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# Expression pattern of (+)-δ-cadinene synthase genes and biosynthesis of sesquiterpene aldehydes in plants of *Gossypium arboreum* L.

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**Abstract.** The cotton (+)-δ-cadinene synthase, a sesquiterpene cyclase, is encoded by a complex gene family which, based on homology, can be divided into two subfamilies: *cad*1-A and *cad*1-C. Southern blots revealed several members of the *cad*1-C subfamily, and a single member of the *cad*1-A subfamily, in the diploid *Gossypium arboreum* genome. One of the *cad*1-C genes, *cad*1-C3, was isolated from this species. According to reverse transcriptase-polymerase chain reaction, transcripts of both *cad*1-C and *cad*1-A genes appeared in roots from the second day post germination and in 1-d-old cotyledons, whereas the transcription levels were too low to be detected in the hypocotyls. Initially, sesquiterpene cyclase activities were found to be high in the seedlings, then dropped in aerial organs but increased in roots during development. Sesquiterpene aldehyde contents followed the same pattern. In fully developed plants, the transcripts of *cad*1-C were detected in stems, leaves and pericarps, as well as in the sepals and petals 3 d before anthesis, but not at the day of anthesis. In contrast, *cad*1-A transcripts were not detected in any of these aerial organs. The sesquiterpene aldehyde contents increased in petals but decreased in sepals after anthesis. Treatment of *G. arboreum* stems with a *Verticillium dahliae* elicitor-preparation activated *cad*1-A transcription, but a significant level of *cad*1-C transcripts was detected both before and after elicitation. In *G. hirsutum* cv. GL-5, a glandless cultivar, the *cad*1-C gene was activated by the same fungal elicitor, followed by the synthesis of the sesquiterpene cyclase, and accumulation of sesquiterpene aldehydes. The *cad*1 gene expression during development and in response to elicitation, as well as the spatial and temporal pattern of sesquiterpene biosynthesis, constitute a chemical defense machinery in cotton plants.

**Key words:** Cadinene – *Gossypium* – Gossypol – Phytoalexin – Sesquiterpene cyclase

## Introduction

Sesquiterpenoids are one of the major groups of isoprenoids widely distributed in plants. Many plant species accumulate sesquiterpenes in specialized secretary structures, such as resin ducts, subepidermal glands and glandular trichomes (McGarvey and Croteau 1995, and references therein), building a constitutive chemical barrier to most microorganisms. In response to phytopathogenic microorganisms, insect attack or wounding, plants may alter their cellular metabolism to produce an array of antimicrobial secondary metabolites (phytoalexins) (Lawton and Lamb 1987; Dixon and Harrison 1990; Dixon and Paiva 1995; Somssich and Hahlbrock 1998). The phytoalexin status of sesquiterpenes in the family Solanaceae has been well documented (Chappell 1995; Ku'c 1995). The bicyclic sesquiterpene capsidiol, for example, accumulated rapidly only in tissues or cell cultures of *Nicotiana tabacum* and *Capsicum annum* challenged with elicitors (Brooks et al. 1986; Vögeli and Chappell 1988). Induced accumulation of diterpene phytoalexins by elicitation has also been detected in *Oryza sativa* (Ren and West 1992) and *Ricinus communis* (Dudley et al. 1986). In *Abies grandis*, increased formation of oleoresin monoterpenes, sesquiterpenes and diterpenes was induced by wounding (Vogel et al. 1996; Bohlmann et al. 1997; Steele et al. 1998b).

Sesquiterpene cyclases catalyze the formation of cyclic sesquiterpene hydrocarbon intermediates from a linear 15-carbon farnesyl diphosphate (FPP), directing the isoprenoid carbon flow into complex sesquiterpenoids. Because of this central function, this enzyme is highly regulated (Chappell 1995; McGarvey and

The gene reported here has the EMBL accession number AF174294

Abbreviations: CAD = (+)-δ-cadinene synthase; dd = double-distilled; EAS = 5-*epi*-aristolochene synthase; FPP = farnesyl diphosphate; PCR = polymerase chain reaction; RT = reverse transcriptase

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Croteau 1995; Starks et al. 1997; Bohlmann et al. 1998b). Investigations of plant terpene cyclases have drawn much attention recently, and cDNAs encoding various sesquiterpene cyclases have been isolated from a number of plant species (Facchini and Chappell 1992; Back and Chappell 1995; Back et al. 1998; Bohlmann et al. 1998a; Colby et al. 1998; Steele et al. 1998a), including two (+)-δ-cadinene synthases (CADs) from *Gossypium arboreum* L. (Chen et al. 1995, 1996). However, up to now only a few studies have been conducted on plant cyclase genes and their expression patterns. The tobacco 5-*epi*-aristolochene synthase (EAS) has been investigated at both the mRNA and gene levels. The tobacco genome has been found to contain 12∼15 copies of *eas* genes (Facchini and Chappell 1992). Yin et al. (1997) analyzed the *eas4* promoter, and found that it directed an induced expression only.

In cotton, (+)-δ-cadinene is a biosynthetic precursor of the cyclic secondary sesquiterpene aldehydes, including gossypol (Davila-Huerta et al. 1995). As in tobacco, the cotton sesquiterpene cyclase is encoded by a multigene family. From an elicitor-induced cDNA library of *G. arboreum*, a diploid A-genome species, four different clones have been isolated. On the basis of sequence similarities, these cDNAs can be grouped into two subfamilies: *cad*1-C and *cad*1-A. The *cad*1-C is plural, of the four cDNAs characterized three belong to this subfamily: *cad*1-C1, *cad*1-C14 (Chen et al. 1995), and *cad*1-C2 (Meng et al. 1999), whereas only one member of *cad*1-A has been isolated (Chen et al. 1996). The three *cad*1-C members are closely related based on an over 95% amino acid sequence identity among each other, whereas *cad*1-A is only distantly related to members of the *cad*1-C subfamily (80% identity). A cDNA recently isolated from *G. hirsutum* (Davis et al. 1998) is also a member of *cad*1-C.

Gossypol and related sesquiterpene aldehydes posses fungistatic properties, insecticidal activities and are toxic to monogastric animals (Bell and Stipanovic 1977). Formation of these secondary sesquiterpenes may be induced by elicitation, thus they behave as phytoalexins (Bell 1984; Heinstein 1985; Essenberg et al. 1990). Increased steady-state levels of *cad*1-C and *cad*1-A mRNAs, the cyclase proteins, and subsequent production of sesquiterpene aldehydes have been detected in *G. arboreum* cell-suspension cultures treated with a fungal elicitor from *Verticillium dahliae* (Heinstein 1985; Chen et al. 1995, 1996). In *G. hirsutum* foliar tissues inoculated with a phytopathogenic bacterium *Xanthomonas campestris*, an elevated δ-cadinene synthase activity was observed (Davis et al. 1996). Similar activation of sesquiterpene cyclase genes by elicitation has been reported for tobacco and *Hyoscyamus muticus* (Facchini and Chappell 1992; Back and Chappell 1995). However, in contrast to tobacco plants that accumulate secondary sesquiterpenes only after induction (Vögeli and Chappell 1990; Yin et al. 1997), cotton plants constitutively accumulate sesquiterpenoids in their pigmented glands. These glands are spread throughout the plant, except seed coat and xylem (Stanford and Viehoever 1918; Bell and Stipanovic 1977). Thus, biosynthesis of sesquiterpenoids in cotton plants is also related to the formation of pigmented glands and is independent of environmental stimuli (Bell 1984). In addition to induction as an activated defense response, the secondary sesquiterpene metabolism of cotton is most likely developmentally regulated as well. Thus investigation of a glanded *G. hirsutum* cultivar has demonstrated an active biosynthesis of sesquiterpenoids in developing seeds, starting at an early cotyledon stage (Meng et al. 1999). However, little is known about the regulation and kinetics of sesquiterpene biosynthesis in cotton seedlings and plants. Here, we report isolation of a *G. arboreum cad*1-C gene, *cad*1-C3, the differential expression patterns of the *cad*1-A and *cad*1-C genes, sesquiterpenoid accumulation in the healthy *G. arboreum* seedlings and plants, and induction by elicitation of *cad*1 expression in cotton stem tissue.

## Materials and methods

*Plant material*. Seeds of *Gossypium arboreum* L. cv. Nanking and *G. hirsutum* L. cv. GL-5 were sown in pots and the plants were grown in a greenhouse, at 28 °C. For seedlings the seeds were germinated in 0.7% agar containing 3% sucrose, at 28 °C.

*Genomic library screening*. Genomic DNA was isolated from cell-suspension cultures of *G. arboreum* L. cv. Nanking, as described by Chen et al. (1995). The DNA was partially digested with *Eco*R I and ligated with the λ-ZAP vector (Stratagene, La Jolla, Calif., USA). A PCR-96 well plate method (Alfandari and Darribere 1994) was used to screen the library. Briefly, the library of about $10^7$ plaque-forming units was spread into the 96 wells. Following overnight incubation with *Escherichia coli* strain XL1-Blue at 37 °C, aliquots from each well were checked by polymerase chain reactions (PCRs), using a forward primer 97400 (CACATCCCTTCGATTCCGAC) and a reverse primer 97T580 (AGGCTTAAATGGTGGGTGGT) that are specific for *cad*1-C1. The PCRs were carried out at 94 °C for 30 s, 51 °C for 30 s and 72 °C for 1 min, 35 cycles. Positive wells were then subjected to subsequent-round selection. After seven rounds a positive clone was isolated and the plasmid excised. The DNA sequences were determined with a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE-ABI, Warrington, UK).

*Southern blotting*. Genomic DNA was digested with *Eco*R I or *Hin*d III, separated on a 0.7% agarose gel, and transferred onto a nitrocellulose membrane. Polymerase chain reaction was performed on the genomic clone of *cad*1-C3 with primers Sou1 (TGACTTCAAATATCACCAC) and Sou2 (CTTGTCGGGACAATTGAGGA) and 97400 and 97T1200 (CATCTATAC ATTTGATATC). For *cad*1-A, primers 98A1 (ATTGCACACATCGTCTCA) and 98T280 (AATTCAGTTGCAGCATCG) were used for PCR amplification of the genomic clone. The products were $^{32}$P-labeled with a Prim-a-Gene Labeling System (Promega, Madison, Wis., USA). A standard protocol was then used for DNA hybridization (Fütterer et al. 1995).

*Isolation and analysis of RNA*. Total RNAs were isolated from various tissues of seedlings or plants by the method of Hughes and Galau (1988). Reverse transcriptase (RT)-PCR was performed as described by Meng et al. (1999). The primers used were 93160 (CACCACTGCTCAACTTACA) and 96T1098 (ATTCGGGAGTTGGTTCATG) for *cad*1-A, and 97400 and 97T580 for *cad*1-C1 and *cad*1-C3. The latter two primers might also work with other members of the *cad*1-C subfamily, due to a high sequence identity (over 95%) among *cad*1-C members. As an internal standard, the

endogenous *histone-3* mRNA (EMBL accession number AF024716) was analyzed in a separate tube, with a forward primer H3F (GAAGCCTCATCGATACCGTC) and a reverse primer H3R (CTACCACTACCATCATGGC).

For further identification, the RT-PCR products amplified by 26 cycles were separated on a 2% agarose gel, and transferred to nitrocellulose membranes. The PCR products obtained with the same primers, using cDNA clones as templates, were then $^{32}$P-labeled, and used for DNA hybridization.

*Elicitation and tissue printing*. The *Verticillium dahliae* elicitor was prepared as described by Heinstein (1985), and the elicitor concentration was adjusted to 50 μg sucrose equivalent/100 μL. The stems of *G. arboreum* L. cv. Qingyangxiaozi and *G. hirsutum* L. cv. GL-5 (a glandless cultivar) were elicited by injecting 200 μL of the elicitor-preparation, or an equal amount of sterile double-distilled (dd) $H_2O$, into the stem of cotton plants, about 5 cm below the stem apex. The stems were then collected 2 d post treatment for RNA analysis, or 8 d post treatment for tissue printing and chemical analysis. For tissue printing (Cappadocia 1993; Varner and Ye 1994), the stem fragment was cut into two halves, one half was placed on a nitrocellulose membrane saturated with phosphate-buffered saline (10 mM K-phosphate, 150 mM NaCl, pH 7.4) for 1 h in a vacuum, then dried at 80 °C overnight. The nitrocellulose membrane was stained with Ponceau S for 5 min to check total proteins transferred, followed by destaining with dd$H_2O$ for 10 min. Then the membrane was incubated in the blocking buffer [10% nonfat dry milk in Tris-buffered saline (TBS = 20 mM Tris-HCl, 500 mM NaCl, pH 8.0) containing 0.05% Tween-20 (TBST)] for at least 2 h at 25 °C, followed by two brief washes in TBST, and two washes in TBS, 10 min each. The blot was challenged first with the rabbit antiserum raised against the bacterially expressed *cad*1-C1, then with alkaline-phosphatase-conjugated anti-rabbit IgG (Sigma, St. Louis, Mo., USA). The signals were developed by incubating the blot with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate.

*Assay of sesquiterpene cyclase*. Proteins were isolated from the seedlings with a buffer containing 50 mM Tris-HCl, 10 mM EDTA (pH 8.0), 1% β-mercaptoethanol, and 20% glycerol. Activities of sesquiterpene cyclase were assayed as described by Vögeli and Chappell (1990) and Meng et al. (1999). Briefly, 15 μg of proteins was added to each reaction mixture of 200 μl, containing 3.5 nmol FPP and 5.55 kB$_2$ [$^3$H]FPP (Sigma). Protein concentrations were determined with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, Calif., USA). For quantitative estimation throughout this investigation, experiments were carried out in triplicate and mean values were taken.

*Quantification of sesquiterpene aldehyde*. Total sesquiterpene aldehydes were extracted from the seedlings with 95% ethanol, and quantified using a phloroglucinol assay, as described by Meng et al. (1999). Standard curves were made with gossypol (Sigma).

## Results

*Isolation of the cad1-C3 gene*. A clone was isolated from a *G. arboreum* genomic library using the 96-well PCR method, with primers specific for the cDNA of *cad*1-C1. Restriction analysis revealed an insert of approximately 5 kb. Nucleotide sequencing showed that the clone contains a fragment of about 710 bp upstream to the translation start codon. Alignment of nucleotide sequences of the genomic insert with the previously isolated cDNAs showed an identity of 97% with *cad*1-C1 (Chen et al. 1995) and *cad*1-C2 (Meng et al. 1999), and an identity of 95.5% with *cad*1-C14 (Chen et al. 1995). Thus, this gene appears to be a new member of the *cad*1-C subfamily of *G. arboreum*, and is assigned *cad*1-C3.

When *G. arboreum* genomic DNA was hybridized with $^{32}$P-labeled DNA fragments flanking the −151- to 125-bp and 437- to 1650-bp regions of the *cad*1-C3 gene, eight *Eco*R I bands and four *Hin*d III bands appeared (Fig. 1). Since the probing region in the *cad*1-C3 clone contained no *Eco*R I sites, eight copies of the *cad*1-C were assumed to exist in the diploid genome of *G. arboreum*. It is also possible that there are fewer than eight copies, especially since some of the bands are smaller than 5 kb (full length of the insert in the genomic clone of *cad*1-C3), indicating the presence of at least one *Eco*R I site within or between the regions recognized by the probes of one or more *cad*1-C members. As mentioned above, three different cDNAs of *cad*1-C have been already isolated from *G. arboreum*. Hybridization with a *cad*1-A probe revealed two *Hin*d III bands and one *Eco*R I band (Fig. 1), and since there was a *Hin*d III site but no *Eco*R I sites in this probe, a single *cad*1-A copy was assumed to be present in *G. arboreum*.

Up to now three *G. arboreum* genes of (+)-δ-cadinene synthase have been isolated, they are *cad*1-C3 reported here, *cad*1-A (EMBL accession number Y18484) and a putative *cad*1-B (EMBL accession number X95323). Although amino acid sequences deduced from the three genes have over 73% identities, the promoter and introns of *cad*1-C3 show little homology with those of the other two genes. A putative TATA-box is located at a position 119 bp upstream to the translation start codon, and a 20-bp domain spanning the TATA-box is completely conserved among the three *G. arboreum cad*1 genes. All plant terpene cyclase genes reported so far have a similar overall structure with six positionally conserved introns (Chappell 1995; Bohlmann et al. 1998b), this too is the case for the three cotton genes.

*Kinetic analysis of seedlings*. Transcription of the CAD genes in *G. arboreum* seedlings was investigated by RT-

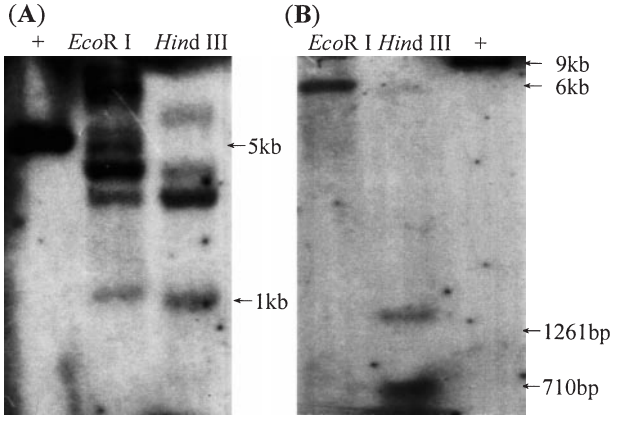


**Fig. 1A,B.** Southern blots of *G. arboreum* genomic DNA hybridized with probes of *cad*1-C3 (**A**) and *cad*1-A (**B**). The genomic DNA was digested with restriction enzymes as indicated. As a positive control (+), genomic clones of *cad*1-C3 and *cad*1-A, respectively, were digested with *Eco*R I

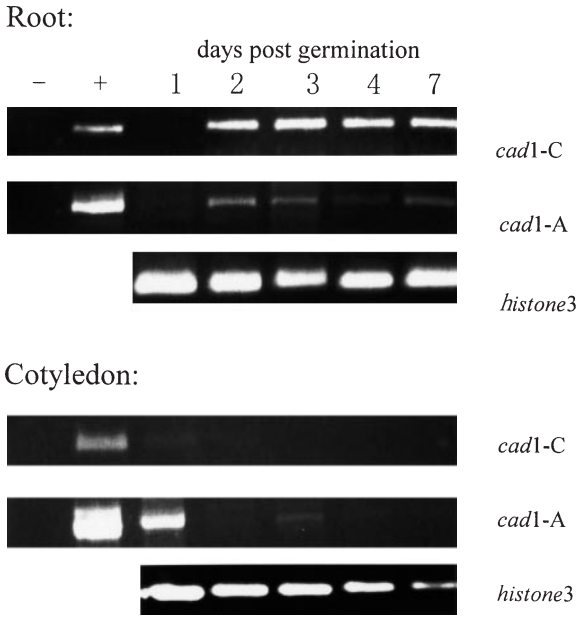


**Fig. 2.** Analysis by RT-PCR of *cad*1-A and *cad*1-C transcripts of *G. arboreum* seedlings. For negative controls (−), no template DNA was added; for positive controls (+), the cDNA clones of either *cad*1-C1 or *cad*1-A (10 ng of plasmid DNA) were used as the template. The *cad*1-A product is 488 bp, and the *cad*1-C1 product 222 bp. The *histone-3* mRNA was analyzed as an internal standard. The PCR was performed by 28 cycles of amplification, with specific primers (see *Materials and methods*)

PCR, with primers specific either for *cad*1-A or for *cad*1-C (including at least at *cad*1-C1 and *cad*1-C3). Transcripts of neither *cad*1-A nor *cad*1-C were detectable in roots of 1-d-old seedlings, but the mRNAs appeared on the second day post germination, and steady-state levels were then maintained relatively stable throughout the investigation period of 7 d (Fig. 2). Amplification by PCR revealed a clear *cad*1-C band and a weak *cas*1-A band, indicating a lower level of *cad*1-A transcripts than *cad*1-C transcripts. Analysis of cotyledons showed an opposite pattern: transcripts of both the *cad*1-A and *cad*1-C were detected in 1-d-old seedlings; thereafter, the transcripts decreased to undetectable levels (Fig. 2). It is not clear whether these transcripts resulted from gene transcription during seed germination, or were remnants in the seeds. The transcripts were not detected in hypocotyls, possibly due to the low level of *cad*1 transcription. However, because of the difficulty of obtaining a good quality RNA from the hypocotyl, low levels of transcripts cannot be excluded, as, with *histone-3* primers, only weak RT-PCR bands were obtained (data not shown).

Specific activities of sesquiterpene cyclases were detectable in 1-d-old seedlings. In roots, the activity clearly increased on the 7th day post germination. In cotyledons and hypocotyls, although variation during the first 2 d post germination was insignificant, the activity decreased to almost undetectable levels on the 7th day (Fig. 3A). Total sesquiterpene aldehydes in seedlings were then quantitatively analyzed with the phloroglucinol/HCl reagent. In roots, sesquiterpene

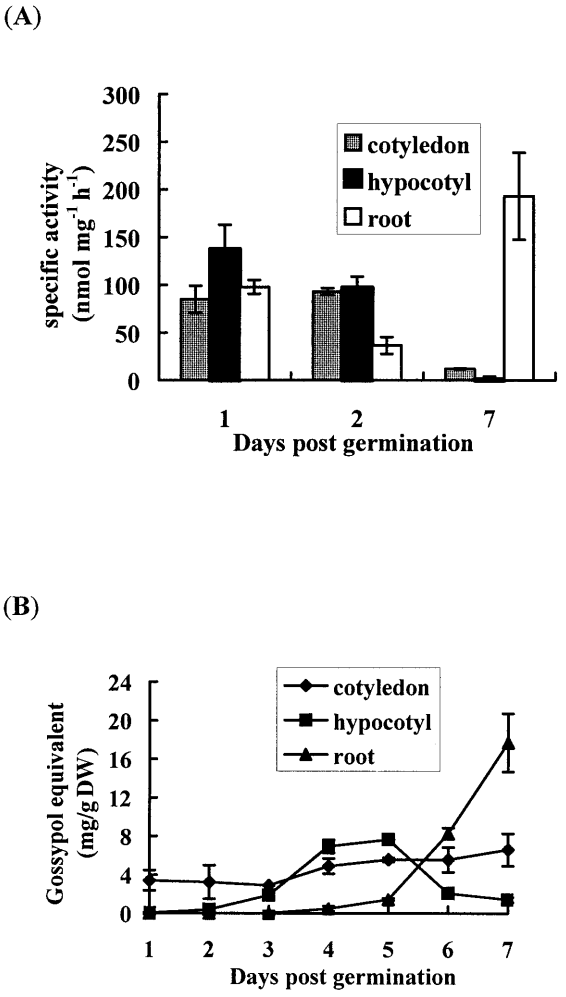


**Fig. 3A,B.** Specific activities of the sesquiterpene cyclase (**A**) and accumulation of sesquiterpene aldehydes (**B**) in *G. arboreum* seedlings. The amount of sesquiterpene aldehydes is given as gossypol equivalent

aldehydes accumulated rapidly after 5 d post germination. In cotyledons, the level was found to be largely stable, whereas in hypocotyls, a slow accumulation of sesquiterpene aldehydes was found up to 5 d post germination, then the level decreased (Fig. 3B).

*Analysis of aerial organs of plants*. The expression of *cad*1-C and *cad*1-A in flowers, pericarps (bolls), stems and leaves were also analyzed by RT-PCR. A significant level of *cad*1-C mRNA was found in sepals and petals collected 3 d before anthesis, but not at the day of anthesis (Fig. 4), nor afterwards (data not shown). The *cad*1-C transcripts were also detected in pericarps 4 d after anthesis, as well as in young stems and leaves. To the contrary, the *cad*1-A transcripts were not detected in these organs, although the gene was found to be expressed in developing seeds (Meng et al. 1999), elicitor-treated suspension cells (Chen et al. 1996) and roots of seedlings as described above. The *cad*1-C mRNA levels were found to be higher in pericarps and petals than in sepals, leaves and stems. Consistently, the sesquiterpene aldehyde contents in sepals and pericarps

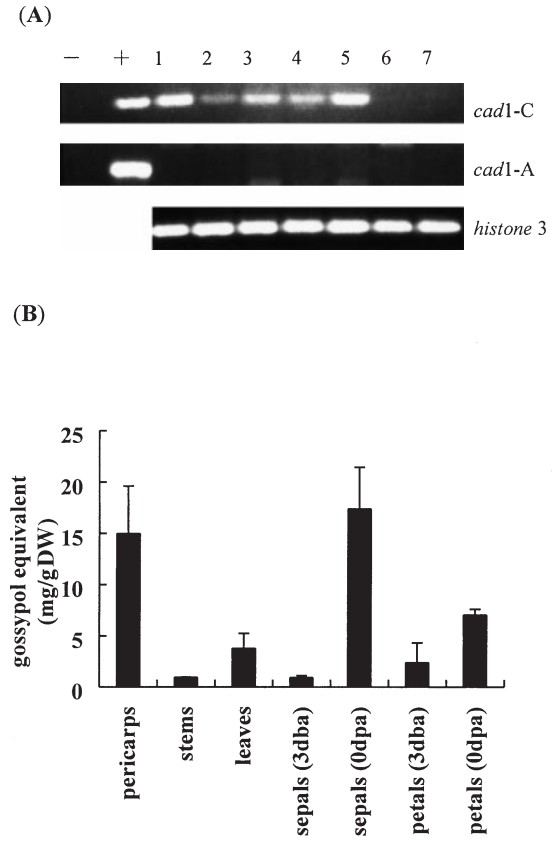


**Fig. 4A,B.** Analysis by RT-PCR of *cad*1 gene transcription (**A**) and accumulation of sesquiterpene aldehydes (**B**) in *G. arboreum* aerial organs. In negative controls (–), the cDNA clone of *cad*1-A, or *cad*1-C1 (10 ng plasmid DNA each), was used as the template in the PCR amplification using primers specific for *cad*1-C (including *cad*1-C1 and *cad*1-C3), or *cad*1-A, respectively. The same positive control (+) as in Fig. 2 was used. Total RNAs, or sesquiterpene aldehydes, were isolated from pericarps 4 d post anthesis (*1*); stems 5 cm below the stem apex (*2*); leaves 3 d post sprouting (*3*); sepals 3 d before anthesis (*3 dba*) (*4*); petals 3 d before anthesis (*5*); sepals at the day of anthesis (*0 dpa*) (*6*); and petals at the day of anthesis (*7*)

were also the highest (Fig. 4). In petals the relative level of sesquiterpene aldehydes dropped to a lower level (about half) at the day of anthesis, whereas in sepals the level slightly increased. These results indicate that the *cad*1-C genes (including at least *cad*1-C1 and *cad*1-C3), but not the *cad*1-A, are expressed in stems, leaves and corollas, and the expression in corollas is highly regulated by development.

*Induced expression in stems*. In stems of *G. hirsutum* L. cv. GL-5, a glandless cotton cultivar, neither the *cad*1 transcripts, nor sesquiterpene aldehydes, were detected. When stems of this cultivar were treated with a *V. dahliae* elicitor preparation, transcription of *cad*1-C was activated. Tissue-printing analysis showed a de-novo formation of the CAD proteins in the treated region, followed by induced accumulation of sesquiterpene aldehydes (Fig. 5). However, when stems of *G. arboreum* were treated with the same elicitor, the amount of cadinene synthase proteins did not increase

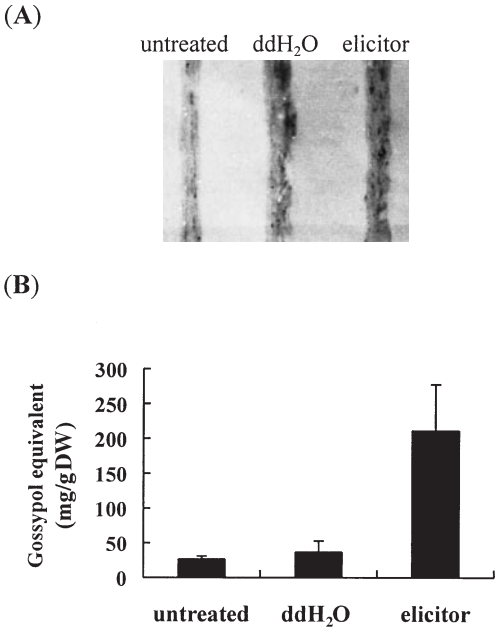


**Fig. 5A,B.** Tissue printing analysis of the CAD1 proteins (**A**) and accumulation of sesquiterpene aldehydes (**B**) in stems of *G. hirsutum* L. cv. GL-5, a glandless cultivar. For treatments see Fig. 6.

significantly, due to a high basal level in untreated stems (Fig. 6); similarly, the sesquiterpene aldehyde contents were increased only slightly after elicitation. The RT-PCR analysis demonstrated that the *cad*1-C genes were transcribed at a similar level in *G. arboreum* stems both before and after elicitor-treatment, but the *cad*1-A mRNA appeared only after elicitation (Fig. 6). A low

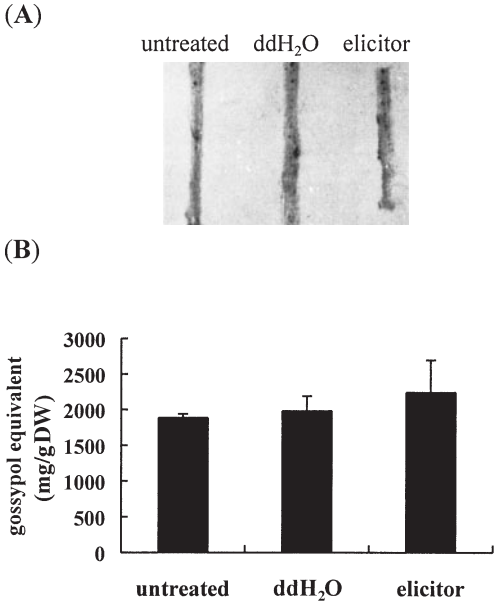


**Fig. 6A,B.** Tissue printing analysis of the CAD1 proteins (**A**) and accumulation of sesquiterpene aldehydes (**B**) in *G. arboreum* stems. The stems (5 cm below the stem apex) were treated by injecting 200 µl of a *V. dahliae* elicitor-preparation, or 200 µl of $ddH_2O$, and collected 8 d post treatment

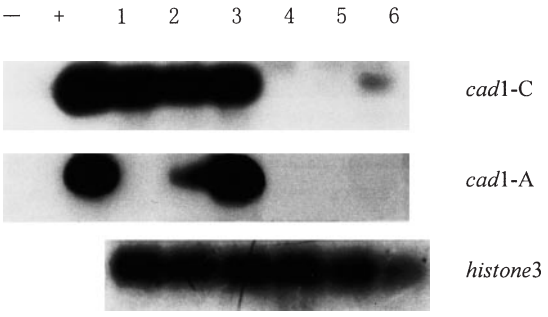


**Fig. 7.** Analysis by RT-PCR of the induced transcription of *cad*1 genes in the stems of *G. arboreum* and *G. hirsutum* L. cv. GL-5. The PCR was performed through 26 cycles of amplification, and the products were hybridized with $^{32}$P-labeled *cad*1-C1 or *cad*1-A probes, respectively. Total RNAs were isolated from untreated *G. arboreum* stems (*1*); dd$H_2O$-treated *G. arboreum* stems (*2*); elicitor-treated *G. arboreum* stems (*3*); untreated *G. hirsutum* L. cv. GL-5 stems (*4*); dd$H_2O$-treated *G. hirsutum* L. cv. GL-5 stems (*5*); and elicitor-treated *G. hirsutum* L. cv. GL-5 stems (*6*). The stems were collected 2 d post treatment. For controls see Fig. 4

level of *cad*1-A transcripts was also detected in *G. arboreum* stems treated with distilled water, and this may be ascribed to wounding-induction, but infection by other pathogens during injection could not be fully excluded.

When the PCRs were performed by 26 cycles of amplification and the products were hybridized with the probes prepared from cDNAs of *cad*1-C1 or *cad*1-A, respectively, specific hybridizing bands confirmed previous RT-PCR analysis (Fig. 7). Thus, these results indicate that the expression of specific members of the sesquiterpene cyclase genes can be induced by fungal elicitation at the transcriptional level. The *cad*1-C genes were constitutively expressed in the stem of *G. arboreum* and expression was induced in the glandless cultivar *G. hirsutum* L. cv. GL-5, whereas *cad*1-A gene expression was induced in the *G. arboreum* stems, but not in stems of *G. hirsutum* L. cv. GL-5, even after elicitation. Whether transcription of *cad*1-A can be activated by other elicitors, or in other glanded or glandless cotton cultivars, requires further investigation.

## Discussion

In many plants the enzymes regulating plant secondary sesquiterpene biosynthesis are encoded by a complex gene family, such as the sesquiterpene cyclase EAS of tobacco (Facchini and Chappell 1992). Different members of the cotton *cad*1 family appear to be differentially regulated. In *G. arboreum*, genes of the *cad*1-C subfamily, such as *cad*1-C3, were found widely expressed in tissues where there was an accumulation of sesquiterpene aldehydes, whereas the *cad*1-A transcripts were not found in aerial parts of the plant except for developing seeds, thus showing a much limited expression. However, the *cad*1-A gene was activated in *G. arboreum* stems by elicitation with the *V. dahliae* elicitor. On the basis of a Northern analysis using a *cad*1-C probe, Alchanati et al. (1998) reported an apparently induced transcription of the CAD genes in cotton stele tissues infected by *V. dahliae*. Similar results were obtained here by RT-PCR analysis of *G. hirsutum* cv. GL-5, but the *cad*1-A transcripts were not detected in this glandless cultivar even after elicitor-treatment.

Different kinetics were observed for the steady-state levels of *cad*1 transcripts, sesquiterpene cyclase activities, and sesquiterpene aldehyde contents in *G. arboreum* seedlings. This may be explained by the fact that active transcription of *cad*1 genes and biosynthesis of the cyclase proteins take place during seed development, resulting in a high concentration of sesquiterpene aldehydes accumulating in pigmented glands of mature seeds (Meng et al. 1999). The enzyme appears to remain active only for a short period after seed germination. In cotyledons, pigmented glands and sesquiterpene aldehydes are formed during seed maturation (Meng et al. 1999). In the hypocotyl there is only a temporal formation of sesquiterpenoids. However, for roots, activation of *cad*1 genes shortly after seed germination and a continuous formation of sesquiterpene aldehydes correlate well with rapid growth and expansion of the root system. The higher mRNA level of *cad*1-C than *cad*1-A detected in roots may be partially due to the fact that there are several members of *cad*1-C, but only one copy of *cad*1-A, and the *cad*1-C-specific primers might also work with other members of the subfamily, in addition to *cad*1-C1 and *cad*1-C3, because of their high sequence identities.

The present investigation found that there was a high level of *cad*1-C mRNAs in sepals and petals before anthesis, and that the transcription ceased after anthesis. The monoterpene synthase, *S*-linalool synthase (LIS), showed a similar developmentally regulated expression in petals of *Clarkia breweri* (Dudareva et al. 1996). The LIS mRNA levels peaked 1 d before anthesis, and dropped to an undetectable level after day 2 post anthesis. The physiological relevance of this down-regulation of biosynthesis of secondary terpenoids, particularly in relation to anthocynin biosynthesis in developing petals, needs further investigation.

Although, a developmentally regulated expression of the tobacco sesquiterpene cyclase gene has not been reported, the *eas4* promoter showed a strong induction in root and stem subepidermal cells and in the cells surrounding the vascular tissues, a pattern consistent with antimicrobial responses (Yin et al. 1997). The pattern of *cad*1 gene expression and sesquiterpene distribution in cotton seedlings similarly points to the plant need to generate a defense barrier. For cotton plants, accumulation of sesquiterpenoids in lysigenous glands of the aerial organs provides an effective protection against insects and pests. In roots, the active transcription of *cad*1-C and *cad*1-A genes, and accumulation of sesquiterpene aldehydes concomitant with root development, form a continuous chemical barrier against invading microorganisms. In *Abies grandis*, two classes of sesquiterpene cyclases have been identified. One class, represented by δ-selinene synthase and γ-humulene synthase, seems to be constitutive and

multi-product enzymes. A second class, including δ-cadinene synthase and α-bisabolene synthase, is characterized by a single dominant product and is induced by stem-wounding (Steele et al. 1998b; Bohlmann et al. 1998a). In *G. arboreum*, however, it appears to be the same sesquiterpene cyclase genes that are responsible for both constitutive and induced formation of sesquiterpene aldehydes. Previous RT-PCR experiments showed an activation of *cad*1-A transcription in developing cotton seeds (Meng et al. 1999), while the present study found that the same species of mRNA accumulated in seedling roots without any environmental stimulation, and in stems only after elicitation. Since Southern blotting identified only one copy of the *cad*1-A subfamily in the *G. arboreum* genome (Fig. 1), it is clear that the same gene is regulated by both developmental and stress-responsible signals. Such complex expression patterns have also been reported for genes encoding enzymes of the general phenylpropanoid pathway, such as phenylalanine ammonia-lyase and 4-coumarate:coA ligase (Liang et al. 1989; Hauffe et al. 1991).

Activation of genes in the sesquiterpenoid pathway is not limited to the cyclase only. In *G. arboreum* suspension-cultured cells treated with fungal elicitors, transcription levels of the FPP synthase and the sesquiterpene cyclase were concomitantly increased, along with induced sesquiterpene accumulation (Liu et al. 1999). Transcriptional induction of 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGR), an enzyme in the core cytosolic isoprenoid pathway, has been reported for plants of *G. barbadence* and *G. hirsutum* infected with *V. dahliae* (Joost et al. 1995). The potato HMGR is also encoded by a complex gene family, and differential expression and induction of different members has been reported (Choi et al. 1992; Bhattacharyya et al. 1995). These data indicate that a complex set of developmental and environmental cues is transduced into an integrated spatial and temporal program for expression of genes encoding enzymes to regulate the synthesis of secondary sesquiterpenes, participating in the multiple defense responses of plants.

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## References

Alchanati I, Patel J, Liu J, Benedict CR, Stipanovic R, Bell A, Cui Y, Magill CW (1998) The enzymatic cyclization of nerolidyl diphosphate by δ-cadinene synthase from cotton stele tissue infected with *Verticillium dahliae*. Phytochemistry 47: 961–967

Alfandari D, Darribere T (1994) A simple PCR method for screening cDNA libraries. PCR Method Appl 4: 46–49

Back K, Chappell J (1995) Cloning and bacterial expression of a sesquiterpene cyclase from *Hyoscyamus muticus* and its molecular comparison to related cyclases. J Biol Chem 270: 7375–7381

Back K, He S, Kim KU, Shin DH (1998) Cloning and bacterial expression of sesquiterpene cyclase, a key branch point enzyme for the synthesis of sesquiterpenoid phytoalexin capsidiol in UV-challenged leaves *of Capsicum annuum*. Plant Cell Physiol 39: 899–904

Bhattacharyya MK, Paiva NL, Dixon RA, Korth KL, Stermer BA (1995) Features of the *hmg* 1 subfamily of genes encoding HMG-CoA reductase in potato. Plant Mol Biol 28: 1–15

Bell AA (1984) Morphology, chemistry and genetics of *Gossypium* adaptations to pests. In: Timmermann BN, Steelink C, Loewus FA (eds) Recent advances in phytochemistry, vol 18, Phytochemical adaptations to stress. Plenum Press, New York, pp 197–232

Bell AA, Stipanovic RD (1977) The chemical composition, biological activity, and genetics of pigment glands in cotton. Proceedings of the Beltwide Cotton Production Research Conference. Atlanta, Georgia, Jan 10–12, pp 244–258

Bohlmann J, Steele CL, Croteau R (1997) Monoterpene synthases from grand fir (*Abies grandis*): cDNA isolation, characterization, and functional expression of myrcene synthase, (−)-(4*S*)-limonene synthase, and (−)-(1*S*,5*S*)-pinene synthase. J Biol Chem 272: 21784–21792

Bohlmann J, Crock J, Reinhard J, Croteau R (1998a) Terpenoid-based defenses in conifers: cDNA cloning, characterization, and functional expression of wound-inducible (E)-α-bisabolene synthase from grand fir (*Abies grandis*). Proc Natl Acad Sci USA 95: 6756–6761

Bohlmann J, Meyer-Gauen G, Croteau R (1998b) Plant terpenoid synthases: molecular biology and phylogenetic analysis. Proc Natl Acad Sci USA 95: 4126–4133

Brooks CJW, Watson DG, Freer IM (1986) Elicitation of capsidiol accumulation in suspended callus cultures of *Capsicum annum*. Phytochemistry 25: 1089–1092

Cappadocia M, Heizmann P, Dumas C (1993) Tissue printing and its applications in self-incompatibility studies. Plant Mol Biol 23: 1079–1085

Chappell J (1995) Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annu Rev Plant Physiol Plant Mol Biol 46: 521–547

Chen XY, Chen Y, Heinstein P, Davisson VJ (1995) Cloning, expression, and characterization of (+)-δ-cadinene synthase: a catalyst for cotton phytoalexin biosynthesis. Arch Biochem Biophys 324: 255–266

Chen XY, Wang MS, Chen Y, Davisson VJ, Heinstein P (1996) Cloning and heterologous expression of a second cotton (+)-δ-cadinene synthase. J Nat Prod 59: 944–951

Choi D, Ward BL, Bostock RM (1992) Differential induction and suppression of potato 3-hydroxy-3-methyl-glutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. Plant Cell 4: 1333–1344

Colby SM, Crock J, Dowdle-Rizzo B, Lemaux PG, Croteau R (1998) Germacrene C synthase from *Lycopersicon esculentum* cv. Vent Cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase. Proc Natl Acad Sci USA 95: 2216–2221

Davila-Huerta G, Hamada H, Davis GD, Stipanovic RD, Adams CM, Essenberg M (1995) Cadinane-type sesquiterpenes induced in *Gossypium* cotyledons by bacterial inoculation. Phytochemistry 39: 531–536

Davis EM, Chen Y-S, Essenberg M, Pierce ML (1998) cDNA sequence of a (+)-delta-cadinene synthase gene (accession No. U88318) induced in *Gossypium hirsutum* L. by bacterial infection. Plant Physiol 116: 1192

Davis EM, Tsuji J, Davis GD, Pierce ML, Essenberg M (1996) Purification of (+)-δ-cadinene synthase, a sesquiterpene cyclase from bacteria-inoculated cotton foliar tissue. Phytochemistry 41: 1047–1055

Dixon RA, Harrison MJ (1990) Activation, structure, and organization of genes involved in microbial defense in plants. Adv Genet 28: 165–234

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7: 1085–1097

Dudareva N, Cseke L, Blanc VM, Pichersky E (1996) Evolution of floral scent in *Clarkia*: novel patterns of S-linalool synthase gene expression in the *C. breweri* flower. Plant Cell 7: 1137–1148

Dudley MW, Dueber MT, West CA (1986) Biosynthesis of macrocyclic diterpene casbene in castor bean (*Ricinus communis* L.) seedlings, changes in enzyme levels induced by fungal infection, and intracellular localization of the pathway. Plant Physiol 81: 335–342

Essenberg M, Grover Jr PB, Cover EC (1990) Accumulation of antibacterial sesquiterpenoids in bacterially inoculated *Gossypium* leaves and cotyledons. Phytochemistry 29: 3107–3113

Facchini PJ, Chappell J (1992) Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. Proc Natl Acad Sci USA 89: 11088–11092

Fütterer J, Gisel A, Iglesias V, Klöti A, Kost B, Scheid MO, Neuhaus-Url G, Schrott M, Schillito R, Spangenberg G, Wang ZY (1995) Standard molecular techniques for the analysis of transgenic plants. In: Potrykus I, Spangenberg G (eds) Gene transfer to plants. Springer, Berlin, pp 215–263

Heinstein P (1985) Stimulation of sesquiterpene aldehyde formation in *Gossypium arboreum* cell suspension cultures by conidia of *Verticillium dahliae*. J Nat Prod 48: 907–915

Hauffe KD, Paszkowski U, Schulze-Lefert P, Hahlbrock K, Dangl JL, Douglas CJA (1991) Parsley *4CL-1* promoter fragment specifies complex expression patterns in transgenic tobacco. Plant Cell 3: 435–443

Hughes DW, Galau G (1988) Preparation of RNA from cotton leaves and pollen. Plant Mol Biol Rep 6: 253–257

Joost O, Bianchini G, Bell AA, Benedict CR, Magill CW (1995) Differential induction of 3-hydroxy-3-methylglutaryl CoA reductase in two cotton species following inoculation with *Verticillium*. Mol Plant-Microbe Interact 8: 880–885

Ku'c J (1995) Phytoalexins, stress metabolism, and disease resistance in plants. Annu Rev Phytopathol 33: 275–297

Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. Mol Cell Biol 7: 335–341

Liang X, Dron M, Schmid J, Dixon RA, Lamb CJ (1989) Developmental and environmental regulation of a phenylalanine ammonia-lyase-$\beta$-glucuronidase gene fusion in transgenic tobacco plants. Proc Natl Acad Sci USA 86: 9284–9288

Liu CJ, Heinstein P, Chen XY (1999) Expression pattern of genes encoding farnesyl diphosphate synthase and sesquiterpene cyclase in cotton suspension-cultured cells treated with fungal elicitor. Mol Plant-Microbe Interact 12: 1095–1104

McGarvey DJ, Croteau R (1995) Terpenoid metabolism. Plant Cell 7: 1015–1026

Meng YL, Jia JW, Liu CJ, Liang WQ, Heinstein P, Chen XY (1999) Coordinated accumulation of (+)-δ-cadinene synthase mRNAs and gossypol in developing seeds of *Gossypium hirsutum* and a new member of the *cad1* family from *G. arboreum*. J Nat Prod 62: 248–252

Ren YY, West CA (1992) Elicitation of diterpene biosynthesis in rice (*Oryza sativa*) by chitin. Plant Physiol 99: 1169–1178

Somssich IE, Hahlbrock K (1998) Pathogen defense in plants – a paradigm of biological complexity. Trends Plant Sci 3: 86–90

Stanford EE, Viehoever A (1918) Chemistry and histology of the glands of the cotton plant, with notes on the occurrence of similar glands in related plants. J Agri Res Washington DC 13: 419–435

Starks CM, Back K, Chappell J, Noel JP (1997) Structural basis for cyclic terpene biosynthesis by tobacco 5-*epi*-aristolochene synthase. Science 277: 1815–1820

Steele CL, Crock J, Bohlmann J, Croteau R (1998a) Sesquiterpene synthases from grand fir (*Abies grandis*): comparison of constitutive and wound-induced activities, and cDNA isolation, characterization, and bacterial expression of δ-selinene synthase and γ-humulene synthase. J Biol Chem 273: 2078–2089

Steele CL, Katoh S, Bohlmann J, Croteau R (1998b) Regulation of oleoresinosis in grand fir (*Abies grandis*): differential transcriptional control of monoterpene, sesquiterpene, and diterpene synthase genes in response to wounding. Plant Physiol 116: 1497–1504

Varner JE, Ye Z (1994) Tissue printing. FASEB J 8: 378–384

Vogel SB, Wildung M, Vogel G, Croteau R (1996) Abietadiene synthase from grand fir (*Abies grandis*) – cDNA isolation, characterization, and bacterial expression of a bifunctional diterpene cyclase involved in resin acid biosynthesis. J Biol Chem 271: 23262–23268

Vögeli U, Chappell J (1988) Induction of sesquiterpene cyclase and suppression of squalene synthase activities in plant cell cultures treated with fungal elicitor. Plant Physiol 88: 1292–1296

Vögeli U, Chappell J (1990) Regulation of sesquiterpene cyclase in cellulase-treated tobacco cell suspension cultures. Plant Physiol 94: 1860–1866

Yin S, Mei L, Newman J, Back K, Chappell J (1997) Regulation of sesquiterpene cyclase gene expression: characterization of an elicitor- and pathogen-inducible promoter. Plant Physiol 115: 437–451