MPMI Vol. 12, No. 12, 1999, pp. 1095–1104. Publication no. M-1999-0927-02R.

Expression Pattern of Genes Encoding Farnesyl Diphosphate Synthase and Sesquiterpene Cyclase in Cotton Suspension-Cultured Cells Treated with Fungal Elicitors

Chang-Jun Liu,[1] Peter Heinstein,[3] and Xiao-Ya Chen[1,2]

[1]National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology, SIBS: [2]Shanghai Research Center of Life Sciences, The Chinese Academy of Sciences, Shanghai 200032, China; and [3]Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907, U.S.A.

Accepted 3 September 1999.

Cotton plants accumulate sesquiterpene aldehydes in pigment glands. The two enzymes farnesyl diphosphate synthase (FPS) and (+)-δ-cadinene synthase (CAD), a sesquiterpene cyclase, are involved in the biosynthesis of these secondary metabolites. A full-length cDNA (*garfps*) encoding FPS was isolated from *Gossypium arboreum* and identified by in vitro enzymatic assay of the *garfps* protein heterologously expressed in *Escherichia coli*. Treatment of *G. arboreum* suspension-cultured cells with an elicitor preparation obtained from the phytopathogenic fungus *Verticillium dahliae* dramatically induced transcription of both FPS and CAD, paralleling the accumulation of the sesquiterpene aldehydes in these cells. For *G. australe*, a wild species from Australia, the *V. dahliae* elicitor preparation also caused an induction of FPS but only a low rate of induction of CAD, apparently because of a constitutive expression of the sesquiterpene cyclase gene in suspension-cultured cells. Two transcripts and proteins of FPS were detected in the elicited *G. australe* cells; the smaller FPS seemed to be de novo synthesized after elicitation. Furthermore, *G. australe*-cultured cells accumulated the cadinene, instead of sesquiterpene aldehydes, indicating that the biosynthetic pathway leading to sesquiterpene aldehydes was absent or blocked after FPP cyclization.

Additional keywords: G. hirsutum.

Plants respond to phytopathogens by altering their cellular metabolism and invoking various defense mechanisms, including accumulation of low molecular weight antimicrobial compounds (phytoalexins), deposition of phenolic materials and hydroxyproline-rich glycoproteins in cell wall, and synthesis of hydrolytic enzymes such as chitinase and 1,3-β-D-glucanase (Dixon and Harrison 1990). Generally, defense response in plants is believed to result from an interaction of elicitor molecules from the pathogen with specific receptors in the host, leading to activation of a range of defense-related genes. This response can be mimicked in suspension-cultured cells exposed to microbial and abiotic elicitors (Dixon and Lamb 1990; Staskawicz et al. 1995; Somssich and Hahlbrock 1998).

Isoprenoids with more than 23,000 different chemical structures represent a very large family of plant metabolites (Chappell 1995; Bohlmann et al. 1998). Some of the secondary terpenoids, especially sesquiterpenoids, have been classified as phytoalexins, because of their induced biosynthesis and their potential role in plant resistance to the phytopathogens (Threlfall and Whitehead 1991). The biosynthesis of sesquiterpene compounds, in general, takes place in the cytosol, with farnesyl diphosphate (FPP) as a common substrate. FPP synthase (FPS), a 1-4′ prenyltransferase, catalyzes two consecutive condensations of isopentenyl diphosphate (IPP) with its isoform dimethylallyl diphosphate (DMAPP) and the resultant 10-carbon geranyl diphosphate (GPP) to form the 15-carbon ultimate product FPP. FPP is a precursor of a structurally diverse class of terpenoids in plants, including phytosterols, electron transfer chain compounds, prenylated proteins, and a range of secondary sesquiterpenes (Chappell 1995; McGarvey and Croteau 1995).

The molecular regulation of the biosynthesis of plant sesquiterpene phytoalexins has drawn much attention in recent years. An array of genes encoding 3-hydroxy-3-methylglutaryl coenzyme A reductases (HMGRs) (e.g., Yang et al. 1991; Choi et al. 1992; Burnett et al. 1993; Weissenborn et al. 1995; Maldonado-Mendoza et al. 1997) and terpene synthases (cyclases) (e.g., Facchini and Chappell 1992; Back and Chappell 1995; Chen et al. 1995; Yin et al. 1997; Bohlmann et al. 1998) have been cloned and characterized at the molecular level. Although the FPS cDNAs or genes have been isolated from a number of plant species (Delourme et al. 1994; Attucci et al. 1995; Adiwilaga and Kush 1996; Cunillera et al. 1996; Li and Larkins 1996; Matsushita et al. 1996; Pan et al. 1996; Sanmiya et al. 1997), in comparison with the extensive literature on the molecular characterization of HMGR and sesquiterpene cyclases, there is a limited understanding of FPS

Corresponding author: X.-Y. Chen; E-mail: xychen@iris.sipp.ac.cn

Nucleotide and/or amino acid sequence data can be found at the EMBL data base as accession no. Y12072.

expression patterns and their regulatory role in plant sesquiterpenoid phytoalexin biosynthesis. Based on an examination of the activity of the enzymes that catalyze the FPP synthesis from IPP, Threlfall and Whitehead (1991) suggested that the rate of FPP formation from mevalonic acid in nonelicited tissues was more than sufficient to account for the synthesis of the sesquiterpene phytoalexins lubimin and rishitin in elicited potato cultures, provided that there was inhibition of squalene synthase. However, it was reported that, in castor bean seedlings infected by the pathogen fungus *Rhizopus stolonifer*, the casbene synthase and geranylgeranyl diphosphate synthase activities increased substantially from very low basal levels in uninfected tissues and the FPP synthase activity approximately doubled from a higher basal level (Dudley et al. 1986). Focusing on the carbon metabolic flux between cytosolic and plastidial isoprenoids, Hugueney et al. (1996) demonstrated a concomitant induction pattern of an FPS gene and a sesquiterpene cyclase gene in the cytosol of the pericarp disks of pepper fruit treated with cellulase.

Verticillium wilt is a serious fungal disease affecting cotton production. Pathogen infection of cotton cultivars or treatment of the suspension-cultured cells with elicitors prepared from *V. dahliae* initiates the de novo synthesis of sesquiterpene phytoalexins, including hemigossypol-related cyclic sesquiterpene aldehydes. These sesquiterpene aldehydes have been shown to possess high antimicrobial activity and toxicity to insects and monogastric animals (Bell 1967; Bell and Stipanovic 1977; Heinstein 1985; Essenberg et al. 1990; Davila-Huerta et al. 1995). A rapid and significant accumulation of these compounds in all tissues aids the cotton plant in its defense against the phytopathogenic invasion, as for example infection by *V. dahliae*. However, if cottonseed meal is to be used as a potential high protein source, synthesis and accumulation of toxic sesquiterpene aldehydes in cottonseeds is not desirable. Therefore, a more thorough investigation of the biosynthesis and a comparison of the regulation of the biosynthesis of sesquiterpenes in *G. arboreum* and *G. australe*, which do not accumulate sesquiterpene aldehydes in seeds, is warranted.

Recently, it has been reported that the transcription levels of the cotton HMGR and (+)-δ-cadinene synthase (CAD), a sesquiterpene cyclase, were apparently increased in tissues infected with *V. dahliae* or in elicitor-treated, suspension-cultured cells (Joost et al. 1995; Chen et al. 1995, 1996; Alchanati et al. 1998). To investigate further the regulatory mechanism of sesquiterpene formation in cotton cells and to define the role of FPS in this sesquiterpene biosynthetic pathway, we isolated and characterized a cDNA encoding FPS from *G. arboreum*, analyzed the steady-state mRNA levels of FPS and CAD, and determined their enzymatic activities, in cotton suspension-cultured cells treated with *V. dahliae* elicitors. The regulatory pattern of sesquiterpene formation in cultured cells of the wild species *G. australe* was found to be different from that in the two cotton species *G. arboreum* cv. Qingyangxiaozi and *G. hirsutum* cv. Sumian-6.

RESULTS

Characterization of the FPS cDNA.

A cDNA library constructed from *G. arboreum* cells treated with *V. dahliae* elicitors (Chen et al. 1995) was used to isolate the cDNA encoding FPS. At first, a cDNA fragment of about 400 bp was amplified by nest polymerase chain reaction (PCR) with a vector-specific forward primer, T3, and two degenerated reverse primers, FPP1 and FPP3. Nucleotide sequences of this fragment showed 77.1% identity with an FPS from *Arabidopsis thaliana* (Delourme et al. 1994). On the basis of this fragment, a cDNA clone, *garfps*, was isolated. Complete DNA sequencing revealed that the clone contained a cDNA insert of 1,286 bp, with a 5′ proximal ATG assumed to be the start codon at +95 bp, and an open reading frame (ORF) coding for a protein of 342 amino acid residues. The identities of predicted amino acid sequences with FPSs of *A. thaliana* (Delourme et al. 1994), *Artemisia annua* (Matsushita et al 1996), and *Zea mays* (Li and Larkins 1996) are 78.9, 80.7, and 71.6%, respectively. Two aspartate-rich domains with the motif DDXXD, which have been found in all isoprenyltransferases reported, and are postulated to be involved in substrate binding and catalysis (Joly and Edwards 1993; Song and Poulter 1994), are also completely conserved in this cotton enzyme. Other conserved amino acid residues, including the region around the first aspartate-rich motif, especially the fourth and fifth amino acids before this motif (Ohnuma et al. 1996, 1997), are also found in *G. arboreum* FPS.

After the cDNA was inserted into a pET-28a(+) expression vector, the *G. arboreum* FPS was overexpressed in the *Escherichia coli* strain BL21(DE3) as a fusion protein. Approximately 2.5 mg of pure homogenous protein was isolated from 24 mg of crude bacterial protein extracts. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed a predicted molecular mass of expressed fused protein of about 40 kDa (data not shown), which was consistent with the deduced molecular mass of 39,485 Da. To check whether the expressed protein was functional, its in vitro enzymatic activity was assayed and was found to catalyze the formation of FPP from IPP and DMAPP. An 18-fold higher specific activity of prenyltransferase was obtained after affinity purification (Table 1). Thin-layer chromatography (TLC) analysis of the hexane-soluble products after treatment with 10 units of calf intestinal alkaline phosphatase revealed farnesol as the main product (data not shown). The authenticity of the synthesized farnesol was documented by TLC.

Induction of FPS and CAD in *G. arboreum* cells.

The *G. arboreum* CAD is encoded by a gene family, which can be divided into two subfamilies, CAD1-A and CAD1-C. Previous investigation indicated that, in suspension-cultured cells treated with *V. dahliae* elicitor, the members of both the CAD1-A and CAD1-C subfamilies showed a similar induction pattern, but the transcription level of CAD1-C was much higher than that of CAD1-A (Chen et al. 1996). In the present work, only CAD1-C was analyzed. Northern (RNA) blotting

Table 1. Specific activity of cotton farnesyl diphosphate synthase (FPS) heterologously expressed in *Escherichia coli*

Protein sources	Specific activity[a]
E. coli BL21 with pET-28a(+),	0.166 ± 0.029
E. coli BL21 with pETFPS	3.968 ± 0.405
Affinity purified proteins from *E. coli* BL21 with pETFPS	69.68 ± 0.922

[a] Expressed as nmol ^{14}C-isopentenyl diphosphate (IPP) incorporated into the hydrolyzed product of E, E-farnesol per min per mg of protein. Dimethylallyl diphosphate (DMAPP) was used as the second substrate.

analysis with *garfps* and *cad1-C1* probes revealed that, when *G. arboreum* suspension-cultured cells were treated with cellular extract of *V. dahliae*, both the FPS and CAD genes were induced at the transcriptional level (Fig. 1A). In control cells only a trace amount of FPS and CAD mRNAs could be detected. Transcripts of both the FPS and CAD increased dramatically and reached the maximal level almost simultaneously around 4 to 8 h after elicitation, then subsequently declined. The FPS mRNA level dropped a little more sharply than that of CAD. The induction was also observed in cells of an upland cotton cultivar, *G. hirsutum* cv. Sumian-6, treated with the same elicitor: the maximal steady-state mRNA level of both FPS and CAD appeared around 12 h post elicitation (data not shown). Thus, the response of *G. hirsutum* was slower than that of *G. arboreum.*

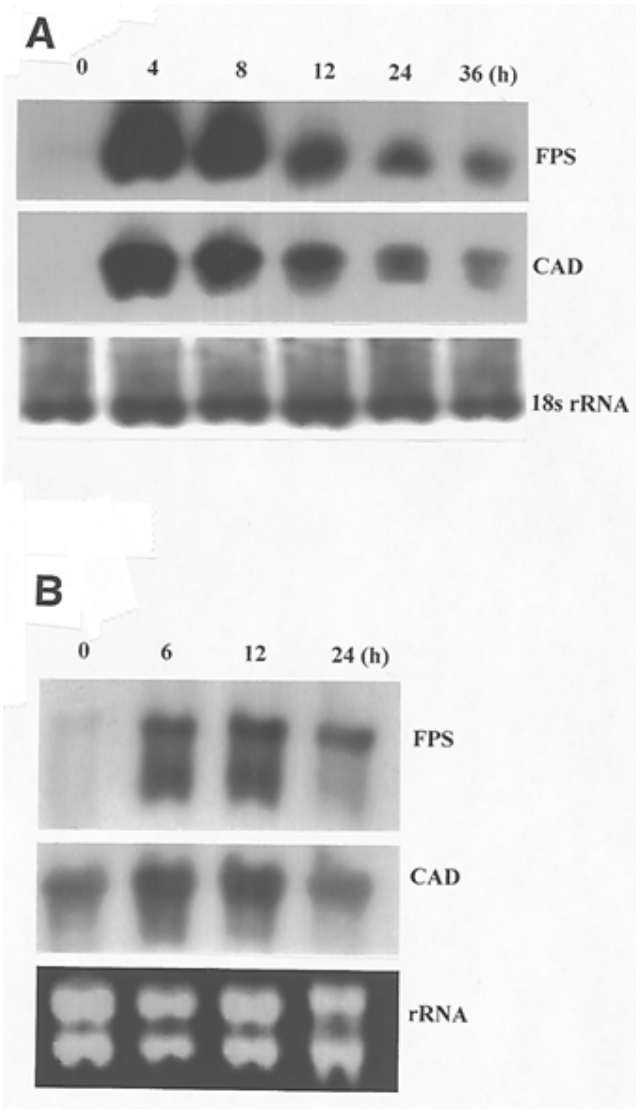

Fig. 1. Northern (RNA) blot analysis of farnesyl diphosphate synthase (FPS) and (+)-δ-cadinene synthase (CAD) mRNAs in suspension-cultured cells of **(A)** *Gossypium arboreum* and **(B)** *G. australe*. Cells were treated with a *Verticillium dahliae* elicitor preparation for different time spans as indicated. Total RNA (10 µg per lane) was blotted and hybridized with ^{32}P-labeled DNA fragments of *garfps*, *cad1-C*, respectively. The 18S rRNA is shown as **(A)** the hybridization band or **(B)** ethidium bromide staining prior to blotting.

Consistent with induced gene transcription, specific activities of FPS and CAD increased concomitantly in suspension-cultured cells of *G. arboreum* and *G. hirsutum* cv. Sumian-6. In *G. arboreum* cells, for both enzymes this increase commenced at 4 h post elicitation and peaked at around 8 to 12 h post elicitation. Specific activities of FPS and CAD increased about sevenfold and ninefold, respectively, over the initial levels measured (Fig. 2A,B). Although specific activities of both the FPS and CAD declined after 12 h post elicitation, the former, which fell to near the basal level in 24 h post elicitation, exhibited a sharper drop than the latter. These results are in accordance with the kinetics of induction and decline of the correspondent mRNA syntheses (Fig. 1A).

Immunological analysis detected only a trace amount of FPS and CAD proteins in control *G. arboreum* cells. However, after elicitation the immuno-detectable protein levels of FPS and CAD were remarkably elevated (Fig. 2A,B). Therefore, increased specific activities of both enzymes were derived from the de novo synthesis of enzymatic proteins, which in turn resulted from the induced transcription of the corresponding genes. Thus, in *G. arboreum* suspension-cultured cells, expression of both the FPS and CAD genes is regulated at the transcriptional level.

A parallel kinetic pattern of accumulation of sesquiterpene aldehydes in elicitor-stimulated *G. arboreum* (Fig. 3A) and *G. hirsutum* cells (data not shown), analogous to the change in steady-state levels of FPS and CAD transcripts and enzymes, was observed. In suspension-cultured cells of the two cotton cultivars, an induced accumulation of these phloroglucinol-reactive phytoalexins appeared 8 h after elicitor treatment, and reached a maximum level of up to 3.34 mg per g dry weight (DW) for *G. arboreum* (Fig. 3A) and 2.57 mg per g DW for *G. hirsutum* (data not shown). The maximum accumulation of sesquiterpene aldehydes in the two species occurred at 36 and 48 h post elicitation, respectively. In comparison with the low and largely stable levels of sesquiterpene aldehydes in control cells, these levels represent an approximate 56-fold increase in *G. arboreum* cells, and a more than 12-fold increase in *G. hirsutum* cells, respectively.

Induction of FPS and CAD in *G. australe* cells.

G. australe is a diploid wild species from Australia. Plants of this species also have pigment glands throughout the plant that contain sesquiterpene aldehydes, such as hemigossypol (Bell and Stipanovic 1977). However, their seeds are glandless and do not contain sesquiterpene aldehydes (Altman et al. 1987). Northern blot analysis as the probe with a *gafps1* fragment showed that, as in cells of *G. arboreum*, the steady-state mRNA levels of FPS were clearly increased in *G. australe* cells treated with the *V. dahliae* elicitor preparations. It is interesting that the blot showed two hybridizing bands of approximately 1.3 and 1.1 kb, respectively (Fig. 1B). The 1.1-kb transcripts presented a more transient induction and were detectable only after elicitation, although the maximal levels of both transcripts appeared almost simultaneously at 12 h post elicitation. In concert with the transcription pattern of FPS, two immunologically reacting bands were detected with an antiserum raised against *G. arboreum* FPS, in electrophoresed extracts of cells at 12 to 36 h post elicitation (Fig. 4A). The induc-

tion of FPS de novo synthesis by fungal elicitors in *G. australe* cells was comparatively slower than that observed with *G. arboreum* cells. For CAD, a relatively high level of transcripts was detected in *G. australe* cells before elicitation; thus, only a low rate of induction was observed (Fig. 1B). Consistent with the steady-state mRNA levels, a significant concentration of the CAD protein and sesquiterpene cyclase activity was present in control cells. Similarly, the fungal elicitation caused only a slight (around twofold) increase in specific sesquiterpene activity (Fig. 4B), a pattern strikingly different from that observed in *G. arboreum* cells.

Acetone extracts of *G. australe* cells were analyzed with the phloroglucinol/HCl reagent, which was demonstrated to recognize the aromatic aldehyde group of cotton sesquiterpene phytoalexins (Bell 1967; Heinstein 1985). Although the induction of FPS activity by elicitation was apparent (Fig. 4A), and a significant level of the sesquiterpene cyclase activity was constantly present (Fig. 4B), sesquiterpene aldehydes were virtually undetectable, either in control or in elicitor-treated cells (Fig. 3B). As mentioned above, hemigossypol has been reported earlier to be present in this plant (Bell and Stipanovic, 1977), and our analysis with phloroglucinol/HCl reagent confirmed the presence of sesquiterpene aldehydes in leaf extracts (data not shown). Thus, we suspected that, in suspension-cultured cells of *G. australe*, the biosynthetic pathway leading to sesquiterpene aldehydes was absent or blocked in the step(s) past the FPP cyclization. Fluorescence TLC showed that the acetone extract of control and elicitor-treated cells of *G. australe* contained a compound that co-migrated with authentic (+)-δ-cadinene (R_f 0.54; see Figure 5A). The standard (+)-δ-cadinene was produced in vitro from FPP by the bacterially expressed *G. arboreum* sesquiterpene cyclase CAD1-C1 (Chen et al. 1995), and showed blue fluorescence under UV light (312 nm). This sesquiterpene hydrocarbon was also detected in leaf extracts of *G. australe*, as well as in *G. ar-*

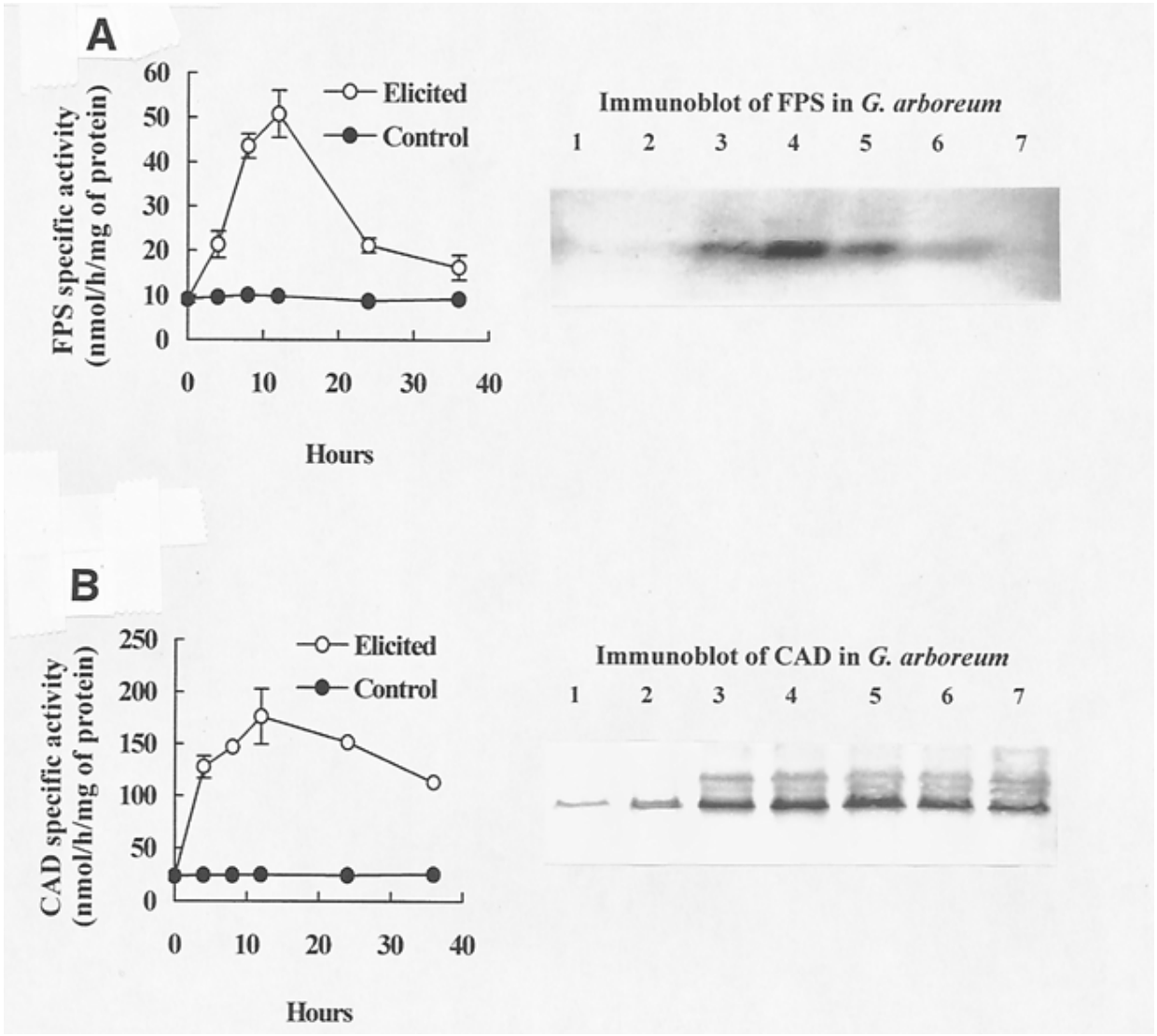

Fig. 2. Specific activity and immunological detection of **(A)** farnesyl diphosphate synthase (FPS) and **(B)** (+)-δ-cadinene synthase (CAD) in *Gossypium arboreum* suspension-cultured cells treated with a *Verticillium dahliae* elicitor preparation. Blots were treated with polyclonal antibodies raised against *G. arboreum* FPS or CAD-C1, respectively. Cross-reacting band was about 40 kDa for FPS and 60 kDa for CAD. Lanes 1 and 2, protein preparations from control cells grown for 0 and 12 h after the initiation of the experiment; lanes 3 through 7, proteins from cells elicited for 4, 8, 12, 24, and 36 h.

boreum-cultured cells, but in all cases at much lower levels (Fig. 5A). Incubation of *G. australe* cells with ^{3}H-FPP as a precursor confirmed the above observations. The radioactivity was indeed incorporated into the compound comigrating with (+)-δ-cadinene, in addition to a compound migrating with the same R_f value as E, E-farnesol (Fig. 5B). Accumulation of alcohols might be the result of the hydrolysis of ^{3}H-FPP. About 1 to 3% of the radioactivity originally added to the cell suspensions was found in pentane extract, of which nearly one half was incorporated into cadinene (Table 2). For the sesquiterpene cyclase activity (Fig. 4B), the incorporation rate of ^{3}H-FPP into cadinene was increased only slightly (about twofold) upon elicitation. In tobacco cells, a comparable rate of incorporation of ^{14}C-acetate and ^{3}H-mevalonate upon elicitation into the sesquiterpene phytoalexin caspidiol was reported, and the de novo synthesis of caspidiol appreciably occurred only after elicitation (Chappell and Nable 1987).

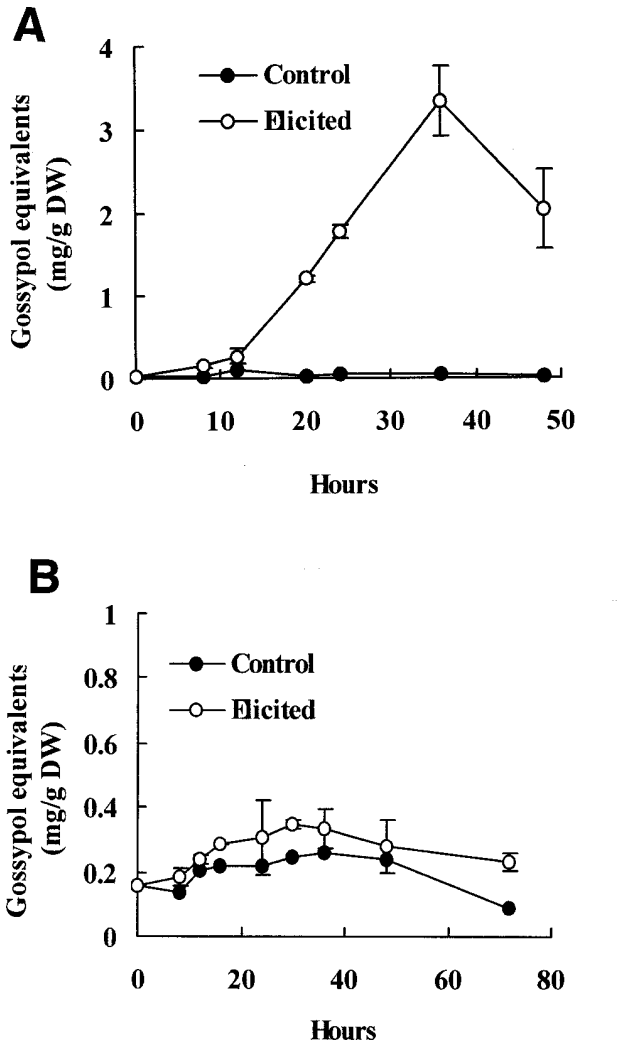

Fig. 3. Induced accumulation of sesquiterpene aldehydes in **(A)** *Gossypium arboreum* and **(B)** *G. australe* suspension-cultured cells treated with a *Verticillium dahliae* elicitor preparation**.** Sesquiterpene aldehydes in 70% acetone extracts of cotton cells were determined with phloroglucinol/HCl.

DISCUSSION

A cDNA encoding FPS was isolated from the diploid cotton species *G. arboreum*. Translation of this cDNA and subsequent analysis of the synthesized protein in an in vitro enzyme assay showed that the cotton mRNA encoded a functional FPS. As in most other plant FPSs, no indication of a transit peptide, targeting the protein to plastids or mitochondria, was found. Therefore, *garfps* encodes a soluble, cytosolic FPS. This, together with our evidence that *garfps* is involved in the biosynthesis of sesquiterpene phytoalexins, is consistent with the conventional hypothesis that sesquiterpenes are synthesized in cytosol (Chappell 1995; Bohlmann et al. 1998; Lange et al. 1998).

In elicitor-treated *G. australe* cells, two mRNA species, 1.3 and 1.1 kb, hybridizing with the FPS probe, as well as their translation products, were detected (Figs. 1B and 4A). It appeared that the 1.3-kb, but not the 1.1-kb, transcript was present in non-elicited cells, and the de novo synthesis of the FPS encoded by the 1.1-kb mRNA was induced upon elicitation. These results agree with the observation by Dudley et al. (1986) in castor bean seedlings exposed to the fungal pathogen *R. stolonifer:* With ion exchange chromatography, they detected two forms of FPS activity in infected seedlings, but only one peak in the extracts of the sterile seedlings prepared under the same conditions. Recently, it was reported that the Arabidopsis *FPS1* gene encoded two mRNA species that generate two isoforms, a soluble FPS protein and an FPS with a transit peptide, which was imported into the cellular mitochondria (Cunillera et al. 1997). Further investigation is required to fully describe the function of the two FPS transcripts identified herein in *G. australe* cells.

Fungal elicitation activates a range of plant defense-related genes and finally leads to a series of defense reactions, including phytoalexin biosynthesis (Somssich and Hahlbrock 1998). In the biosynthetic pathway leading to terpenoid phytoalexins, terpene cyclases, catalyzing reactions at branch points, have been shown to play a regulatory role (Chappell 1995; Bohlmann et al. 1998). However, induction was not limited to the synthesis of these branch-point enzymes. As mentioned above, fungal infection of castor bean seedlings or elicitor treatment of pepper pericarp caused a remarkable increase of the specific activities of both the terpene synthases and prenyltransferases for isoprenoid phytoalexin biosynthesis (Dudley et al. 1986; Hugueney et al. 1996). The concomitant induction of FPS and sesquiterpene cyclase is clearly supported by our data obtained from cells of *G. arboreum* and *G. hirsutum*. In this case, transcription of not only CAD but also FPS was induced by a fungal elicitor preparation, resulting in elevated levels of immunologically detectable FPS and CAD proteins, their specific activities, and the subsequent accumulation of sesquiterpene aldehydes. Induction of HMGR mRNA synthesis, coding for an enzyme in the common terpenoid biosynthetic pathway, has been reported by Joost et al. (1995) to occur in *Gossypium* spp. following inoculation with *V. dahliae* spores. Together, these results show that a coordinated up-regulation of enzymes catalyzing reactions in the general isoprenoid pathway and the branch pathway occurs at transcription and translation levels, during the fungal elicitation of *G. arboreum* and *G. hirsutum* cells. This coordinated induction leads to a rapid accumulation of the toxic sesquiter-

pene aldehydes in the cells challenged with the elicitor. The coordinated induction of secondary metabolism in plants apparently is not limited to the isoprenoid pathway. When parsley cell suspension cultures were challenged with a fungal elicitor or a UV stimulus, the transcription levels of phenylalanine ammonia-lyase, 4-coumarate: CoA ligase, and cinnamate 4-hydroxylase, three enzymes in the core phenylpropanoid pathway, were significantly up-regulated, and this induction was correlated with the formation of cell wall-bound phenolic furanocoumarin phytoalexins (Hahlbrock et al. 1995).

Induction of FPS in elicitor-treated cotton cells might fit with the requirement of an excess of FPP as a substrate for biosynthesis of sesquiterpene phytoalexins. In cultured cells of *Nicotiana tabacum*, a solanaceous plant that also produces sesquiterpene phytoalexins, induction of sesquiterpene cyclase was coordinated with suppressed squalene synthase activities (Threlfall and Whitehead 1988; Vögeli and Chappell 1988). The suppression of squalene synthase activity may result from a post-transcriptional modification, as fungal elicitor treatment did not alter the steady-state level of squalene synthase mRNA in tobacco cells (Devarenne et al. 1998). In suspension-cultured cells of the rubiaceous plant *Cinchona robusta*, treatment with *Phytophthora cinnamomi* elicitors resulted in synthesis of anthraquinone-type phytoalexins, in which DMAPP was incorporated into the C ring. Induction of IPP isomerase activity was correlated with a concomitant reduction of FPS activity, leading to an efficient channeling of C5 precursors into anthraquinone biosynthesis (Ramos-Valdivia et al. 1997). It may be assumed that, in the history of evolution, differential up- and down-regulation of enzymes in the isoprenoid pathway has adapted to production of specific secondary metabolites in different plant species.

Coordinated up-regulation at the transcriptional level of the sesquiterpene biosynthetic enzymes and accumulation of sesquiterpene phytoalexins were observed in both the *G ar-*

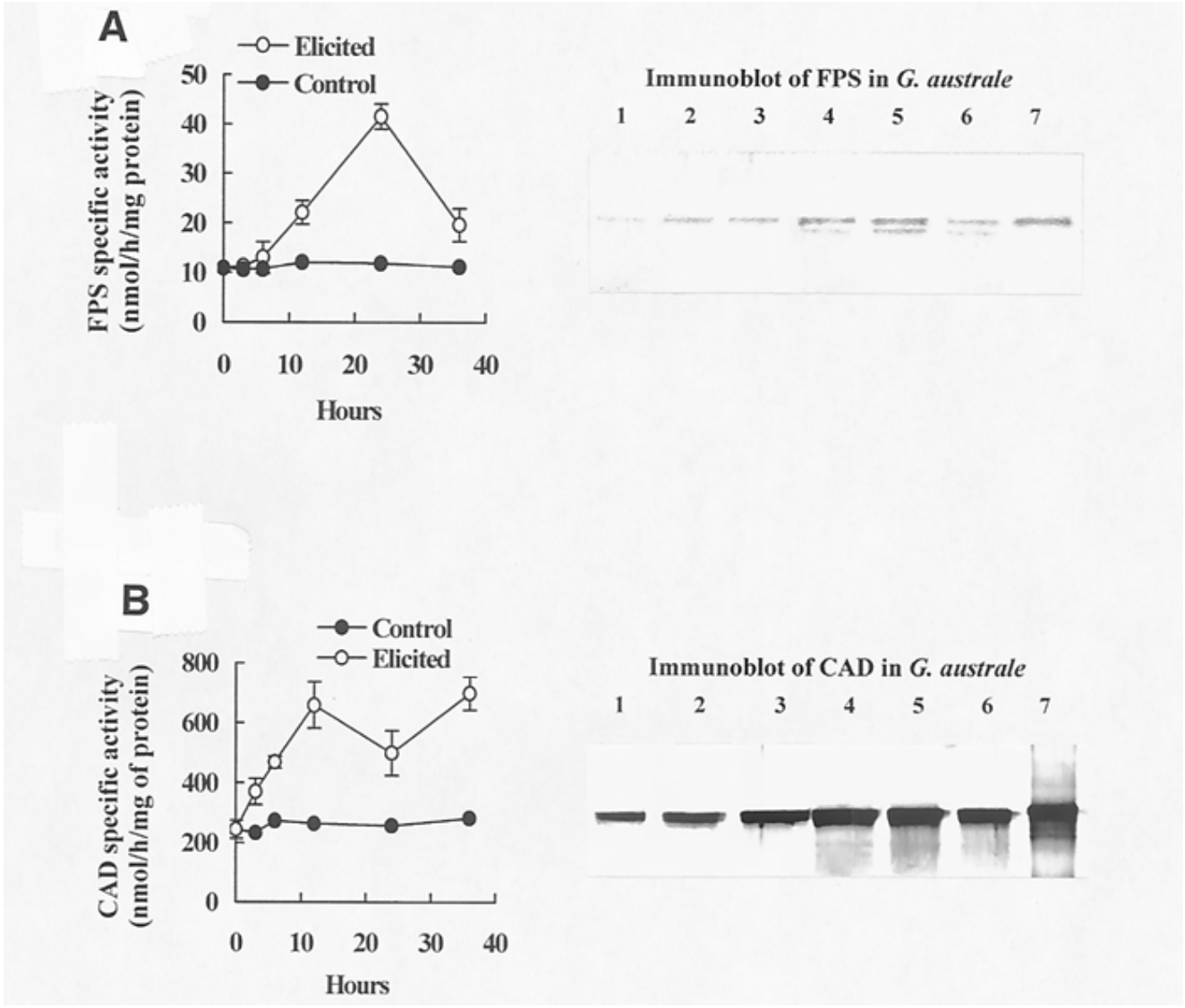

Fig. 4. Specific activity and immunological detection of **(A)** farnesyl diphosphate synthase (FPS) and **(B)** (+)-δ-cadinene synthase (CAD) in *Gossypium australe* suspension-cultured cells treated with a *Verticillium dahliae* elicitor preparation. Lanes 1 to 6, proteins form the cells elicited for 0, 3, 6, 12, 24, 36 h; lane 7, bacterially expressed fusion proteins of **(A)** *garfps* and **(B)** *cad-C1*.

boreum and *G. hirsutum* cells. On the other hand, elicitation of *G. australe* suspension-cultured cells presented a somewhat different induction pattern. While transcription of FPS was clearly induced after elicitation (Fig. 1B), the induction of CAD in *G. australe cells* was not as profound because a certain level of CAD transcripts and sesquiterpene cyclase activities was constitutively present in the control cells of this wild *Gossypium* sp. Thus, in suspension-cultured cells of *G. australe*, FPP cyclization is unlikely to be a rate-limiting step for sesquiterpene biosynthesis, but the formation of FPP may play a more important regulatory role. Furthermore, the biosynthetic pathway of sesquiterpene aldehydes in suspension-cultured cells of *G. australe* was absent or blocked after FPP cyclization, leading to the accumulation of the sesquiterpene hydrocarbon. This distinct regulatory pattern of sesquiterpene formation in *G. australe* cell cultures is reminiscent of the specific accumulation pattern of sesquiterpene aldehydes in these plants. Unlike most cotton cultivars, which accumulate a high amount of sesquiterpene aldehydes in seed glands during seed maturation (Meng et al. 1999), *G. australe* have glandless seeds that do not contain sesquiterpene aldehydes, and these toxic secondary metabolites are formed only after germination (Altman et al. 1987). Therefore, these seeds may rely more on the antibacterial activity of (+)-δ-cadinene as a chemical defense (Essenberg et al. 1990). These results also suggest that a more detailed analysis is warranted of the genes and gene expression of sesquiterpene biosynthesis in *G. australe* cells, as a model system for the development of glandless seed varieties of cultivated cotton for human nutrition.

MATERIAL AND METHODS

Cell suspension culture and elicitation.

Cell suspension cultures of *G. arboreum* L. 'Nanking', *G. hirsutum* L. 'Sumian-6', and *G. australe* F. Muell were initiated and maintained in MS medium (Murashige and Skoog 1962). Cells were transferred every 7 days. After three to five subculturings in liquid medium, cells were used for elicitation. Elicitor of *V. dahliae* was prepared as described (Heinstein 1985; Davis et al. 1992), and applied at a final concentration of 1 µg of sucrose equivalent per ml of culture. For control, an equal volume of sterile, distilled water was added.

Isolation of *garfps* cDNA.

Two degenerate reverse primers, FPP1 (5′-RTCCATNATR TCRTC-3′; R = A,G; N = A,G,C,T) and FPP3 (5′-TARTCR TCYTGNAYYTGRAA-3′; Y = T,C), encompassing two conserved aspartate-rich domains of FPS of *Arabidopsis thaliana* (Delourme et al. 1994) and yeast (Ashby and Edwards 1990), were synthesized. The two primers were used respectively

Table 2. Incorporation rate of ^{3}H-farnesyl diphosphate synthase (FPS) into the sesquiterpenes in pentane extracts of *Gossypium australe* suspension-cultured cells

	Incorporation	
Treatment	**Pentane extract**	**Cadinene**
Water (control)	22,536 (1.0 to 2.2%)	10,589 (0.3 to 1.0%)
Elicitor	38,281 (2.1 to 3.1%)	16,546 (0.6 to 1.5%)

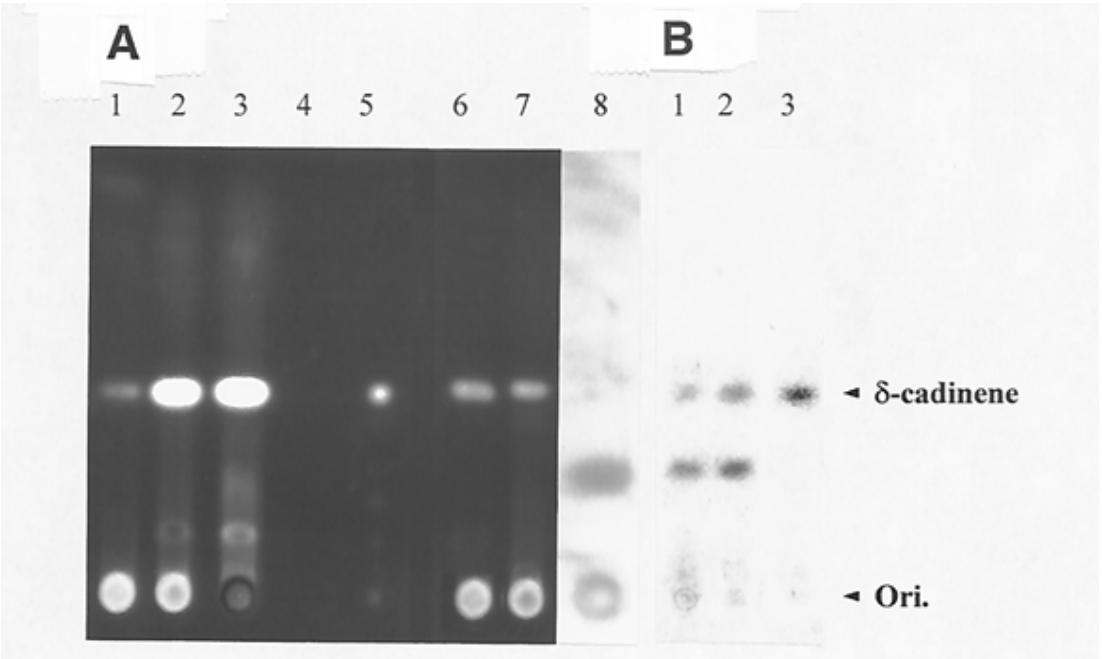

Fig. 5. A, Accumulation of cadinene in *Gossypium australe* and *G. arboreum* suspension cells. Thin-layer chromatography (TLC) plate (silica gel $HSGF_{254}$) was developed with toluene:ethylacetate (9:1) and visualized under UV light at a wavelength of 312 nm. The following samples were applied onto the TLC plate: lane 1, acetone extract of *G. australe* leaves; lane 2, *G. australe* cells treated with ddH_2O; lane 3, *G. australe* cells treated with a *Verticillium dahliae* elicitor preparation; lane 4, authentic E, E-farnesol; lane 5, a pentane extract of an enzymatic reaction with farnesyl diphosphate (FPP) as substrate and bacterially expressed *G. arboreum* (+)-δ-cadinene synthase CAD1-C1; lane 6, an acetone extract of *G. arboreum* cells treated with ddH_2O; lane 7, *G. arboreum* cells treated with a *V. dahliae* elicitor preparation; and lane 8, E, E-farnesol, visualized with 5% conccentrated H_2SO_4 in ethanol. **B,** Autoradiography of the incorporation of ^{3}H-FPP into sesquiterpenes by *G. australe* cells treated with (lane 1) ddH_2O, and (lane 2) a *V. dahliae* elicitor preparation, and (lane 3) radio-labeled (+)-δ-cadinene produced in vitro by incubation of ^{3}H-FPP with the bacterially expressed CAD1-C1 of *G. arboreum*.

with the vector universal primer T3 (Stratagene, La Jolla, CA) in nest PCR amplification of an FPS cDNA fragment from a λ-UniZAP cDNA library of *G. arboreum* cv. Nanking (Chen et al. 1995). The PCR was carried out at 94°C for 15 s, 48°C for 30 s, and 72°C for 45 s, 25 cycles for the first round and 35 cycles for the second. The product was then TA cloned. Based on nucleotide sequences of this fragment, a specific reverse primer, FPP1-1 (5′-GTCCATGATGTCGTCAAG-3′) was synthesized, followed by screening the cDNA library by a PCR 96 well plate method (Alfandari and Darribere 1994). Briefly, the library of about 10^7 PFU was mixed with the *E. coli* strain XL1-Blue and spread into the 96 wells. After overnight incubation at 37°C, aliquots from each well were examined by PCR with the forward primer T3 and the reverse primer FPP1-1. Positive wells were selected and subjected to next round screening. After four rounds a positive clone, designated as *garfps*, was isolated and the plasmid was excised. DNA sequences were determined with a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE-ABI, Warrington, UK).

Expression of *garfps* in *E. coli*.

For insertion of the ORF of *garfps* cDNA into expression vectors, an N-terminal forward primer, FPP2-1 (5′-GCTTT GAATTCATGGCGGATCTCAGG-3′), was designed to incorporate an *Eco*RI site, and used together with a T7 primer in PCR amplification of the cDNA insert. After digestion with *Eco*RI and *Xho*I, the PCR product was ligated into plasmid pET-28a(+) to create pETFPS for expression in the *E. coli* strain BL21(DE3). The bacteria harboring pETFPS were induced by IPTG (isopropyl-β-D-thiogalactopyranoside; at 1 mM) for fusion protein production. The bacterial protein extract was filtrated and purified by a His-Tag affinity column (Novagene, Madison, WI). Total and purified proteins were examined by SDS-PAGE, and used for enzymatic assay as described below. Protein concentrations were determined with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

RNA isolation and analysis.

Total RNA was extracted from cotton suspension-cultured cells by a cold phenol method (Fütterer et al. 1995). For Northern blotting analysis, 10 μg of total RNA per lane was electrophoresed on a 1.2% formaldehyde-agarose gel, transferred capillarily to a nitrocellulose membrane, and cross-linked by baking at 80°C for 2 h. An FPS probe was obtained by PCR amplification of *garfps* cDNA with primers FPP2-1 and FPP1-1. For the CAD probe, the PCR was conducted on *cad1-C1* (pXC1) with primers 93160 and 93T800 (Chen et al. 1995, 1996). After purification with a Wizard PCR Purification System (Promega, Madison, WI), PCR products were labeled with ^{32}P-dATP by a random primer labeling system (Promega). Prehybridization and hybridization were performed as described (Chen et al. 1995), and the RNA blot was finally exposed to an X-ray film for 12 to 24 h.

Cotton cellular protein preparation and enzyme assay.

Proteins were extracted from cultured cells with a buffer containing 50 mM Tris/HCl, 10 mM EDTA (pH 8.0), 1% β-mercaptoethanol, and 20% glycerol. Sesquiterpene cyclase was assayed with 0.15 μCi ^{3}H-FPP (15 to 30 Ci/mmol; Sigma, St. Louis, MO) and 3.5 nmol of FPP (Sigma) as described (Vögeli and Chappell 1990; Chen et al. 1995).

FPS was assayed as described (Attucci et al. 1995; Delourme et al. 1994). The reaction was initiated by adding 15 μg of plant protein into a final 100-μl incubation mixture containing 50 mM Tris/HCl (pH 7.6), 2 mM DTT (dithiothreitol), 1 mM $MnCl_2$, 40 μM ^{14}C-IPP (12.5 mCi/mmol; Sigma), and 80 μM DMAPP (Sigma). After incubation at 30°C for 30 min, the reaction mixture was adjusted to pH 9.0, and hydrolyzed with 10 units of calf intestinal alkaline phosphatase for 3 h at 37°C, followed by incubation overnight at room temperature. After addition of 0.1 μmol of E, E-farnesol (Sigma) as a carrier, the alcohol was extracted twice with 150 μl of hexane, and the pooled hexane was concentrated. The residue was separated on a TLC plate (silica gel $HSGF_{254}$) developed with a solvent of benzene:ethyl acetate (9:1). The farnesol spot, which was visualized on the TLC plate with iodine vapor, was scraped and the radioactivity was determined by liquid scintillation counting. Co-chromatography with standard farnesol was used to authenticate the enzymatically synthesized farnesol.

Immunoblotting.

The purified fusion proteins of FPS and CAD1-C (Chen et al. 1995) produced in bacteria were used to raise the polyclonal antibodies in New Zealand rabbits according to a standard procedure. The FPS immune serum was further purified by binding with Protein A-Sepharose CL6B (Sigma), followed by selective elution of IgG with 50 mM glycine, pH 3.0, 0.5 mM NaCl, neutralized with 1 M Tris/HCl to pH 7.0. Fifteen micrograms of total proteins from cotton cells was separated with 12% SDS-PAGE and electrotransferred to Hybond-C membranes (Amersham, Buckinghamshire, UK). Blots were developed with either anti-FPS serum (1:200) or anti-CAD serum (1:400), respectively. Immunodetection was carried out with alkaline phosphatase conjugated anti-rabbit antiserum as the secondary antibody, and nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium as substrates.

Analysis of sesquiterpenes.

Total sesquiterpene aldehydes were quantitated with a phloroglucinol/HCl assay (Bell 1967). Cotton cells of about 1.5 g (fresh weight) were immersed in liquid nitrogen, ground into a fine powder, and extracted with 70% acetone for 30 min. After centrifugation, the acetone extract was adjusted to 2 ml. An equal volume of the reagent (1% phloroglucinol, 2 N HCl in 95% ethanol) was added to the acetone extract, which was then incubated at 55°C for 5 min, and the absorbency at 555 nm was immediately measured. Standard curve was prepared with gossypol (Sigma).

For TLC analysis, about 20 μl of the total acetone extract was spotted onto a TLC plate (silica gel $HSGF_{254}$), and was developed by toluene:ethylacetate (9:1). The plate was firstly visualized with a UV illuminator (312 nm), then was sprayed with 5% of concentrated H_2SO_4 in ethanol and heated at 100°C until full development of colors occurred. As a reference, (+)-δ-cadinene was in vitro produced by incubation of FPP with a bacterially expressed *G. arboreum* sesquiterpene cyclase CAD1-C1 (Chen et al. 1995).

In vivo labeling experiment.

The labeling experiment was based on a procedure described by Chappell and Nable (1987). Subcultures of *G. aus-*

trale cells were treated with *V. dahliae* elicitor, as described above. At 45 min post elicitation, 1 to 1.5 μCi of ^{3}H-FPP was added to the cell cultures and continuously incubated for 48 h. The cells were then extracted with 70% acetone, and the acetone extract was concentrated to a largely aqueous solution, which was re-extracted with pentane. The pooled pentane extracts were concentrated, followed by separation with TLC, as described above. The plate was first visualized under UV to mark the blue fluorescent spot, then exposed to an X-ray film for 2 to 3 weeks. The areas resembling the radiographic spots were scraped for determination of radioactivity by scintillation counting.

ACKNOWLEDGMENTS

We thank Joe Chappell of the University of Kentucky and Meng-Ming Hong of the Shanghai Institute of Plant Physiology for their valuable suggestions and discussion. Francis Karst and Kariona Grabinska of the University of Poitiers, France, helped us in characterization of the *garfps* cDNA clone with the yeast mutant complementation system. This work was supported by the Natural Science Foundation of China (grants 39570460 and 39670473) and by the National Biotech Program of China (grant Z-17-02-04).

LITERATURE CITED

Adiwilaga, K., and Kush, A. 1996. Cloning and characterization of cDNA encoding farnesyl diphosphate synthase from rubber tree (*Hevea brasiliensis*). Plant Mol. Biol. 30:935-946.

Alchanati, I., Patel, J. A. A., Liu, J., Benedict, C. R., Stipanovic, R. D., Bell, A. A., Cui, Y., and Magill, C. W. 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., and Darribere, T. 1994. A simple PCR method for screening cDNA libraries. PCR Methods Appli. 4:46-49.

Altman, D. W., Stelly, D. M., and Kohel, R. J. 1987. Introgression of glanded-plant and glandless-seed trait from *Gossypium strutianum* Willis into cultivated upland cotton using ovule culture. Crop Sci. 27: 808-884.

Ashby, M. N., and Edwards, P. A. 1990. Elucidation of a deficiency in two yeast coenzyme mutant. Characterization of the structural gene encoding hexaprenyl pyrophosphate synthase. J. Biol. Chem. 265: 13157-13164.

Attucci, S., Aitken, S. M., Ibrahim, R. K., and Gulick, P. J. 1995. A cDNA encoding farnesyl pyrophosphate synthase in white lupin. Plant Physiol. 108:835-836.

Back, K., and 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.

Bell, A. A. 1967. Formation of gossypol in infected or chemically irritated tissue of *Gossypium* species. Phytopathology 57:759-764.

Bell, A. A., and Stipanovic, R. D. 1977. The chemical composition, biological activity, and genetics of pigment glands in cotton. Pages in 244-258: Beltwide Cotton Prod. Res. Conf.

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

Burnett, R. J., Maldonado-Mendoza, I. E., McKnight, T. D., and Nessler, C. L. 1993. Expression of a 3-hydroxy-3-methyl-glutaryl coenzyme A reductase gene from *Camptotheca acuminata* is differentially regulated by wounding and methyl jasmonate. Plant Physiol. 103:41-48.

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

Chappell, J., and Nable, R. 1987. Induction of sesquiterpene biosynthesis in tobacco cell suspension cultures by fungal elicitor. Plant Physiol. 85:469-473.

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

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

Choi, D., Ward, B. L., and Bostock, R. M. 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.

Cunillera, N., Arro, M., Delourme, D., Karst, F., Boronat, A., and Ferrer, A., 1996. *Arabidopsis thaliana* contains two differentially expressed farnesyl diphosphate synthase genes. J. Biol. Chem. 271:7774-7780.

Cunillera, N., Boronat, A., and Ferrer, A. 1997. The *Arabidopsis thaliana FPS1* gene generates a novel mRNA that encodes a mitochondrial farnesyl-diphosphate synthase isoform. J. Biol. Chem. 272:15381-15388.

Davila-Huerta, G., Hamada, H., Davis, G. D., Stipanovic, R. D., Adams, C. M., and Essenberg, M. 1995. Cadinene-type sesquiterpenes induced in *Gossypium* cotyledons by bacterial inoculation. Phytochemistry 39:531-536.

Davis, D., Merida, J., Legender, L., Low, P. S., and Heinstein, P. 1992. Independent elicitation of the oxidative burst and phytoalexin formation in cultured plant cells. Phytochemistry 32:907-915.

Delourme, D., Lacrote, F., and Karst, F. 1994. Cloning of an *Arabidopsis thaliana* cDNA coding for farnesyl diphosphate synthase by functional complementation in yeast. Plant Mol. Biol. 26:1867-1873.

Devarenne, T. P., Shin, D. H., Back, K., Yin, S., and Chappell, J. 1998. Molecular characterization of tobacco squalene synthase and regulation in response to fungal elicitor. Arch. Biochem. Biophys. 349:205-215.

Dixon, R. A., and Harrison, M. J. 1990. Activation, structure, and organization of genes involved in microbial defense in plants. Adv. Genet. 28:165-234.

Dixon, R. A., and Lamb, C. J. 1990. Molecular communication in plant: Microbial pathogen interactions. Ann. Rev. Plant Physiol. Plant Mol. Biol. 41:339-367.

Dudley, M. W., Dueber, M. T., and West, C. A. 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, P. B., Jr., and Cover, E. C. 1990. Accumulation of antibacterial sesquiterpenoids in bacterially inoculated *Gossypium* leaves and cotyledons. Phytochemistry 29:3107-3113.

Facchini, P. J., and 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., Klögi, A., Kost, B., Scheid, M. O., Neuhaus-Url, G., Schrott, M., Schillito, R., Spangenberg, G., and Wang, Z. Y. 1995. Standard molecular techniques for the analysis of transgenic plants. Pages 215-263 in: Gene Transfer to Plants. I. Potrykus and G. Spangenberg, eds. Springer-Verlag, Berlin.

Hahlbrock, K., Scheel, D., Logemann, E., Nurnberger, T., Parniske, M., Reinold, S., Sacks, W. R., and Schmeizer, E. 1995. Oligopeptide-elicitor mediated defense gene activation in cultured parsley cells. Proc. Nat. Acad. Sci. USA 92:4150-4157.

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.

Hugueney, P., Bouvier, F., Badillo, A., Quennemet, J., d'Harlingue, A., and Camara, B. 1996. Developmental and stress regulation of gene expression for plastid and cytosolic isoprenoid pathways in pepper fruits. Plant Physiol. 111:619-626.

Joly, A., and Edwards, P. A. 1993. Effect of site-directed mutagenesis of conserved aspartate and arginine residues upon farnesyl diphosphate synthase activity. J. Biol. Chem. 268:26983-26989.

Joost, O., Bianchini, G., Bell, A. A., Benedict, C. R., and Magill, C. W. 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.

Lange, B. M., Wildung, M. R., McCaskill, D., and Croteau, R. 1998. A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. Proc. Natl. Acad. Sci. USA 95:2100-2104.

Li, C. P., and Larkins, B. A. 1996. Identification of a maize endosperm-specific cDNA encoding farnesyl pyrophosphate synthetase. Gene 171:193-196.

Maldonado-Mendoza, I. E., Vincent, R. M., and Nessler, C. L. 1997. Molecular characterization of three differentially expressed members of the *Camptotheca acuminata* 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) gene family. Plant Mol. Biol. 34:781-790.

Matsushita, Y., Kang, W. K., and Charlwood, B. V. 1996. Cloning and analysis of a cDNA encoding farnesyl diphosphate synthase from *Artemisia annua*. Gene 172:207-209.

McGarvey, D. J., and Croteau, R. 1995. Terpenoid metabolism. Plant Cell 7:1015-1026.

Meng, Y. L., Jia, J. W., Liu, C. J., Heinstein, P., and Chen, X. Y. 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.

Murashige, J., and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 115:473-497.

Ohnuma, S., Hirooka, K., Ohto, C., and Nishino, T. 1997. Conversion from archaeal geranylgeranyl diphosphate synthase to farnesyl diphosphate synthase. Two amino acids before the first aspartate-rich motif solely determine eukaryotic farnesyl diphosphate synthase activity. J. Biol. Chem. 272:5192-5198.

Ohnuma, S., Narita, K., Nakazawa, T., Ishida, C., Yakeuchi, Y., Ohto, C., and Nishino, T. 1996. A role of the amino acid residue located on the fifth position before the first aspartate-rich motif of farnesyl diphosphate synthase on determination of the final product. J. Biol. Chem. 271:30748-30754.

Pan, Z., Herickhoff, L., and Backhaus, R. A. 1996. Cloning, characterization, and heterologous expression of cDNAs for farnesyl diphosphate synthase from the guayule rubber plant reveals that this prenyltransferase occurs in rubber particles. Arch. Biochem. Biophys. 332: 196-204.

Ramos-Valdivia, A. C., Heijden, R., and Verpoorte, R. 1997. Elicitor mediated induction of anthraquinone biosynthesis and regulation of isopentenyl diphosphate isomerase and farnesyl diphosphate synthase activities in cell suspension cultures of *Cinchona robusta* How. Planta 203:155-161.

Sanmiya, K., Iwasaki, T., Matsuoka, M., Miyao, M., and Yamamoto, N. 1997. Cloning of a cDNA that encodes farnesyl diphosphate synthase and the blue-light-induced expression of corresponding gene in the leaves of rice plants. Biochem. Biophys. Acta 1350:240-246.

Somssich, I. E., and Hahlbrock, K. 1998. Pathogen defense in plant - a paradigm of biological complexity. Trends Plant Sci. 3:86-90.

Song, L., and Poulter, C. D. 1994. Yeast farnesyl-diphosphate synthase: Site-directed mutagenesis of residues in highly conserved prenyltransferase domains I and II. Proc. Natl. Acad. Sci. USA 91:3044-3048.

Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. Science 268:661-667.

Threlfall, D., and Whitehead, I. M. 1988. Co-ordinated inhibition of squalene synthase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in cultures of *Nicotiana tabacum*. Phytochemistry 27:2567-2580.

Threlfall, D., and Whitehead, I. M. 1991. Terpenoid phytoalexins: Aspects of biosynthesis, catabolism, and regulation. Pages 159-208 in: Ecological Chemistry and Biochemistry of Plant Terpenoids. J. B. Harborne and F. A. Tomas-Barberan, eds. Oxford Science, Oxford.

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

Weissenborn, D. L., Denbow, C. J., Laine, M., Lang, S. S., Yang, Z., Yu, X., and Cramer, C. L. 1995. HMG-CoA reductase and terpenoid phytoalexins: Molecular specialization within a complex pathway. Physiol. Plant. 93:393-400.

Yang, Z., Park, H., Lacy, G. H., and Cramer, C. L. 1991. Differential activation of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes by wounding and pathogen challenge. Plant Cell 3:397-405.

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