with a *V. dahliae* conidial suspension or a water control by a stem puncture method and harvested 48 h later. CDNS expression was assessed at the transcript and protein level.

CDNS transcripts were detected by hybridization of total RNA with a full-length antisense RNA probe of *cdn*1-C4. The RNA was immobilized to a membrane in dot-blot format to enable equivalent hybridization across all 36 samples, including the untransformed controls, on a single filter. Electrophoresis of the RNA from biological replicates and subsequent northern hybridization with the *cdn*1-C4 probe revealed a transcript of 1.9 kb and gave the same results as the dot blot (data not shown). Based on the Southern-hybridization and genomic-library screening, the *cdn*1-C4 probe used was expected to hybridize to all CDNS family members at high stringency, although the cdn1-A subfamily may be weakly hybridizing. CDNS transcripts were significantly up-regulated in Coker 315 cotyledons within 36 h of inoculation with *Xcm* (Fig. 5A). No up-regulation of CDNS transcripts was observed in the mock-inoculated control cotyledons and indicates that wounding does not induce expression of CDNS (Fig. 5A). Consistent with the up-regulation of CDNS gene transcripts, CDNS protein (64 kD) was induced by *Xcm* infection of cotyledons detected using the CDNS-Ab described earlier. Induced CDNS protein was detectable within 36 h of

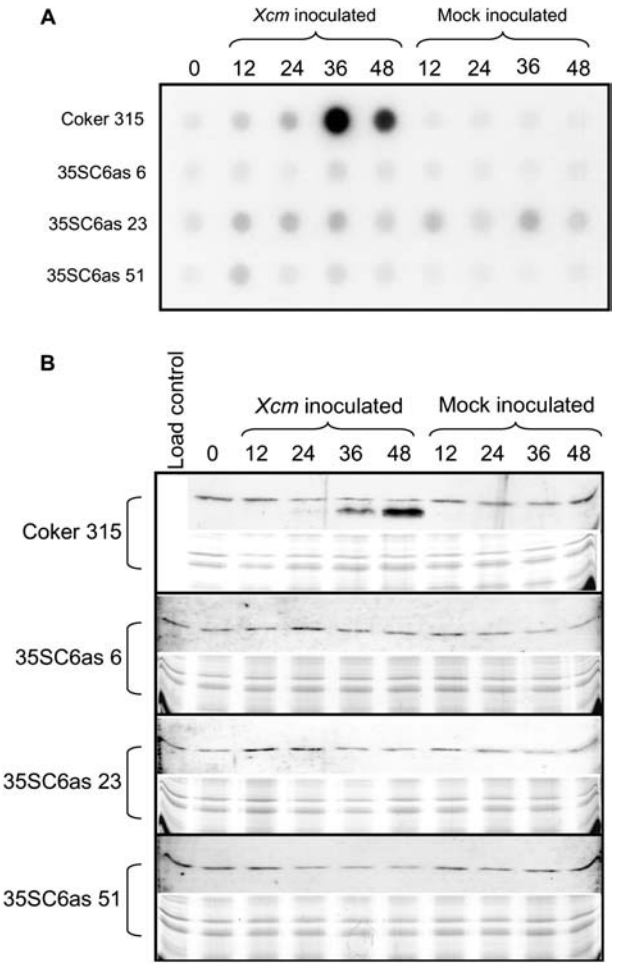

Figure 5. CDNS expression in cotyledons is induced by the *Xcm* interaction with Coker 315 but not in the 35SC6as transgenics, nor by wounding. A, Hybridization of 10 μg total RNA in dot-blot format with a radiolabeled full-length *cdn*1-C4 RNA probe. B, Chemiluminescent western blotting of 20 μg of total soluble protein with the CDNS-Ab showing the 64-kD CDNS band and a 68-kD unknown band (top section). The bottom sections are duplicate gels stained with Coomassie Brilliant Blue. Numbers refer to hours postinoculation. The load control in the three antisense lines is the Coker 315 cotyledon 0-h sample.

inoculation with *Xcm* and increasing to a higher level by 48 h, and was not induced in mock-inoculated samples (Fig. 5B). In contrast, the induction of CDNS transcripts and protein was severely attenuated or absent in all three of the 35SC6as transformed lines examined in replicate experiments (Fig. 5).

Infection of stems of 35SC6as transformants with *V. dahliae*, however, gave very different results to the *Xcm* experiment. In agreement with previous studies, CDNS transcript and protein levels were highly induced in the untransformed Coker 315 stems 48 h after inoculation with the fungal pathogen, and there was no observed response to wounding (Fig. 6). The same strong induction of CDNS was also observed in the three 35SC6as transformed lines infected with *V. dahliae* (Fig. 6).

DISCUSSION

The major phytoalexins of cotton are the TAs and their cadalene derivatives. Gossypol is one important such phytoalexin, but is present in high levels in seeds to the detriment of the quality of cottonseed oil and meal. Both groups of phytoalexins share a common biosynthetic origin from (+)-δ-cadinene, which is synthesized from FPP by CDNS (Fig. 1). This study aimed to investigate the potential for the modification of the chemical defense response and developmental regulation of terpene levels in cotton by manipulating CDNS gene expression using a genetic engineering approach.

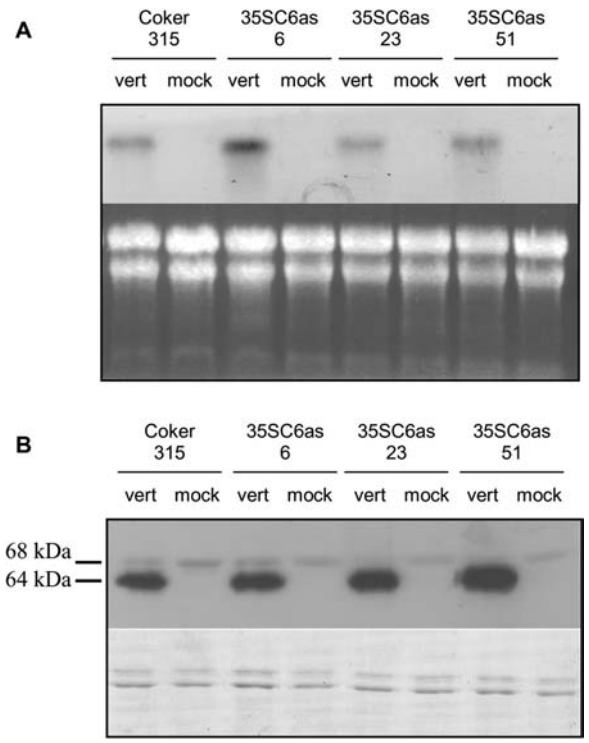

Figure 6. CDNS expression is induced in Coker 315 and 35SC6as transgenic cotton stems 48 h after infection with *V. dahliae*. A, Northern hybridization of 20 μg of total RNA probed with a radiolabeled full-length *cdn*1-C4 RNA probe (top); ethidium bromide stained RNA gel prior to capillary blotting (bottom). B, Chemiluminescent western blotting of 20 μg of total soluble protein with the CDNS-Ab showing the 64-kD CDNS band and a 68-kD unknown band (top); duplicate SDS-PAGE gel stained with Coomassie Brilliant Blue (bottom). Mock indicates inoculation with water, vert indicates inoculation with a *V. dahliae* conidial suspension.

Cotton Contains a Large Multigene Family of CDNS Genes

A CDNS cDNA clone, *cdn*1-C4, was isolated from developing embryos of the major cultivated species of cotton, *G. hirsutum*. Production of recombinant protein in *E. coli* combined with enzymatic analysis and product identification was used to confirm that it encodes a functional CDNS enzyme. Various Gossypium species and varieties were shown to contain a dozen or more CDNS genomic sequences related to *cdn*1-C4 (Fig. 2), indicating the presence of a large multigene family of closely related genes. The *cdn*1-C4 gene belongs to the cdn1-C subfamily of CDNS genes. Four cdn1-C genes, as well as a single gene each of cdn1-A and cdn1-B subfamilies, all occur in the A-genome diploid *G. arboreum*, so the diversity of CDNS genes in *G. hirsutum* is not necessarily just connected to its polyploid origins. *cdn*1-B and the cdn1-D subfamily member, *cdn*1-D1, have yet to be isolated as ESTs or expressed in *E. coli* for enzymatic characterization. Chen et al. (1996) showed that the *G. arboreum cdn*1-A gene product induced by Verticillium cell wall elicitors also catalyzes the cyclization of FPP to (+)-δ-cadinene with almost identical kinetics to the various cdn1-C types, but with slightly different pH and cofactor optima. The prevalence of CDNS genes in cotton may therefore not have so much a biochemical basis as a functional one in regulating the synthesis of sesquiterpenes in response to specific developmental or environmental cues. We are exploring this by the analysis of genomic sequences and gene knockouts in transgenic cotton plants and have so far partially characterized five of the predicted 12 to 14 CDNS genes in *G. hirsutum* and, not surprisingly, a number are predicted to be pseudogenes.

The isolation and characterization of *cdn*1-C5, the genomic equivalent of *cdn*1-C4, and four other genomic clones has confirmed a high degree of conservation of both coding sequence and genetic structure among these CDNS genes and subfamilies (see supplemental material). If the different genes play diverse functional roles, then it is not surprising to find that the promoter sequences of the various genomic clones are significantly different, with the most notable homology occurring in the regions within and around the TATA box and predicted transcription start sites. This would suggest that the genes may share some common transcription factor-binding sites. The promoters may also contain other as yet unidentified

motifs for transcription factor binding important in the coordinated regulation of subsets of CDNS genes under particular environmental or developmental signals. Fusion of the *cdn*1-C5 and *cdn*1-D1 promoters to reporter genes and analysis in transgenic cotton is in progress to investigate any differences in expression and activity of these two members of the CDNS multigene family.

Discrete Functional Roles for CDNS Multigene Family Members?

Cotton plants transformed with an antisense copy of *cdn*1-C4 were expected to have a block in CDNS gene activity, thereby reducing constitutive gossypol levels in the transformed plants, as well as attenuating the induced sesquiterpene pathway. *cdn*1-C4 is highly expressed in cotton embryos at the time of the initial synthesis and deposition of gossypol and this expression overlaps that of the cauliflower mosaic virus 35S and lectin promoters (Townsend and Llewellyn, 2002) used to drive the expression of the antisense constructs. Despite this reasoning, neither the constitutive 35S promoter nor the seed-specific soybean (*Glycine max*) lectin promoter-driven antisense expression of *cdn*1-C4 significantly reduced the quantity of free gossypol in the mature seeds of transformants. Martin et al. (2003) recently reported a 70% reduction in gossypol in seeds and similar reductions in gossypol and heliocide levels in leaves of cotton transformed with a similar 35S promoter-driven antisense copy of the *G. arboreum cdn*1-C3 gene that is highly homologous to *cdn*1-C4. While this suggests that constitutive suppression of the major CDNS genes may be possible, no analysis of CDNS transcript or protein levels in leaves or seeds were reported in this study. Additionally, this group recovered an apparently somaclonal variant from the same line that also has reduced levels of gossypol but in the absence of the transgene (Benedict et al., 2004). There is therefore insufficient evidence for coinheritance of the low gossypol trait and the antisense transgene to conclude that the mechanism of gossypol reduction achieved was via PTGS.

The role of CDNS in providing a phytoalexin defense response has been demonstrated in both bacterial and fungal cotton plant-pathogen interactions (Benedict et al., 1995; Chen et al., 1995; Davis and Essenberg, 1995; Davis et al., 1996; Liu et al., 1999a; Tan et al., 2000; this study). The 35S antisense *cdn*1-C4 transgenic plants enabled us to study the effects of blocking CDNS expression in other plant parts besides the seeds. Analysis of CDNS induction in *Xcm*- and *V. dahliae*-infected untransformed plants verified the high levels of induction of both the CDNS gene and protein with either pathogen in a susceptible host (Figs. 5 and 6). Similarly, antisense *cdn*1-C4 transformants showed strong up-regulation of CDNS transcripts and protein in stems in response to inoculation with *V. dahliae*. However, the same transformants did not show any significant induction of CDNS transcripts or protein when inoculated with the bacterial pathogen *Xcm* despite the appearance of extensive water-soaked lesions characteristic of *Xcm* infection in cotton.

The differential induction of CDNS with the two pathogens may reflect a case where different members of the CDNS multigene family are responsible for specific developmental or defense roles in the plant. One or more members may be responsible for the branch of TA biosynthesis involved in seed gossypol deposition, while other members could play a specific role in constitutive deposition of gossypol or heliocides in the aerial glands. Similarly, specific CDNS genes may be involved in production of the TAs or cadalene derivatives for defense against bacterial pathogens, and others for defense against fungi.

Differential Silencing of Individual CDNS Genes

Silencing of multigene families appears to be more complex than can often be explained by current models of PTGS. Silencing of the glutelin seed storage protein multigene family in rice in the natural-deletion mutant LGC-1, for example, has recently been established as a posttranscriptional phenomenon resulting from the creation of an inverted-repeat transcript from the very similar and adjacent GluB4 and GluB5 genes (Kusaba et al., 2003). The two most similar genes, GluB4 and GluB5, were almost completely silenced, and a closely related gene, GluB2, was significantly silenced by the GluB4/5 inducer of PTGS. However, GluA1 and GluA2, which are 66% and 65% identical to GluB4/5, respectively, were only partially silenced, indicating that silencing was related to the extent of identity of the inducer and target genes.

Antisense transgene constructs have classically worked best when multiple-copy insertions generate inverted repeats that produce hairpin transcripts that fold into double-stranded RNAs that in turn trigger PTGS (e.g. Wang and Waterhouse, 2000). Crossing plants with sense and antisense transgenes can also result in gene silencing through the production of large amounts of dsRNA (Waterhouse et al., 1998). Small 21 to 25mers (siRNAs) generated from cleavage of the dsRNA molecules by Dicer proteins then become part of a nuclease containing RNA-induced silencing complex that uses these siRNAs to target sequence-specific degradation of mRNAs with the efficiency of degradation dependent on the presence of near-perfect matches to the siRNAs (for review, see Waterhouse and Helliwell, 2003). Antisense suppression, when activated, should therefore target all expressed genes sharing at least one of these siRNA domains, so silencing of all members of multigene families by constructs containing a single family member is highly likely, unless specific less conserved domains are selected to generate the initial dsRNA.

Given the high DNA sequence conservation among the different CDNS gene families the ability of the antisense *cdn*1-C4 constructs to silence only bacterial

blight-induced CDNS genes and not the seed or Verticillium-induced CDNS was surprising. Certain members of the cdn1-C and cdn1-A families are known to be expressed in seeds and/or induced by Verticillium (Chen et al., 1996; Davis et al., 1998; Meng et al., 1999), so sense transcripts should have been coexpressed in the same cells or at the same time as the antisense *cdn*1-C4 transcripts, leading to dsRNA production. All of our transgenic plants analyzed were single copy. In fact, all the 35S promoter plants initially produced that survived transfer to soil were low-copy number, so there could have been some selection against fully silenced plants with multiple inserts. This may suggest that there is sufficient sequence divergence among different CDNS genes expressed in the seed that the sequence-specific PTGS of *cdn*1-C4 and closely related CDNS genes is ineffective in silencing all family members. Silencing was therefore unlikely to result from production of the hairpin-type dsRNAs that are known to be very efficient at inducing PTGS. By the same token, we may not have reached a critical threshold for antisense expression to trigger the silencing process. dsRNA would be induced in these plants only through the hybridization of separate sense and antisense transcripts, a process that would be concentration and sequence dependent and most likely to occur when the endogenous CDNS gene(s) were induced to very high levels (as is the case during the bacterial blight infection when induction is over 500-fold). Why silencing did not also occur with the Verticillium-induced expression of CDNS genes is unknown but may suggest there is some critical threshold for induction of PTGS or, as discussed above, that divergent CDNS genes are involved. New approaches using the more efficient inverted-repeat technology developed recently (Waterhouse and Helliwell, 2003) with specific unique regions of the CDNS genes are being carried out to investigate the potential to silence individual members or whole subfamilies. Similarly, it would be of interest to silence the *cdn*1-C4 gene in a *G. hirsutum* cultivar that was resistant to *Xcm* Race 1 to determine if there is a direct effect on plant susceptibility to infection when CDNS induction is blocked, and whether this was linked to the gene-for-gene resistance mechanisms for this pathogen that are found in cotton.

The occurrence of large multigene families for most terpene synthases studied so far, and the work on CDNS presented in this study, has important implications for the future analysis and engineering of these enzymes and their metabolic pathways. Gossypol gland morphogenesis and TA deposition are distinct processes in the cotton plant (Brubaker et al., 1996; Abraham et al., 1999, Benedict et al., 2004), and the regulatory machinery involved in initiation of TA deposition could prove to be an interesting new avenue for research in this field. The enormous diversity of secondary metabolites in plants has arisen by as yet unknown genetic mechanisms, but research into discrete genes and proteins all performing the same biochemical role and belonging to the same multigene family will provide insight into the development of such diversity between plant species and particularly in allotetraploids, like cotton, that have brought together two discrete biochemical pathways into a single pathway for multifaceted protection against pests and pathogens.

MATERIALS AND METHODS

Isolation and Manipulation of Nucleic Acids

Seeds were provided by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Cotton Research Unit, Narrabri, Australia. DNA manipulations were carried out as described in Sambrook et al. (1989). Genomic DNA was isolated from cotton (*Gossypium hirsutum*) cotyledons or young leaves by the method of Paterson et al. (1993). Total RNA was extracted by the method of Jacobsen-Lyon et al. (1995) except extraction and LiCl precipitation was followed by DNase I treatment according to the manufacturer (Promega, San Luis Obispo, CA).

Plasmids were prepared for DNA sequencing and subcloning using the Qiagen Plasmid Midi kit as recommended (Qiagen, Hilden, Germany). Sequencing was carried out using Dye Terminator chemistry and an ABI 377 automated-sequencing machine (Applied Biosystems, Foster City, CA). DNA sequences were analyzed and manipulated with the Wisconsin GCG Package version 9.1 (Genetics Computer Group, Madison, WI, 1997), and BLAST searches were conducted via the National Center for Biotechnology Web site (http://www.ncbi.nlm.nih.gov).

Isolation of CDNS Genes by PCR

Genomic DNA from *G. hirsutum* cv DP16 was partially digested with *Eco*RI and 20 ng was used as a template for a 20-μL PCR reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units *Amplitaq* DNA polymerase (Perkin-Elmer, Boston), and 0.8 μM of degenerate primers 9,315 [5′-A(A/G)(A/G)TC(C/T)TTCCACCACCT-3′; Chen et al., 1995] and 9,320 [5′-GA(C/T)GA(A/G)CA(A/G)GGIAA(C/T)TT(C/T)AA-3′]. Cycling conditions were 30 cycles of 94°C for 30 s, 45°C for 1 min, 72°C for 30 s, and a final cycle of 72°C for 5 min (Corbett Research FTS-1 capillary thermal sequencer, Mortlake, Australia). Nucleotides were removed using a Wizard PCR purification column (Promega), the products were cloned into pBluescript SK(−) (Stratagene, La Jolla, CA), digested with *Eco*RV, and sequenced.

A 0.75-kb *Sal*I/*Eco*RI fragment of CSZ-7 was labeled with [α-^{32}P]dCTP (10 μCi μL^{-1}; New Megaprime Random Labeling kit, Amersham Biosciences, Uppsala). A total of 450,000 plaques from a *G. hirsutum* cv DP16 21- to 30-DPA embryo cDNA library (Liu et al., 1999b) were screened with the CSZ-7 insert according to Sambrook et al. (1989). Positive phagemids were excised in vivo according to the manufacturer (Stratagene) and sequenced. To simplify downstream cloning procedures the cDNA insert of C6 was subcloned into the *Eco*RI site of a kanamycin-resistant version of pBluescript SK(−) and named C6kan.

Seventy thousand plaques were screened from an unamplified *G. hirsutum* cv DP16 partial *Sau*3AI genomic library in λEMBL4 (Stratagene; J. Norman and R. Chapple, CSIRO Plant Industry, Canberra, Australia) using the C6 insert probe. Lambda DNA was purified as recommended using a Lambda Mini kit (Qiagen) and 1 μg used for DNA sequencing by a primer-walking strategy.

Heterologous Protein Production

The *Xho*I/*Bam*HI insert of C6kan was cloned into pET-19b (Novagen, Madison, WI) to create pET-C6, which was transformed into *Escherichia coli* BL21(DE3) pLys*S* as recommended (Novagen). Expression of the CDNS protein was induced by the addition of isopropylthio-β-galactoside to 1 mM. Protein purification under both denaturing and native conditions was performed using Talon Metal Affinity Resin (CLONTECH Laboratories, Palo Alto, CA) as recommended for the large-scale batch format procedure. Under denaturing conditions the His-tagged protein was eluted with 50 mM imidazole buffer followed by 100 mM EDTA, pH 8.0. Pooled eluates were concentrated (Amicon concentrator model 52, Millipore, Billerica, MA) and dialyzed against

0.2× phosphate-buffered saline in a Slide-A-Lyser cassette (10,000 molecular weight cutoff; Pierce, Rockford, IL). Polyclonal antibodies against the CDN1-C4 protein (CDNS-Ab) were generated in rabbits using denatured-purified-recombinant protein. For purification of native-recombinant protein, the His-tagged protein was eluted from the resin with 100 mM EDTA, pH 8.0, and concentrated. The eluate was desalted and fractionated on a PD-10 Sephadex G-25 desalting column (Amersham Biosciences) as recommended using 15 mM HEPES Na^+, pH 7.0, 50 mM NaCl. Fractions containing protein were pooled and concentrated with a Centricon-30 (Amicon, Millipore). The native-recombinant protein was stored in 15 mM HEPES Na^+, pH 7.0, 50 mM NaCl, 40% glycerol, and 1 mM dithiothreitol (DTT) at −20°C. Protein purification was monitored by anti-His western blotting (Ausubel et al., 1994) using the chemiluminescent SuperSignal HisProbe western-blotting kit (Pierce). Protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA) with bovine serum albumin standards.

Recombinant Enzyme Assays

CDNS enzyme assays were based upon the methods of Chen et al. (1995) and Davis and Essenberg (1995). Radiochemical enzyme assays were performed at 30°C for 20 min in 100 μL with 2 μg of purified protein in reaction buffer (25 mM HEPES Na^+, pH 7.5, 15 mM $MgCl_2$, and 5 mM DTT). Unlabeled FPP ammonium salt was spiked with [1-*n*-^{3}H]FPP triammonium salt (21 Ci $mmol^{-1}$), both supplied by Sigma (St. Louis), to produce a [^{3}H]FPP substrate with a specific activity of 11.1 μCi μmol^{-1}. To determine enzyme kinetics the final substrate concentrations varied between 5 and 200 μM, with the specific activity determined at 70 μM FPP. Reactions were stopped with the addition of 50 mM EDTA and extracted twice in 200 μL hexane. Radioactivity in the pooled hexane extracts was quantified by liquid scintillation counting (Minaxiβ Tri-carb 400 Series, United Technologies Packard, Pangbourne, UK) in organic scintillation fluid (Sambrook et al., 1989).

Product Identification

For product analysis by capillary GC-MS, enzyme reactions were carried out for 2 h at 30°C in 1-mL reaction volumes with 20 to 50 μg of protein and 70 μM FPP. Reactions were stopped and extracted as before. Pooled hexane extracts were analyzed on a gas chromatograph (model HP5890 series II, Hewlett-Packard, Palo Alto, CA) coupled to a mass-selective detector (model 5971, Hewlett-Packard). Injections of 1 μL were made in the splitless mode onto a 25-m × 0.22-mm i.d. × 0.25-μm film thickness BPX-5 column (SGE, Ringwood, Victoria, Australia), with helium carrier gas delivered at a constant column velocity of 30 cm s^{-1}. The initial oven temperature of 60°C was held for 1.5 min and then increased to 200°C at 25°C min^{-1} and then to 300°C at 5°C min^{-1} (GC program 1).

Chiral capillary GC employed a 30-m × 0.25-mm i.d. × 0.2-μm film thickness β-cyclodextrin column (Agilent J&W Scientific, Folsom, CA) with helium carrier gas delivered at a velocity of 30 cm s^{-1}. After manual injection of 2 μL with a split ratio (1:10) the initial oven temperature of 50°C was held for 5 min before increasing to 70°C at 5°C min^{-1} then increasing to 200°C at 2.5°C min^{-1} (GC program 2). (+)-δ-Cadinene (Fluka, Seelze, Germany) was used as a standard for GC-MS and chiral GC. The essential oil from the root of *Angelica archangelica* was used as a standard to test the resolution of the (+) and (−) enantiomers of δ-cadinene by chiral GC (Davis et al., 1996; Cornwell et al., 2001).

NMR spectra were recorded on a Varian Inova 500 instrument with a ^{1}H nanoprobe and ^{1}H 5-mm indirect-detection pulsed-field gradient probe at 500 MHz using $CDCl_3$ solvent and reference for ^{1}H and double-quantum-filtered correlation spectroscopy. The commercially available (+)-δ-cadinene was also analyzed by NMR. The sample for NMR was prepared as described by Chen et al. (1995) except that 1.2 mg of purified fusion protein was incubated with 2.0 mM FPP in a 2-mL reaction volume.

T-DNA Vector Construction and Cotton Transformation

The LeC6as transformation construct was generated by *Not*I digestion of C6 and ligation into the *Not*I site pGLe-10, a plasmid containing the soybean (*Glycine max*) lectin 5′-promoter and 3′-terminator regions (Cho et al., 1995), and selecting for the antisense orientation. A 4-kb *Xba*I fragment of the expression cassette was ligated into the *Xba*I site of the binary vector pGA482 (An, 1987). For the 35SC6as construct the *Eco*RI insert of C6kan was ligated into the expression cassette pJ35SN (Landsmann et al., 1988) containing the 35S promoter of cauliflower mosaic virus and the terminator from the *Agrobacterium tumefaciens* nopaline synthase gene, and the antisense orientation selected. The expression cassette was digested with *Sal*I, filled with Klenow DNA polymerase (New England Biolabs, Beverly, MA), and cloned into the *Bgl*II-digested and filled-in pGA482.

The introduction of the construct into *A. tumefaciens* AGL1, transformation of *G. hirsutum* cv Coker 315, and subsequent analysis of segregating transgenic plants by Southern hybridization and NPTII assays was carried out as described by Townsend and Llewellyn (2002). All lines were checked for uniform segregation by Southern hybridization of genomic DNA in the T_1, T_2, and T_3 generations using a radiolabeled probe to the NPTII gene near the right border of the T-DNA. The T-DNA insertions of the antisense construct were tested to be complete in the T_1 generation using probes designed to the opposite end of the predicted T-DNA (data not shown). For 35SC6as lines, the probe was a 360-bp *Bgl*II/*Sca*I fragment of pJ35SN that flanks the antisense construct hybridized to *Hin*dIII-digested genomic DNA. For the LeC6as lines, the probe was a 1.2-kb *Hpa*I fragment of pGLe-10 containing the lectin promoter and 3′ end and hybridized to *Xba*I-digested genomic DNA.

Determination of Seed Gossypol Levels

Gossypol levels in seed were determined by the HPLC method of Benson et al. (2001). Mature seeds with seed coat removed from four plants of the T_3 generation of transformants and Coker 315 were frozen in liquid N_2 and lyophilized for 24 h. Ten embryos from each plant were pooled for extraction and quantification of gossypol with duplicate 25-μL injections performed for each extract. The HPLC system consisted of autosampler (WISP710B), pump (model 510), and UV detector (model 441) measuring 254 nm, linked to the Waters 840 Data and Chromatography Control Station software (v4.0, Waters, Milford, MA). Samples were isocratically eluted from a 150- × 3.9-mm i.d. Nova-Pak C18 column (Waters) with acetonitrile/water/phosphoric acid (80:20:0.1) mobile phase at 1 mL min^{-1} and a 7-min total run time. Gossypol acetic acid (Sigma) was used to determine the retention time for the gossypol peak (4.55 min) and to generate a calibration curve.

Pathogen Isolation and Growth

Xcm isolate BT07 was streaked onto peptone/yeast extract/Glu agar and grown for 3 d at 29°C. For plant infection, 25 mL of peptone/yeast extract/Glu broth (DeFeyter et al., 1990) was heavily inoculated with BT07 and incubated for around 24 h at 29°C with vigorous shaking. The culture was diluted 1 in 15 with sterile saturated $CaCO_3$ to approximate 10^8 colony-forming units per milliliter. Characterization of the *Xcm* race of BT07 was performed by Dr. R. DeFeyter (CSIRO Plant Industry, Canberra) using a standard set of differential cotton lines with known resistance (R) genes (Hunter et al., 1968).

A virulent strain of *Verticillium dahliae* was provided by Dr. S. Allen (New South Wales Agriculture, Narrabri, Australia). Conidia were spread on Difco Czapeck-Dox agar (BD, Franklin Lakes, NJ) and grown in the dark at 22°C for 1 to 2 weeks. Agar plugs were used to inoculate fresh plates, which were grown for 3 weeks before collecting conidia in sterile distilled water. The inoculum was diluted to approximately 10^7 conidia per milliliter, using a hemocytometer to determine the concentration of conidia.

Plant Growth and Harvest for Infection Experiments

In all experiments, the untransformed Coker 315 control was grown and treated under equivalent conditions as the homozygous 35SC6as transformed lines 6, 23, and 51. For the *Xcm* infection experiment, seeds of the T_4 generation were grown for 2 weeks in a glasshouse in wooden boxes of cotton potting mix (Townsend and Llewellyn, 2002). The *Xcm* suspension was used to inoculate the intercellular spaces from the underside of cotyledons with a needleless 1-cc syringe. Sterile saturated $CaCO_3$ was used as the mock-inoculated control. Cotyledons were inoculated on each side of the main vein. One cotyledon was harvested from three individual seedlings and pooled for RNA and protein extraction. A zero-time sample was taken prior to inoculation and the *Xcm*-inoculated or mock-inoculated cotyledons were sampled at intervals thereafter.

Infection of the vasculature with *V. dahliae* was carried out on 5-week-old plants in pots in a glasshouse (30°C days [14 h] and 22°C nights [10 h]). At least 2 d prior to infection, the plants were transferred to a growth cabinet (25°C days and 18°C nights) to encourage infection by the pathogen. A stem puncture technique introduced 25 μL of *V. dahliae* conidial suspension into the vasculature at the base of internodes 1 and 2. Sterile distilled water was used as the mock-inoculated control. After 48 h, elapsed internodes 1, 2, and 3 were

harvested from triplicate plants and stripped of the bark before storage and extraction of RNA and protein.

Analysis of CDNS Expression in Transgenic Plants

Northern blotting in an agarose-formaldehyde gel and transfer to Hybond-N membrane (Amersham Biosciences) was as previously described (Sambrook et al., 1989). Total RNA from the *Xcm* infection experiment was fixed to a single Hybond-N membrane using a 96-well format dot-blot apparatus with 2× SSC washing buffer. The *cdn*1-C4 antisense RNA probe was prepared using the Riboscribe T7 RNA probe synthesis kit (Epicentre Technologies, Madison, WI) using [α-^{32}P]UTP (20 μCi μL^{-1}, Amersham) by the in vitro transcription of C6kan linearized with *Pst*I. Filters were prehybridized at 55°C for 2 h and hybridized at 55°C for 20 to 40 h in hybridization solution B (Promega). The membranes were washed twice at 55°C in PSE (0.25 M sodium phosphate buffer [pH 7.2], 2% [w/v] SDS, 1 mM EDTA) and PES (0.04 M sodium phosphate buffer [pH 7.2], 1% [w/v] SDS, 1 mM EDTA) buffers (Promega), followed by rinsing at room temperature in 2× SSC three times for 5 min each, treatment with RNase A (10 μg mL^{-1} in 2× SSC; Sigma), and a final stringent wash in 0.1× SSC/0.1% SDS for 30 min at 50°C.

Plant protein was extracted in 25 mM HEPES Na^+, pH 7.2, 10% glycerol, 15 mM $MgCl_2$, and 5 mM DTT. Total soluble protein was separated with stacking SDS-PAGE. Duplicate gels were prepared, one was stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and the other used for transfer onto nitrocellulose BA 85 (Schleicher & Schuell, Dassel, Germany) using a Transphor Electrophoresis Unit with transfer buffer (25 mM Tris, 192 mM Gly, 0.2% SDS, and 20% ethanol; Hoefer Scientific Instruments, San Francisco). For the detection of CDNS protein in plant extracts, the CDNS-Ab (1:1,000) primary antibody was used in conjunction with the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5,000; GAR-HRP; Bio-Rad). Bound antibodies were detected with the chemiluminescent Supersignal West Pico Substrate (Pierce) as recommended.

An extra band of 68 kD and unknown identity was routinely detected with the CDNS-Ab, particularly from cotyledon and leaf tissue and less so from stems. Antibody eluted from the nitrocellulose membrane bound to this band was capable of binding to itself again but did not cross-react with purified CDNS protein in a western blot. Conversely, the antibody eluted from the 64-kD CDNS band bound to CDNS in a western blot but not the 68-kD band (data not shown). This indicated the antibodies and epitopes detected by each were different as a product of the polyclonal nature of the antisera and that the antibody detecting the 68-kD band was nonspecific but served as a convenient marker to monitor equivalent protein loading in western blots.

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Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AF270425, AY800007, AY800008, AY800006, AY800106, and AY800107.

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