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THE ENZYMATIC CYCLIZATION OF NEROLIDYL DIPHOSPHATE BY δ-CADINENE SYNTHASE FROM COTTON STELE TISSUE INFECTED WITH *VERTICILLIUM DAHLIAE*

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Key Word Index—*Gossypium barbadense*; malvaceae; cotton; enzymology; sesquiterpenoid phytoalexins; nerolidyl diphosphate; δ-cadinene; δ-cadinene synthase mRNA.

Abstract—Soluble preparations of cotton stele tissue infected with *Verticillium dahliae* containing δ-cadinene synthase convert (1-*RS*)-[1-^{2}H]-*E*,*E*-farnesyl diphosphate to [5-^{2}H]- and [11-^{2}H]-δ-cadinene and convert [4,4,13,13,13-$^2H_5$]-nerolidyl diphosphate to [8,8,15,15,15-$^2H_5$]-δ-cadinene. These data imply that nerolidyl diphosphate is an intermediate in the enzymatic cyclization of the natural substrate *E*,*E*-farnesyl diphosphate to δ-cadinene by δ-cadinene synthase and involves the conversion of *E*,*E*-farnesyl diphosphate to nerolidyl diphosphate followed by cyclization to *cis*-germacradienyl cation, a 1,3-hydride shift, a second cyclization to a cadinanyl cation and deprotonation to δ-cadinene. Kinetic analyses of induced δ-cadinene synthase mRNA, δ-cadinene synthase activity and formation of sesquiterpenoid phytoalexins in cotton stele tissue infected with *Verticillium dahliae* show that 12 hr after fungal inoculation the δ-cadinene synthase mRNA was at a maximum level. The tissue injected with H_2O in place of fungal inoculation showed no detectable δ-cadinene synthase mRNA or δ-cadinene synthase activity after 12 to 96 hr. After 12 hr, 54% of the δ-cadinene synthase activity had developed, but no phytoalexins were detected, the midpoint in the formation of the phytoalexins was 48 hr. These data, together with the enzyme analyses, support the conclusion that *Verticillium dahliae* initiates a signal in the stele tissue that results in an increased steady-state level of δ-cadinene synthase mRNA and an increased activity of δ-cadinene synthase which functions in the conversion of *E*,*E*-farnesyl diphosphate → nerolidyl diphosphate → δ-cadinene that is metabolically converted to desoxyhemigossypol, desoxyhemigossypol-6-methyl ether, hemigossypol and hemigossypol-6-methyl ether.

INTRODUCTION

Infection of the *Gossypium barbadense* cotton cultivar Seabrook Sea Island 12B2 (SBSI) with *Verticillium dahliae* induces the formation of a cadalene group of phytoalexins: desoxyhemigossypol (dHG), desoxyhemigossypol-6-methyl ether (dMHG), hemigossypol (HG) and hemigossypol-6-methyl ether (MHG) [1, 2]. Gossypol (G), a dimer of HG, is formed in the vegetative tissues of Seabrook Sea Island but is not induced by infection with *V. dahliae*. 2,7-Dihydroxycadalene (DHC) is induced by *Xanthomonas campestris* pv *malvacearum* in *G. hirsutum* cotton cotyledons [3].

Biosynthetic studies with intact cotton cotyledons and cotton roots have demonstrated that the formation of these cadalenes from acetate or mevalonate involves the isomerization of *E*,*E*-farnesyl diophosphate (FDP) to *Z*,*E*-FDP (or an equivalent nerolidyl diphosphate) followed by cyclization to 10-membered ring cations and an associated hydride shift [4, 5, 6, 7]. Enzyme studies with δ-cadinene synthase isolated from SBSI cotton stems infected with *V. dahliae* demonstrated the formation of [5-^{2}H]- and [11-^{2}H]-δ-cadinene from (1-*RS*)-[1-^{2}H]-(*E*,*E*)-FDP [8]. The labelling of δ-cadinene can be accounted for by the formation of a *cis*-germacradienyl cation (a 10-membered ring intermediate), a 1,3-hydride shift, a second cyclization and a deprotonation to form δ-cadinene.

In this paper we present evidence showing that δ-cadinene synthase utilizes [4,4,13,13,13-$^2H_5$]-nerolidyl diphosphate (NDP) to form [8,8,15,15,15-$^2H_5$]-δ-cad-

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inene indicating an enzymatic mechanism that involves a conversion of *E,E*-FDP to NDP followed by cyclization to *cis*-germacradienyl and to cadinanyl cations. This mechanism is consistent with the enzymatic formation of the hydroxylated cadalene, epicubenol, by epicubenol synthase from *Streptomyces* [9, 10] and liverworts [11] which requires an initial isomerization of *E,E*-FDP to NDP prior to cyclization of *cis*-germacradienyl cation. The evidence that NDP can serve as a substrate and is probably an intermediate in this terpene cyclization adds to similar evidence for tertiary allylic diphosphate intermediates in terpene cyclizations [12, 13].

Northern blots of *V. dahliae* treated diploid *Gossypium arboreum* tissue cultures indicate increased levels of δ-cadinene synthase mRNA [14]. Tetraploid *G. hirsutum* cotton cotyledons inoculated with *X. campestris* show an induction of δ-cadinene synthase activity [15] and convert δ-cadinene to DHC and other structurally related terpenoids [16]. In this paper we have attempted to correlate δ-cadinene synthase mRNA levels with enzyme activities and sesquiterpenoid phytoalexin accumulations in a *G. hirsutum* stele tissue infected with *V. dahliae*.

RESULTS AND DISCUSSION

A soluble extract of cotton stele tissue infected with *V. dahliae* that had high δ-cadinene synthase activity and converted (1-*RS*)-[1-^{2}H]-*E,E*)-FDP to [5-^{2}H]-and [11-^{2}H]-δ-cadinene, was incubated with [$^2H_5$]-NDP to determine the utilization of NDP by δ-cadinene synthase. The deuterated hydrocarbons enzymatically synthesized in the reaction mixtures were extracted into hexane–ethyl acetate (3:1), collected between 17.0 and 18.0 min. from HPLC columns and analysed by GC mass spectrometry. The GC separation resulted in one significant peak with a R_t of 52.45 minutes. The major ions and the relative intensities from the mass spectrum of this hydrocarbon are given in the Experimental section. The mass spectrum agreed with that reported for δ-cadinene [17]. There was no deuterated carophyllene or humulene detected in the hydrocarbon fraction and it is probable that the major sesquiterpene cyclase that was induced by *V. dahliae* in the infected stele tissue was δ-cadinene synthase.

A proposed fragmentation scheme which accounts for the major peaks in the mass spectrum of the deuterated δ-cadinene is shown in Fig. 1. A single ion at *m/z* 209 accounts for the formation of [8,8,15,15,15-$^2H_5$]-δ-cadinene. The loss of a methyl group from the isopropyl side chain produces the ion at *m/z* 194. The peak at *m/z* 166 results from the loss of the isopropyl side-chain. A reverse Diels–Alder reaction provides the peak at *m/z* 139. A significant peak at *m/z* 134 is observed in naturally occurring δ-cadinene and this peak has been used to distinguish δ-cadinene from other cadinenes [17]. The loss of a methyl group from *m/z* 139 provides the *m/z* 124. This fragmentation pattern supports the conclusion that δ-cadinene synthase utilizes [4,4,13,13,13-$^2H_5$]-NDP to catalyse the formation of [8,8,15,15,15-$^2H_5$]-δ-cadinene. It is to be noted that there was no loss in the deuterium from the methyl carbon (carbon-13) of NDP in the formation of the δ-cadinene. The formation of [8,8,15,15-$^2H_4$]-δ-cadinene would have indicated germacrene D as an intermediate in the formation of δ-cadinene from *E,E*-FDP as suggested by Arigoni [18]. These data agree with previous work of Davis and Essenberg [16] who detected no ^{3}H-germacrene D during the conversion of ^{3}H-δ-cadinene to DHC in cotton cotyledons infected with *X. campestris*.

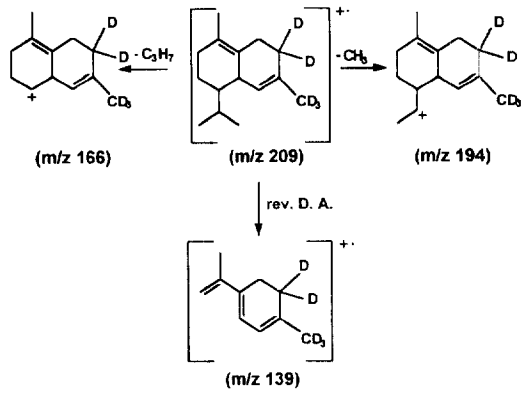

Fig. 1. A proposed fragmentation scheme for [8,8,15,15,15-$^2H_5$]-δ-cadinene.

The proposed pathway (Fig. 2) for the formation of δ-cadinene from [4,4,13,13,13-$^2H_5$]-NDP includes the formation of *cis*-germacradienyl cations, a 1,3-hydride shift, formation of a cadinanyl cation and deprotonation to [8,8,15,15,15-$^2H_5$]-δ-cadinene. The enzymatic formation of *cis*-germacradienyl cations from NDP by δ-cadinene synthase is similar to the reaction catalysed by epicubenol synthase involving cyclization of NDP to *cis*-germacradienyl cation [9, 10, 11]. Following the formation of the cadinanyl cation the mechanism for the formation of the epicubenol and the δ-cadinene are different.

An alternate pathway, suggested by a reviewer, accounting for the formation of [8,8,15,15,15-$^2H_5$]-δ-cadinene from the cyclization of [4,4,13,13,13-$^2H_5$]-NDP by δ-cadinene synthase is presented in Fig. 3. The first cyclization would form a bisabolyl cation which following a 1,3-hydride shift and second cyclization would form a cadinanyl cation which following two more 1,3-hydride shifts and a deprotonation would be converted to [8,8,15,15,15-$^2H_5$]-δ-cadinene. This proposed pathway would form a bisabolyl cation from the first cyclization similar to the cyclization of NDP to bisabolyl cation catalysed by trichodiene synthase [9]. It is also important to note that [5-^{2}H]- and [11-^{2}H]-δ-cadinene would be formed from (1-*RS*)-[1-^{2}H]-*E,E*)-FDP by this alternate pathway. This alternative is a more complicated mechanism and is inconsistent with the hydride shift patterns shown for 7-hydroxycalamenene synthesis in *Heterosciphus planus* [19] and for (+)cubenene synthesis in *Streptomyces*

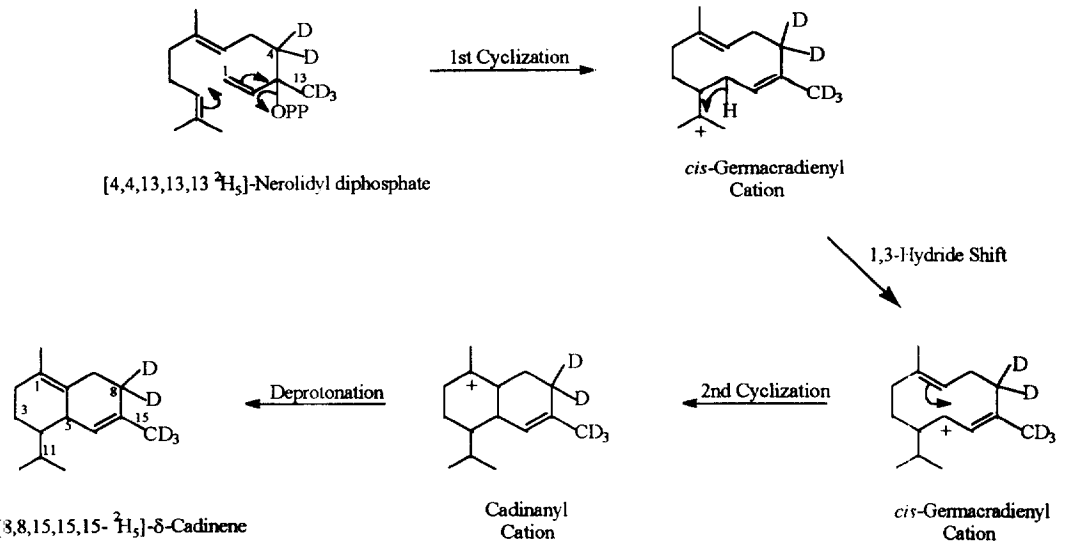

Fig. 2. A proposed reaction scheme for the enzymatic formation of [8,8,15,15,15-$^2H_5$]-δ-cadinene from [4,4,13,13,13-$^2H_5$]-NDP by δ-cadinene synthase in cotton stele extracts.

[9] and is mechanistically unreasonable for epicubenol synthesis [9, 11]. At the present time there is no evidence excluding it for δ-cadinene synthesis in cotton.

The kinetic analyses of the induction of δ-cadinene synthase mRNA, δ-cadinene synthase activity, dHG, dMHG, HG and MHG by *V. dahliae* in cotton stele tissue is shown in Fig. 4. Twelve hours after inoculation of the tissue with *V. dahliae*, the δ-cadinene synthase mRNA was already at or near its peak level and the synthase activity was 54% of its total activity at 48 hr. Separate experiments have demonstrated that the δ-cadinene synthase mRNA was barely detectable in Northern blots of the cotton stele tissue from 12 to 96 hr following the injection of H_2O in place of the fungal inoculation and that no δ-cadinene synthase activity or dHG, dMHG, HG, or MHG was detect-

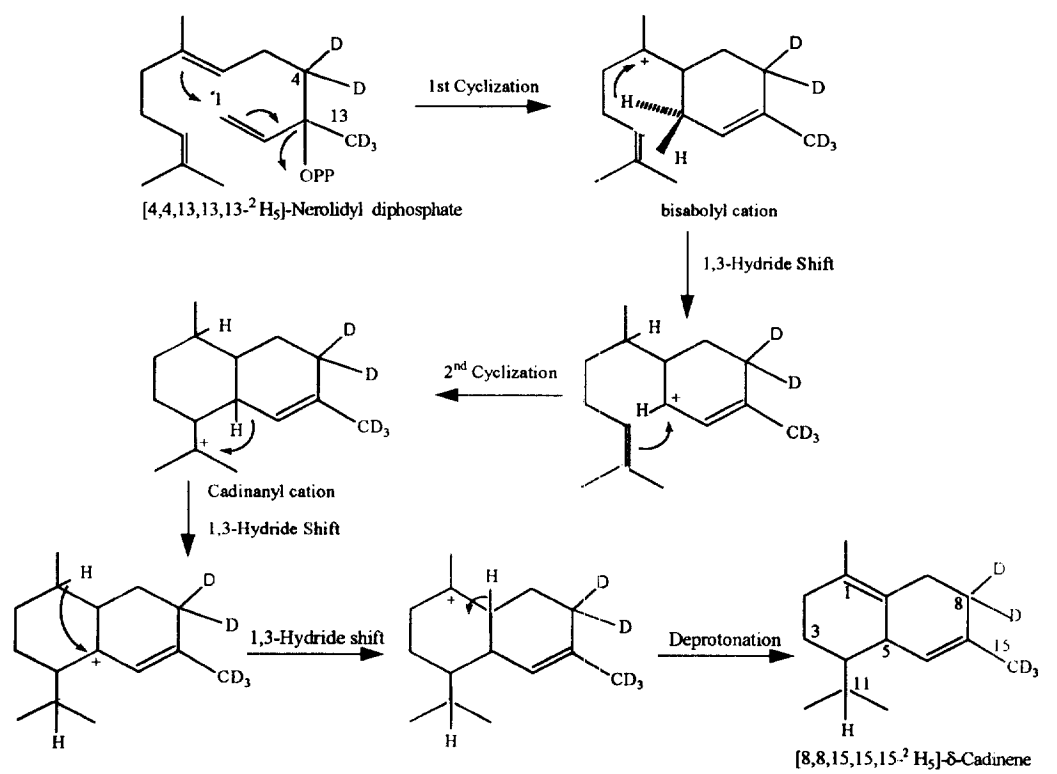

Fig. 3. An alternate reaction scheme for the enzymatic formation of [8,8,15,15,15-$^2H_5$]-δ-cadinene from [4,4,13,13,13-$^2H_5$]-NDP by δ-cadinene synthase in cotton stele extracts.

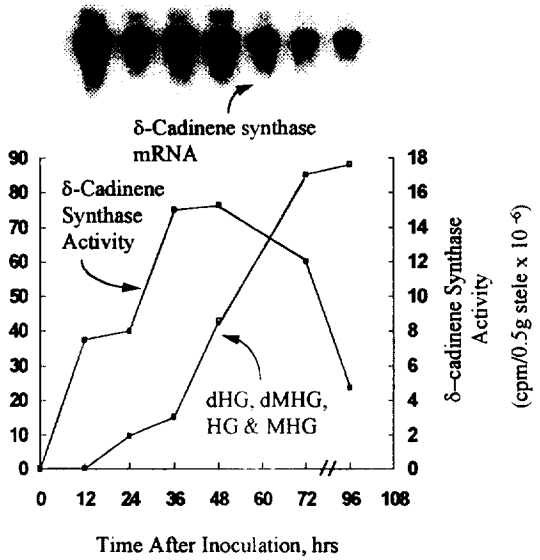

Fig. 4. The kinetic analyses of the induction of δ-cadinene synthase mRNA, δ-cadinene synthase activity and total phytoalexins [deoxyhemigossypol (dHG), hemigossypol (HG), deoxyhemigossypol-6-methyl ether (dMHG) and hemigossypol-6-methyl ether (MHG)] in cotton stele tissue (SBSL) inoculated with conidia of *Verticillium dahliae*.

able in the H_2O inoculated tissue. The δ-cadinene synthase activity peaks at 48–60 hr after fungal inoculation. After 48 hr the formation of dHG, dMHG, HG, and MHG has reached a midpoint. The kinetic analysis and enzyme data support the conclusion that *V. dahliae* initiates a signal in the cotton stele tissue which leads to an increase in the steady-state level of δ-cadinene synthase mRNA and δ-cadinene synthase activity which functions in the enzymatic conversion of *E,E*-FDP → NDP → δ-cadinene that is subsequently converted to dHG, dMHG, HG, and MHG. These data demonstrating the elicitation of the steady-state level of δ-cadinene synthase mRNA followed by increase in δ-cadinene synthase activity and phytoalexin formation are important for experiments designed to regulate the pathway: *E,E*-FDP → NDP → δ-cadinene → dHG → HG → G by antisense constructs of δ-cadinene synthase gene.

It is important to relate these findings to reported results of others on the same enzymatic reaction and its determining genes in cotton. Chen *et al.* [14, 20] have cloned, sequenced and entered in Genebank three genes for (+)-δ-cadinene synthase which they called cad1-A, cad1-C1, and cad1-C14. These genes have since been renamed cdn1-A, cdn1-C1, and cdn1-C14. The Northern blots in Fig. 4 with the cdn1-C1 probe would probably detect mRNA of cdn1-C1 and cdn1-A. Chen *et al.* [14] have demonstrated that treatment of diploid *G. arboreum* tissue culture with *V. dahliae* initiates a signal resulting in an increased level of mRNA for δ-cadinene synthase. Davis *et al.* [15] reported a time course of the induction of δ-cadinene synthase in intact *G. hirsutum* cotton cotyledons infiltrated with *X. Campestris*. Davis *et al.* [16] have also reported that the infiltration of radioactive δ-cadinene into intact cotyledons labels dHG, HG, and DHC. These data demonstrate that in different species of cotton and different tissues of cotton that elicitors generated by fungal and bacterial infection induce δ-cadinene synthase mRNA and δ-cadinene synthase activity. The results in Fig. 4 correlate the kinetics of induction of δ-cadinene synthase mRNA, δ-cadinene synthase activity and sesquiterpenoid phytoalexins in a tetraploid *G. hirsutum* cotton stele tissue. These events may be used to model the regulation of the gossypol pathway.

EXPERIMENTAL

Chemicals. Geranylacetone was purchased from Aldrich. Deuterium oxide (99.9 atom % D) was obtained from Cambridge Isotope Laboratory. Deuterated MeOH (99.5 atom % D) was purchased from Sigma Chemical Company. Sodium deuteroxide (40 wt. % soln in deuterium oxide, >99 atom % D) was obtained from E. Merck Laboratories, Inc.

Radiochemical. [1-^{3}H]-Farnesyl diphosphate (FDP), 832.5 Gbq mmol^{-1} was purchased from New England Nuclear.

Instrumentation. ^{1}H (300 MHz), ^{13}C (75 MHz) and ^{31}P (121 MHz) NMR spectra were recorded on a Bruker ARX-300 instrument in the specified deuterated solvents with residual $CHCl_3$ as int. standard unless otherwise specified.

Plant material. Cotton (*Gossypium barbadense* cv Seabrook Sea Island 12B2) seeds were germinated in paper rolls at 30° for 48 hr and then transplanted into greenhouse soil mix in 16 ounce plastic cups. The seedlings were grown in the greenhouse to the 6–8 true leaf stage, transplanted to gallon containers and placed in an environmental growth chamber programmed for a 14 hr day and 10 hr night at 27°. The plants were equilibrated in the environmental chamber 1 week prior to inoculation with *Verticillium dahliae*.

Inoculum preparation. Verticillium dahliae defoliating strain V-76 isolated from cotton plants grown in Sonora, Mexico was grown on potato dextrose agar plates at room temperature (*ca* 22°). The agar plates were inoculated by spreading conidia over the agar surface and the fungus was allowed to grow for 3 to 4 days before the conidia were washed from the plates with sterile H_2O. The conidia were washed in H_2O, centrifuged, and diluted to an absorbance of 0.5 at 600 nm. This suspension was used as an inoculum for the cotton plants.

Inoculation of the plants. A 20 μl droplet of inoculum was placed at each of 3 locations equally spaced around the stem 1/4″ below the cotyledons. A puncture wound was made through the droplets with a 23-gauge needle so that the inoculum was taken up by the xylem vessels.

Preparation of δ-cadinene synthase. Three days after inoculation, the plants were removed from the

environmental chambers and the first internode was excised. The bark was removed, and 1 gm of the stele tissue was ground to a powder in a mortar in liquid N_2. The powder was further ground in 8 ml of 0.1 M Tris–HCl buffer (pH 7.5) containing 5 mM GSH, 0.1% Triton X-100 and 5% insoluble PVP. The homogenate was centrifuged in a Beckman J2-21 refrigerated centrifuge at 10000 rpm for 20 min and the supernatant fr. was centrifuged in a Beckman L8-55M Ultracentrifuge at 100000 *g* for 60 min. The soluble supernatant fr. was used as the source of the δ-cadinene synthase.

Assay of δ-cadinene synthase. The reaction mixt. for the enzymatic cyclization of [^{3}H]-(*E,E*)-FDP to [^{3}H]-δ-cadinene by δ-cadinene synthase contained: 200 μl of soluble enzyme, 20 μl 0.1 M GSH pH 7.5, 40 μl 0.2 M KF, 20 μl [1-^{3}H]-(*E,E*)-FDP containing 0.45 nmol and 22.6×10^6 dpm of radioactivity in a total volume of 300 μl. The reaction mixt. was incubated for 20 min at 30° and the reaction was stopped by extracting the aq. fr. with 2.0 ml hexane–EtOAc (3:1). The aq. fr. was extracted × 2 with 2.0 ml hexane–EtOAc and the extracts combined. A 200 μl aliquot of the hexane–EtOAc extracts was assayed for radioactivity in a Beckman Liquid Scintillation Spectrometer.

To verify that the stele extracts contained δ-cadinene synthase an aliquot of the 100000 *g* supernatant fr. was incubated with (1-*RS*)-[1-^{2}H]-(*E,E*)-FDP as previously described [8]. The reaction mixts were incubated for 1 hr at 30°. The reactions were stopped with the addition of 2.0 ml of hexane–EtOAc (3:1). The aq. phase in each reaction tube was vigorously extracted with this solvent. The organic phase was then removed and the extraction repeated twice. The organic phases were combined and evapd to dryness under a stream of N_2 and the residue dissolved in 20 μl EtOAc and 60 μl of Me_2CO–0.1% ascorbic acid (9:1). A 25 μl sample of the [^{2}H]-hydrocarbon was sepd on a 250 × 4.6 mm Scientific Glass Engineering MOS-hypersil-1 C-8 column at a column temp. of 40° with a flow rate of 1.25 ml min^{-1} using a Hewlett Packard 1090 HPLC equipped with a photodiode array detector. A linear MeOH–H_2O gradient containing 0.07% phosphoric acid was used with an initial ratio of 1:4 progressing to 7:3 over 7 min, to 4:1 over the next 5 min and to 9:1 over the next 7 min and to 100% MeOH over the last 4 min. The [^{2}H]-hydrocarbon eluting between 17.0 and 18.0 min was collected and the eluent evapd to dryness under a stream of N_2. The residue was dissolved in 10 μl of hexane–EtOAc (3:1) and subjected to GC-MS analysis. The mass spectrum of the [^{2}H]-hydrocarbon showed ions at *m/z* (%) 206 (15), 205 (84), 190 (29), 163 (21), 162 (100), 161 (90), 160 (10), 159 (12), 135 (58), 134 (58), 133 (15), 131 (11), 129 (11), 120 (48), 119 (51), 118 (13), 117 (13), 115 (12), 106 (31), 105 (52) which identifies it as [5-^{2}H]- and [11-^{2}H]-δ-cadinene. These data verify that the major sesquiterpene cyclase present in the infected stele tissue is δ-cadinene synthase. The same soluble supernatant fr. used to verify the presence of δ-cadinene synthase was used to determine the utilization of [$^2H_5$]-NDP by δ-cadinene synthase.

(1,1,3,3,3-$^2H_5$)-(E)-6,10-*Dimethyl*-5,9-*undecadien*-2-*one* (*pentadeuterogeranylacetone*). Geranylacetone was deuterated by repeated exchange reaction in a basic solvent mixt. of 40% NaOD–CH_3OD–D_2O (1:2500:500) to provide the pentadeuterogeranylacetone. The residual H content is less than 5% as measured by NMR and GC-MS.

(3RS)-(4,4,13,13,13-$^2H_5$)-(E)-3,7,11-*Trimethyl*-1,6,10-*dodecatrien*-3-*ol*(*pentadeuteronerolidol*). Vinylmagnesium bromide (23 ml, 0.023 mol, 1 M soln in THF) was dissolved in 100 ml of dry THF, and pentadeuterogeranylacetone (4.0 g, 0.02 mol) dissolved in 25 ml of dry THF was added dropwise under N_2 at room temp. over a 30 min period. The reaction mixt. was stirred at room temp. for an additional 30 min. The reaction was terminated by the addition of satd ammonium chloride (2.6 ml). The reaction mixt. was dried by addition of anhydrous Na_2SO_4. The solvent was evapd by rotary evapn under diminished pressure and the resulting residue was dissolved in a solvent mixt. of EtOAc–hexane (1:1). After washing with distilled H_2O and brine, the soln was concd to give the pentadeuteronerolidol (4.4 g, 96%) as a pale yellow liquid. ^{1}H NMR ($CDCl_3$), δ 5.89 (1H, *dd*, $J_{1,2(trans)} = 17.4$ Hz, $J_{1,2(cis)} = 10.7$ Hz, H2), 5.18 (1H, *dd*, $J_{gem} = 1.4$ Hz, H1 (*trans*)), 5.12 (1H, *qt*, $J_{5,6} = 7.1$ Hz, $J_{6,14} = 1.3$ Hz, H6), 5.06 (1H, *sept.t*, $J_{9,10} = 8.2$ Hz, $J_{10,12} = 1.3$ Hz, $J_{10,15} = 1.3$ Hz, H10), 5.04 (1H, *dd*, H1 (*cis*)), 1.93–2.05 (6H, *m*, H5, H8, H9), 1.66 (3H, *br s*, H12), 1.58 (3H, *br s*, H14), 1.58 (3H, *br s*, H15).

(3RS)-(4,4,13,13,13-$^2H_5$)-(E)-3,7,11-*Trimethyl*-1,6,10-*dodecatrien*-3-*yl triammonium diphosphate* ((3RS)-(4,4,13,13,13-$^2H_5$)-*Nerolidyl Diphosphate*, [$^2H_5$]-*NDP*). Pentadeutero-NDP was prepd from pentadeuteronerolidol according to the method of Popjak *et al.* [21]. The procedure of Holloway and Popjak [22] was used to purify the [$^2H_5$]-NDP. ^{1}H NMR (D_2O–ND_4OD, pH 8.5, DOH signal is taken as 4.84 ppm), δ 6.11 (1H, *dd*, $J_{1,2(trans)} = 17.4$ Hz, $J_{1,2(cis)} = 10.8$ Hz, H2), 5.25 (1H, *dd*, $J_{gem} = 1.1$ Hz, H1 (*trans*)), 5.14–5.26 (2H, *m*, H6, H10), 5.15 (1H, *dd*, H1 (*cis*)), 2.01–2.17 (6H, *m*, H5, H8, H9), 1.70 (3H, *s*, H12), 1.63 (6H, *s*, H14, 15), 1.63 (3H, *s*, H15); ^{13}C NMR [D_2O–ND_4OD, pH 8, CD_3OD (int.) signal is taken as 49.0 ppm], δ 143.4 (C2), 137.4 (C7), 134.4 (C11), 125.6 (C6), 125.4 (C10), 114.3 (C1), 83.2 (C3), 39.7 (C8), 26.7 (C9), 25.7 (C12), 23.0 (C5) 17.9 (C15), 16.1 (C14); ^{31}P NMR (D_2O, H_3PO_4 as external standard at 0.00 ppm), δ −7.30 (*d*, $J_{P,P} = 20.3$ Hz, external P), −13.37 (*d*, internal P).

The formation of [^2H]-*δ-cadinene from* (3RS)-(4,4,13,13,13-$^2H_5$)-*Nerolidyl diphosphate by δ-cadinene synthase.* The following reaction mixt. was used to measure the enzymatic incorporation of [$^2H_5$]-NDP into deuterated δ-cadinene: 200 μl of soluble δ-cadinene synthase, 20 μl 0.1 M GSH pH 7.5, 20 μl 0.1 M $MgCl_2$, 40 μl 0.2 M KF and 20 μl 1 mM (3*RS*)-

[4,4,13,13,13-$^2H_5$]-NDP. Forty individual reactions were incubated at 30° for 20 min. The reactions were stopped by repeatedly extracting the individual aq. reaction mixts each with 2.0 ml hexane–EtOAc (3:1). The hexane–EtOAc fr. was removed and the extraction repeated with an additional 2.0 ml hexane–EtOAc. The extracts were combined and evapd to 30–50 μl. A 25 μl sample of the deuterated-hydrocarbon was sepd on a 250 × 4.6 mm Scientific Glass Engineering MOS-Hypersil-1 C-8 column (5μ) at a column temperature of 40° and a flow rate of 1.25 ml min^{-1} in a Waters HPLC with 990 photodiode array detector with a linear MeOH–H_2O gradient containing 0.07% phosphoric acid as described previously. The column eluate was collected at 1 min intervals between 1 and 12 min and every 15 sec from 12 to 26 min. The deuterated δ-cadinene was eluted from the column between 17.0 to 18.0 min. These frs were pooled and extracted with hexane–EtOAc (3:1). The organic phase was evapd to 25 μl and analysed by GC-MS.

The deuterated δ-cadinene isolated from the reaction mixts was identified using a Hewlett-Packard 5971A GC-Mass Spectrometer equipped with a split-/splitless injector and a DB-1 fused-silica capillary column (60 × 0.32 mm) (0.25 μm film thickness). The linear velocity was 30 cm sec^{-1} at 150°. Three μl of deuterated sample was injected manually. The following temp. regime was followed: 40°, hold 5 min; to 100° at 3.5°C min^{-1} hold 5 min; to 150° at 2.0° min^{-1} hold 5 min; to 250° at 5.0°C min^{-1} hold for 15 min (total run time 110.47 min). Other than background peaks only one significant peak was observed at 52.45 min. The mass spectrum of the deuterated δ-cadinene showed ions at *m/z* (%) 209 (55), 194 (9.8), 167 (31), 166 (100), 139 (62), 124 (25), 121 (19), 119 (19), 113 (30), 112 (68).

Determination of induced phytoalexins in cotton stele tissue infected with V. dahliae. The HPLC analysis of the induced photoalexins in cotton stele tissue infected with *V. dahliae* over a period of 12 to 96 hr was as follows: the first internode of the stems was excised from the plant, the bark peeled off and the stele tissue cut into small sections with a razor blade. Randomly selected stele sections (yielding 150–350 mg fr. wt) were weighed and a mixt. of Me_2CO: 1% aq. ascorbic acid (9:1) was added to give a ratio of 3 μl of extractant per mg of stele tissue. The samples were held at 4° for 24 hr after which the extractant was removed and placed in a sealed vial. A 50 μl aliquot of the extracted phytoalexins was sepd on a 250 × 4.6 mm Keystone Scientific MOS Hypersil-1 C8 column at a temp. of 40° with a linear gradient of MeOH–H_2O containing 0.07% phosphoric acid at a flow rate of 1.25 ml min^{-1} in a Hewlett Packard Model 1090 HPLC equipped with a photodiode array detector as described previously

Samples of dHG, dMHG, HG and MHG were purified from cotton plants and the identity of each phytoalexin proven by UV, IR, NMR and MS spectroscopy. Standard curves for different amounts of each phytoalexin were constructed by calculating the area of the elution profile for known quantities of dHG, dMHG, HG and MHG. The amount of each phytoalexin induced in the stele tissue with *V. dahliae* was calcd from the standard curves relating to elution profile area to phytoalexin concentration and expressed as μg phytoalexin g^{-1} stele tissue.

RNA extraction and northern blotting. The amount of δ-cadinene synthase mRNA induced in the cotton stele tissue infected with *V. dahliae* was determined over a period of 12 to 96 hr. Total cellular RNA was isolated from 1 g of fresh cotton stele tissue immediately frozen in liquid N_2 using an Rneasy® plant total RNA kit (catalogue number 74904) developed by Qiagen, Inc [23]. *A* at 260 and 280 nm was used to determine purity and concn of RNA. Aliquots containing 4 μg of RNA for denaturing gel electrophoresis were used to quantify specific mRNAs in each sample [24] and 4 μg RNA samples were loaded into the wells of a 1.2% agarose gel for electrophoresis at 35 V. Gels were then blotted onto Zeta-Probe GT membranes following the alkaline Northern blotting protocol recommended by BIO-RAD [25]. The δ-cadinene synthase PCAD-C1 probe now renamed to cdn1-C1 probe (a gift from Dr Peter Heinstein, Purdue University) was cut from the Blue Script plasmid vector with the restriction enzyme BamH1 and XhoL1 and was labelled by the random prime method [26]. Hybridization followed the standard procedure recommended by BIO-RAD [25]. After the final post hybridization wash, the blots were exposed to Kodak XAR5 film at −80°. The cdn1-C1 probe will also probably detect mRNA of cdn1-A as well as mRNA of cdn1-C1.

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