PURIFICATION OF THE NATIVE ENZYME AND CLONING AND CHARACTERIZATION OF A cDNA FOR (+)-δ-CADINENE SYNTHASE FROM BACTERIA-INOCULATED COTTON FOLIAR TISSUE

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ABBREVIATIONS

bp	base pairs
C	degrees celsius
CDN1	(+)-δ-cadinene synthase
cfu	colony-forming units
CPP	copalyl diphosphate
CPS	*ent*-copalyl diphosphate synthase
CSPD	disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo]decan)-4-yl) phenyl phosphate
Da	daltons
DHC	2,7-dihydroxycadalene
DTT	dithiothreitol
dHG	desoxyhemigossypol
DIG	digoxigenin
EDTA	ethylenediaminetetraacetic acid
EEO	electroendosmosis
EtOH	ethanol
FPLC	fast-protein liquid chromatography
FPP	farnesyl diphosphate
g	grams
GGPP	geranylgeranyl diphosphate
GPP	geranyl diphosphate
GUS	β-glucuronidase

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HG	hemigossypol
HMGR	3-hydroxy-3-methylglutaryl CoA reductase
hpi	hours post inoculation
HR	hypersensitive response
IPTG	isopropyl-β-thiogalactoside
kDa	kilodalton
KS	*ent*-kaurene synthase
LB	Luria-Bertani medium
LC	laciniline C
LCME	laciniline C 7-methyl ether
LIS1	*S*-linalool synthase
ml	milliliters
mM	millimolar
$N_2(l)$	liquid nitrogen
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PCR	polymerase chain reaction
PHAST	an electrophoresis apparatus manufactured by Pharmacia
PMSF	phenylmethylsulfonyl fluoride
Psm	*Psuedomonas syringae* pv. *maculicola*
PVPP	polyvinylpolypyrrolidone
SDS	sodium dodecyl sulfate

SEC	size-exclusion chromatography
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEAS	5-*epi*-aristolochene synthase
TE	Tris-EDTA
TEN	Tris-EDTA-sodium chloride
Tris	Tris(hydroxymethyl)aminoethane
µg	micrograms
µl	microliters
µM	micromolar
UV	ultraviolet absorbance spectroscopy
Vd	*Verticillium dahliae*
WbM*gl*	Westburn *M* gl_1 gl_1 gl_2 gl_2, a glandless cotton line
XAD	a beaded non-polar adsorbent
Xcm	*Xanthomonas campestris* pv. *malvacearum*

CHAPTER 1

INTRODUCTION

A. Preface

Higher plants have a considerable array of defense strategies which when induced in a timely manner, prevent microbes from causing disease. The ability of the plant to prevent the microorganism from growing and causing disease depends not only upon the response itself but also upon the speed with which it is induced (Kúc and Rush, 1985). In a compatible interaction, it is likely that the plant does not perceive the invader and mount a defense quickly enough, allowing the microbes sufficient opportunity to become established. In an incompatible interaction, the plant initiates the defense response and ultimately prevents disease by preventing pathogen spread (Kúc and Rush, 1985). The hypersensitive response (HR) is defined as localized plant cell death, which is induced by and occurs in cells neighboring the pathogen. Associated with the HR is the generation of reactive oxygen species, the release of molecules with potential roles in signal transduction (i.e. salicylate and methyl jasmonate), the induction of plant cell wall reinforcement by phenols, lignins, and hydroxyproline rich glycoproteins, and an increase in accumulation of phytoalexins, pathogenesis-related proteins, and protease inhibitors. The induction of a multicomponent defense response makes it very difficult to determine the role of any one factor. However, considerable progress is being made in the study of phytoalexins. A central question being addressed in the laboratory of Margaret Essenberg and Margaret Pierce concerns the role of the sesquiterpene phytoalexins produced in response to bacterial infection in Upland cotton. The following sections of this introduction, though by no means exhaustive, will attempt to provide an overview of the topics pertaining to sesquiterpene phytoalexins. Section B will consider some complementary experimental

approaches utilized to ascertain the role of phytoalexins in plant defense. Section C provides a brief introduction to plant terpene synthases. Section D discusses the regulation of two well-studied terpenoid synthases from castor bean and tobacco, which are involved in the production of phytoalexins. Section E considers the cotton -*Xcm* interaction as it relates to the involvement of terpenoid phytoalexins and the sesquiterpene cyclase.

B. Phytoalexins

Phytoalexins are low molecular weight, antimicrobial compounds synthesized by and accumulated in plants after exposure to microorganisms (Paxton, 1981). More than 300 unique phytoalexins have been identified originating from diverse biosynthetic pathways (Kúc and Rush, 1985) and representing at least 20 different plant families (Smith, 1996). Many studies have been undertaken to determine the role of phytoalexins and several will be considered here that provide complementary approaches.

Inhibitor studies have contributed information about the importance of phytoalexins in plant-pathogen interactions; however, these studies are often confounded because inhibitors are rarely specific for one enzyme and often affect an entire pathway. Two inhibitor studies of sesquiterpenoid phytoalexins were undertaken after observations indicated a transient induction of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), an early enzyme in the pathway leading to the synthesis of isoprenoids (Figure 1), in elicitor-challenged tobacco (Chappell et al., 1991) and *Verticillium*-challenged cotton (Joost et al., 1995). The HMGR inhibitors, compactin and mevinolin were applied and in both studies a decrease in phytoalexin accumulation was correlated with an increase in susceptibility (Eldon and Hillocks, 1996; Chappell and Nable, 1987). However, later studies demonstrated that mevinolin also was an inhibitor of the tobacco sesquiterpene cyclase (Vögeli and Chappell, 1991). Thus, it seems quite plausible that the inhibitory activity of mevinolin may also affect other enzymes including those involved in pathogen defense. Further studies in mevinolin-treated resistant tobacco showed that partial resistance was maintained after

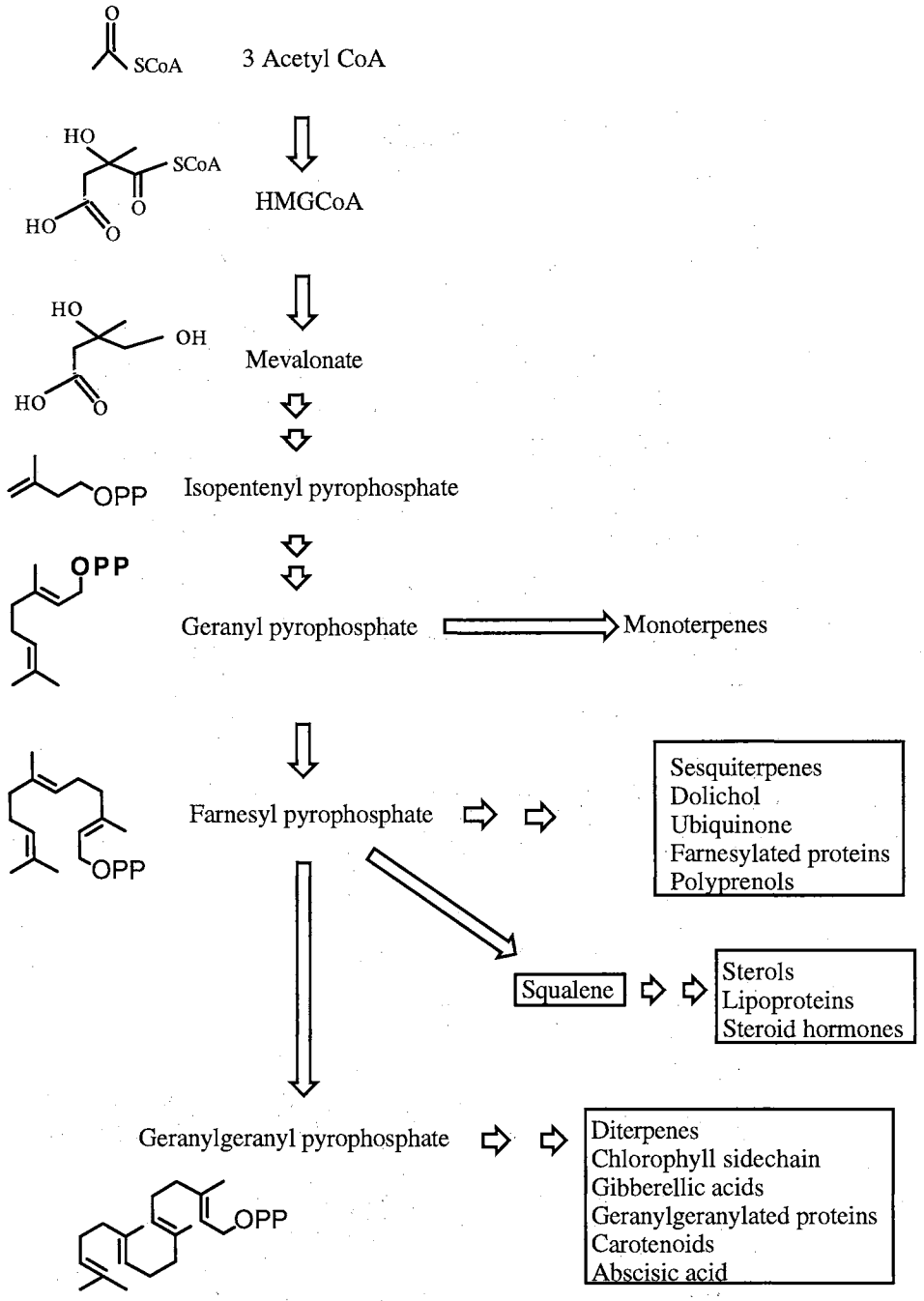

Figure 1. A pathway showing isoprenoid biosynthesis in plants. Two arrowheads indicates more than one step is required to reach at least one of the products listed.

using fosetyl-Al, a phosphonate compound known to enhance phenylalanine ammonia lyase activity, lignin deposition, and ethylene biosynthesis (Nemestothy and Guest, 1990). Though these results are consistent with the proposed role of the phytoalexins in the incompatible interaction, they also reinforce the concept of a multicomponent defense response and the inherent difficulties associated with determining the role of any one factor. Studies attempting to correlate the direct effects of phytoalexins on the plant-pathogen interaction using inhibitors or enhancers are problematic unless the target enzyme is committed only to phytoalexin biosynthesis and the inhibitor or enhancer has a known specificity. Inhibitors of HMGR cause not only a decrease in isoprenoid biosynthesis but also an increase in protein synthesis and decrease in protein prenylation (Shipton et al., 1995). Furthermore, protein prenylation may function in the regulation of diverse cellular events in plants as has been shown in other eukaryotes (Hall, 1990; Epstein et al., 1991).

Investigations were made by several groups using the *Arabidopsis thaliana-Pseudomonas syringae* interactions as a model to dissect the importance of phytoalexins. In response to the incompatible interaction with *P. syringae* pv. *maculicola* (*Psm*), *A. thaliana* produces a phytoalexin, camalexin (Tsuji et al., 1992), an indole derivative with a sulfur-containing moiety, which functions by compromising membrane integrity of the pathogen (Rogers et al., 1996). Five different phytoalexin deficient (*pad*) mutants have been isolated and reported to date which lack the ability to produce wild-type levels of camalexin after infection with *Psm* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). Relative to wild-type levels, three of the mutants had increased susceptibilities to virulent strains but identical resistance to avirulent strains; one of these mutants produced wild-type levels of camalexin during some interactions but not in others, suggesting that it may represent a mutation in a regulatory gene (Glazebrook et al., 1997). The two other mutants reported showed no differences relative to wild-type in either the compatible or the incompatible interaction; however, the possibility of a buildup of a toxic precursor has not been ruled out (Glazebrook et al., 1997). None of these genes has been cloned yet and so

the determination of whether they are regulatory genes or camalexin biosynthetic genes must await further studies. However, based on these results, it does appear that camalexin has a minor role in the *A. thaliana-Psm* incompatible interaction (Glazebrook et al., 1997).

Resveratrol is a stilbene-like phytoalexin found in grapevine, pine, and peanut, which is toxic to many fungi. The stilbene synthase gene from grapevine was transferred into tobacco to determine whether the heterologous induction of a foreign phytoalexin gene was possible in tobacco by fungal elicitors and to determine if this transgenic tobacco would have an increased resistance to fungi (Hain et al., 1993). Two very interesting results came from this study regarding the importance of phytoalexins in the incompatible interaction. First, two independent transformants showed highly variable resistance to a fungal pathogen, *Botrytis cinerea*; the more resistant line had maximum transcript accumulation between 6 and 24 hours post inoculation (hpi) while the less resistant line had maximum accumulation between 24 and 48 hpi, though the final transcript levels were comparable. Second, the amount of extracted phytoalexin at 24, 48, 72, and 96 hpi was directly correlated with the level of resistance in the transgenic tobacco. These two results provide evidence of the important role for the phytoalexin, resveratrol, in fungal resistance. Resveratrol synthase has been introduced into rape, tomato, potato, and rice with similar results (Stark-Lorenzen et al., 1997).

A different perspective on the importance of phytoalexins was gained by studying the pathogens rather than the hosts in interactions of pea with *Nectria haematococca* and *Cochliobolus*. Crosses between pathogenic and non-pathogenic fungi showed that a pisatin-detoxifying gene is closely linked to pathogenicity (Tegtmeier and VanEtten, 1982). Subsequent studies identified that other closely linked pathogenicity genes are also required (VanEtten et al., 1994). In addition, avirulent and saprophytic fungi were transformed with the ability to degrade pisatin and only the avirulent fungi became virulent indicating that other factors, in addition to the pisatin-detoxifying gene, are required for pathogenicity (Schäfer et al., 1989).

Many examples of correlative studies have been conducted to determine whether phytoalexins are present at the right time, in the right concentrations, or in the right location to inhibit pathogen growth. One of the most complete studies has been conducted in the cotton-*Xcm* interaction and is considered in Section D.

C. Plant terpene synthases

Plant terpene synthases have been identified in angiosperms and gymnosperms and are classified according to their substrate (i.e. monoterpene (C10), sesquiterpene (C15), or diterpene (C20) synthases). Most cyclize the substrate into one or more rings, though some produce acyclic products. A few have been identified which do not remove the diphosphate moiety and others that yield oxygenated products. Over two dozen terpene synthases have been partially or completely purified and characterized to date from plants, and while their products are structurally diverse, the enzymes which catalyze these reactions are quite similar in their biochemical properties.

Plant terpene synthases are functionally soluble enzymes with molecular weights ranging from 50 to 100 kDa. Most are monomers though two homodimer synthases have also been described (Alonso and Croteau, 1991; Munck and Croteau, 1990). All require a cation, usually Mg^{2+} (Mn^{2+}, Fe^{2+}, or K^{+} can be substituted with some cyclases), a reducing agent (i.e. β-mercaptoethanol or DTT) and have pH optima between 6-9. The Michaelis constants for the isoprenoid diphosphate substrates usually range from 0.1 to 5 μM; a notable exception is the sesquiterpene synthase β-selinene synthase from *Citrofotunella mitis* fruits with a Km of 45 μM (Belingheri et al., 1992). The turnover numbers, $k_{cat,}$ range from 0.05 to 0.5 s^{-1}. Treatment of many of the terpene synthases with amino acid-modifying reagents has helped to identify residues that are important for catalysis. Most of the terpene synthases show susceptibilities to cysteinyl, arginyl, and histidyl-directed reagents. Several of the synthases can be protected from these reagents by the presence of the substrate and cation, indicating that these residues are likely to be at or

near the active site (Rajaonarivony et al., 1992; Lewinsohn et al., 1992; Savage et al., 1994; Croteau et al., 1994; Salin et al., 1995; Savage et al., 1995). Some cyclases have been reported to utilize more than one isoprenoid diphosphate substrate *in vitro* (Colby et al., 1998; Steele et al., 1998; Crock et al., 1997; Wildung and Croteau, 1996) and many generate multiple products from a single substrate (Gambliel and Croteau, 1984; Munck and Croteau, 1990; Alonso and Croteau, 1991; Wagschal et al., 1991; Lewinsohn et al., 1992; Colby et al., 1993; Savage et al., 1994; Adam et al., 1996; Hill et al., 1996). Remarkable examples are the overexpressed cDNA clones for the sesquiterpene synthases δ-selinene synthase and γ-humulene synthase, which were shown to generate 11 and 15 total sesquiterpenes, respectively. This result is consistent with the product profiles identified in cell free extracts (Steele et al., 1998). The majority of the terpenoid products function in plant defense as herbivore deterrents, attractants of herbivore parasites, and as phytoalexins, though some are precursors of phytohormone biosynthesis (e.g. gibberellin and abscisic acid).

D. Regulation of terpenoid phytoalexins

Regulation of the terpene synthases appears to be a coordinated event and has been closely studied in two elicitor-inducible systems, tobacco and castor bean. Casbene synthase, a diterpene cyclase from *Ricinus communis* L. (castor bean), is a good model for studying phytoalexin synthesis because its product is the phytoalexin, casbene. Studies in the regulation of enzyme activity have demonstrated that casbene synthase and two direct precursors, geranyl transferase and farnesyl transferase, are upregulated after fungal infection and are all localized to proplastids (Dudley et al., 1986). Though transcriptional regulation of genes involved in the pathways leading to the production of phytoalexins has been demonstrated (Dixon and Harrison, 1990); the potential of introducing a novel phytoalexin into a foreign plant by transformation of a single transcriptionally regulated gene is of significance (as noted for resveratrol in section B).

Perhaps the best-studied terpene synthase involved in the production of phytoalexins is the sesquiterpene cyclase, 5-*epi*-aristolochene synthase (TEAS) from tobacco. Farnesyl diphosphate is a precursor to both squalene and the sesquiterpene phytoalexins (Figure 1) and two independent groups observed that squalene synthase activity was *ca.* 80% below its constitutive levels at the time that phytoalexin accumulation began (Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988). Further, an elicitor-inducible HMGR was transiently upregulated (Chappell et al., 1991) while the overexpression of a hamster HMGR did not affect phytoalexin production, rather sterol synthesis increased (Chappell et al., 1995). These results indicated that the expression of the sesquiterpenoid phytoalexins was coordinately regulated with at least two other enzymes of isoprenoid biosynthesis (HMGR and squalene synthase) and implied the possibility of specific HMGR isozymes controlling the distribution of isoprenoids. Control of TEAS was determined to be at the transcriptional level, as both the protein and the mRNA were made *de novo* (Vögeli and Chappell, 1990). Studies using calmodulin antagonists were consistent with a calcium-based signal transduction mechanism for phytoalexin induction in elicitor-treated tobacco cell suspensions (Vögeli et al., 1992). Elicitation of tobacco cell suspension with a 10 kDa fungal protein resulted in the release of a chemically unidentified diffusible signal (less than 1kb) within four hours which is capable of inducing TEAS, chitinases, and a hypersensitive-responsive gene (hsr203) (Chappell et al., 1997).

To better understand the regulation and control of the transcriptional activation of TEAS, it was cloned, sequenced and determined to belong to a gene family of 12-15 members by Southern analysis (Facchini and Chappell, 1992). Expression of GUS fusions with one TEAS gene promoter showed induction after wounding, elicitor treatments, and bacterial and fungal challenges, but not during any plant developmental stages consistent with TEAS assays of non-transformed tobacco. These fusions also showed no tissue specificity (induced in the roots, leaves, and stems) or cell type specificity (induced in mesophyll, subepidermal, trichome, phloem, and xylem cells) and

had barely detectable induction after H_2O_2, salicylate, or methyl jasmonate treatments (Yin et al., 1997). With the characterization of this defense-specific promoter it will be interesting to learn the specificities of the promoters of the other gene family members.

E. Cotton-*Xcm* interactions and the sesquiterpene synthase

Gossypium hirsutum L. (Upland cotton) produces at least five sesquiterpenoid phytoalexins in response to *Xanthomonas campestris* pv. *malvacearum* (*Xcm*); 2,7-dihydroxycadalene (DHC), laciniline C (LC), its methyl ether, laciniline C methyl ether (LCME), desoxyhemigossypol (dHG) and hemigossypol (HG) (Figure 2). DHC is the most potent *Xcm*-induced phytoalexin identified in cotton (Essenberg et al., 1990) and has shown inhibitory activity against *Verticillium dahliae* (*Vd*) (Mace et al., 1987) and Cauliflower mosaic virus (Sun et al., 1988). *In vitro* bioassays indicated DHC has a greater potency in blue or far UV light against *Xcm* than in the dark (Sun et al., 1989).

Initial studies showed that the response of cotton to *Xcm* was a local response resulting in localized plant cell death, evidenced by brown necrotic cells which had a yellow-green fluorescence adjacent to *Xcm* colonies (Essenberg et al., 1979a; Essenberg et al., 1992b) and that the leaves produce a small bacteriostatic zone surrounding each colony (Essenberg et al., 1979b). Thin-layer chromatography identified fluorescent compounds principally from inoculated-resistant cotton and *in vitro* bioassays confirmed an inhibitory activity against *Xcm* for LC, LCME, and DHC (Essenberg et al., 1982; Essenberg et al., 1990). Using the fluorescence of the phytoalexins as a marker, fluorescence-activated cell sorting experiments determined that the fluorescent cells contained in excess of 75, 80 and 90% of the total LC, LCME, and DHC. Fluorescent and brown necrotic cells were used as markers in other experiments designed to evaluate the phytoalexin concentration in these cells at the time that *Xcm* multiplication stopped. The results indicated that the concentrations necessary for inhibition were attained at the time of cessation of *Xcm* growth in the resistant cotton, but were not attained in the susceptible plants (Pierce et al.,

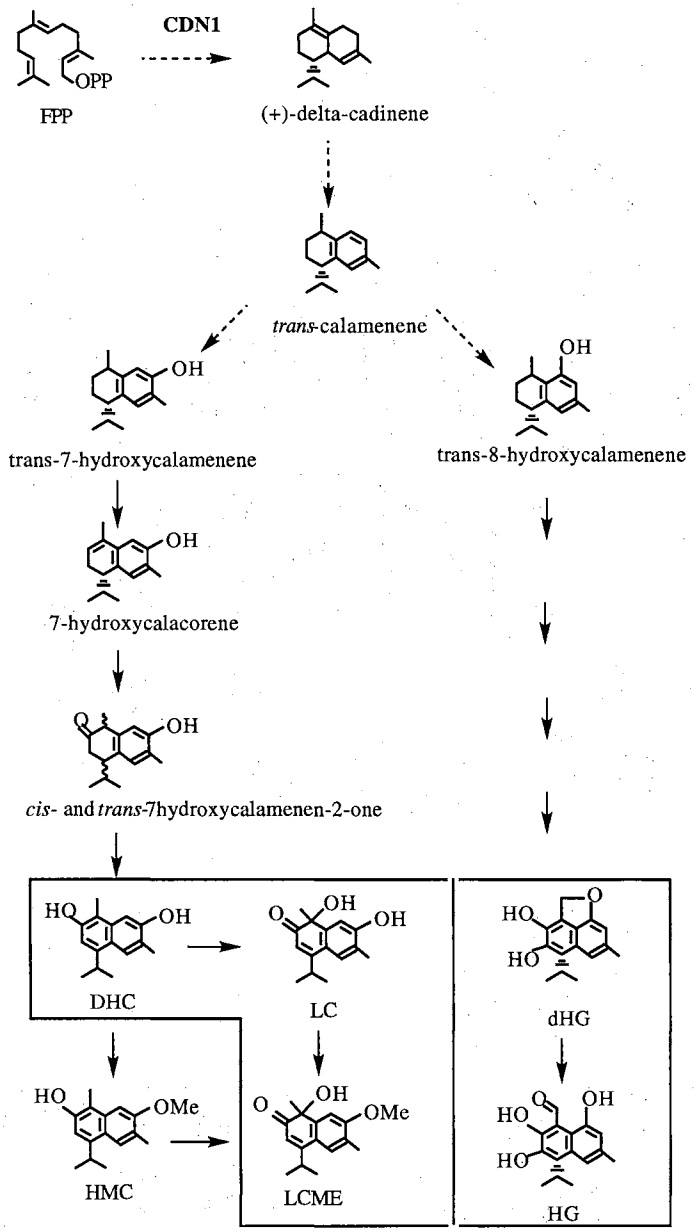

Figure 2. Proposed biosynthetic pathway for the production of phytoalexins. Phytoalexins induced in cotton foliar tissue after *Xcm* inoculation are boxed. Potential points where the two pathways diverge are indicated with dashed arrows.

1996). Phytoalexin levels from a highly resistant cotton line (Im216) and a less resistant line (OK1.2) were compared and it was found that OK1.2 produced more total phytoalexins than Im216. This prompted a study to determine the ratio of necrotic cell clusters to bacterial colonies. The results indicated that the ratios for Im216 reached unity before OK1.2 and that both obtained this value at the same time as bacteriostasis occurred (Pierce et al., 1996). Further, immunohistochemical studies using polyclonal antibodies raised against the sesquiterpene cyclase showed that detectable cyclase was found earlier in Im216 than in OK1.2 and that it preceded HR (Park, 1997). These data indicate that the sesquiterpenoid phytoalexins produced in cotton are localized at the right place, accumulate in sufficient concentrations, and are at inhibitory levels at the right time to account for the resistance of cotton to *Xcm*. Further, the timing of the phytoalexin accumulation may be a key factor determining the level of resistance.

In addition to the *Xcm*-cotton interaction, studies of fungal wilt diseases in cotton have identified sesquiterpenoid phytoalexins which are capable of inhibiting the growth of *Vd* (Bell, 1969; Mace et al., 1985), or *Fusarium oxysporum* f. sp.*vasinfectum* (Zhang et al., 1993). The most potent of these is desoxyhemigossypol (dHG) which was found at fungicidal levels adjacent to fungal conidia during the incompatible interaction (Mace et al., 1985). dHG has also been identified in the cotton-*Xcm* incompatible interaction (Davis and Essenberg, 1995) and has exhibited *in vitro* toxicity to *Xcm* (Abraham et al., 1996).

Central to all the known sesquiterpenoid phytoalexins in cotton is the common enzymatic cyclization of farnesyl diphosphate to (+)-δ-cadinene by (+)-δ-cadinene synthase (CDN1; EC 4.6.1.11) (Davis and Essenberg, 1995). CDN1 activity has been associated with the incompatible interactions of cotton with *Xcm* (Davis and Essenberg, 1995) and *Vd* (Benedict et al., 1995). Two types of sesquiterpene synthase clones were obtained, sequenced and characterized from *Vd* elicitor-treated cell suspension cultures (Chen et al., 1995; Chen et al., 1996). Expression studies of the transcripts (*cdn1*-A and *cdn1*-C) showed that they were differentially expressed in roots and cotyledons after inoculation

below the internode with*Vd*-elicitor or after leaf infiltration with *Xcm* (Heinstein et al., 1996). Results showed that both *cdn1*-A and C were induced in the cotyledons to about the same level after either treatment, but *Xcm* infiltration resulted in *ca.* 4-fold higher induction than did *Vd* elicitor. *Xcm* infiltration gave little induction of either transcript in the roots (though only a single 8-hour time point was analyzed) but *Vd*-elicitor induced *cdn1*-A 16 fold. The conclusion drawn from this study was that CDN1-A is involved in the production of the aromatic sesquiterpene aldehydes dHG, HG and G in the root tissue, while the CDN1-C is involved in the production of the cadalene derivatives in leaf tissue (Heinstein et al., 1996). Further evidence that the two pathways are differentially induced was shown by the findings that in *Xcm*-inoculated cotton cotyledons the peak accumulation of the terpene aldehydes occurs between 42 and 78 hpi while the cadalene peak occurs between 96 and 108 hpi (Abraham et al., 1996). Though consistent with the control for the two pathways occurring at the cyclization step, neither experiment rules out the possibility for control at a distal enzyme in the pathway.

The research presented in this dissertation was begun prior to the cloning of cDNAs for *cdn1* (Chen et al., 1995; Chen et al., 1996) and subsequent expression studies (Heinstein et al., 1996). At this time there were many unanswered questions. For instance, is this a typical plant terpene cyclase? Are its kinetic constants similar to other cyclases? Will amino acid sequence comparisons of this cyclase with others help to define important catalytic residues? Will antibodies raised against this enzyme help to determine where and when phytoalexins are produced after *Xcm* challenge? What is the intercellular location of this enzyme? Are there different cyclases, one leading to the production of the terpenoid aldehydes and another to the cadalenes? Or do these pathways branch at a later step? Maybe there are differentially regulated isozymes. If so, are they spatially and/or temporally regulated? Is this enzyme regulated at the level of transcription or translation? Or is it post-translational modifications that regulate its activity? Can the cDNA for this enzyme be utilized to determine the role of phytoalexins in the cotton-*Xcm* interaction? The

work presented in this dissertation will provide the means to answer all of these questions. CDN1 was purified to apparent electrophoretic homogeneity using a combination of salt-induced phase separation, batch-mode hydroxylapatite fractionation, hydrophobic-interaction and strong anion-exchange chromatography, and renaturation following denaturing polyacrylamide gel electrophoresis. Amino acid sequences for three tryptic peptides were determined and used in the cloning of two isozymes, one of which, when overexpressed in *E. coli,* demonstrated CDN1 activity. Preliminary transcript expression studies using plants inoculated in the cotyledons with *Xcm*, showed the induction of both *cdn1*s to peak at *ca.* 24 hpi in the cotyledons, while a systemic induction of one *cdn1* occurred by *ca.* 36 hpi in the roots.

CHAPTER 2

PURIFICATION OF (+)-δ-CADINENE SYNTHASE FROM BACTERIA-INOCULATED COTTON FOLIAR TISSUE

A. Preface

Why purify an enzyme from plant tissue rather than clone the cDNA using sequence information available from other cloned terpene synthases? Three key results suggested that (+)-δ-cadinene synthase was different from many of the other plant terpene synthases. First, amplification by PCR failed to yield any cyclase-like products using cDNA generated from 40 hpi cotton cotyledons and multiple combinations of primers designed to conserved cyclase sequences (Tsuji, 1993). Second, (+)-δ-cadinene synthase appeared as a *ca.* 39 kDa enzyme as determined by size-exclusion chromatography (SEC) and non-denaturing PAGE (Davis, 1993). Other terpene cyclases range from 50-100 kDa, a notable exception is the native molecular weight of 44 kDa for TEAS (Vögeli et al., 1990). Third, a non-denaturing PAGE used for a western blot and detected with a pool of monoclonal antibodies raised against TEAS, cross reacted with two proteins from a cotton extract with molecular weights of 62-66 kDa but not with a 39 kDa protein. Assays of a duplicate lane of this gel showed activity that did not align with the 62-66 kDa band. Possibly, the cotton extract contains another cyclase-like protein or the native and denatured (+)-δ-cadinene synthase have different electrophoretic mobilities and the antibodies only detect the denatured enzyme preventing the activity from comigrating with the immunodetectable band.

Given these results and the development of a simple and reliable assay for the cyclase (Davis, 1993) the surest approach was to purify the enzyme from cotton foliar tissue.

Previously, *ca.* 8 µg of cyclase was purified *ca.* 318 fold from 500 g of *Xcm*-infiltrated cotyledons using various combinations of anion-exchange chromatography and SEC (Davis, 1993); however, two attempts at obtaining sequence information from this preparation were unsuccessful. Further attempts at the purification focused on the optimization of this protocol in an attempt to increase the yield and purification but had little success. Thus it was decided that an entirely new approach must be designed.

This chapter reports the purification of CDN1-C from cotton foliar tissue. As with the purification of any enzyme of suspected low abundance and high lability, great care and consideration must be given to the optimization and the planning of the protocol. The purification was begun without initially realizing this basic tenet and much time and energy were spent without many valuable results. After this realization, the focus was directed to the optimization of two key areas: the homogenization buffer and fractionation techniques. The final conditions utilized in the homogenization alone were based on over 20 different trials. Fractionation techniques were systematically tested on a crude homogenate; the criteria used to assess these methods were yield, fold purification and convenience of incorporation into a master purification scheme. Methods attempted and later discarded included: ammonium sulfate precipitation (effective but caused phase partitioning of the PEG), heat denaturation (effective but difficult to manage in larger volumes), PEG precipitation (not effective), dye-affinity chromatography (most media resulted in modest purification with unacceptable yields), SEC (all types tried resulted in very low yields), preparative native PAGE (never successful with a crude homogenate), and salt-induced multi-phase partitioning using PEG, Dextran and Ficoll (yields and fold purification were similar to PEG alone).

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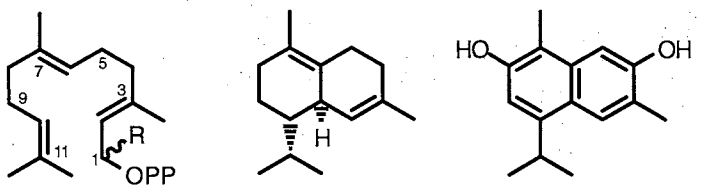
contributed equally and are therefore co-first authors. This manuscript is presented with little modification.

B. Introduction

Xanthomonas campestris pv. *malvacearum* (Smith) Dye (*Xcm*) is the causal agent of bacterial blight of cotton. In leaves of susceptible cultivars of cotton, *Xcm* multiplies to levels greater than 10^8 colony-forming units cm^{-2} and causes water-soaked, angular lesions. In contrast, *Xcm* elicits a hypersensitive response, and its growth is restricted in resistant cultivars of cotton. During this incompatible interaction in resistant cotton foliar tissue, *Xcm* also induces the accumulation of sesquiterpenoid phytoalexins, of which 2,7-dihydroxycadalene (DHC) (**1**) has the greatest antibacterial activity (Essenberg et al., 1982). Previous work has indicated that the phytoalexins accumulate at infection centers soon enough and in sufficient quantities to play a role in the resistance response of cotton to *Xcm* (Pierce and Essenberg, 1987; Essenberg et al., 1992a; Essenberg and Pierce, 1994).



	R
1 FPP	H
4 [1-^{3}H]FPP	^{3}H

2 (+)-δ-Cadinene **3** DHC

Cyclization of a prenyl diphosphate is often the first committed step in biosynthesis of cyclic terpenes (Croteau, 1987; Cane, 1990). A bacteria-induced enzyme that cyclizes (*E,E*)-farnesyl diphosphate (FPP) (**2**) to a hydrocarbon was detected in cotton cotyledon extracts using (*E,E*)-[1-^{3}H]FPP (**3**) as substrate (Davis and Essenberg, 1995). When tritiated product was infiltrated into *Xcm*-inoculated cotton cotyledons, label was incorporated into **1** and structurally related terpenoids (Davis and Essenberg, 1995).

Cochromatography with an authentic standard in four GLC and two HPLC systems plus mass spectral and NMR analysis showed that the product formed from (*E,E*)-FPP was the bicyclic sesquiterpene δ-cadinene. It was identified as (+)-δ-cadinene (**4**) by chiral GC-EIMS (Davis and Essenberg, 1995). Failure to detect the monocyclic hydrocarbon germacrene D in cell-free sesquiterpene cyclase assays or in extracts of inoculated cotyledons suggested that germacrene D does not function as an enzyme-free intermediate in the cyclization of (*E,E*)-FPP to (+)-δ-cadinene. Thus an appropriate name for this enzyme is (+)-δ-cadinene synthase. Here we report the purification of (+)-δ-cadinene synthase from cotyledon extracts, the first purification of an inducible sesquiterpene cyclase from a whole-plant/pathogen system.

A glandless line of cotton was used to reduce interference from the cyclic terpenes and terpene cyclases found in glanded cotton. The glands in leaves and stems of most cotton varieties contain a mixture of defense compounds, many of which, like the antibacterial phytoalexins, have cadinane carbon skeletons (Minyard et al., 1966; Elzen et al., 1985). The line used in this study, Westburn M *glandless* (WbM*gl*), is completely glandless and contains virtually no extractable terpenes in healthy tissues, yet accumulates high levels of sesquiterpenoid phytoalexins following inoculation with *Xcm* (Davis and Essenberg, 1995).

C. Time of appearance of sesquiterpene cyclase activity after inoculation

The maximum sesquiterpene cyclase activity occurred in WbM*gl* cotyledons at 60 hr following inoculation with *Xcm* (Figure 3). The activity increased between 20 and 60 hr and then declined rapidly. The sesquiterpene cyclase activity remained low in mock-inoculated cotyledons. Cotyledons harvested at *ca.* 60 hr post-inoculation were used for all enzyme isolations.

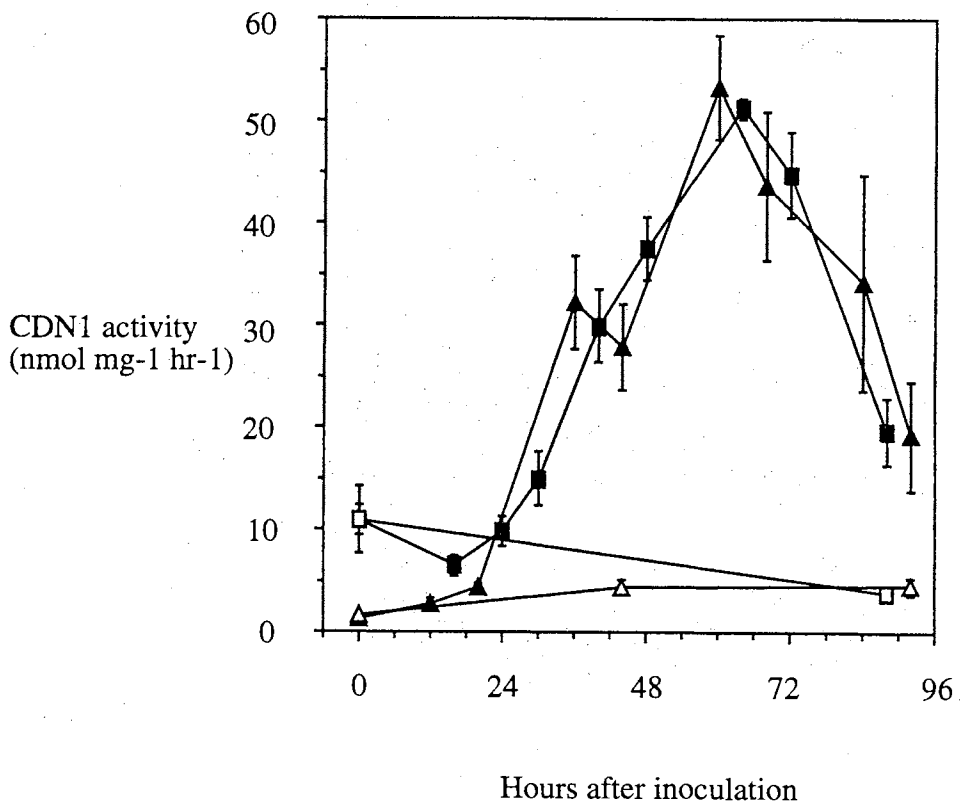


Figure 3. Sesquiterpene cyclase activity in WbM*gl* cotton cotyledons as a function of time after inoculation. Cotyledons were infiltrated with a suspension of *Xcm* (■ ▲) or suspension media alone (□ △), as described. Extracts of individual cotyledons were prepared and assayed as described. Results from two experiments are shown, represented by (▲ △) and (■ □). Each value represents the mean of three cotyledons. Error bars indicate standard error.

D. Enzyme isolation and purification

Secondary products make up a considerable percentage of the dry weight of many parts of the cotton plant, including leaves, but cotyledons have been shown to contain lower levels than foliage leaves (Bell, 1986). We worked with cotyledons of a glandless line and avoided interference from constitutive terpenes; however, flavonoids, tannins, infection-induced terpenes and unusually high levels of polysaccharide, in the form of bacterial exopolysaccharide, were present. These substances along with oxidative enzymes make the purification of enzymes from cotton tissue difficult. To overcome some of these problems we developed an extraction procedure, which yields high sesquiterpene cyclase activity, by including a non-ionic detergent (Tween 80), polyethylene glycol (PEG), and a substrate analog (tetrasodium diphosphate) in the extraction buffer. In addition, the purification includes a salt-induced phase separation (Busby and Ingham, 1980), which rapidly removes polyphenols.

Most extraction buffers used for plant terpene cyclases contain polyvinylpolypyrrolidone (PVPP) and XAD-4 adsorbent to bind phenolic substances (Croteau and Cane, 1985; Loomis et al., 1979). In our initial trials, we found that PVPP was not essential, that including XAD-4 actually lowered recovery of sesquiterpene cyclase activity, and that PEG 3350 significantly increased recovery of activity. We did not test PEGs of different molecular weights. PEG has been reported to bind polyphenols (Jones, 1965) and has been used to improve extraction of protein from cotton leaves (Lane and Weeks, 1966). In addition to binding polyphenols, PEG may act by preventing aggregation and precipitation of partially folded proteins (Cleland et al., 1992). During experimentation with a range of concentrations for the components in the extraction buffer, we also found that recovery of sesquiterpene cyclase activity, plus ease of handling, was increased by each of the following: 150 mM Tris and 10% glycerol (higher concentrations of either marginally enhanced yields), $\geq$ 0.1% Tween 80, and tetrasodium diphosphate in

the presence of $MgCl_2$. Inclusion of dithiothreitol (DTT) was essential for sesquiterpene cyclase activity.

Sesquiterpene cyclase was purified from *Xcm*-inoculated cotton cotyledons to apparent homogeneity by a sequence of phase partitioning, hydroxylapatite batch separation, hydrophobic interaction chromatography, anion exchange HPLC on Mono Q, and preparative polyacrylamide gel electrophoresis. Purification is shown in Table 1. During purification, a loss of activity was observed during freezing/thawing which was gradually restored during incubation at 0C, much like that observed for aristolochene synthase (Cane et al., 1993). Complete recovery of activity occurred by 4–5-hr; therefore, a 5-hr wait was incorporated into the activity assay for frozen/thawed samples. Following the batch-wise separation on hydroxylapatite, the enzyme preparation could be stored at –20C for at least one year without loss of activity.

When $(NH_4)_2SO_4$ was added to the crude enzyme extract (to 20% w/v), followed by a brief centrifugation, two phases were observed: a dark yellow, PEG-rich, upper phase (*ca.* 10% of the original volume) containing polyphenols, and a pale yellow, lower phase containing most of the sesquiterpene cyclase activity. A white precipitate was observed at the interphase. The recovery of sesquiterpene cyclase activity was 70%–90%. Although no protein purification was achieved, we judged this step to be valuable for removing non-proteinaceous contaminants such as pigments and phenolics. After the addition of more $(NH_4)_2SO_4$ and PEG 3350 to the lower phase followed by a brief centrifugation, two new phases were observed: a yellow, PEG-rich, upper phase containing the majority of sesquiterpene cyclase activity, and a lower phase. A white precipitate was again observed at the interphase. Neither of these precipitates contained significant amounts of cyclase activity. In early experiments the recovery of sesquiterpene cyclase activity in this step was low (20%–40% of the crude extract). However, adding more DTT (5 mM) to the first lower phase, prior to adding more $(NH_4)_2SO_4$ and PEG, resulted in much higher

Table 1. Purification of (+)-δ-cadinene synthase from 200 grams of *Xcm*-inoculated WbM*gl* cotyledons.

Fractionation step	Total protein (mg)	Total activity (μmol/hr)	Specific activity (μmol/hr mg)	Yield (%)	Purification (fold)
14,000 x *g* supernatant	910	620	0.68	100	1
20% $(NH_4)_2SO_4$ phase	820	490	0.60	80	0.89
2nd 5% PEG phase	76	450	6.0	74	8.8
Hydroxylapatite *	75	230	3.1	38	4.6
Phenyl agarose *	18	73	4.0	12	6.0
Mono Q *	1.8	47	26	7.6	38

* Values represent a pool of ≥ 90% of the recoverable activity.

recoveries (70%–85% of the crude extract). The second salt-induced phase separation achieved *ca.* 8-fold purification of sesquiterpene cyclase.

Phase partitioning has been used to localize the plant sesquiterpene cyclase β-selinene cyclase to the endoplasmic reticulum (Belingheri et al., 1988) by using a dextran–PEG two-phase system. The enzyme was later purified by other means (Belingheri et al., 1992), but the use of PEG phase partitioning in the purification of enzymes on laboratory and industrial scales is increasing (Walter and Johansson, 1994). The partitioning of phenolics into the PEG phase upon salt-induced phase separation proved to be a good means of separating them from the cotton sesquiterpene cyclase.

After removing PEG and associated phenolics by a hydroxylapatite batch separation, the enzyme preparation was applied to a phenyl-agarose column. Proteins were eluted from the column with a descending step gradient of $(NH_4)_2SO_4$. Typically, sesquiterpene cyclase activity was found in phenyl-agarose fractions containing 50 mM or 0 mM $(NH_4)_2SO_4$ in 50 mM Tris buffer, indicating that the enzyme is, like other terpene cyclases, rather hydrophobic. While recovery of cyclase activity was *ca.* 30% of that loaded, additional elution with 50% ethylene glycol did not increase the yield. After chromatography on phenyl agarose, *ca.* 98% of the total protein had been removed. However, because of the substantial loss in sesquiterpene cyclase activity, apparent net purification of the enzyme was only about 6 fold.

The phenyl-agarose fractions containing high levels of sesquiterpene cyclase activity were pooled, dialyzed, loaded onto a Mono Q quaternary amine anion exchange HPLC column and chromatographed with an ascending salt gradient (Figure 4). Sesquiterpene cyclase eluted in 230 mM NaCl. Previous purification steps did not resolve more than one sesquiterpene cyclase activity. However, on anion exchange HPLC, a second, minor peak of activity was observed (Figure 4), amounting to *ca.* 12% of the activity in the major peak. Fractions comprising the major peak were used for further purification.

Final purification was achieved by preparative, denaturing polyacrylamide gel

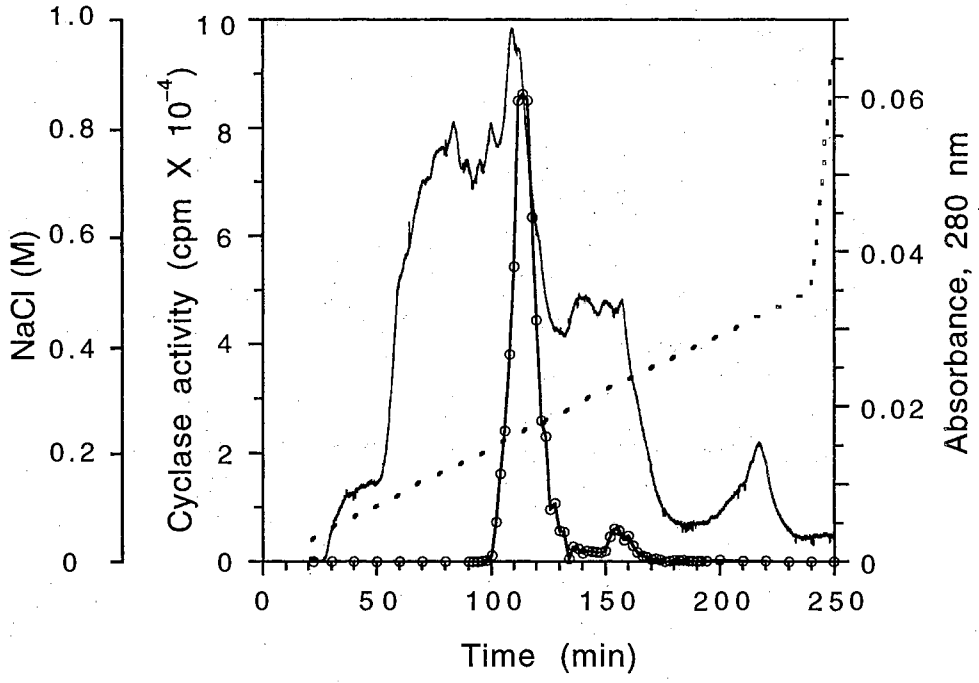


Figure 4. Anion exchange HPLC of (+)-δ-cadinene synthase from cotton. A partially purified sample from phenyl-agarose chromatography was obtained and prepared for anion exchange HPLC, as described. Sesquiterpene cyclase activity (O), the salt gradient (---), and elution of protein indicated by UV absorbance at 280 nm (—) are presented. One-min (0.5 ml) fractions were collected.

electrophoresis (SDS-PAGE) of individual fractions from the anion-exchange column. Following electrophoresis, 1-mm-thick gel slices were incubated in HEPES buffer containing 0.1% Tween 80 to displace the SDS and allow the cyclase to renature. The (+)-δ-cadinene synthase activity coincided with the most intensely stained protein band on the gel (Figure 5). About 10% of the cyclase activity applied to the gel was recovered. The product identified as described below was produced by enzyme eluted from SDS-PAGE and renatured. Polyacrylamide gel electrophoresis in the presence of SDS followed by renaturation in nonionic detergent was also used in the purification of (-)-pinene cyclase from stems of grand fir (*Abies grandis*) (Lewinsohn et al., 1992).

Analysis by SDS-PAGE of the preparation at different stages in the purification of (+)-δ-cadinene synthase is presented in Figure 6. Lane 7, designated 'Mono Q', shows a pool of aliquots from the fractions representing the major peak of sesquiterpene cyclase activity obtained from anion exchange HPLC. The major protein band in this lane co-migrated with the 64–65 kDa band in lane 8 that was confirmed to be (+)-δ-cadinene synthase following renaturation from a gel slice from preparative SDS-PAGE (Figure 5). Densitometric analysis of lane 7 (Figure 6) indicated the band representing (+)-δ-cadinene synthase contained 50% of the protein loaded. Therefore, from slicing a single band from an SDS-polyacrylamide gel, the final purification was approximately twice the 38 fold shown at the Mono Q stage. Straightforward interpretation of the *ca.* 76-fold purification suggests that (+)-δ-cadinene synthase comprises about 1/76th of the total soluble protein of infected cotton cotyledons. We believe this to be an over-estimate, since the SDS-PAGE pattern of the crude extract (Figure 6, lane 2) does not include a protein of such abundance with the mobility of (+)-δ-cadinene synthase. A distinguishable band at this mobility is first evident in lane 5 of Figure 6A, the fraction obtained from batch-wise separation on hydroxylapatite, and judging from densitometric analysis, the band represents *ca.* 1.8% of the protein loaded. Comparison with the value of 50%, which (+)-δ-cadinene synthase represents in lane 7 (Mono Q), indicates roughly a 28-fold purification of the cyclase

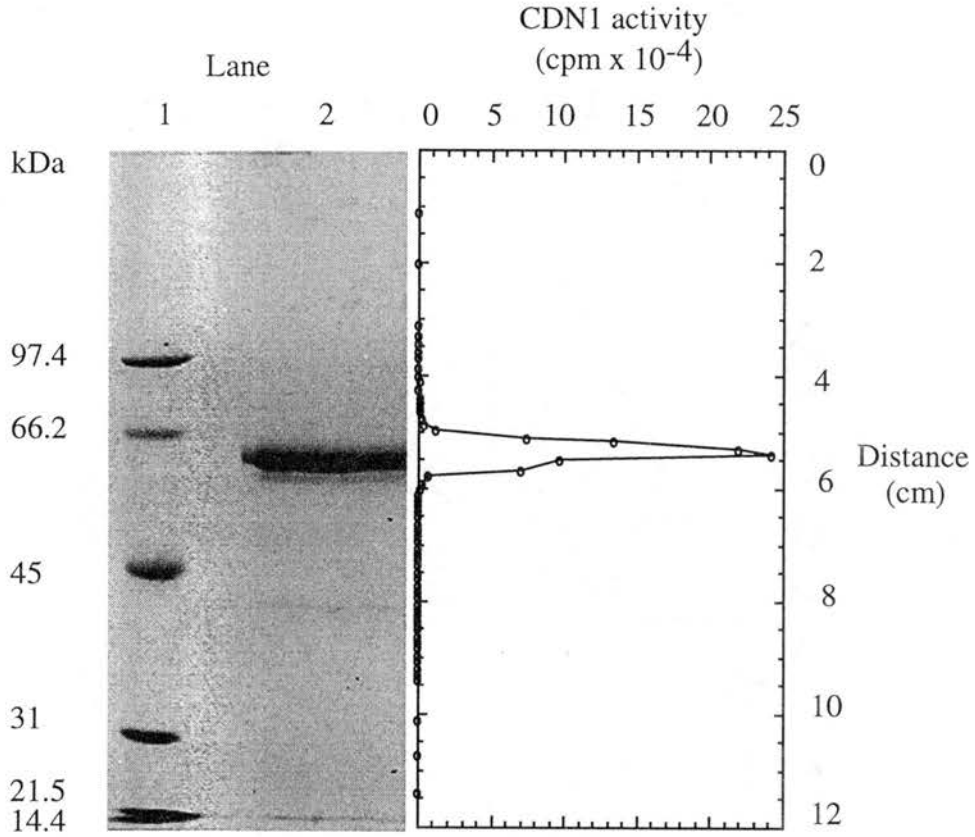


Figure 5. Purification of (+)-δ-cadinene synthase by preparative SDS-PAGE. Electrophoresis in an 18 cm long, 9% polyacrylamide gel was carried out as described; proteins in the gel were stained with Coomassie R250. Lane 1, protein standards in order from top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor and lysozyme at the front. Lane 2, protein from fraction 117 (117 min in Figure 4) from anion-exchange FPLC. Unstained slices of a portion of lane 2 were incubated in buffer, as described, to allow renaturation of protein and assay of (+)-δ-cadinene synthase activity, shown graphically.

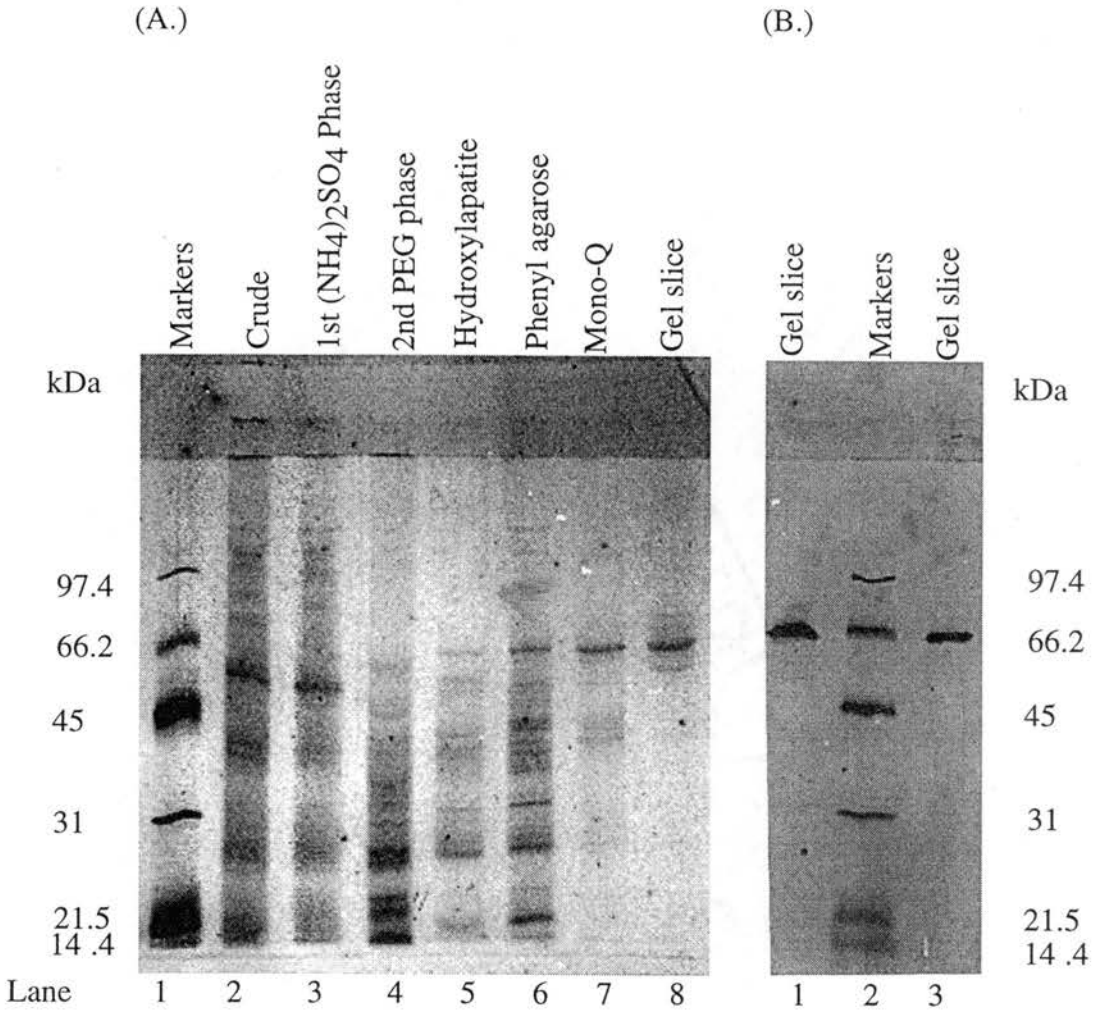


Figure 6. (A) Electrophoretic analysis of fractions in the progressive purification of (+)-δ-cadinene synthase from *Xcm* inoculated WbM*gl* cotyledons. Analytical SDS-PAGE in 12.5% polyacrylamide gels was carried out as described with 10 mM DTT in the sample buffer, and proteins were stained with silver. Lane 1, protein standards (the same as shown in Figure 5); lane 2, 14,000 x *g* supernatant from the crude extract; lane 3, the initial 20% $(NH_4)_2SO_4$ phase; lane 4, the second 5% PEG phase; lane 5, pooled sample from hydroxylapatite fractionation; lane 6, pooled sample from phenyl agarose chromatography; lane 7, pooled sample from anion-exchange chromatography; lane 8, (+)-δ-cadinene synthase eluted from the gel slice which corresponded to the peak activity shown in Figure 5. Twenty-five to 100 ng of protein was loaded per well. (B) Electrophoretic analysis of (+)-δ-cadinene synthase and protein standards carried out with 300 mM DTT in the sample buffer. Lanes 1 and 3 are aliquots from the same sample as in Figure 6 (A), lane 8. Lane 2 is an aliquot from the same sample as in Figure 6 (A), lane 1.

protein between those two steps in the purification, while the purification table (Table 1) indicates only an 8-fold increase in specific activity between those steps. Most likely, active and inactive conformations of enzyme co-purified, and/or enzyme was purified which had impaired activity.

We expected a single, sharp protein band for the gel-purified enzyme in lane 8 (Figure 6A) since it was sliced from a single band on a preparative gel, and were surprised by silver-staining material above and below the principal band. Neither addition of DTT to the sample over a range of concentrations up to 80 mM, nor addition of β-mercaptoethanol at 2.5 or 5%, nor addition of 20 mM iodoacetamide in the presence of 20 mM DTT to carboxymethylate cysteine residues on the enzyme eliminated the extraneous bands (data not shown). However, when other aliquots of the same gel-purified sample were subjected to SDS-PAGE in 300 mM DTT, artifactual bands disappeared (*e.g.*, Figure 6B, lanes 1 and 3), and there was a concomitant sharpening of the M_r standards (Figure 6B, lane 2). The sample also showed a single, sharp band following SDS-PAGE on a 7.5% polyacrylamide gel. A similar sharpening of bands on SDS-PAGE in 300 mM DTT can be seen in (Senda et al., 1988). Further evidence of the purity of the final preparation is the successful sequencing of three tryptic peptides obtained from it, all of which showed strong homology to other plant terpene cyclases (see below).

E. Molecular weight

The molecular weight for (+)-δ-cadinene synthase of 64–65 kDa determined by analytical SDS-PAGE is similar to those of many other monomeric terpene cyclases (Moesta and West, 1985; Seckler and Poralla, 1986; Dehal and Croteau, 1988; Vögeli et al., 1990; Gijzen et al., 1991; Alonso et al., 1992; Back and Chappell, 1995). Those cited range in M_r from 56–75 kDa. The native molecular weight suggested by gel permeation HPLC was M_r 45,000 (data not shown). Thus, (+)-δ-cadinene synthase appears to be a monomer. Why gel permeation chromatography and non-denaturing PAGE indicated a

smaller molecular weight than SDS-PAGE is not known; interaction with the column matrix or a compact enzyme structure is possible. Very similar behavior was observed with the sesquiterpene cyclase purified from tobacco (Vögeli et al., 1990).

F. Product identification

The product of the most highly purified (and renatured) cotton sesquiterpene cyclase, like the product from cell-free tissue extracts (Davis and Essenberg, 1995), was shown to be δ-cadinene: the tritiated reaction product co-chromatographed with an authentic standard on octadecylsilane reverse phase HPLC, and the chromatogram exhibited a single radioactive peak at the elution position of δ-cadinene, which was baseline resolved from the other sesquiterpenoid standards. Chiral GC-EIMS has established that the reaction product is (+)-δ-cadinene of high enantiomeric purity (Davis and Essenberg, 1995).

Recently Benedict *et al.* reported that δ-cadinene synthase activity is induced in stele tissue of *G. barbadense* cv. Seabrook Sea Island by infection with the fungal pathogen *Verticilium dahliae* (Benedict et al., 1995). They did not determine the enantiomeric configuration of the δ-cadinene produced. Since we observe formation of (+)-δ-cadinene by an enzyme from the WbM*gl* line of *G. hirsutum*, whereas isolation of (-)-δ-cadinene from the Deltapine Smoothleaf variety of *G. hirsutum* has been reported (Minyard et al., 1966), the chirality of the *G. barbadense* enzyme product cannot be predicted on the basis of present knowledge.

G. Amino acid sequences of trypsin-hydrolyzed peptides

Three peptides of 9, 7, and 4 amino acids were prepared and sequenced from 20–50 μg of (+)-δ-cadinene synthase. These sequences were compared to other known sequences in terpene cyclases and found to have strong identity to specific regions of the aligned sequences (Table 2). Furthermore, the (+)-δ-cadinene synthase peptide sequences are

Table 2. Identities and similarities of partial amino acid sequences from (+)-δ-cadinene synthase to deduced sequences from other plant terpene cyclases. The aligned amino acid sequences are from the monoterpene cyclase limonene synthase from *Mentha spicata* (Colby et al., 1993), the sesquiterpene cyclases (+)-δ -cadinene synthase from *G. hirsutum* and *G. arboreum* (Chen et al., 1995; Chen et al., 1996), 5-*epi*-aristolochene synthase from *Nicotiana tabacum* (Facchini and Chappell, 1992), and vetispiradiene synthase from *Hyoscyamus muticus* (Back and Chappell, 1995), and the diterpene cyclase casbene synthase from *Ricinus communis* (Mau and West, 1994). Similarities between amino acid sequences were computed using a rescaled Dayhoff table (Gribskov and Burgess, 1986).

Plant	Residue No.	Amino Acid Sequence	Identity (%)	Similarity (%)
M. spicata	179	F K N E E G E F K	67	100
G. hirsutum	---	F K D E Q G N F K	---	---
G. arboreum cdn1-C	136	F K D E Q G N F K	100	100
G. arboreum cdn1-A	137	F K D E A G N F K	89	89
N. tabacum	131	F Q D E N G K F K	67	67
H. muticus	136	F Q D A N G K F K	56	56
R. communis	184	F K D S D G K F K	67	78
M. spicata	309	K L P F	75	100
G. hirsutum	---	K L P Y	---	---
G. arboreum cdn1-C	265	K L P Y	100	100
G. arboreum cdn1-A	266	K L P F	75	100
N. tabacum	261	T L P Y	75	75
H. muticus	265	T L P Y	75	75
R. communis	314	D I P Y	50	75
M. spicata	574	N G D G H G T	43	57
G. hirsutum	---	E G D G Y T Y	---	---
G. arboreum cdn1-C	529	E G D G Y T Y	100	100
G. arboreum cdn1-A	530	E G D G Y T H	86	86
N. tabacum	524	N L D G Y T H	57	71
H. muticus	529	N Q D G Y T H	57	71
R. communis	578	Y G D G Y T D	71	71

100% identical to amino acid sequences deduced from the nucleotide sequences of two (+)-δ-cadinene synthase cDNA clones from a *G. arboreum* cell suspension culture (Chen et al., 1995; Chen et al., 1996). Western blots and northern analysis showed the *G. arboreum* cell culture synthase to be inducible by elicitor preparations from *V. dahliae*.

H. Possible biosynthetic importance of (+)-δ-cadinene synthase

The incorporation of [^{3}H](+)-δ-cadinene into the antibacterial cadalene and lacinilene phytoalexins of cotton foliar tissues and also into the antifungal phytoalexins desoxyhemigossypol and hemigossypol (Davis and Essenberg, 1995) suggests that cyclization of FPP to (+)-δ-cadinene is the first step committed to the biosynthesis of a large group of sesquiterpenes and bisesquiterpenes of *Gossypium* spp. that share the cadinane carbon skeleton (Bell, 1986). Regulation of this large, branched pathway is probably complex. In addition to their elicitation by pathogens, antifungal members of the group accumulate in epidermal cells of young, healthy roots (Bell et al., 1993). Quinone derivatives of the group are stored in the glands of green tissues and appear to be important in defense against herbivorous insects and mammals (Altman et al., 1990). The presence of these toxic compounds in the glands of cotton embryos impairs the value of cottonseed as food and feed (Lusas et al., 1978). It will be interesting to learn whether there is a differentially regulated family of δ-cadinene synthase genes in *Gossypium*.

I. Materials and methods

Chemicals

PEG 3350 (Sigma) is polyethylene glycol of average M_r 3350 g mol^{-1}. PVPP (G.A.F.) and XAD-4 polystyrene adsorbent (Sigma) were washed before use, PVPP as per (Loomis et al., 1979) and XAD-4 with organic solvents, deionized water, and buffer. High purity Tris, NaCl, and $(NH_4)_2SO_4$ (all Sigma) and ultrapure H_2O were used.

Plants and bacteria.

The glandless cotton line used in this study, WbM*gl* was developed for us by B. E. Greenhagen and L. M. Verhalen (Davis and Essenberg, 1995). It has high resistance to most races of *Xcm*. Plants were grown in a growth chamber as previously described (Pierce et al., 1993). *Xcm* strain 3631 (Pierce et al., 1993) was cultured in nutrient broth, and cotyledons were inoculated *ca.* 7 days after seedling emergence with a suspension of *Xcm* in sterile water saturated with $CaCO_3$ (*ca.* 5 x 10^6 colony-forming units ml^{-1}) as previously described (Pierce and Essenberg, 1987). Mock-inoculated control cotyledons were infiltrated with the sterile $CaCO_3$ solution.

Preparation of substrate.

(*E,E*)-[1-^{3}H]FPP obtained from DuPont NEN was diluted in sp. act to 32 μCi/μmol (1 nmol/μl) with non-radioactive (*E,E*)-FPP in buffer containing 25 mM $(NH_4)_2CO_3$ (pH 7.0) and 40% glycerol (v/v); this substrate stock solution was stored at –20C. The non-radioactive (*E,E*)-FPP was synthesized according to (Davisson et al., 1986) and purified by chromatography on a combination of anion exchange and reverse phase cartridges.

Sesquiterpene cyclase assays

Sesquiterpene cyclase activity was measured as previously described (Davis and Essenberg, 1995), with some modifications. In a typical assay, enzyme preparations (1 μg or less of total protein) were diluted in assay buffer containing 30 mM HEPES (pH adjusted to 7.0 with NaOH), 10% glycerol, 1 mM $MgCl_2$, and 5 mM DTT to a final volume of 245 μl. The assay mixture was warmed at 30C for 5 min. The reaction was started by the addition of 5 μl of substrate stock solution. After incubation at 30C for 15 min, the reaction was stopped by the addition of 3 ml of hexane, followed by vigorous mixing. The hexane extract was transferred to a test tube containing *ca.* 0.1 g of silica gel,

to remove remaining traces of oxygen-containing products, and mixed vigorously. One ml of the hexane extract was transferred to a scintillation cocktail for radioactivity assay. Protein content of the enzyme preparations was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as standard.

Time course of sesquiterpene cyclase activity

Inoculated and mock-inoculated cotyledons were harvested at various times after treatment, frozen in liquid nitrogen, and stored at –70C. Individual cotyledons were ground to a fine powder in liquid nitrogen with a mortar and pestle chilled to –20C, and the powder was suspended in 5 ml of a homogenization buffer containing 50 mM HEPES (pH 7.0), 10% glycerol, 30 mM $MgCl_2$, and 5 mM DTT. PVPP and XAD-4 (*ca.* 0.3 g of each/g tissue) were added. The homogenates were vortexed and centrifuged at 12,000 x *g* for 10 min. An aliquot of the supernatant was diluted in homogenization buffer and assayed for sesquiterpene cyclase activity as described above.

Isolation and purification of (+)-δ-cadinene synthase

Unless otherwise stated, all procedures were performed at 4C or on ice. In a standard preparation, 200 g of cotyledons were harvested at *ca.* 60 hr post-inoculation, frozen in liquid nitrogen, and temporarily stored (< 6 hr) at –20C. The frozen cotyledons were ground to a fine powder as described above. The powder was suspended in 1 l of cold extraction buffer containing 150 mM Tris·HCl (pH 7.76 determined at 5C), 10% glycerol (v/v), 5% PEG (w/v), 0.1% Tween 80 (v/v), 5 mM $MgCl_2$, 2 mM tetrasodium diphosphate, 1 mM PMSF, and 5 mM DTT. The homogenate was filtered through eight layers of cheesecloth, transferred to 250-ml bottles, and centrifuged at 14,000 x *g* for 20 min. The supernatant was transferred to a 1.5-l beaker, and $(NH_4)_2SO_4$ was slowly added to 20% (w/v). After the $(NH_4)_2SO_4$ had completely dissolved, the extract was centrifuged

for 10 min as before, resulting in phase separation. The PEG-rich, upper phase was removed using a disposable pipet and discarded. The lower phase was transferred to a 1.5-l beaker and treated with additional DTT (5 mM), PEG (5%, w/v), and $(NH_4)_2SO_4$ (10%, w/v). After the DTT, PEG, and $(NH_4)_2SO_4$ had completely dissolved, the mixture was centrifuged as before, resulting in another phase separation. The PEG-rich, upper phase, which contained the cyclase this time, was transferred to 30-ml glass tubes and centrifuged at 10,000 x *g* for 10 min.

The total PEG-rich phase containing the sesquiterpene cyclase activity was added to 20 g of hydroxylapatite (BioRad) equilibrated in 50 mM Tris·HCl (pH 7.76), 10% glycerol, and 5 mM DTT. The hydroxylapatite was allowed to bind to proteins for at least 30 min, with occasional swirling, and then washed extensively with equilibration buffer to remove PEG and remaining polyphenols. A wash with 10 mM tetrasodium diphosphate plus 5 mM $MgCl_2$ was not very effective in eluting sesquiterpene cyclase activity. Activity was eluted from the hydroxylapatite with 200 mM and/or 300 mM K_2HPO_4. The eluates containing substantial enzyme activity were pooled, and $(NH_4)_2SO_4$ was added to 1 M (no precipitate).

The sample was loaded onto a phenyl-agarose (BRL) column (4.8 cm x 3.0 cm i.d.) previously equilibrated in 50 mM Tris·HCl (pH 7.76), containing 10% glycerol, 5 mM DTT, and 1 M $(NH_4)_2SO_4$. The column was washed with 60 ml of this buffer. Proteins were eluted from the column with this buffer containing a descending step gradient of $(NH_4)_2SO_4$ [20 ml of each with the following $(NH_4)_2SO_4$ concentrations: 500 mM, 250 mM, 200 mM, 150 mM, 100 mM, 50 mM], followed by 100 ml without $(NH_4)_2SO_4$, and 20 ml of 50% ethylene glycol (v/v) in buffer without $(NH_4)_2SO_4$.

Active fractions were pooled, dialyzed for 3–4 hr in 10% glycerol and 5 mM DTT, and loaded onto a Mono Q HR 5/5 anion exchange HPLC column (Pharmacia) equilibrated in 10 mM Tris·HCl buffer (pH 7.76), containing 10% glycerol and 5 mM DTT. Proteins

were eluted during 4 hr (0.5 ml min^{-1}) with a linear gradient of 0 to 500 mM NaCl in this buffer and detected by absorbance at 280 nm; 0.5-ml fractions were collected.

Final purification was achieved by preparative SDS-PAGE, which was carried out according to Laemmli (Laemmli, 1970) in 9% and 12% polyacrylamide vertical slab gels (16 cm x 16 cm x 3 mm, and 8.2 cm x 7.2 cm x 1.5 mm, respectively). SDS, glycerol, and DTT were added, to 0.1%, 10%, and 5 mM, respectively, to the HPLC fractions containing sesquiterpene cyclase activity. Samples were loaded onto the gels, flanked by lanes containing M_r standards (Bio-Rad), and subjected to electrophoresis (100–150 V) for *ca.* 1.5 hr. To localize the sesquiterpene cyclase, a 2-cm-wide portion running the length of the gel was sliced *in situ* horizontally every 1-mm. Slices were removed leaving behind slice marks on the remainder of the gel to assist in correlating the activity with the stained gel. To renature the cyclase, each slice was transferred to a microcentrifuge tube, homogenized with a plastic pestle, and incubated for 2–3 hr in 100 µl of 30 mM HEPES buffer (pH 7.0), containing 0.1% Tween 80, 10% glycerol, and 5 mM DTT. The samples were centrifuged for 5 min at 12,000 x *g*, and 20 µl of the supernatant was assayed for sesquiterpene cyclase activity. The unassayed portions of the gels were stained with 0.2% Coomassie R250 in 20% MeOH and 0.5% HOAc.

Preparation of enzyme for protein sequencing

Half-ml samples were prepared as described above for preparative SDS-PAGE. One half of a sample was subjected to electrophoresis until all of it had just entered the stacking gel. The current was interrupted while the other half was loaded and then the entire sample was subjected to electrophoresis at constant voltage (100–150 V). After staining (above), the band corresponding to sesquiterpene cyclase was excised from the gel and partially destained in 30% MeOH. Protein was electroeluted at 24–25 mA for *ca.* 4.5 hr into 500 µl of 50 mM NH_4HCO_3 and 0.1% SDS, using 10–15 kDa M_r cut-off membranes. The

electroeluted protein was dried in a vacuum spin concentrator, and residual SDS and salts were removed by ion-pair extraction (Henderson et al., 1979).

Trypsin-catalyzed hydrolysis and purification of peptides

The dried protein, 20–50 μg, was suspended in 200 mM NH_4HCO_3 and dissolved overnight at 37C with a 40:1 (w/w) ratio substrate:tosyl-L-phenylalanine chloromethyl ketone-treated bovine trypsin. Resulting peptides were dried, dissolved in 0.1% TFA, loaded onto a octadecylsilane HPLC column (15 cm x 1.0 mm i.d.), and eluted in 0.1% TFA with a CH_3CN gradient of 0 to 5% in 0.02 min, followed by 5 to 75% over 80 min at 40 μl/min. Although well-separated peaks were obtained, those chosen for sequence analysis contained multiple sequences. Additional peaks were chosen and rechromatographed on the same column after incubation with 2.5 mM DTT in 2% CH_3CN, 0.1% each TFA and Et_3N (pH 4.2). Peptides were eluted in 0.09% each TFA and Et_3N (pH 4.2), with a CH_3CN gradient of 2 to 9% in 0.04 min, followed by 9 to 36% over 60 min. Peptides were detected by absorbance at 214 nm.

Protein sequencing

Amino acid sequence analyses were performed using a Model 470A gas-phase protein sequencer equipped with a Model 120A on-line phenylthiohydantoin amino acid analyzer (Perkin Elmer, Applied Biosystems Division) according to standard procedures (Hewick et al., 1981).

Identification of enzyme product

Purified enzyme renatured from SDS-PAGE was used to convert (*E,E*)-[1-^{3}H]FPP to a tritium-labeled product as described above. The hexane extract containing the product was reduced to near dryness (Davis and Essenberg, 1995) and combined with a mixture of terpenoid standards. The standards used were (+)-δ-cadinene [generated from (-)-α-

cubebene], (*E,E*)-farnesol, (*R*)-(+)-α-pinene, α-humulene, (-)-β-caryophyllene, (-)-α-copaene, and (-)-α-cubebene, all obtained as previously described (Davis and Essenberg, 1995). The tritium-labeled product was identified by co-chromatography of radioactivity with a known standard in analytical reverse phase HPLC as previously described (Davis and Essenberg, 1995). The eluting solvent was H_2O–CH_3CN–H_3PO_4 (25:75:0.1).

Gel permeation chromatography

The native molecular weight of (+)-δ-cadinene synthase was determined, following anion-exchange HPLC, by HPLC on a GPC-300 (SynChrom) column (30 cm x 7.8 mm i.d.), which was calibrated with bovine thyroglobulin (M_r 669,000), bovine serum albumin (M_r 66,000), chicken ovalbumin (M_r 45,000), and horse skeletal muscle myoglobin (M_r 17,200), all from Sigma. The void volume was determined with DNA, and the included volume with Ala-Phe. The column was equilibrated in 100 mM Tris·HCl (pH 7.6), 5 mM DTT, 5 mM $MgCl_2$, 15% glycerol (v/v), and run at a flow rate of 0.25 ml min^{-1}. Protein was detected by absorbance at 280 nm.

Analytical polyacrylamide gel electrophoresis

Analytical SDS-PAGE was performed with 7.5% and 12.5% polyacrylamide gels using the Pharmacia Phast system according to the manufacturer's instructions. Samples were incubated at 100C for 5 min in 2% SDS, 10 mM Tris (pH 8.0), and DTT at 10 mM or 300 mM as indicated, and centrifuged at 12,000 x *g*. Gels were stained with silver and destained following the protocol described in the Bio-Rad silver staining kit, and restained according to Pharmacia's instructions, with modification, using 0.25% $AgNO_3$ for 13 min at 40C, followed by two rinses with water at room temp for 45 sec and development with 0.015% (v/v) formaldehyde in 2.5% Na_2CO_3. This detailed staining protocol facilitated removal of excessive background. M_r standards were obtained from Bio-Rad. Densitometric analysis of the gels was performed with the computer program Image

(version 1.57 by Wayne Rasband, NIH, USA) on gels scanned using a Scan Jet IIcx (Hewlett Packard).

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CHAPTER 3

SEQUENCE AND CHARACTERIZATION OF A (+)-δ-CADINENE SYNTHASE cDNA INDUCED IN *GOSSYPIUM HIRSUTUM* L. BY BACTERIAL INFECTION

A. Introduction

When challenged with the plant pathogen *Xanthomonas campestris* pv. *malvacearum* (*Xcm*), *Gossypium hirsutum* L. (Upland cotton) produces sesquiterpenoid phytoalexins, the most potent of which is 2,7-dihydroxycadalene (Essenberg et al., 1982). *In planta* infiltration of [^{3}H](+)-δ-cadinene resulted in tritium incorporation into these cadinane phytoalexins (Davis and Essenberg, 1995). (+)-δ-cadinene synthase (CDN1; EC 4.6.1.11), the enzyme responsible for the cyclization of *trans,trans*-farnesyl diphosphate (FPP) to (+)-δ cadinene, has been purified to homogeneity from cotton foliar tissue, and three tryptic peptides from the enzyme have been sequenced (Davis et al., 1996). Three *G. arboreum cdn1* cDNA clones from *Verticillium dahliae*-elicitor-induced cells and one genomic clone have been sequenced. We describe herein the identification from *G. hirsutum* of two *cdn1* cDNAs. Further one cDNA was sequenced, characterized, and overexpressed in *E. coli* and showed a deduced amino acid sequence and catalytic activity corresponding to the purified (+)-δ-cadinene synthase.

B. Identification of *cdn1* cDNAs

The identification and cloning of three (+)-δ-cadinene synthase cDNAs from *V. dahliae*-elicitor treated *G. arboreum* suspension cultured cells (Chen et al., 1995; Chen et al., 1996), along with one genomic clone (GenBank accession no. X95323), facilitated our search for the *G. hirsutum* cyclase cDNA. The three *G. arboreum* cDNA clones are of two arbitrarily named types: a *cdn1*-C type (two cDNA clones found with 97% nucleotide

identity) and a *cdn1*-A type (*ca.* 80% nucleotide identity to the *cdn1*-C type). The genomic clone was named *cdn1-B* and is *ca.* 93% identical to the *cdn1*-C type and 80% identical to the *cdn1*-A in the deduced coding region. Based on the sequences of the *G. arboreum* clones, the tryptic peptide sequences of the *G. hirsutum* enzyme matched perfectly with the deduced amino acid sequences of the *cdn1*-C types but not with the *cdn1*-A; each peptide differed at one residue (Table 2). This prompted us to determine whether the *cdn1*-A and *cdn1*-B are also present during the *Xcm-G. hirsutum* incompatible interaction.

RNA was isolated from a pool of cotyledons harvested at 30 to 60 hpi using a method developed specifically for cotton foliar tissue (Wan and Wilkins, 1994). RNA quality was based on integrity of the rRNA as indicated by denaturing formaldehyde agarose gels (Sambrook et al., 1989) and absorbance ratios (260nm/280nm). The rRNA bands were clearly visible; however, smearing indicated some degradation had occurred. The absorbance ratios were 2.0, indicating the isolation from contaminants was successful. RT-PCR using two degenerate primers (Table 3, primers 918 and 919) based on the sequenced tryptic peptides obtained from the purified (+)-δ-cadinene synthase (Table 2, the 9mer and 7mer,respectively) amplified a 1200 bp product. This agreed with the expected size based upon the *G. arboreum cdn1* clones (Chen et al., 1995; Chen et al., 1996), and partial sequencing showed greater than 90% identity of this product with the *cdn1*-Cs and less than 80% identity with the *cdn1*-A. To determine if a *cdn1*-A was also present, we used a primer which was specific for *cdn1*-A (Chen et al., 1996) and our degenerate primer (Table 3, primers 3186 and 919, respectively). A product of *ca.* 1050 bp was amplified, sequenced and showed greater than 90% identity with the *G. arboreum cdn1*-A and less than 80% identity with the *cdn1*-Cs. To determine if a *cdn1*-B was present, a new primer based on the 3' end of the *G. arboreum* genomic sequence (GenBank accession no. X95323) was synthesized (Table 3, primer 1926). Initial attempts failed to amplify a product from the cDNA using primers 918 and 1926, so genomic DNA was used as

Table 3. Primers used for the identification, cloning, and sequencing of *cdn1*s from *G. hirsutum* L. All primers are listed from 5' to 3'. Underlined sequences are restriction endonuclease sites. The locations for *G. arboreum cdn1*-A specific primers (*) and *cdn1*-B specific primers (**) are indicated. The following primers were based on (Chen et al., 1995; Chen et al., 1996): 3187 (93126), 3186 (93160), 2937 (93T1600), and 3188 (9315). Abbreviations used are: R (A or G), S (C or G), Y (C or T), and I (inosine).

Sense Primers	Location in *G. hirsutum cdn1*-C	Sequence
1427	5' RACE	ACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
2034	5' terminus	CGGGATCCGAATTCAAAGGCASCAAATTAAGC
3187	78-97	ATAAGGATGAAATGCGTCC
918	433-456	GGCTGCAGTTYAARGAYGARCARGGIAA
2369	688-708	TATACCAAGATATTGAGTCCC
2375	1538-1555	GAAACCAACAGAAATGCC
2503	* 42-60	TGCTTCCAAAACCCTACAC
2562	* 117-136	CACCCCATCCCGCCATTTCA
3186	* 621-637	CCACTGCTCAACTTACA

Antisense Primers	Location in *G. hirsutum cdn1*-C	Sequence
2937	272-245	CTGGACTGAATCAATGAAG
3188	809-792	AAATCTTTCCACCACCT
2370	1354-1337	GCTTGAATGATCTTAGGG
919	1633-1614	CTCGAATTCTAIGTRTAICCRTCICCYTC
2033	3' terminus	CCCTCGAGTCTAGATTTCCACAAATGAAAGC
2938	* 1763-1742	ATGTTGAATTTCAAATTTGTAT
1926	** 3271-3255	TCTTCCATCCCTTAAAC
AUAP	3' RACE	GGCCACGCGTCGACTAGTACGAC
AP	3' RT primer	GGCCACGCGTCGACTAGTACGAC$(T)_{17}$

template to test the primers. A *ca.* 2100 bp product was amplified from the genomic DNA and the partial sequence showed highest identity with *cdn1*-B; however, under no circumstance was a PCR product amplified under a variety of conditions (including the identical conditions used for genomic DNA amplification) using the cDNA as template. Positive controls using both the *cdn1*-A and *cdn1*-C specific primers worked in both the genomic DNA and cDNA amplifications. It is likely that *cdn1*-B is a pseudogene; the deduced amino acid sequence of the *G. arboreum* gene indicates a 460 amino acid protein while the other CDN1s are all 554-555 residues. Our finding that the *cdn1*-B is not expressed in cotyledons at 30 to 60 after *Xcm* inoculation, is consistent with *cdn1*-B being a pseudogene, but other possibilities can not be ruled out. We conclude that *cdn1*-C and *cdn1*-A are expressed in *G. hirsutum* cotyledons during the incompatible interaction.

C. Cloning, sequencing, and expression of *cdn1*-C

Using the RNA purified from a pool of cotyledons harvested between 30-60 hpi, several attempts were made to obtain the full coding region of the *cdn1*-C cDNA. The first approach attempted was circular-RACE (Murayama et al., 1995). This method uses RNA ligase to circularize or concatenate the cDNA before PCR amplification with a sense primer that anneals downstream relative to the antisense primer. Successful amplification is possible only if the cDNA in question is joined together. Circular-RACE did not succeed using primers 2937 and 918 (Table 3) though this protocol was successfully followed in another laboratory using the same buffers and RNA ligase with different plant cDNA and primers (Liu, 1997). Prior to ligation, the cDNA must be phosphorylated and degradation of the ATP stock solution is a likely reason for circular-RACE failure. Different experiments conducted nearly two years later requiring ATP were not successful until a fresh ATP solution was used.

3' RACE was the second method attempted. This approach was unsuccessful when the cDNA was reverse transcribed from RNA using an oligo dT_{18} primer followed by PCR and

nested PCR amplifications using the oligo dT_{18} as the antisense primer with gene specific sense primers (Table 3, primers 3187 and 918). However, using 3' RACE kit primers (GIBCO-BRL) for reverse transcription (Table 3, AP) and for amplification (Table 3, AUAP) products of the correct size (described below) were amplified. The AP or adapter primer anneals to the RNA poly-A tail providing a priming site for reverse transcriptase. The AUAP primer, which is complementary to the 5' end of the AP primer is used in a PCR reaction with a gene-specific sense primer (Table 3, primer 3187). A second PCR amplification using AUAP and a nested gene-specific sense primer (Table 3, primer 918) resulted in a *ca.* 700 bp product. The yield for this product was quite low, and the presence of many nonspecific products prevented cloning or sequencing. Nevertheless, judging that the 3' end was successfully obtained, an attempt was made with a 5' RACE protocol. 5' RACE involves a terminal transferase-catalyzed tailing of the 3' end of the first-strand cDNA with dCTP followed by PCR amplification with a gene-specific antisense primer and a modified oligo dG sense primer (GIBCO-BRL) (Table 3, primer 919 and 1427, respectively). This amplification resulted in a product of *ca.* 1300 bp, which was the expected size based upon the *G. arboreum* cDNA sequences but was not abundant or specific enough for cloning.

In order to confirm these RACE products as (+)-δ-cadinene synthase cDNA fragments, the PCR products were each gel purified and used together as template for another PCR reaction without any primers. This method, called marathon PCR (Chenchick et al., 1995), relies on the overlapping sequences of two complementary fragments to behave as primers for the polymerase. After cycling, the product was diluted and used as template in a final PCR reaction using the two primers specific for the 5' and 3' ends (Table 3, primers 1427 and AUAP, respectively). The result was a *ca.* 1750 bp product that was ligated into a T-tailed cloning vector (pCRII, Invitrogen) and cloned into *E. coli* XL1-Blue. Partial sequences from each end showed greater than 90% identity with the *G. arboreum cdn1*-C cDNAs.

This clone was overexpressed in *E. coli,* and a crude protein extract was assayed for sesquiterpene cyclase activity. A tritium-labeled, hexane-extractable product was generated from this clone using 1-[^{3}H]-FPP as substrate. No detectable activity was identified from the assay of an extract from an empty-vector control. Because the marathon PCR method does not exclude the possibility of the amplification of a chimeric cDNA, two new primers were designed based upon the sequences of the 5' and 3' ends of this cDNA (Table 1, 2034 and 2033, respectively). These primers included two different restriction endonuclease sites in the 5' ends of each primer to facilitate subcloning. PCR amplification resulted in a 1872 bp product which was cloned and sequenced (GenBank accession no. U88318) (Figure 7). Plasmid from a single clone was utilized for sequencing; the cDNA was sequenced at least twice, and where primers permitted, from both directions. Ambiguities were resolved by multiple sequences and/or sequences of PCR-amplified products. PCR amplification with two primer combinations (Table 3, primers 918 and 919 and primers 3187 and 919) resulted in products with different sequences compared to the cloned *cdn1*-C. The sequences of these PCR products had higher identity to *cdn1*-C1 while the cloned cDNA had higher identity to *cdn1*-C14, indicating the possibility of multiple *cdn1*-C genes in *G. hirsutum*, as seen in *G. arboreum* (Chen et al., 1995). The sequences obtained using these PCR products were not used for determination of the submitted sequence. The regions corresponding to the primer sequences were not included in the submitted sequence as it is possible that their sources may be different cDNAs from the one amplified and cloned. The *cdn1*-C cDNA was also cloned into the pCRII vector behind a portion of the lacZ gene, and the expressed protein catalyzed the conversion of *trans, trans*-FPP into a hydrocarbon which co-chromatographed with a (+)-δ-cadinene standard in C_{18} reverse phase HPLC.

D. Cloning of *cdn1*-A

The cloning of the *cdn1*-A type cDNA proved more challenging than *cdn1*-C. Two

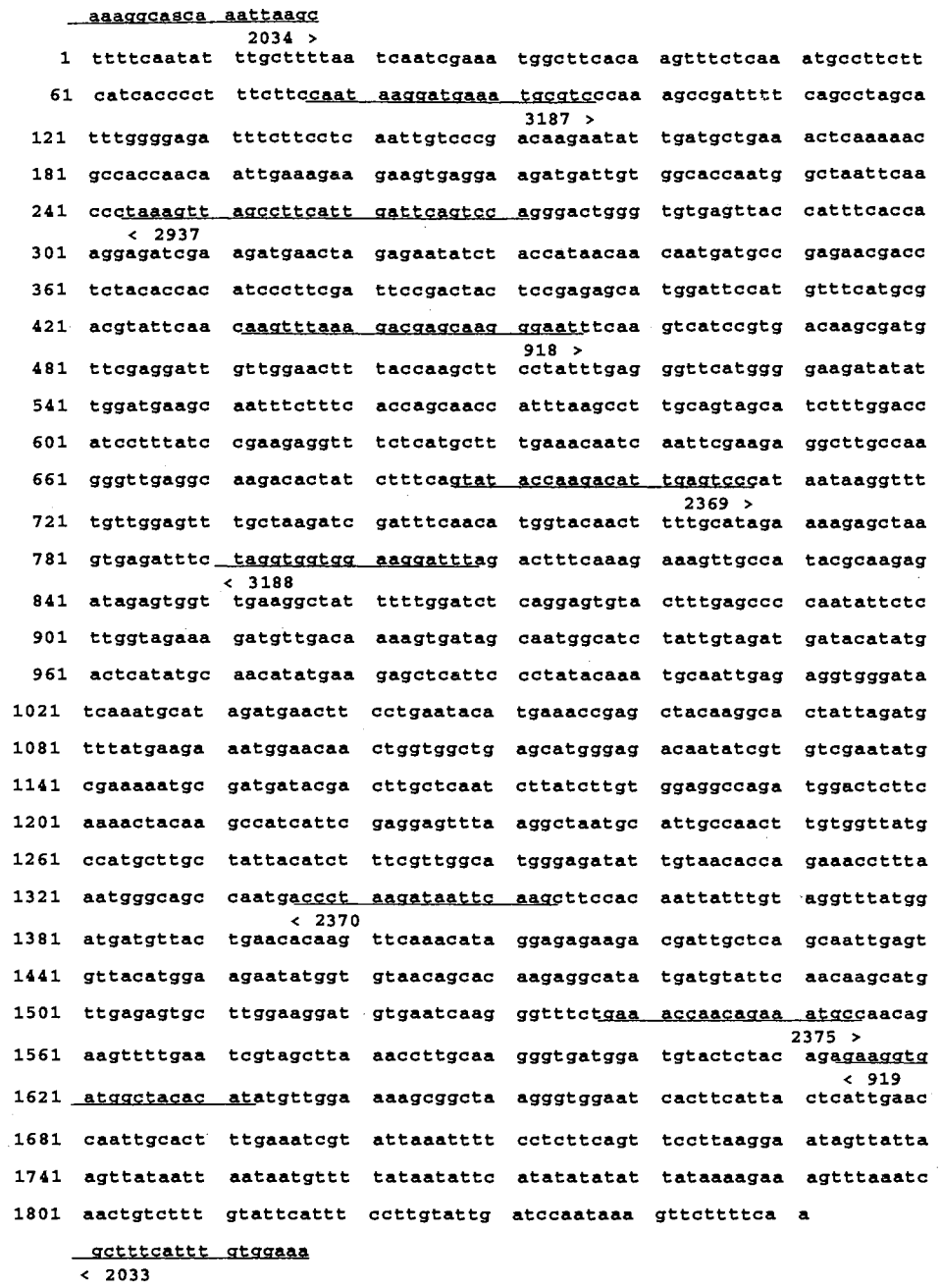


Figure 7. Nucleotide sequence of a *cdn1*-C cDNA from *G. hirsutum*. Underlined sequences denote the primer sequences used for the cloning or sequencing of *cdn1*-C; the arrowheads indicate sense (>) and antisense (<) primers. The sequences that represent the two terminal primers (2034 and 2033) have been offset to indicate that they are not a part of the GenBank entry.

partial *cdn1*-A PCR products were amplified with ease and consistency using the *cdn1*-A specific primer and either one of two antisense primers, resulting in products of *ca.* 1050 bp or 200 bp (Table 3, primer 3186 with 919 or 3188, respectively); however, 5' and 3' RACE never succeeded. All combinations of primers were attempted with the 5' and 3' RACE primers under a variety of conditions without success. Many PCR amplifications produced products of the expected size which were ligated into pCRII, cloned, and subsequently identified as something other than *cdn1*-A. On numerous occasions, PCR amplification of the clones gave a positive result of approximately the expected size when amplified using *cdn1*-A specific primers; however, plasmid preps yielded vectors containing very small or no inserts. The amplification of untransformed *cdn1*-A that is present on the agar plate after the plating of the transformation reaction, provides a likely explanation for this result. Three new primers were synthesized, two of which corresponded to the 5' end and one to the 3' end of the *G. arboreum cdn1*-A cDNA (Table 3, primers 2503, 2562 and 2938, respectively). These primers worked well, and a clone was obtained which included the entire open reading frame plus 37 bp of the 5' and 13 bp of the 3' untranslated regions. The rigorous identification of this clone as a (+)-δ-cadinene synthase cDNA has not been determined; however, partial sequence analysis of the 5' and the 3' ends (*ca.* 400 bp from each end) showed greater than 90% identity with the *G. arboreum cdn1*-A cDNA.

E. Cloning rationale

Since the initial identification and limited sequencing of the *G. hirsutum cdn1*-A and *cdn1*-C partial cDNAs showed greater than 90% identity with their *G. arboreum* homologs, the rationale for cloning the full length *G. hirsutum* cDNAs should be made. There were four reasons for cloning nearly full-length *cdn1* cDNAs. First, cloning a full-length *cdn1* would provide a cDNA for use in the sense and antisense transformation of cotton. Second, this cDNA could be used for the overexpression of active CDN1 in *E. coli* thus

confirming the identity of the clone s as (+)-δ-cadinene synthases and providing a *G. hirsutum* source of (+)-δ-cadinene. Third, complete sequencing of the cDNAs would allow a comparison of nucleotide and amino acid sequences between the *G. arboreum* and *G. hirsutum* CDN1s. Fourth, the full-length cDNAs could be used as controls for the quantitative analysis of *cdn1* transcript expression.

The initial plan was to study the expression of the *cdn1*-A and *cdn1*-C transcripts using quantitative RT-PCR. Accurate quantitation is possible if the relative efficiencies of reverse transcription and PCR amplification with each transcript are determined. Full-length *cdn1* templates would ensure that these values were accurately determined.

Though an *Xcm*-induced (40 hpi) *G. hirsutum* cDNA library was available in our laboratory, a PCR-based cloning approach was used. The basis for this decision was twofold. First, it seemed (based on the information described below) that the *cdn1* cDNAs were in very low abundance or not present in this library and second, the relative ease with which RT-PCR products were amplified from 30-60 hpi RNA made PCR-based cloning an attractive option. The reasons against using the cDNA library were based on the following results. Using the cDNA generated for the library, attempts to PCR amplify a *cdn1* product using various primers designed to conserved regions of three-cloned terpene cyclases was unsuccessful (Tsuji, 1993). Further, using the same cDNA generated for the library (stored at -20C for *ca.* 1.5 years) many futile attempts were made to PCR amplify a *cdn1* product using degenerate primers (Table 3, primer 918 and 919) designed from the tryptic peptide sequences obtained from the purified protein. These experiments were successful only when fresh cDNA was made from a pool of tissue harvested from 30-60 hpi (after 2 years and many freeze-thaw cycles, this cDNA was still a good template for the amplification of *cdn1*-C with these primers). The lack of success with the library cDNA can be explained if the *cdn1*-C and *cdn1*-A transcripts are being expressed at very low levels at 40 hpi. This explanation is supported by qualitative RT-PCR experiments which

showed expression of the two transcripts in cotyledons to peak at 24 hpi and to be barely detectable at 36 hpi and 48 hpi (data discussed below).

There are two negative aspects associated with the PCR-based cloning approach. First, the potential for incorporation of errors by the thermostable polymerases is significant (Cushman, 1996). Though, this pitfall can be overcome by using less error-prone polymerases and/or sequencing multiple clones this can be expensive. The cloning and sequencing of the *G. hirsutum cdn1*-C cDNA did not adequately address this problem. Second, unlike a cDNA library, the PCR-based approach does not provide a future resource for the laboratory; a cDNA library can be screened for other expressed gene family members and defense-related cDNAs.

F. Qualitative expression of *cdn1*-A and *cdn1*-C transcripts

Are the *cdn1*-A and *cdn1*-C transcripts tissue or inducer specific? Or, are they pathway specific, one CDN1 for the cadalenes and another for the terpenoid aldehydes? To address these questions regarding the interactions of cotton with the pathogens *Xcm* and *Vd*, a collaboration was begun with Dr. Peter Heinstein's laboratory at Purdue. RNA was collected from roots and leaves of four-week old *G. arboreum* plants inoculated below the internode with *Vd*-elicitor. Quantitative RT-PCR showed *ca.* 16-fold induction of *cdn1*-A transcripts in the root and a 2-fold induction in the leaves, while *cdn1*-C transcript accumulation was 2 and 1.2 fold higher in roots and cotyledons, respectively (Heinstein et al., 1996). Additional studies in *G. arboreum* with *Xcm*-inoculated leaves have revealed an 8 and 6-fold induction of *cdn1*-A and *cdn1*-C, respectively in the cotyledons; however, not enough data were collected to assess the induction of *cdn1*-A and *cdn1*-C in the roots after *Xcm* inoculation of the cotyledons (Heinstein et al., 1996). Qualitative RT-PCR data using *G. hirsutum* inoculated with *Xcm* in the cotyledons has shown an induction of both transcripts in the cotyledons to occur by 24 hpi while mock inoculated controls showed essentially no accumulation (Figure 8). These data are consistent with the observed CDN1

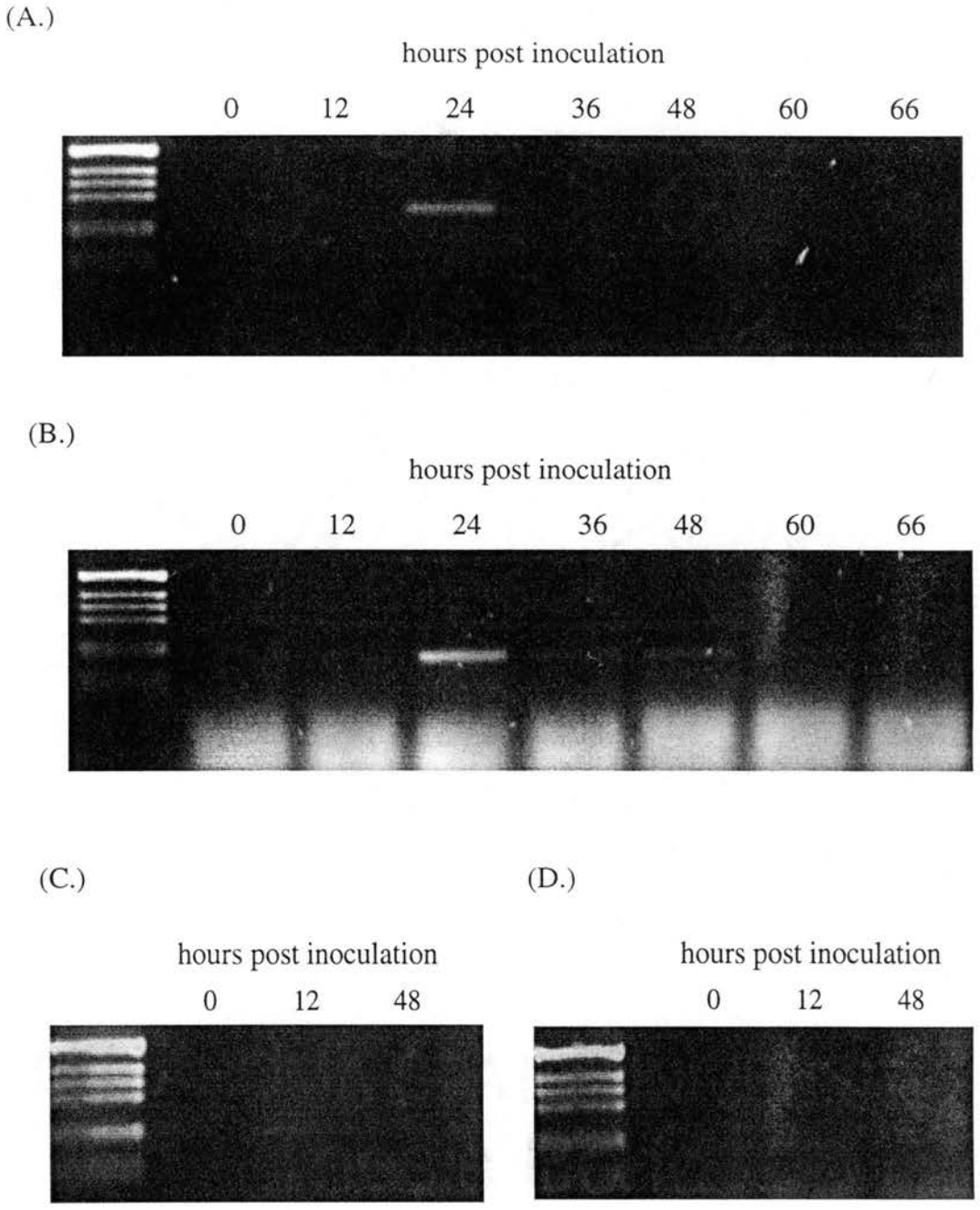


Figure 8. Qualitative RT-PCR analysis of *cdn1*-A and *cdn1*-C from *G. hirsutum* cotyledons. RNA was isolated from whole cotyledons at the indicated times after *Xcm* inoculation (panels A and B) or after mock inoculation (panels C and D). RT-PCR amplification using primers to specifically amplify *cdn1*-A (3186 and 3188; panels A and C) or *cdn1*-C (3187 and 2937; panels B and D) was performed.

activity time course (Figure 3) which revealed the greatest rate of increase of cyclase activity in *Xcm*-inoculated cotyledons to occur between 24 and 36 hpi. Interestingly, *cdn1*-A transcript levels are induced in the root at 36 hpi (Figure 9). Though the potential for systemic induction of the sesquiterpene cyclase is quite interesting, this experiment has not been repeated. *Cdn1*-C transcripts in the root were detectable at very low levels from all time points; however, recent no-template controls also show similar levels.

No detectable cross amplification of *cdn1*-A and *cdn1*-C transcripts using the transcript-specific primers (Table 3, 3186 and 3188 for *cdn1*-A and 3187 and 2937 for *cdn1*-C) was shown in *G. arboreum*, (Chen et al., 1996). Genomic DNA fragments (containing *ca.* 100 bp introns) introduced into the PCR reaction provided the internal standards for quantitation. A major factor not addressed in their study is the variability of reverse transcriptase activity, determined to range between 40 to 50% for different templates and preparations of RNA (Bouaboula et al., 1992; Berger et al., 1983). Our qualitative RT-PCR experiment contained no standards or controls.

The value of quantitative RT-PCR data for assessing transcript levels is often criticized (Hengen, 1995), but when adequate controls are performed the technique offers a *ca.* 1,000 fold increase in sensitivity and greater speed compared to northern blotting (Wang et al., 1989). Quantitative RT-PCR experiments should always include the following important controls and preliminary experiments. First, each RNA sample should be PCR amplified without being reverse transcribed, to detect whether genomic DNA and/or contaminating DNA is being amplified. Second, the internal RNA standards should be reverse transcribed and PCR amplified in the same tube as the sample RNA. Third, the assessment of reverse transcriptase efficiency and PCR amplification efficiencies of the standards and desired transcripts should be determined (Seiffert et al., 1994). Fourth, heterologous product formation during PCR amplification should be identified and eliminated (Schneeberger et al., 1995). Fifth, the proper conditions necessary to ensure

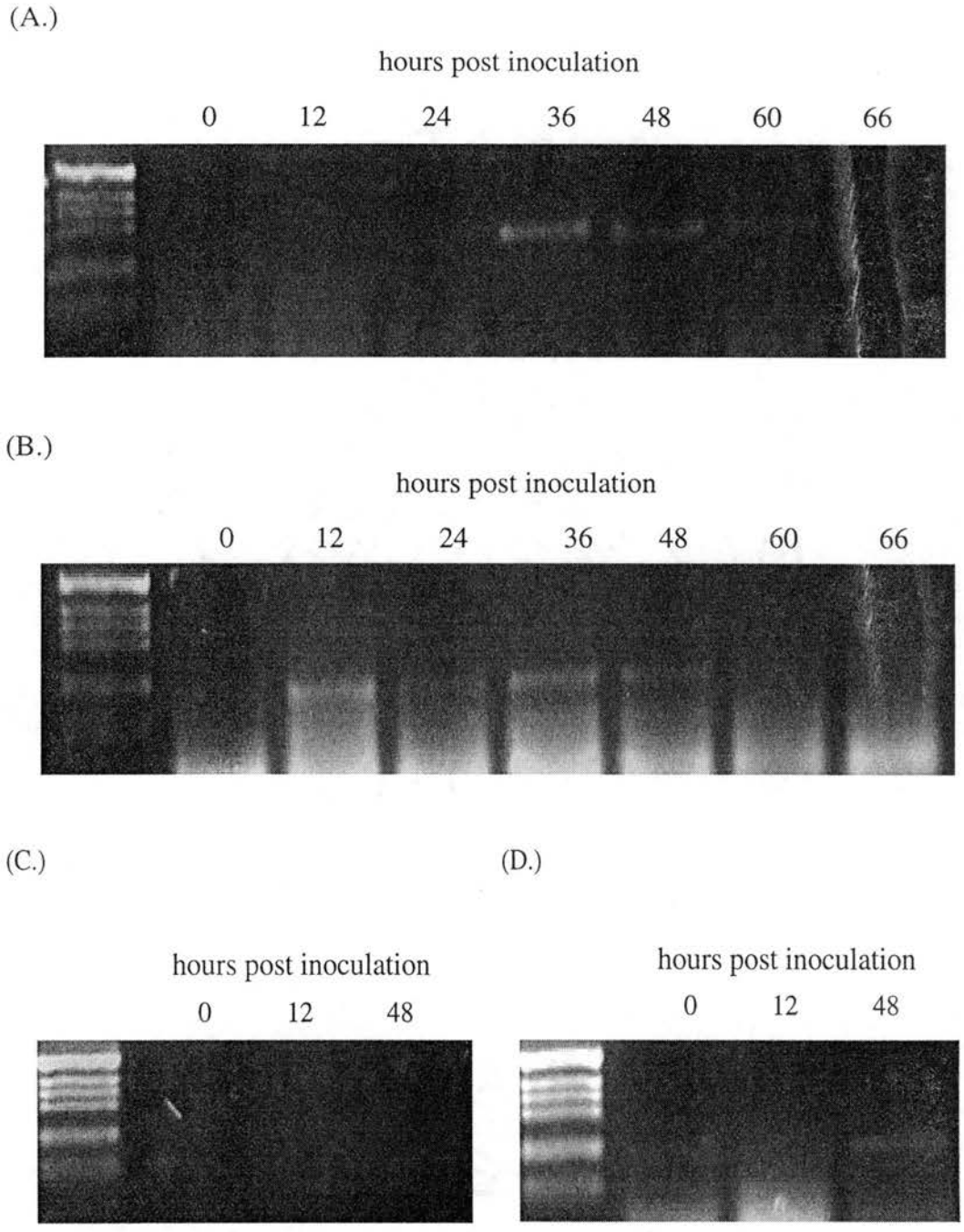


Figure 9. Qualitative RT-PCR analysis of *cdn1*-A and *cdn1*-C from *G. hirsutum* roots. RNA was isolated from whole roots at the indicated times after *Xcm* inoculation (panels A and B) or after mock inoculation (panels C and D). RT-PCR amplification using primers to specifically amplify *cdn1*-A (3186 and 3188; panels A and C) or *cdn1*-C (3187 and 2937; panels B and D) was performed.

primer specificity should be determined (Wang et al., 1989; Saed and Fivenson, 1994; Bouaboula et al., 1992).

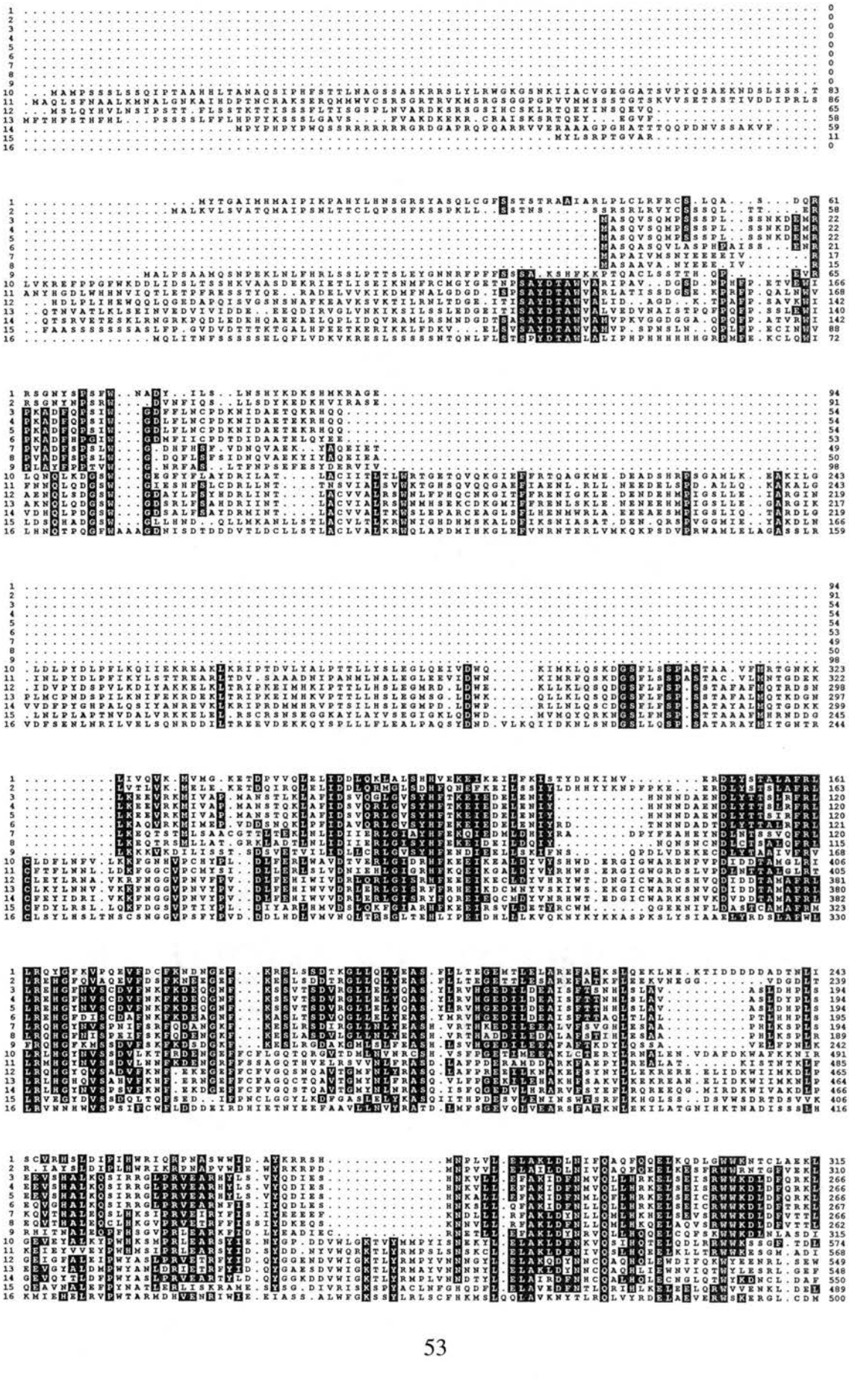
Cdn1-A and *cdn1*-C heterologous mimics were designed using a fragment of DNA from *Brucella abortus* (Accession no. U43785). Primers specific for the *B. abortus* cDNA were used to amplify 300 bp products. Each primer had a 5' linker that allows reamplification of this construct with the *cdn1*-A and *cdn1*-C specific primers. Thus, these mimics can be *in vitro* transcribed, the mimic RNA added to the RNA samples and RT-PCR amplified in the same tube. The resultant mimic products are distinguishable from the amplified *cdn1*-A and *cdn1*-C products on an agarose gel, have similar melting temperatures and hairpin structures, and have no significant sequence homology. Although heterologous mimics and the nearly full-length *cdn1*-A and *cdn1*-C cDNAs were cloned, the preparation and controls for this experiment are still far from complete. The no-template *cdn1*-C controls began giving positive PCR products even when using fresh reagents, buffers and clean pipetmen, and the potential for other pitfalls was still present. In addition, the reviewers of a quantitative RT-PCR manuscript might expect northern blot analysis to corroborate the quantitative RT-PCR data. Thus, it was decided that designing transcript-specific probes and testing them for specificity with *in vitro* transcribed *cdn1*-A and *cdn1*-C RNA would be faster. These probes have been successfully constructed and preliminary northern blot experiments are underway. It is hoped that the northern analysis will begin to address the questions regarding transcript induction relative to timing, tissue specificity, phytoalexin type (i.e., cadalenes and terpenoid aldehydes), and systemic responses.

G. Characterization of CDN1-C

Numerous plant terpene synthases have been partially or completely purified, and the genes encoding twelve distinct enzymes have been sequenced and reported to date. Each of these enzymes employs an isoprenoid precursor: geranyl diphosphate (monoterpene

synthases), farnesyl diphosphate (sesquiterpene synthases), or geranylgeranyl diphosphate or copalyl diphosphate (diterpene synthases), and eleven of them catalyze cyclizations of these precursors into a diverse array of terpenes. The exception to these cyclases is the acyclic monoterpene synthase, *S*-linalool synthase (LIS1) from *Clarkia breweri*. Although ten plant families are represented among the cloned genes, the different terpene synthases share many features and are highly conserved. The deduced amino acid sequence of the *G. hirsutum* clone shares with the other cloned terpene synthases 12 invariant residues and three highly conserved regions, in CDN1 they are residues 110 to 182, 192 to 221, and 228 to 349 (Figure 10). LIS1, *ent*-copalyl diphosphate synthase (*ent*-kaurene synthase A, CPS), and *ent*-kaurene synthase (*ent*-kaurene synthase B, KS) are the least conserved among the terpene synthases, which is not surprising given the acyclic product of LIS1 and the two-step conversion of GGPP to *ent*-kaurene via CPP by CPS and KS. Removing these from the alignment, there are 55 invariant residues. Secondary structure predictions of the terpene cyclases indicate the potential for high amounts of alpha helix (47-65%) and low amounts of beta strand (5-12%). This prediction was confirmed by the recent crystal structure of a plant sesquiterpene synthase, 5-*epi*-aristolochene synthase (TEAS), both in the presence and absence of substrate analogs (Starks et al., 1997).

The TEAS crystal structure revealed residues that bind the divalent cation magnesium and ones that interact with the substrate FPP during the catalysis of cyclization. An alignment with CDN1-C (Figure 10) shows nearly all these residues of TEAS to be conserved in CDN1-C. In CDN1-C they are: arg 270, 272, and 448; trp 279; asp 307, 311, 451, and 531; glu 385; thr 407; and tyr 527 and 533. Included in that list are amino acids whose catalytic functions indicated by the crystal structure of TEAS are also needed for the FPP cyclization by CDN1: to pull the diphosphate away from the carbocation intermediate, to stabilize a positive charge on C1, C11, and C7, and to remove a proton from C6. However, also conserved in CDN1-C are an asp 451, tyr 527, asp 531 triad, proposed in TEAS to assist deprotonation of C13 and protonation of the double bond at



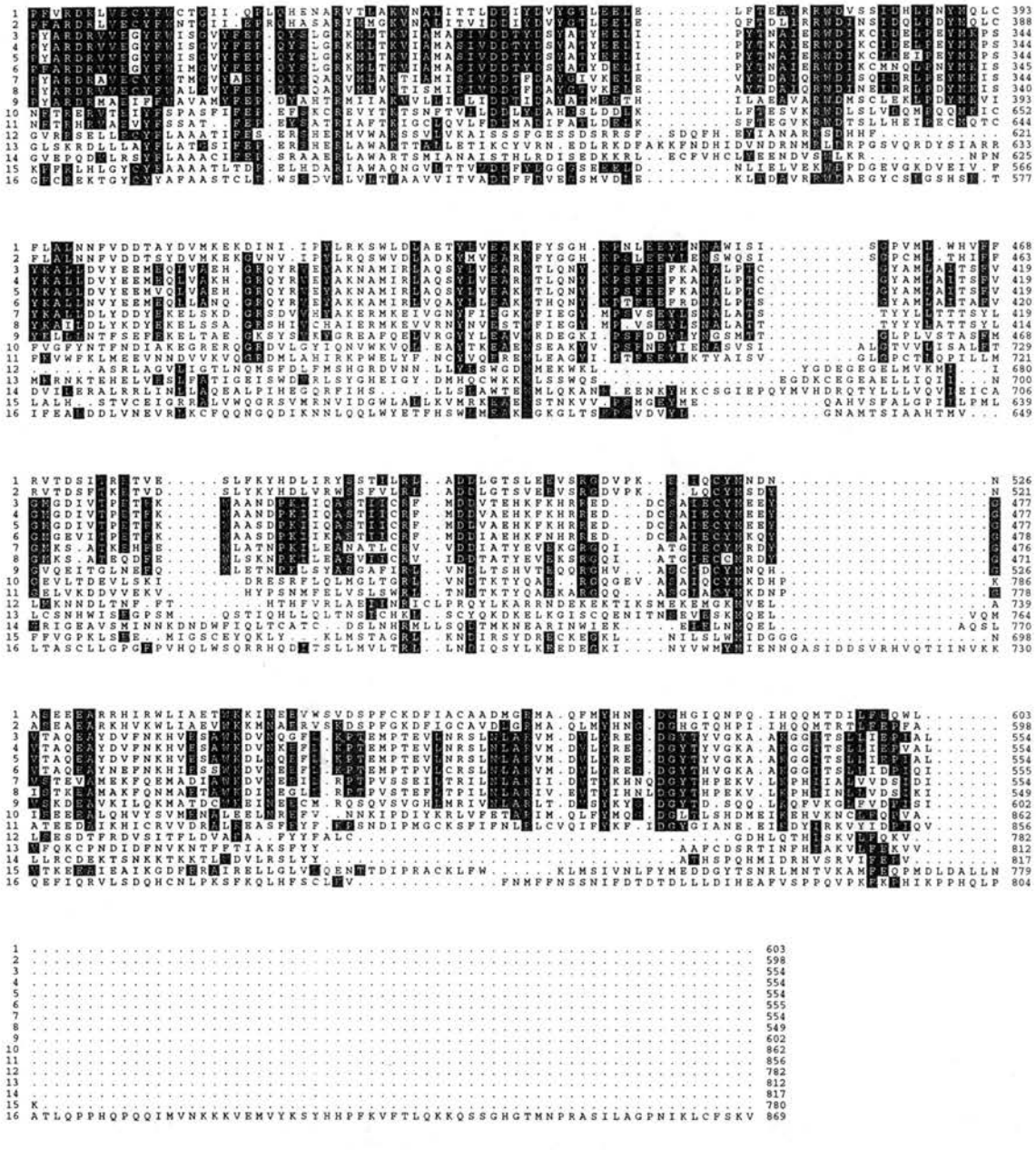


Figure 10. Alignment of the deduced amino acid sequences of plant terpene synthases. 1, *Perilla frutescens* (-)-limonene synthase(Yuba et al., 1996); 2, *Mentha spicata* (-)-limonene synthase (Colby et al., 1993); 3, *G. hirsutum cdn1*-C (Davis et al., 1998); 4, *G. arboreum cdn1*-C14 (Chen et al., 1995); 5, *G. arboreum cdn1*-C1(Chen et al., 1995); 6, *G. arboreum cdn1*-A (Chen et al., 1996); 7, *Hyoscyamus muticus* vetispiradiene synthase (Back, et al., 1995); 8, *Nicotiana tabacum* TEAS (Facchini and Chappell, 1992); 9, *Ricinus communis* casbene synthase(Mau and West, 1994); 10, *Abies grandis* abietadiene synthase (Vogel et al., 1996); 11, *Taxus brevifolia* taxadiene synthase (Wildung and Croteau, 1996); 12, *Arabidopsis thaliana ent*-copalyl diphosphate synthase (Sun and Kamiya, 1994); 13 *Pisum sativum* L. *ent*-copalyl diphosphate synthase (Ait-Ali et al., 1997); 14, *Zea mays ent*-copalyl diphosphate synthase (Bensen et al., 1995); 15, *Cucurbita maxima* L. *ent*-kaurene synthase (Yamaguchi et al., 1996); 16, *Clarkia brewerii* linalool synthase (Dudareva et al., 1996).

C6. These proton movements are not needed during the cyclization to (+)-δ-cadinene if it proceeds through a nerolidyl diphosphate intermediate (Gaydou et al., 1986)(Figure 11). The conservation of the triad is consistent with the deprotonation of C15 to form a germacrene D intermediate, bond rotations to permit C-6, C1 closure and reprotonation of C15 (Arigony, 1975) (Figure 12).

Previous studies using residue-specific inhibitors have also identified arginines, histidines and cysteines as being critical residues for monoterpene cyclase activity, indicating that they are either at the active site or maintain the active tertiary structure (Rajaonarivony et al., 1992; Savage et al., 1995). A recent alignment (Bohlmann et al., 1997) has shown certain arg, his, and cys residues to be highly conserved; these are candidates for the critical residues. An alignment of CDN1-C with other terpene synthases confirms the presence of eleven highly conserved arginines at positions 119, 122, 211, 215, 270, 272, 293, 328, 448, 462, and 521; three highly conserved histidines at positions 88, 124, and 246; and one cysteine within a highly conserved CYM motif. In an alignment of the terpene cyclases with the recently identified monoterpene synthase active-site peptide (McGeady and Croteau, 1995), an LXLYXAS motif, two asp/glu residues, an ala, and a leu (found in CDN1-C at positions 155-161, 168, 172, 174, and 182, respectively) were highly conserved.

H. Features of the deduced amino acid sequence

The deduced amino acid sequence consists of 554 amino acids encoding a protein with a calculated molecular weight of 64,019 daltons and an isoelectric point of 5.3. The N-terminal region is predicted to function as a plastid transit peptide with an estimated certainty of 0.88; however, the cellular location of this enzyme is unknown. Sesquiterpenes are not found in plastids (Kleinig, 1989), though that does not preclude (+)-δ-cadinene synthase from being imported into the chloroplast and functioning as a monoterpene synthase. Four sesquiterpene cyclases are capable of utilizing GPP as a

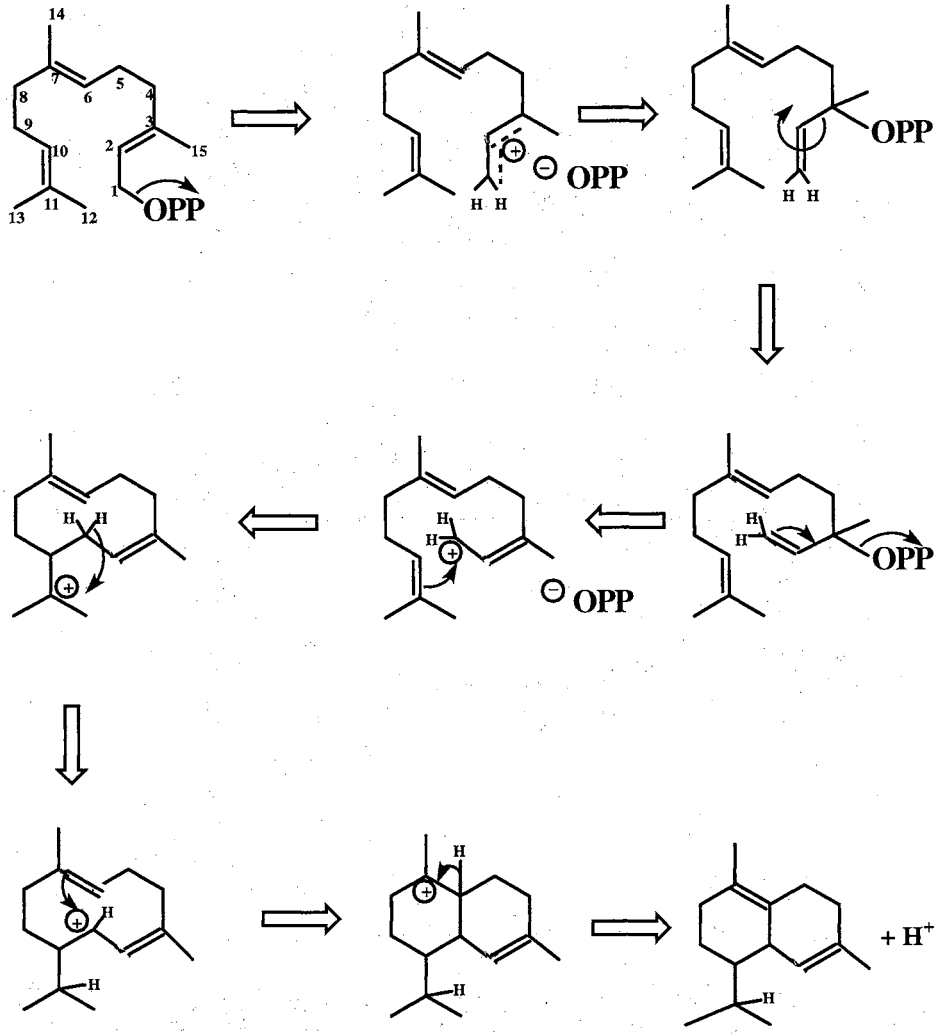


Figure 11. Conversion of farnesyl diphosphate to δ-cadinene through a nerolidyl diphosphate intermediate.

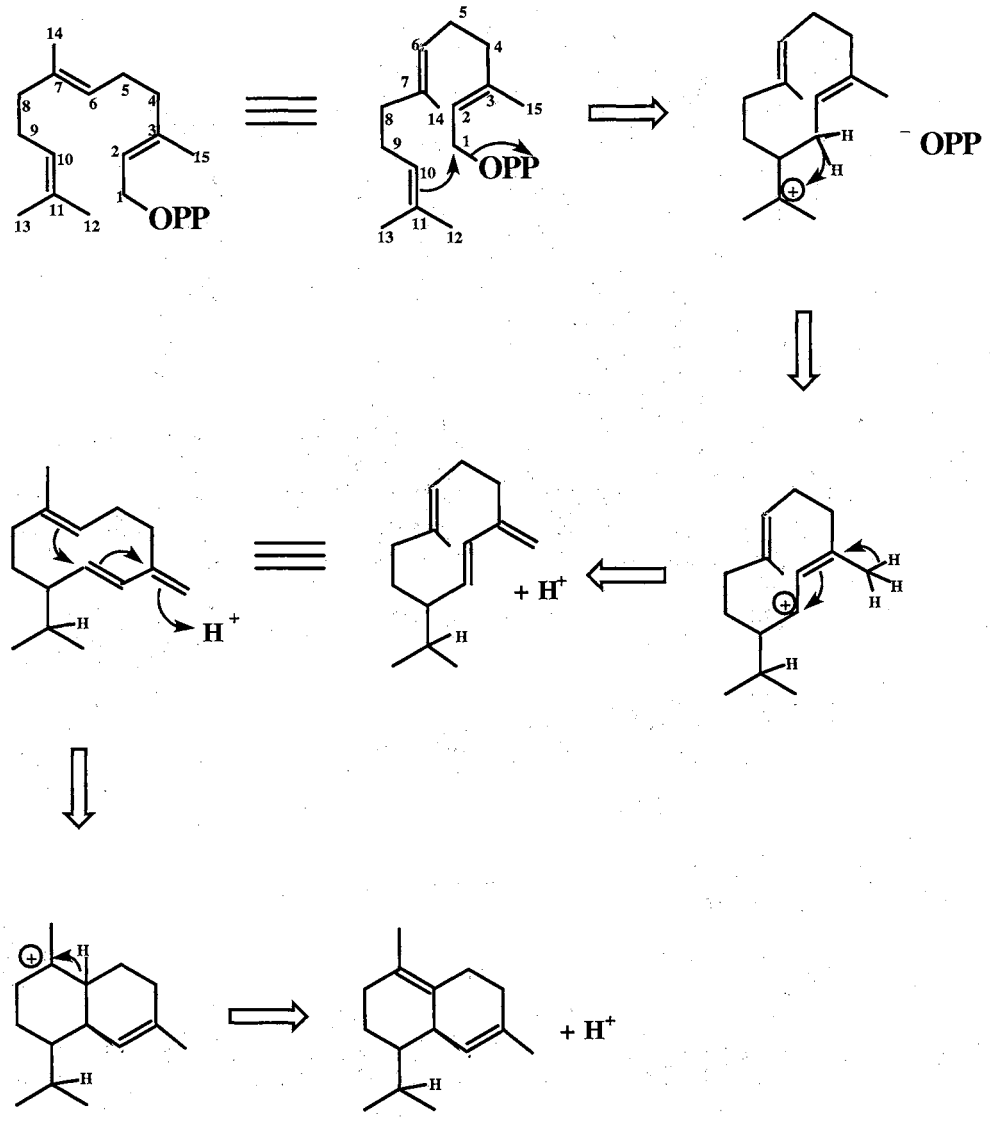


Figure 12. Conversion of farnesyl diphosphate to δ-cadinene through a germacrene D intermediate.

substrate for cyclization (Steele et al., 1998; Colby et al., 1998; Crock et al., 1997) and all produce limonene as the principle product. Secondary structure predictions indicate greater than 60% of the protein is alpha helix with less than 10% beta strand.

I. Materials and methods

Plants and bacteria

The glandless (i.e., lacking gossypol glands) cotton line used in this study, WbM*gl* was developed for us by B. E. Greenhagen and L. M. Verhalen and has high resistance to most races of *Xcm* (Davis and Essenberg, 1995). Plants were grown in a growth chamber as previously described (Pierce et al., 1993). *Xcm* strain 3631 (Pierce et al., 1993) was cultured in nutrient broth, and cotyledons were inoculated *ca.* 7 days after seedling emergence with a suspension of *Xcm* in sterile water saturated with $CaCO_3$ (5.4×10^6 colony-forming units ml^{-1}) as previously described (Essenberg et al., 1982). Mock-inoculated control cotyledons were infiltrated with the sterile $CaCO_3$ solution.

Nucleic acid purification

Cotyledons stored at -70C were ground in $N_2(l)$ to a fine powder in a mortar and immediately transferred to an 80C borate buffer (5ml of buffer per gram fresh weight tissue). RNA was purified exactly as described (Wan and Wilkins, 1994), treated with 5U DNase, phenol/chloroform extracted (Sambrook et al., 1989), ethanol precipitated, and stored as a 70% ethanolic suspension at -70C. The RNA was quantitated by absorbance at 260 nm and quality was evaluated by A_{260}/ A_{280} ratios and by electrophoresis on 1% formaldehyde agarose gel. Genomic DNA was extracted from cotyledons using the method of (Jobes et al., 1995). The DNA was then further precipitated by a 2M NaCl precipitation in ethanol (Fang et al., 1992) and stored as a 70% ethanolic suspension.

Reverse transcription

Five µg of total RNA were reverse transcribed with Superscript reverse transcriptase following the manufacturer's suggested protocol (GIBCO-BRL). Briefly, *ca.* 5 µg RNA, stored at -70C as a 70% ethanolic suspension, was precipitated by centrifugation at 12,000 x *g* for 10 min, dried in a vacuum desiccator for 30 min, resuspended in 12 µl water with 0.5µg oligo dT_{18} or 75 pM of a gene-specific antisense primer and heated to 70C for 10 min, chilled on ice for 2 min, and spun to collect the liquid at the bottom of the tube. Seven µl of the RT mix (final reaction concentrations were: 50 mM Tris, pH=8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 0.5 mM dNTPs) and 200 U reverse transcriptase were added, mixed, and spun to collect the liquid at the bottom of the tube. After a 10-min incubation at 25C, the reaction was continued for 50 min at 42C. The reaction was stopped by an incubation of 15 min at 70C, iced for two min, and spun to collect the liquid at the bottom of the tube. RNA was removed by the addition of 2 U RNase H for 30 min at 37C and the 20-µl reaction was stored at -20C.

Template circularization and circular RACE

Total RNA was reverse transcribed and digested with RNase H as described above. Approximately 0.5 µg cDNA was phosphorylated with T-4 polynucleotide kinase with 2 mM ATP as suggested by the manufacturer (Promega) and incubated at 37C for 90 min. The reaction was precipitated with 0.3 M sodium acetate and 2.5 volumes cold ethanol (absolute) for one hour on ice and spun at 12,000 x *g* for 30 min. The pellet was rinsed with 70% ethanol, dried, and resuspended in 2 µl water. The single stranded cDNA was circularized as described (Murayama et al., 1995) with 1.5 U T-4 RNA ligase, 1X reaction buffer (70 mM Tris, pH=7.6, 10 mM $MgCl_2$, and 5 mM β-mercaptoethanol), 10 µg/ml BSA, 25% PEG-8000, 50 µM ATP, and 1 mM hexamine cobalt chloride and incubated overnight at *ca.* 25C (room temp).

cDNA tailing for 5' RACE

Total RNA was reverse transcribed and digested with RNase H as described. Single-stranded cDNA was precipitated with 0.3 M sodium acetate and 2.5 volumes cold ethanol (absolute) for one hour on ice and spun at 12,000 x *g* for 30 min. The pellet was rinsed with 70% ethanol, dried, and resuspended in 14 µl water. The cDNA was heated at 94C for one min, iced for one min, and spun to collect the liquid at the bottom of the tube. The cDNA was tailed at the 3' end in 1X tailing buffer (0.2 M potassium cacodylate, 2 mM $MnCl_2$, and 0.2 mM DTT), 0.25 mM dCTP, and 100 U terminal transferase and incubated for five min at 30C and terminated by a five min incubation at 65C (Deng and Wu, 1981). The reaction was diluted to 50 µl with 0.2X TE.

Standard PCR

PCR amplifications were done using the MJ, the Thermoline, or the Perkin Elmer thermocycler. Each reaction contained $MgCl_2$, (1.5 mM with all primer combinations except 2033 and 2034 which required 2 mM), dNTPs (0.2 mM), primers (0.8 µM), template (0.5 µL of first-strand cDNA or 0.5 µL of a 10- or 100-fold dilution of a gel purified PCR product in water), and 5 U Taq DNA polymerase (Promega Corp). The amplification program consisted of 95C for 45 sec, 30-40 cycles (94C for 45 sec, 46C for 45 sec, 72C for 1 min), and 72C for 10 min.

Marathon PCR amplification

Marathon PCR was performed as described (Chenchick et al., 1995) with a few modifications. The PCR products were gel purified from a TAE agarose gel (see below) and 8 µl of each was added to a 50 µl PCR mixture containing 1X Taq polymerase buffer A (Promega Corp), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 5 U Taq polymerase and overlaid with mineral oil. The DNA was denatured at 95C for 1 min and amplified with 20 cycles

as follows: 94C for 30 sec and 68C for 20 min. This reaction mixture was diluted 100 fold and reamplified using standard PCR conditions with the 5'- and 3'-specific primers (Table 3, primers 1427 and AUAP, respectively).

DNA purification after agarose gel electrophoresis

Agarose gels were run in 1X TAE or TBE buffer. For fragments greater than 700 bp 1.0% to 1.2% gels were cast with Fisherbrand agarose (low EEO) while for smaller fragments a 2.0%-2.5% gel was cast using Nu-Sieve GTG agarose (FMC Corp). Gels (3 cm x 5 cm) were run until the bromophenol blue had migrated halfway at 50-75 volts, stained with 0.3 µg/ml ethidium bromide, and bands of interest were quickly excised on a UV transilluminator. The bands were weighed and extracted using QIAGEN gel extraction kits (as described by the manufacturer).

Ligations using T-tailed cloning vectors

T-tailed cloning vectors were prepared as described (Hadjeb and Berkowitz, 1996). Ten µg of pBluescript S/K or pSP64 (polyA) (Promega Corp) was digested for at least 12 hours at the appropriate temperature in 50 µl of 1X reaction buffer with EcoRV (pBluescript S/K) or PstI (pSP64 (polyA)) to generate blunt ends followed by inactivation at 70C for 10 min. The volume was brought up to 100 µl and the DNA was incubated at 72C for 15 min with 1 U Taq polymerase and 2 mM dTTP for 3' T-tailing. After phenol/chloroform extraction (Sambrook *et al.*, 1985), the nontailed vector was religated with T-4 DNA ligase, and the successfully T-tailed linear vector was gel purified and extracted using QIAGEN gel extraction kit as per the manufacturers instructions. This was aliquoted and stored at -20C. Ligation reactions were generally prepared with 0.5 µl of the T-tailed vector and 1, 3, and/or 5 µl of a PCR product. The DNA was ligated for at least 12 hours at 14C in 1X ligation buffer with 1U of T-4 DNA ligase (GIBCO-BRL). The ligation reaction was recut with the blunt-end generating enzyme for at least 3 hours at the

appropriate temperature before transformation to reduce the number of religated blunt-end vectors. A vector only control was routinely included to monitor background.

DNA sequencing and oligonucleotide synthesis

PCR products were gel purified as described above from 1-1.2% agarose gels. Plasmids were prepared using a modified PEG/alkaline lysis (Sambrook et al., 1989). DNA (in Tris, pH=8) was quantitated by absorbance at 260nm and between 0.5 and 1 µg was sequenced. Nucleotide sequencing using the dideoxy chain-termination method and oligonucleotide synthesis was done by the Oklahoma State University Recombinant DNA/Protein Resource Facility.

Transformation of *E. coli*

Competent XL1-Blue cells were made by the $CaCl_2$ method (Sambrook et al., 1989). All transformations were performed as recommended by Invitrogen. A 50 µl aliquot of competent cells was thawed on ice and 2 µl of 0.5 M β-mercaptoethanol was added, and the cells were split into two tubes containing 1-4 µl of a ligation mixture, incubated on ice for 30 min, 42C for 30 sec, and ice for 2 min. After addition of 300 µl SOC, the mixture was shaken at *ca.* 225 rpm for 60 min at 37C and plated on LB plates with 50 µg/ml ampicillin (up to 325 µl per plate depending on the efficiency of the transformation).

Identification of positive transformants and insert orientation

Transformants were selected by growth on ampicillin (50 µg/ml) LB agar plates. For clones in pBluescript or PCR2.1, blue/white colony screening was used to distinguish between cells containing empty vectors and cells containing vectors with an insert, respectively. For pSP64 (polyA), plasmids were isolated by a phenol/chloroform extraction of a liquid culture of each potential clone and analyzed on a 1% agarose gel to confirm the presence and size of the plasmid (Akada, 1994). All positive transformants

(distinguished by size) were screened (Costa and Weiner, 1995) by PCR using 0.5 µl of the liquid culture and amplification using two gene-specific primers, then another amplification using vector-specific primer (SP6, M13 universal or reverse, T3, or T7) and a gene-specific primer to determine the orientation of the insert. Positive clones were sequenced.

Overexpression of cloned cDNAs in E. coli

Approximately 50 µl of a glycerol stock of the *cdn1*-C clone was used to inoculate 10 ml of terrific broth (Sambrook et al., 1989) with 50 µg/ml ampicillin and allowed to grow at 37C at 225 rpm. After 9 hours, 5ml of fresh terrific broth with 150 µg/ml ampicillin was added to the turbid culture with IPTG (1 mM final concentration) and permitted to grow for 12 additional hours. The culture was centrifuged in a 50 ml tube for 10 min at 5,000 x *g* at 4C. Pellets were resuspended in 2 ml of assay buffer (30 mM HEPES, pH=7.0, 10%glycerol, 1 mM $MgCl_2$, 5 mM DTT) with 1 mM PMSF and sonicated with a microprobe at a setting of 4.5 for seven 5-10 sec bursts. After centrifugation at 23,500 x*g* for 20 min at 4C the pellet was resuspended in assay buffer with 0.1% Tween 80, and the pellet and supernatant fractions were assayed for sesquiterpene cyclase activity.

Preparation of substrate

(*E,E*)-[1-^{3}H]FPP obtained from DuPont NEN was diluted in sp. act to 32 µCi/µmol (1 nmol/µl) with non-radioactive (*E,E*)-FPP in buffer containing 25 mM $(NH_4)_2CO_3$ (pH 7.0) and 40% glycerol (v/v); this substrate stock solution was stored at –20C. The non-radioactive (*E,E*)-FPP was synthesized according to (Davisson et al., 1986) and purified by chromatography on a combination of anion exchange and reverse phase cartridges by GD Davis.

Sesquiterpene cyclase assay and product identification

The sesquiterpene cyclase assay was performed as described (Davis and Essenberg, 1995) with the following modifications: the assay was allowed to continue for 60 min, the reaction was terminated by the addition of 1.5 ml hexane, mixed with silica, and 300 µl of the hexane was assayed by scintillation counting. The remaining 1.2 ml hexane extract was reduced to 75 µl after addition of 50 µl isopropanol. The entire sample was injected on a 250 X 4.6 mm C_{18} column (Lichrosphere 100) and separated across a 46% to 100% gradient in acetonitrile over 1 hr. The eluate was split (HP splitter model) with 66% assayed by scintillation and 33% detected by absorbance at 215 nm. The chromatographic separation was performed by G. Davila-Huerta.

Sequence alignments, identities, and similarities

The sequences used in alignments and comparisons were: 5-*epi*-aristolochene synthase, L04680; abietadiene synthase, U50768; (+)-δ-cadinene synthase, U23205, U23206, U27535, U88318; casbene synthase, L32134; *ent*-copalyl diphosphate synthase, L37750, U11034, U63652; *ent*-kaurene synthase, U43904; 4*S*-limonene synthase, D49368, L13459; *S*-linalool synthase, U58314; taxadiene synthase, U48796; and vetispiradiene synthase, U20187. Nucleotide sequence comparisons were made using the Basic Local Alignment Search Tool (BLAST) program accessed through the National Center for Biotechnology Information WWW server. Amino acid sequence comparisons and sequence alignments were generated using Bestfit, Pileup, and Prettybox all from the Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin. The Pileup output was carefully examined and gaps were manually added or subtracted to the sequences to optimize the alignment. Protein features were determined using the programs Prosite and Profile Network Prediction of Secondary Structure (PHDsec) accessed through the ExPASy WWW server maintained at the Geneva University Hospital and the University of Geneva, Geneva, Switzerland.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

(+)-δ-Cadinene synthase is the first committed enzyme in the production of broad-spectrum antimicrobial phytoalexins; therefore it is an attractive candidate for further studies regarding the importance of phytoalexins involved in plant-pathogen interactions. This enzyme has been purified to apparent electrophoretic homogeneity from *Xcm*-inoculated cotton foliar tissue and three tryptic peptides have been sequenced. Two clones have been identified from the pathogen-challenged tissue, and one has been completely sequenced and overexpressed in *E. coli* and shown to generate (+)-δ-cadinene.

The purification of this CDN1-C isozyme of (+)-δ-cadinene synthase has allowed production of polyclonal antibodies in chickens. Immunohistochemical studies have been conducted to determine where the cyclase accumulates in relation to *Xcm* and whether the differences between a highly resistant line and a less resistant line can be correlated to the speed of cyclase induction (Park, 1997).

Cotton and tobacco plants have been transformed with antisense and sense constructs of *cdn1*-C and are presently being evaluated to determine the relative effects of constitutive expression of these constructs on disease resistance. This project has the potential for addressing the role of phytoalexins in different cotton-pathogen interactions. Preparative-scale overexpression of the cloned *cdn1*-C is being pursued for the production of large amounts of ^{3}H-(+)-δ-cadinene for use as a substrate in the quest for the next enzyme in the pathway leading to the phytoalexins.

Spatial and temporal expression patterns of *cdn1*-A and *cdn1*-C transcripts are being investigated by northern analysis. Total RNA has been extracted from roots, cotyledons, hypocotyls, and emerging leaves at various time points after *Xcm*-inoculation of the

cotyledons. In addition, RNA has been isolated from the same tissue types 36 hours after infiltration with salicylate or H_2O_2. This experiment may suggest whether either of these chemicals has a role in local or systemic signaling of *cdn1*-A or *cdn1*-C. Preliminary results from this experiment indicate that salicylate does have a role in systemic signaling in cotton.

Future experiments may also involve the transfer of *cdn1* into plants which do not generally produce (+)-δ-cadinene and allow existing enzymes (oxidoreductases and isomerases) to further transform this sesquiterpene olefin into a novel and unique metabolite which may inhibit the growth of pathogens.

There are still many unanswered questions regarding the regulation and control of the two pathways that lead to phytoalexins in cotton. Is the regulation of these pathways at *cdn1* or at another enzyme in the pathway? Why do the two CDN1 isozymes have substantially different pH optima? Perhaps they function in separate intracellular compartments or in different tissues. If *cdn1* transcripts are involved in a systemic response, would exposure of the roots to a root pathogen result in the accumulation of *cdn1* transcript in the cotyledons? What is the significance of the transit peptide-like amino terminus? Is this an indication that these sesquiterpene cyclases evolved from plastidial monoterpene or diterpene cyclases and have retained non-functional transit peptide-like sequences? Or is CDN1 being delivered to the chloroplast? Perhaps it is targeted to the chloroplast and produces defense-related monoterpenes. The production of volatile mono- and sesquiterpenes has been shown in cotton after wounding and perhaps, *cdn1* has a role in this response (Tumlinson and Pare, 1997).

These are all intriguing questions that may provide a lot of fun and interesting answers.

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APPENDIX A

GENBANK ENTRY FOR *cdnl*-C
(Accession number U88318)

```
LOCUS       GHU88318     1851 bp    mRNA            PLN       07-APR-1998

DEFINITION  Gossypium hirsutum (+)-delta-cadinene synthase (cdn1) mRNA,
            complete cds.

ACCESSION   U88318

NID         g1843646

KEYWORDS    .

SOURCE      upland cotton.
  ORGANISM  Gossypium hirsutum
            Eukaryota; Viridiplantae; Charophyta/Embryophyta group;
            Embryophyta; Tracheophyta; seed plants; Magnoliophyta;
            eudicotyledons; Rosidae; Malvales; Malvaceae; Gossypium.

REFERENCE   1  (bases 1 to 1851)
  AUTHORS   Davis,E.M., Chen,Y.-S., Essenberg,M. and Pierce,M.L.
  TITLE     cDNA sequence of a (+)-delta-cadinene synthase gene (Accession No.
            U88318) induced in Gossypium hirsutum L. by bacterial infection
            (PGR98-040)
  JOURNAL   Plant Physiol. 116 (3), 1192 (1998)

REFERENCE   2  (bases 1 to 1851)
  AUTHORS   Davis,E.M., Chen,Y.-S., Essenberg,M. and Pierce,M.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-FEB-1997) Biochemistry & Molecular Biology, Oklahoma
            State University, 246 Noble Research Center, Stillwater, OK
            74078-3035, USA

FEATURES             Location/Qualifiers
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```

BASE COUNT 592 a 332 c 387 g 540 t

ORIGIN

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//
```

APPENDIX B: PROTOCOLS

A. Introduction

Where possible the original source for a protocol is given. An invaluable resource for troubleshooting methods can be found at the following site: bionet.molbio.methds-reagnts. Basic solution recipes can be found in (Sambrook et al., 1989) andwill not be given here. If recipes are different or not found in this manual, they will be specified.

B. Preparation of competent cells (from a Dr. U. K. Melcher protocol which originated in (Sambrook et al., 1989)).

1. Cultures were grown *ca.* 4 to 6 hours from a small scoop of ice from a previous competent cell batch with the appropriate antibiotic in 20 mL of LB.
2. When turbid ($A_{600} < 0.3$) the culture is put on ice for 10 min in a sterile 50 ml oakridge tube.
3. Spin at low speed (1,500 x *g*) in the Sorvall SS-34 for 10 min at 4C.
4. The supernatant is decanted as completely and quickly as possible, the last drops by tapping the tube upside down on a Kim-wipe, until the loose pellet slides toward the rim.
5. The pellet is gently resuspended in 10 ml 0.1 M cold $CaCl_2$ and stored on ice for 20 min.
6. Spin again as in 3.
7. Resuspend in 1.2 ml 0.1 M $CaCl_2$ and add to 164 µl prechilled glycerol in a 1.5 ml microfuge tube (warm glycerol is easier to pipet). Vortex to mix.
8. Aliquot 50 µL into microfuge tubes and either put directly on dry ice or on ice until transport to the -70C freezer.

C. Transformation of *E. coli* (From the Stratagene instructions accompanying the TA cloning kit)

1. Thaw competent cells on ice.
2. Add 2 µL 0.5 M β-mercaptoethanol and mix by stirring with the pipet tip.
3. Split the contents equally into two tubes and add to each tube the appropriate amount of the ligation mixture (generally, 1-5 µL of a PCR mixture).
4. Ice for 30 min, heat shock at 42C for 30 sec, and ice for 2 min.
5. Add 300 µL room temperature SOC to each tube and shake at 225 rpm at 37C for 60 min. During this time add X-gal if necessary and dry LB plates in the transfer hood for 20 to 50 min depending upon how wet they are.
6. Chill cells on ice briefly and spread the entire contents (or if the cells are fresh and the ligation was efficient add less) on a plate and grow overnight at 37 C.

D. Identification of positive transformants: Quick method (adapted from UKM and (Akada, 1994))

1. If blue/white colony selection is available select white colonies if not select colonies of different size and morphology and circle and number them on the back of the plate for later identification and backup.
2. Grow cultures from selected colonies for *ca.* 8 hours in 5 mL TB (with antibiotic).
3. Pipet 200 µL of culture into a sterile microfuge tube containing 200 µL of 30% glycerol:water and store at -70C (this is the glycerol stock).
4. Add 100 µL of culture to 50 µL phenol:chloroform:isoamyl alcohol (25:24:1v/v at pH=8) and 20 µL of loading buffer. Vortex for 30 sec and spin for 2 min. Load 10-30 µL on a 1% TBE agarose gel.
5. See (Akada, 1994) for an example result after staining with ethidium bromide.

NOTE: It is of considerable help in selecting the correct size plasmid if there is a supercoiled empty vector run as a reference.

E. Identification of positive transformants: Slow method

This method has the advantage of providing a purified plasmid at the completion.

1. If blue/white colony selection is available select white colonies if not select colonies of different size and morphology and circle and number them on the back of the plate for later identification and backup.
2. Grow cultures from selected colonies for about 8 hours in 5 mL TB (with antibiotic).
3. Pipet 200 µL into a sterile microfuge tube containing 200 µL of 30% glycerol:water and store at -70C (this is the glycerol stock).
4. Pellet 3-4.5 mL culture in one microfuge tube by repeated centrifugations.
5. Purify plasmid using the method of choice (QIAGEN columns are fast and reliable).

NOTE: A quick and cheap method to recycle QIAGEN columns (either from gel extraction or from the mini-plasmid kits) is as follows:

a. Add 1 ml Nanopure H_2O to a used column, leave for at least an hour and spin for 1 min.

b. Pour off the flow through and add another 1 ml H_2O and spin for 1 min.

c. Repeat b. once more, add 1mL 100% EtOH and repeat.

d. Dry for 2 hours to overnight at 70-80C. Columns are ready for use

e. Follow the recommended protocol from the kit. One column has been reused two times with no difference as determined by analytical agarose gel electrophoresis. Side by side plasmids were purified and sequenced with no detectable difference in the quality of sequence data.

6. Electrophorese plasmids on an agarose gel for size selection. Plasmids of the right size can be PCR amplified using gene-specific primers or sequenced to determine the insert identity.

F. Typical PCR amplification

1. Make a master mix (MM) in a microfuge tube as follows:
   624 µL nanopure H_2O
   6.8 µL 25 mM dNTPs
   51 µL $MgCl_2$
   80 µL 10X buffer

2. Add 22 µL of MM to a 0.5 µL microfuge tube and add 1 µL each from a 20 µM primer stock.

4. Add a small amount of template (usually without quantitating, 0.5 µL of a 1:10 dilution of a gel-purified PCR product or plasmid prep).

5. Mix and quick spin to collect contents at the bottom and take at room temp to the thermocycler.

6. Start your favorite program and put tubes in when the temperature reaches 95C. The polymerase can be added anytime but specificity is better if it is added when the temperature is greater than the annealing temperature.

Sample program (written as input for the MJ Thermocycler):

Cycle No.	Temperature	Time
1 (melt)	95	45 sec
2 (melt)	94	45 sec
3 (anneal)	46-55	45 sec
4 (extend)	72	1-2 min
5 GOTO 2 30-40 times		
6 (extend)	72	10 min
7 (storage)	4	00 min
8 END		

NOTE: My experience has been to choose a low annealing temperature and high cycle number regardless of the recommendations when trying a new primer-template combination. If no product or a smear of products is produced then it is advisable to alter the $MgCl_2$ concentrations (usually a range of 1, 2, 3, and 4 mM is adequate). Factors that increase the specificity of a PCR reaction are less template, higher annealing temperature, less $MgCl_2$, less dNTP, and less primers. Usually it is adequate to alter only the annealing temperature, cycle number, and amount of template to obtain the desired results. One can also speed up the technique by shortening the melt, anneal, and elongation times and by increasing the annealing temperature.

G. Generation of single-stranded DNA probes for northern analysis (Hannon et al., 1993)

1. Dilute primers to 20 μM with H_2O. For sense primer phosphorylation heat 44 μL of the primer to 70C for 10 min then chill on ice. Add ATP to 2 mM, 5 μL 10X, and 1.5U polynucleotide kinase to the primer and incubate at 37C for 60 min then 70C for 10 min.
2. Make a mixture of 1 mM dNTP as follows:
   5 μL each of 10 mM dATP, dCTP and dGTP
   2.14 μL of 10 mM dTTP
   17.5 μL 1 mM DIG-dUTP
   15 μL H_2O
3. PCR amplify (see note below) with a final dNTP concentration of 0.2 mM. Use a 2 min elongation time per 300-600 bp product.
4. Run a preparative agarose gel (2% Nusieve GTG for a 300-800 bp fragment) by loading the entire product in as few lanes as possible. After running the gel about halfway at 50-60V, stain the gel 10 min with ethidium bromide (0.3μg/mL), and quickly excise the product from the gel and put it into a preweighed microfuge tube.
5. Follow the recommended protocol for the QIAGEN gel extraction kit (using a fresh column) except to be sure to elute in 50 μL of 10 mM Tris, pH=8-8.5.
6. Concentrate the eluate so the total volume is 50 μL. Remove 6 μL for gel analysis. Add 5 μL 10X λ-exonuclease buffer and 1.5 U λ-exonuclease. Incubate for the predetermined time (see note below) and stop the reaction at 70C for 10 min.
7. Chill on ice and spin to collect the liquid at the bottom of the tube.
8. Add entire contents of reaction to a Sephadex G-50 column and spin for two minutes at 2,000 x *g*. The eluate contains the probe and is ready for use and should be stored at -20C.

9. The yield of DIG-labeled probe can be determined following the protocol suggested by the manufacturer (Genius System User's Guide for Membrane Hybridization Version 3.0: 33-37).

NOTE: The DIG-dUTP (alkali labile) substrate was purchased from Boehringer Mannheim. Initial optimization of this protocol can be done using dTTP instead of DIG-dUTP (expensive). The yield of PCR amplification should be maximized. λ-Exonuclease will preferentially digests 5' phosphorylated DNA; however, it can also digest nonphosphorylated DNA. Therefore, it is critical to determinethe minimum time for the λ–exonuclease digestion necessary for generation of a single-stranded DNA (5-20 min). Agarose gel electrophoresis can be used to detect the conversion of double-stranded DNA to single-stranded DNA, though ethidium bromide stains the single-stranded DNA much less efficiently.

H. Preparation of nucleic acid desalting columns (Sambrook et al., 1989)

1. Rinse about 5 g of Sephadex G-50 (medium) in a 2.0-l beaker with several changes of sterile nanopure H_2O (DEPC-treated for RNA probes).
2. Put media into 50 ml falcon tube (media should occupy less than 20 mL) and rinse several times with sterile TEN (DEPC-treated for RNA probes).
3. During the rinses, prepare the columns as follows:
   a. Push the bottoms out of used QIAGEN quick spin columns with the thin end of a p10 pipet tip.
   b. Throw away the inserts but keep the small o-ring.
   c. For RNA probes soak the column and insert in DEPC-H_2O.
   d. Insert a small amount of glass wool (baked at 180C overnight) and pack it down lightly.
   e. Using the big end of a p10 pipet tip, push the o-ring firmly down to the bottom.
   f. Place the column into an empty 1.5 ml microfuge tube.
4. With a p1000 draw out the media suspended in TEN and fill the columns.
5. Spin the columns in a microfuge for 4 min at 1,600 rpm.

6. Add more media until the column is nearly full.
7. Rinse the columns several times with TEN, and spin as in 5.
8. Store at 4C until ready for use. Autoclaved if the columns will be stored for more than one week.

I. RNA purification (Taken with minor modifications from (Hartson, 1991; Chomczynski et al., 1987)).

1. Plant part(s) were frozen in N_2(l) and ground to a powder.
2. The ground tissue (still in a N_2(l) bath) was poured into oakridge centrifuge tubes (precooled in N_2(l)).
3. As the N_2(l) just begins to evaporate, add one volume buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH=7, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) and vortex vigorously.
4. Quickly add 1 volume H_2O-saturated phenol, 0.2 volumes chloroform:isoamyl alcohol (24:1 v/v), and 0.1 volume of 2 M sodium accetate (pH=4) and vortex vigorously.
5. Incubate on ice for 20 min. Centrifuge the tubes and collect the aqueous phase.
6. Add one volume of isopropanol to the aqueous phase and incubate at –20C for 60 min.
7. Pellet RNA by centrifugation at 12,000 x *g* for 30 min. Pour off the supernatant fraction and rinse the pellet with 70% EtOH.
8. Resuspend the moist pellet in 200 µl TE-SDS (10 mM Tris, pH=7, 1 mM EDTA, 0.1% SDS) and heat to 70C for 10 min, cool on ice for 15 min and spin at 12,000 x *g* for 10 min.
9. Add 1/9 volume of 3 M sodium acetate, pH=6 and spin for 10 min at 12,000 x *g*. Add 2.5 volumes of EtOH to the supernatant fraction and incubate at –20C for at least 1 hour.

10. Pellet the RNA by centrifugation, rinse the pellet with 70% EtOH (this suspension can be stored for later use). Dry the pellet in a desiccator for 10-15 min, don't overdry just evaporate the EtOH.
11. Resuspend the pellet in water or TE and quantitate.

J. Preparing RNA formaldehyde gels (Sambrook et al., 1989)

1. Clean the gel box, comb, tray, and stir bars and soak overnight in fresh DEPC-H_2O.
2. Remove the water and shake out remaining drops.
3. Dissolve 0.48 g agarose in 28.5 ml DEPC-H_2O in the microwave (swirl often and be sure agarose is completely dissolved). After dissolution heat until boiling.
4. Working in the hood, add 7 ml deionized formaldehyde and swirl quickly then add 5ml 10X MOPS and swirl. Cast the gel as soon as bubbles from swirling are gone.
5. When the gel solidifies remove comb and put the casting tray into the electrophoresis box with 1X MOPS buffer (with stir bars at each end) and put into 4C until ready for use.

NOTE: Gel preparation and electrophoresis should be carried out in a fume hood.

K. Preparing RNA samples for electrophoresis (Sambrook et al., 1989)

1. Quantitate RNA using quartz cuvettes at 260 and 280 nm. Calculate the A_{260}/ A_{280} ratio (it should be close to 2.0). Subtract the blank A_{260} and A_{280} from the sample readings. Determine the quantities using 40 µg/ml = 1.0 (A_{260}). If the ratio is less than 2.0, there is interference from phenol or proteins. A ratio of 1.7 means that there is about half as much RNA as predicted by the A_{260} (an estimate).

2. Prepare a premix as follows:

number of samples	10X (µl)	formaldehyde (µl)	formamide (µl)
8	18	31.5	90
12	26	45.5	130
16	34	59.5	170
24	50	87.5	250

3. To each sample (5-10 µg RNA) add 15.5 µl premix and water to bring volumes up to 20 µl, heat samples at 70C for 20-30 min, put samples on ice and add 3 µl loading buffer.

4. Load the lanes and run gel at 40V. Turn on the stir bars after samples have migrated into the gel.

5. Stop electrophoresis when samples have migrated about halfway.

L. Blotting by capillary transfer (Sambrook et al., 1989)

1 Stain gel for 10-15 min with 0.4 µg/ml ethidium bromide in DEPC-H_2O.

2. Destain briefly in DEPC-H_2O (10 min).

3. Wipe the transilluminator with RNase Away (Invitrogen). Photograph gel quickly on the UV transilluminator, save the image being sure that none of the bands are saturated (click the view saturation button on the computer screen).

4. If samples are visually degraded or if any are underloaded or overloaded, adjust and rerun the gel.

5. Fill a reservoir with 20X SSC (lid to pipet box) and put a raised small box inside.

6. Place two wet 3MM sheets over the raised box and down into the 20X SSC.

7. Put the gel face down onto these sheets and remove lanes if they stick out too high.

8. Put a mask of parafilm around the gel to prevent a short circuit of buffer flow.

9. Place Hybond N+ (Amersham Inc) on the gel and avoid bubbles.

10. Place 3 X 3MM sheets on top of the membrane.

11. Place a stack of paper towels on top of this (2-3 inches).
12. Place a plate on top with a 1 kg weight on this (temp block).
13. Leave overnight with an occasional check on the reservoir level (i.e. before going home and first thing in the morning).
14. Restain gel if desired to monitor the efficiency of transfer.
15. View the membrane on the transilluminator, face down after cleaning the glass.
16. UV crosslink at 1,200 μJ/sec with the membrane face up (if the lights are above).
17. Store membrane dry and in the dark until ready to use.

M. Northern hybridization (Engler-Blum et al., 1993)

1. Prehybridize (0.25 M Na_2HPO_4 pH=7.2, 1 mM EDTA, 20% SDS) for 3 hours at 68C.
2. Hybridize (dilute probe in prehybridization solution) overnight at 68C.
3. Wash three times (in 20 mM Na_2HPO_4, pH=7.2, 1 mM EDTA, 1% SDS) at 68C for 20 min.
4. Wash for 5 min (0.1 M maleic acid, pH=8, 3M NaCl, 0.3% Tween-20).
5. Block for 2 hours (maleic acid buffer from step 4 and 0.5% blocking reagent (GIBCO-BRL).
6. Incubate with antibody (diluted 1:15,000 in block buffer) for 30 min.
7. Wash four times 10 min with maleic acid buffer from step 4.
8. Incubate for 5 min in detection buffer (0.1 M Tris-HCl, pH=9.5 and 0.1 M NaCl).
9. Remove excees liquid from membranes and drop CSPD onto membranes (until wet) between cellulose acetate sheets (precleaned with 70% EtOH).
10. Incubate for 10 min and remove excess CSPD by wicking with paper towels.
11. Incubate at 37C for 4 hours in the dark with the edges sealed (can be with the film if signal is low).
12. Place film against an acetate sheet for 10 min, then adjust exposure.

N. Sesquiterpene cyclase assays (modification of (Davis and Essenberg, 1995))

1. For consistency, maintain all samples in the same conditions for the same amount of time, this is especially important if the extract was frozen and thawed. A loss in the enzymatic activity of a crude extract after thawing is restored after a *ca.* 3-5 hour incubation on ice.
2. Dilute 1 µg or less of protein extract in a total volume of 245 µl assay buffer (30 mM HEPES pH=7, 10% glycerol, 1 mM $MgCl_2$, 5 mM DTT) in a 13 mm x 100 mm test tube. Generally, less than 10 µl of a crude extract is sufficient.
3. Warm the assay mixture to 30C for 5 min.
4. Add 5 µl of the diluted 1-^{3}H-FPP to each tube and vortex with a lid for 3 sec. Add the FPP to a different tube every 30 sec continuing until all tubes have substrate added.
5. Incubate the assay mixture for a set time (15 min will allow 30 samples to be assayed).
6. At the end of the incubation time, stop the reaction by adding 3 ml of 100% hexane with a p5000 (use the same tip but only if it does not contact the inside of the tube) and vortex carefully for 12 sec with a lid. The hexane should be added sequentially every 30 sec.
7. Remove as much of the hexane as possible (upper layer) with a pasteur pipet and add it to another tube containing a small capful of silica (*ca.* 0.1 mg) and vortex with a lid for 12 sec. Continue with all tubes using a fresh pasteur pipet for each sample.
8. Remove 1 ml of the hexane from each tube and add it to 5 ml of scintillation fluid, cap the tube, and shake vigorously. Use any of the programs available on the scintillation counter designated for counting tritium.

NOTE: Use extra care and precautions when handling tritium. All waste goes in a special bag and any spills should be cleaned immediately and swipe tests performed until background levels are obtained. Although vortexing samples was used routinely, one should be extremely careful and consider the hazards involved. It may be advisable to mix with a disposable pasteur pipet and to perform the assays under the fume hood, to avoid vapors (δ-cadinene and hexane are very volatile). Protective clothing and eyewear is advised.

O. Bradford protein assays (Bradford, 1976)

1. Label enough 13 x 100 mm test tubes to assay all samples plus five standards.
2. Because this assay is sensitive to many buffer additives and detergents (check the BioRad catalog for a complete listing), it is advisable to insure that each tube, including the standards has the same concentration of each component found in all samples. For instance, if you add 100 µl of your most dilute protein, then all other tubes should receive an equivalent amount of buffer. If samples are in different buffers then an attempt should be made to compensate so that all tubes have the same concentration of buffer components.
3. Dilute protein standard (BSA) to 20 µg/ml and add 0, 100, 200, 300, 400 µl to five tubes for the standard curve (giving 0, 2, 4, 6, and 8 µg/ml final concentration). Make twice the volume of the 0 µg/ml standard for use as a blank.
4. Add the sample and the necessary buffer components and bring the volume up to 800 µl with water.
5. Add 200 µl of the Bradford reagent (BioRad) and vortex each tube for 10 sec.
6. Leave samples for at least 15 min but less than 60 min and read absorbance at 595 nm.

NOTE: The standards should range from straw colored (0 µg/ml) to blue (8 µg/ml). If any samples fall visibly outside of this color range, new tubes should quickly be prepared before spectrophotometric analysis. The samples and standards should be made at nearly the same time. The absorbance should be greater than 0.07 for all samples containing protein otherwise the reproducibility will suffer.

VITA

Edward M. Davis

Candidate for the Degree of

Doctor of Philosophy

Dissertation: PURIFICATION OF THE NATIVE ENZYME AND CLONING AND CHARACTERIZATION OF A cDNA FOR (+)-δ-CADINENE SYNTHASE FROM BACTERIA-INOCULATED COTTON FOLIAR TISSUE

Major Field: Biochemistry and Molecular Biology

Biographical:

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