Characterization of Novel Sesquiterpenoid Biosynthesis in Tobacco Expressing a Fungal Sesquiterpene Synthase[1]

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The gene encoding trichodiene synthase (*Tri5*), a sesquiterpene synthase from the fungus *Fusarium sporotrichioides*, was used to transform tobacco (*Nicotiana tabacum*). Trichodiene was the sole sesquiterpene synthase product in enzyme reaction mixtures derived from unelicited transformant cell-suspension cultures, and both trichodiene and 5-epi-aristolochene were observed as reaction products following elicitor treatment. Immunoblot analysis of protein extracts revealed the presence of trichodiene synthase only in transformant cell lines producing trichodiene. In vivo labeling with [³H]mevalonate revealed the presence of a novel trichodiene metabolite, 15-hydroxytrichodiene, that accumulated in the transformant cell-suspension cultures. In a trichodiene-producing transformant, the level of 15-hydroxytrichodiene accumulation increased after elicitor treatment. In vivo labeling with [¹⁴C]acetate showed *that the biosynthetic rate of trichodiene and 15-hydroxytrichodiene* also increased after elicitor treatment. Incorporation of radioactivity from [¹⁴C]acetate into capsidiol was reduced following elicitor treatment of a trichodiene-producing transformant as compared with wild type. These results demonstrate that sesquiterpenoid accumulation resulting from the constitutive expression of a foreign sesquiterpene synthase is responsive to elicitation and that the farnesyl pyrophosphate present in elicited cells can be utilized by a foreign sesquiterpene synthase to produce high levels of novel sesquiterpenoids.

Sesquiterpenoids are thought to be prominent in plant-fungal interactions. Members of this structurally diverse group of cyclic terpenoids appear to function both as defensive compounds in plants and as virulence factors in fungal plant pathogens. The best characterized examples of this include the accumulation of antimicrobial sesquiterpenoids in tobacco (*Nicotiana tabacum*) and other Solanaceae following exposure to fungi or fungal cell-wall fragments (Vögeli and Chappell, 1988; Zook and Kuc, 1991) and the production of phytotoxic sesquiterpenoids by fungal plant pathogens that enhance their virulence on certain plant hosts (Proctor et al., 1995). Sesquiterpenoids also frequently occur as components of plant essential oils and contribute to the flavor and fragrance of some agricultural products (Templeton, 1969).

The first unique step in sesquiterpenoid biosynthesis involves a group of enzymes known as sesquiterpene synthases. These enzymes catalyze cyclization reactions involving farnesyl diphosphate that yield an estimated 200 different products (Croteau and Cane, 1985). Subsequent steps in sesquiterpenoid pathways modify sesquiterpene synthase products and generate thousands of compounds that exhibit diverse bioactivities. In plants these modification steps frequently involve various oxygenation reactions, some of which are catalyzed by Cyt P450-type monooxygenases (Karp et al., 1987; Funk et al., 1994). Sesquiterpene cyclases from fungi (Hohn and VanMiddlesworth, 1986; Proctor and Hohn, 1993), plants (Dehal and Croteau, 1988; Vögeli et al., 1990), and actinomycetes (Cane et al., 1994) have been purified and characterized. The gene coding for trichodiene synthase has been cloned from several different fungi (Hohn and Beremand, 1989; Hohn and Desjardins, 1992; Proctor et al., 1995). Trichodiene is the parent compound for the family of cytotoxic sesquiterpenoids known as trichothecenes (Zamir et al., 1989). It is interesting that trichothecenes appear to belong to the small group of fungal toxins that are produced by both plants and fungi. Trichothecenes accumulate to high levels in two species of the plant genus *Baccharis* (Jarvis, 1991) and may function as growth regulators (Kuti and Jarvis, 1992) in addition to their possible role as defensive compounds.

Capsidiol is a fungitoxic sesquiterpenoid (Fig. 1) that is produced in tobacco cell-suspension cultures in response to specific elicitors (Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988). Studies of capsidiol biosynthesis have revealed that biosynthetic steps beyond 3-hydroxy-3-methylglutaryl-CoA reductase may also play important roles in regulating sesquiterpene biosynthesis. The activities of two enzymes that utilize farnesyl diphosphate, 5-epi- aristolochene synthase and squalene synthase, have been observed to change immediately following the induction of capsidiol biosynthesis by elicitor treatment. There is an increase in 5-epi-aristolochene synthase activity and rapid decreases in the activity of squalene synthase (Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988). A similar type of regulation involving sesquiterpenoid phytoalexins also appears to occur in potato tuber tissue after elicitation (Zook and Kuc, 1991).

Abbreviation: FPP, farnesyl PPi.

Acetyl CoA

↓

HMG CoA

↓

Mevalonate

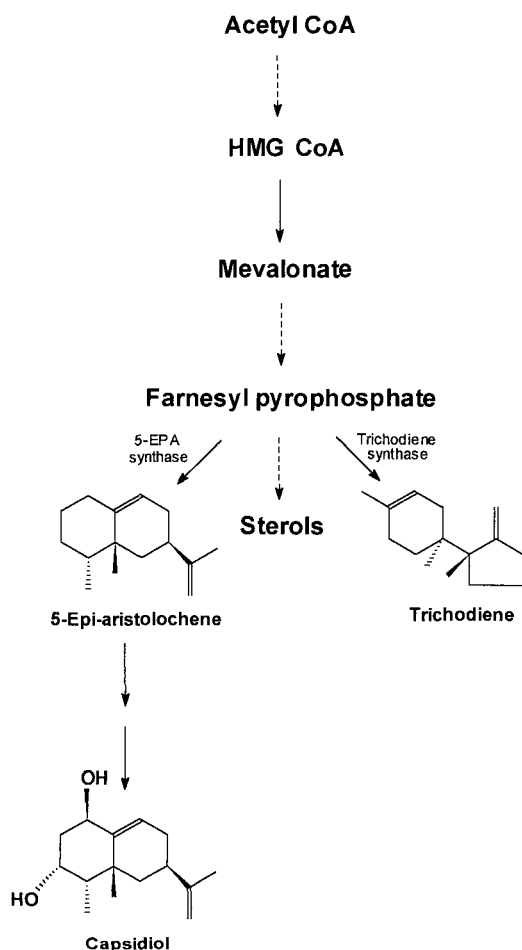↓

Farnesyl pyrophosphate

Figure 1. Abbreviated diagram of the isoprenoid pathway responsible for the synthesis of capsidiol, 5-epi-aristolochene (5-EP A), and trichodiene. HMG, 3-Hydroxy-3-methylglutaryl-CoA.

To investigate the heterologous expression of sesquiterpene synthase genes in plants a fungal gene for trichodiene synthase was recently introduced into tobacco (Hohn and Ohlrogge, 1991). Expression of trichodiene synthase was regulated by the cauliflower mosaic virus 35S promoter, and leaves from the transgenic plants were shown to contain trichodiene synthase polypeptide and active enzyme. In this paper we describe the expression of trichodiene synthase in derivative tobacco cell-suspension cultures. We report here that expression of the trichodiene synthase gene results in the accumulation of both trichodiene and a monooxygenated trichodiene metabolite, 15-hydroxytrichodiene.

MATERIALS AND METHODS

Tobacco (*Nicotiana tabacum* cv Petite Havana) cells were grown in a liquid medium of Murashige-Skoog salts, Gamborg's B-5 vitamin mixture, 3% (w/v) Suc, 1 mg L^{-1} Mes (pH 5.80), 1 mg L^{-1} 2,4-D, and 50 μg mL^{-1} kanamycin. Cell cultures were subcultured on a weekly basis. Tobacco cells were used for experiments 3 to 4 d after subculturing.

Cell-suspension cultures were generated from callus cultures grown on the same medium described above with 0.7% agar. The callus cultures were generated from sterilized seed from third-generation individual cell lines of transgenic tobacco plants.

Elicitor Treatment

Cell cultures were treated with cellulase R10 (1.0 μg mL^{-1} cells; Karlan Research Products, Santa Rosa, CA) from a 10 mg mL^{-1} stock solution in H_2O.

Sesquiterpene Synthase Assay

Cells (20 mL) were harvested 16 h after treatment with cellulase. The cells were suction-filtered from the liquid medium using a single sheet of premoistened Miracloth (Calbiochem) and homogenized in 2 mL of buffer containing 100 mM potassium phosphate (pH 7.0), 10 mM sodium *meta*-bisulfite, 10 mM sodium ascorbate, 10 mM magnesium chloride, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol, and 0.5 g of polyvinylpolypyrrolidone. After homogenization, 0.5 g of methanol-washed Amberlite XAD-4 (Sigma) was added, the suspension was mixed gently, and the slurry was then allowed to stand on ice for 3 min. The homogenate was filtered through premoistened Miracloth and centrifuged at 13,500g for 15 min. A 10-μL aliquot of the supernatant (10–15 μg of protein) was assayed for sesquiterpene synthase activity. The volume of the reaction mixture (120 mM potassium phosphate buffer [pH 7.0], 2.0 mM sodium *meta*-bisulfite, 2.0 mM sodium ascorbate, 12.0 mM 2-mercaptoethanol, 12 mM magnesium chloride, and 40 μM [^{3}H]farnesyl diphosphate [87 μCi μmol^{-1}]) was 50 μL. The mixture was incubated at 35°C for 15 min. The reaction products were extracted twice with 500 μL of 3% (v/v) diethyl ether in hexane. The organic phase from each extraction was applied to a 3-mL silica column (Supelco, Bellefonte, PA) preconditioned with 1.0 mL of 3% (v/v) diethyl ether in hexane, and the silica column was then rinsed with 500 μL of the same solvent. The effluent from the 3-mL silica column containing the olefin reaction product of the sesquiterpene synthase was collected with the use of a vacuum manifold (Supelco) and counted for radioactivity. Protein concentrations were determined according to the method of Bradford (1976).

Comparison of Cellulase-Treated Wild Type and Untreated Transgenic Tobacco Sesquiterpene Synthase Products by Argentation Chromatography

A silica gel G60 TLC plate (Fischer Scientific) was sprayed to the point of saturation with a 15% (w/v) solution of $AgNO_3$ in water using a chromatography sprayer (Sigma). The TLC plate was placed in an oven at 110°C for 4 h and then allowed to cool to room temperature. ^{3}H-labeled sesquiterpene synthase products from cellulase-treated wild type or the untreated transgenic cell line TH8 were isolated as described previously for the assay of sesquiterpene synthase activity. On the same Ag^+-silica gel TLC plate the following were spotted: lane 1, the wild-type sesquiterpene synthase product (5-epi-aristolochene); lane 2,

untreated TH8 sesquiterpene synthase product; and lane 3, an equal mixture of 5-epi-aristolochene and the untreated TH8 sesquiterpene synthase product. After the TLC plate was developed with hexane:benzene:diethyl ether (50:50: 0.25, v/v), 1-cm segments were scraped from each lane and counted for radioactivity.

In Vivo Labeling of Capsidiol and 15-Hydroxytrichodiene from Cellulase-Treated and Untreated Wild-Type and Transgenic Tobacco Cell-Suspension Cultures

[^{3}H]Mevalonate (2 × 10^6 dpm) was added 6.5 h after cellulase treatment to 40 mL of tobacco cells in a volume of 5 μL. Following treatment with cellulase, capsidiol was present mainly in the liquid culture medium. Trichodiene and 15-hydroxytrichodiene, however, were present mainly within transformant cells rather than in the culture medium following elicitor treatment (data not shown). For simultaneous analysis of these terpene metabolites, both the culture cells and the culture medium were extracted with chloroform and methanol. After 1.5 h the cells were harvested by suction filtration using a single sheet of pre-moistened Miracloth and immediately homogenized in 30 mL of chloroform:methanol (2:1, v/v) using a mortar and pestle. The homogenate was filtered through Miracloth, and the retentate was washed with a fresh 20-mL portion of the same solvent mixture. The organic filtrates were combined with the filtrate from the cell-suspension culture. Chloroform (30 mL) was added to the combined filtrates, and the solution was shaken vigorously. After the phases had separated, the aqueous phase was re-extracted with 150 mL of chloroform. The organic extracts were pooled, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The remaining residue was redissolved in CHCl$_3$ and applied to a silica gel TLC plate. The TLC plate was developed with hexane:ethyl acetate (1:1, v/v), and the separated metabolites were visualized after a vanillin/H$_2$SO$_4$ reagent (a methanolic solution containing 6% [v/v] concentrated sulfuric acid and 1% [w/v] vanillin) was sprayed and the TLC plate was heated at 110°C for 2 min. Spots corresponding to capsidiol and 15-hydroxytrichodiene were scraped separately and counted for radioactivity.

In Vivo Labeling of Trichodiene from Cellulase-Treated and Untreated Transgenic Tobacco Cell-Suspension Cultures

Transgenic tobacco cell cultures were labeled with [^{3}H]mevalonate and extracted with organic solvents as described above. The organic extracts were pooled, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was redissolved in 3% (v/v) ethyl ether in hexane and applied to a 3-mL silica gel column as described above for the sesquiterpene cyclase assay. The eluant from the column in 3% (v/v) ethyl ether in hexane was collected, evaporated under a stream of nitrogen, redissolved in 50 μL of the same solvent mixture, and applied to a 15% AgNO$_3$ silica gel TLC plate. The TLC plate was then developed with hexane:benzene:diethyl ether (50:50:0.25, v/v). The region of the plate correspond-

ing to the R$_F$ of trichodiene was scraped and counted for radioactivity. The more polar material that remained on the 3-mL silica column was eluted with two 750-μL portions of ethyl acetate. The ethyl acetate eluant was collected, evaporated under a steam of nitrogen, redissolved in 50 μL of 3% ethyl ether in hexane, and applied to a silica gel G60 TLC plate. After the TLC plate was developed with hexane: isopropanol (9:1, v/v), the TLC plate was sprayed with ENH^{3}ANCE (NEN-DuPont) for autoradiography.

Immunoblot Analysis

Immunoblot analysis was performed as previously described (Hohn and Ohlrogge, 1991). Blots were probed simultaneously with three different tobacco sesquiterpene synthase monoclonal antibodies (each at a 1:1000 dilution) and a trichodiene synthase polyclonal antibody (1:1000 dilution). Both goat anti-mouse and goat anti-rabbit alkaline phosphatase conjugate antibodies (1:1000 dilution) were included in the secondary antibody incubation. The tobacco sesquiterpene synthase monoclonal antibodies were generously provided by Dr. Joseph Chappell (Department of Agronomy, University of Kentucky, Lexington).

Quantitation of 15-Hydroxytrichodiene Accumulation in Transgenic Tobacco Cultures

Cellulase-treated and untreated transgenic cell-suspension culture TH3 was extracted with chloroform:methanol (2:1, v/v) as described above. After the organic phase was evaporated to dryness under reduced pressure, the remaining residue was redissolved in chloroform and applied to a silica gel TLC plate, which was developed with hexane:ethyl acetate (1:1, v/v). A 1.5-cm region of the TLC plate corresponding to the R$_F$ of 15-hydroxytrichodiene (Zook et al., 1996) was scraped into a scintered glass funnel. 15-Hydroxytrichodiene was eluted from the silica gel with ethyl acetate. The ethyl acetate eluant was evaporated to dryness under a stream of nitrogen, and the remaining residue was redissolved in 100 μL of hexane:isopropanol (93:7, v/v) just prior to HPLC analysis. The HPLC mobile phase (hexane:isopropanol [93:7, v/v]) was pumped through a 5-μm 150- × 4.6-mm silica column (Econosphere; Alltech, Deerfield, IL) at a flow rate of 1.0 mL min^{-1}. The eluant from the column was monitored at 210 nm using a variable wavelength detector. The amount of 15-hydroxytrichodiene in each sample was determined by comparison to the HPLC detector response of injections of known amounts of pure 15-hydroxytrichodiene.

RESULTS

Expression of Trichodiene Synthase in Transformant Cell Cultures

Wild-type tobacco cell-suspension cultures have a low level of sesquiterpene synthase activity that can be induced approximately 10-fold after treatment with cellulase (Table I). A similar induction of sesquiterpene cyclase activity was observed by Vögeli and Chappell (1988). Since the expression construct used to transform tobacco plants used the

Table I. *Sesquiterpene cyclase activity in cellulase-treated and untreated wild-type and transgenic tobacco cell suspension cultures*

Tobacco Cell Line	Treatment[a]	Sesquiterpene Cyclase Activity[b]
		nmol product h^{-1} mg^{-1} protein
Wild type	H_2O	4.64 ± 0.22
Wild type	Cellulase	48.2 ± 2.6
TH1	H_2O	38.5 ± 17.0
TH1	Cellulase	50.7 ± 2.0
TH2	H_2O	88.8 ± 1.5
TH2	Cellulase	105 ± 4.0
TH3	H_2O	75.4 ± 13.9
TH3	Cellulase	88.3 ± 15.4
TH8	H_2O	63.5 ± 5.7
TH8	Cellulase	95.2 ± 0.1
TH10	H_2O	2.57 ± 0.17
TH10	Cellulase	47.5 ± 4.0
TH11	H_2O	84.7 ± 2.1
TH11	Cellulase	125 ± 19

[a] Twenty milliliters of cell-suspension culture was treated with either H_2O or cellulase (1.0 μg mL^{-1} cells) 3 to 4 d after subculturing.   [b] Sesquiterpene cyclase activity was assayed 9 h after treatment application. Data are presented as the mean of two determinations $\pm$ the range.

cauliflower mosaic virus 35S promoter, a constitutive promoter in tobacco, it was anticipated that sesquiterpene synthase activity would be elevated in the unelicited transformants. Transformant and wild-type cell-suspension cultures were assayed for sesquiterpene synthase activity 18 h after treatment with either H_2O (unelicited) or cellulase (elicited). Cell homogenates from unelicited transformant cell cultures contained 10- to 25-fold higher levels of sesquiterpene synthase activity than were observed in similarly treated wild-type cultures, with the exception of TH10 cultures (Table I). In contrast, activity levels were similar in cell homogenates from elicited transformant and wild-type cell cultures. These data demonstrate that, with the exception of TH10, the transformant cell cultures constitutively express elevated levels of sesquiterpene synthase activity. PCR analysis indicated that the failure of unelicited TH10 cell-suspension cultures to produce elevated levels of sesquiterpene synthase activity is due to the absence of an intact copy of the trichodiene synthase open reading frame (data not shown).

To characterize the sesquiterpene synthase reaction products, it was necessary to separate the primary reaction product of wild-type tobacco, 5-epi-aristolochene, from trichodiene. Argentation TLC was found to be suitable for this purpose. The ^3H-labeled sesquiterpene synthase products present in reactions to which either an elicited wild-type cell homogenate or an unelicited TH8 homogenate had been added were analyzed by TLC. Following chromatography and subsequent autoradiography of the TLC plate the sesquiterpene synthase product from the elicited wild-type culture was observed to co-migrate with authentic 5-epi-aristolochene (R_F 0.50), whereas the sesquiterpene synthase product from the unelicited TH8 cell line co-migrated with authentic trichodiene (R_F 0.71). The profile of radioactivity from a

third lane, containing an equal mixture of the two sesquiterpene synthase products, showed clear chromatographic separation of the two sesquiterpene synthase products. These data provide strong evidence that the transgenic cell lines, with the exception of TH10, produce active trichodiene synthase.

Confirmation of trichodiene synthase expression in transformants was obtained by immunoblot analysis of soluble protein extracts from cell-suspension cultures (Fig. 2). Antibodies specific for the tobacco enzyme, 5-epi-aristolochene synthase, detected higher levels of this enzyme following elicitor treatment as previously reported for wild-type cell-suspension cultures (Vögeli and Chappell, 1988). Using antiserum specific for trichodiene synthase, we detected a polypeptide that co-migrated with purified trichodiene synthase in protein extracts from TH1, TH3, TH8, and TH11. This polypeptide was not detected in protein extracts from transformant TH10 or wild type. Estimates of the relative trichodiene synthase levels in individual transformants based on band intensity (Fig. 2) were consistent with previous data concerning trichodiene synthase activity levels (Table I) and the level of [^3H]mevalonate incorporation into trichodiene (Table II). Thus, the production of trichodiene by transformant cell cultures appears to be related to the level of trichodiene synthase expression.

Production of Trichodiene and Trichodiene Metabolites

To test whether the transgenic cell lines produce trichodiene in vivo, wild-type and transformant cell lines were pulse-labeled with [^3H]mevalonate 6.5 h after elicitor presentation (Table II). The wild type and transformant TH10 incorporated low levels of radioactivity into compounds migrating on argentation TLC plates in the region where trichodiene was found. Much higher levels of radioactivity were observed in this region of the TLC plate for extracts from transformants TH3, TH8, and TH11. The relative amounts of radioactivity observed are consistent with
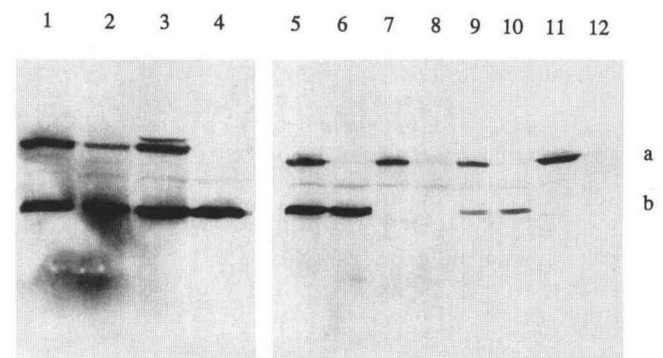

Figure 2. Immunoblot analysis of protein extracts from elicited and unelicited wild-type and transformant cell-suspension cultures separated by SDS-PAGE. The letter a indicates the 62-kD tobacco sesquiterpene cyclase, whereas the letter b indicates the 45-kD subunit of trichodiene synthase. The even-numbered lanes were untreated, and the odd-numbered lanes were cellulase-treated. Lanes 1 and 2, TH11; lanes 3 and 4, TH3; lanes 5 and 6, TH8; lanes 7 and 8, TH10; lanes 9 and 10, TH1; lanes 11 and 12, wild type.

Table II. *In vivo labeling of trichodiene with 5-[³H]mevalonate in cellulase-treated and untreated wild-type and transgenic tobacco cell suspension cultures*

Tobacco Cell Line	Treatment[a]	Radioactivity Incorporated into Trichodiene[b]
		dpm
Wild type	H_2O	185
Wild type	Cellulase	160
TH1	H_2O	3,170
TH1	Cellulase	3,145
TH2	H_2O	11,185
TH2	Cellulase	16,495
TH3	H_2O	38,900
TH3	Cellulase	55,975
TH8	H_2O	14,195
TH8	Cellulase	23,295
TH10	H_2O	800
TH10	Cellulase	855
TH11	H_2O	29,845
TH11	Cellulase	26,405

[a] Twenty milliliters of cell suspension culture was treated with either H_2O or cellulase (1.0 μg mL^{-1} cells) 3 to 4 d after subculturing.   [b] 5-[³H]-Mevalonate (2.1 × 10⁶ dpm) was added to 40 mL of tobacco cells in a volume of 5 μL 6.5 h after cellulase treatment and 1.5 h prior to harvesting cells for determination of the incorporation of radioactivity into trichodiene as described in "Materials and Methods."

the levels of sesquiterpene synthase activity found in the individual transformants. Additional evidence of trichodiene production by transformants was obtained by GC-MS analysis. The olefin fraction from each transformant was separated by argentation chromatography, and the region of the TLC plate corresponding to trichodiene was eluted and analyzed by GC-MS (data not reported). A comparison of these spectra with that of authentic trichodiene confirmed the presence of trichodiene in extracts from transformants that have in vitro trichodiene synthase activity.

After establishing that transformant cell lines produced trichodiene in vivo, we attempted to determine whether foreign sesquiterpene synthase expression altered the profile of inducible metabolites. Following elicitation, cell cultures were labeled with [³H]mevalonate and the chloroform-soluble, relatively polar metabolites were extracted. This fraction, which includes capsidiol, was then analyzed by autoradiography following TLC (Fig. 3). Spots corresponding to capsidiol increased in intensity for each cell line after elicitation in agreement with previous reports for wild-type cell-suspension cultures (Vögeli and Chappell, 1988). Closer inspection of the autoradiogram revealed a compound in extracts from the trichodiene-producing transformants that was not present in the wild-type or TH10 extracts. The intensity of this spot increased after elicitation and appeared more intense in the extracts from transgenic lines that produced higher levels of trichodiene, such as TH3.

Effects of Elicitor Treatment on 15-Hydroxytrichodiene Biosynthesis

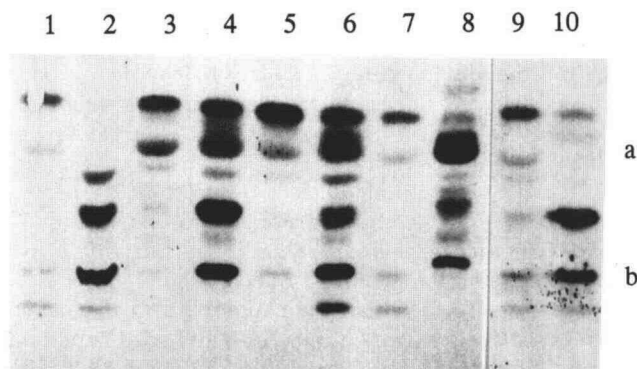The accumulation of 15-hydroxytrichodiene (Zook et al., 1996) was analyzed following elicitor treatment of

Figure 3. Autoradiographic analysis of metabolites from elicited and unelicited cell-suspension cultures labeled with [³H]mevalonate. Extracted metabolites were separated on a silica gel G60 TLC plate developed with hexane:isopropanol (9:1, v/v). 5-[³H]Mevalonate (2.2 × 10⁶ dpm) was applied to 20 mL of cell-suspension cultures in a volume of 5 μL. The letter a represents the R$_F$ of 15-hydroxythrichodiene, and the letter b represents the R$_F$ of capsidiol. The odd-numbered lanes were untreated, and the even-numbered lanes were cellulase-treated. Lanes 1 and 2, Wild type; lanes 3 and 4, TH1; lanes 5 and 6, TH2; lanes 7 and 8, TH3; lanes 9 and 10, TH10.

transgenic tobacco cell cultures. Treatment of transgenic cell line TH3 with elicitor resulted in an increase in the levels of 15-hydroxytrichodiene as compared to unelicited TH3 cell cultures that were not treated with cellulase (Fig. 4). The levels of 15-hydroxytrichodiene began to increase above control levels between 3 and 9 h after elicitor treatment, and this increase continued throughout the course of the experiment. The amount of 15-hydroxytrichodiene (45 μg in 20 mL of culture) that accumulated 24 h after elicitor treatment was approximately equal to the levels of capsidiol that accumulate in wild-type tobacco cell-suspension cultures after elicitor treatment (Chappell and Nable, 1987).

To investigate trichodiene, 15-hydroxytrichodiene, and capsidiol biosynthesis following elicitor treatment, cell cultures were pulse-labeled with [¹⁴C]acetate. Elicitation of
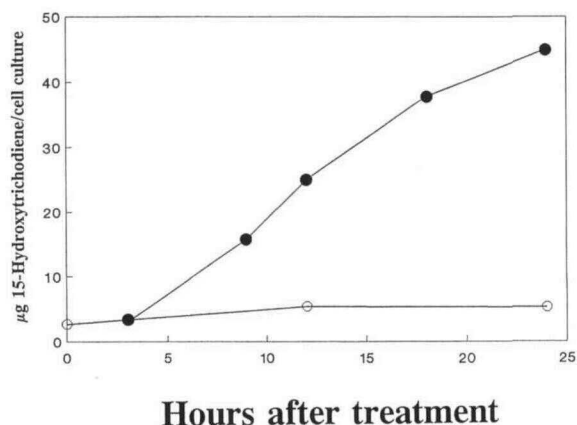

Hours after treatment

Figure 4. Accumulation of 15-hydroxytrichodiene in transformant TH3. Cell-suspension cultures were treated with cellulase (●) or H_2O (○) as described in Table I. The extraction and quantitation of 15-hydroxytrichodiene is described in "Materials and Methods."

TH2 cell cultures resulted in a rapid increase in the percentage of radioactivity incorporation from [^{14}C]acetate into both trichodiene and 15-hydroxytrichodiene compared with untreated cell cultures (Fig. 5). Incorporation of radioactivity into trichodiene peaked 3 h after treatment and then declined over the next 9 h until it reached the level observed for untreated cell cultures. In a similar manner, the percentage of incorporation of label into 15-hydroxytrichodiene also peaked 3 h after elicitor treatment but decreased at a much slower rate.

Comparison of 15-hydroxytrichodiene and capsidiol biosynthesis was performed with TH3 and wild-type cell cultures. Transgenic cell line TH3 and wild-type cell cultures were pulse-labeled with [^{14}C]acetate after either elicitor treatment or no treatment, and the percentage of radioactivity incorporation into 15-hydroxytrichodiene and capsidiol was measured (Fig. 6). The kinetics of radioactivity incorporation in TH3 were similar for both 15-hydroxytrichodiene and capsidiol, but the increase in
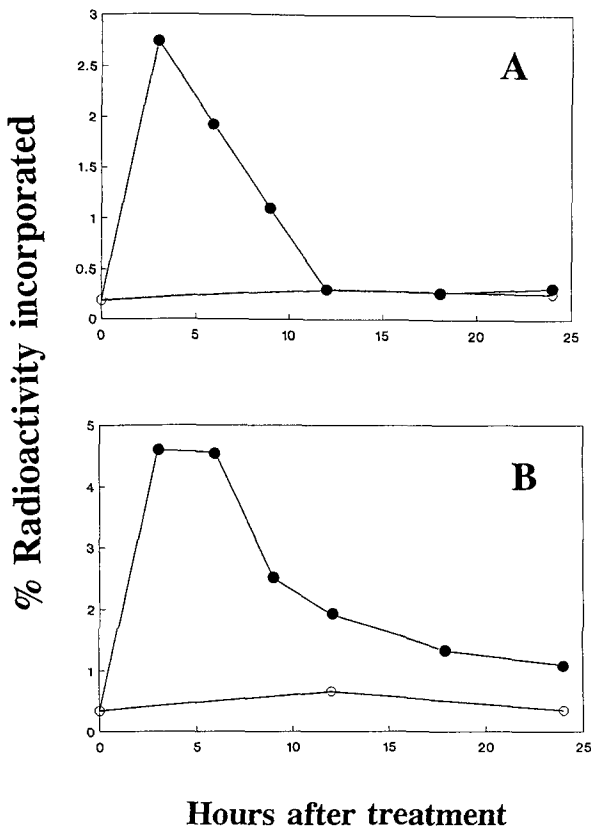

Figure 5. Incorporation of radioactivity from 2-[^{14}C]acetate into trichodiene (A) and 15-hydroxytrichodiene (B) in transgenic tobacco cell line TH2. Cell-suspension cultures were treated with cellulase (●) or H$_2$O (O) as described in Table I. [^{14}C]Acetate (2.0 × 10^6 dpm) was applied to 20 mL of cell-suspension culture in a volume of 5 μL 1.5 h before the cells were harvested for the determination of radioactivity incorporated into trichodiene or 15-hydroxytrichodiene. Regions of either silica G60 TLC plates or 15% AgNO$_3$ silica G60 TLC plates corresponding to the R$_F$s of 15-hydroxytrichodiene or trichodiene, respectively, were scraped and analyzed for radioactivity as described in "Materials and Methods."
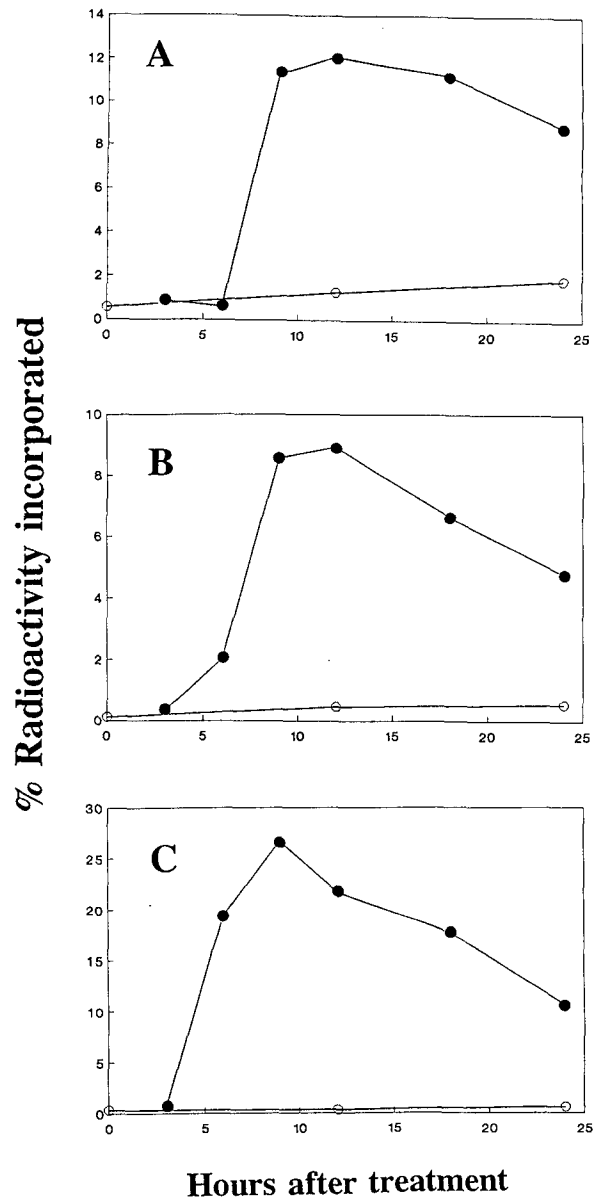

Hours after treatment

Figure 6. Incorporation of radioactivity from 2-[^{14}C]acetate into 15-hydroxytrichodiene in transformant TH3 (A), capsidiol in transformant TH3 (B), or capsidiol in a wild-type cell line (C). Cell-suspension cultures were treated with cellulase (●) or H$_2$O (O) as described in Table I. 2-[^{14}C]Acetate (1.1 × 10^6 dpm) was applied to 20 mL of cell-suspension culture in a volume of 5 μL 1.5 h before the cells were harvested for determination of radioactivity incorporated into capsidiol or 15-hydroxytrichodiene. Each point in A and B is derived from the same extract. Regions of silica G60 TLC plates corresponding to the R$_F$s of 15-hydroxytrichodiene or capsidiol were scraped and counted for radioactivity as described in "Materials and Methods."

percentage of incorporation into capsidiol occurred earlier in wild-type cultures. The effect of elicitor treatment on the radioactivity incorporation from [^{14}C]acetate into capsidiol was consistent with the effects previously reported with wild-type tobacco cell-suspension cultures (Vögeli and Chappell, 1988). The percentage of radioac-

tivity incorporation from [^{14}C]acetate into capsidiol in the transgenic cell line TH3 was greatly reduced compared with wild-type cell cultures. However, the combined percentage of radioactivity incorporation into 15-hydroxytrichodiene and capsidiol 9 h after elicitor treatment in transgenic cell line TH3 was similar to the percentage of radioactivity incorporated into capsidiol 9 h postelicitation in the wild-type cell cultures.

DISCUSSION

In this report we have shown that it is possible to alter plant sesquiterpenoid metabolism through the introduction of a foreign sesquiterpenoid biosynthetic gene. Cell-suspension cultures derived from transformed tobacco plants expressed a functional trichodiene synthase and accumulated both trichodiene and a novel oxygenated trichodiene metabolite, 15-hydroxytrichodiene. The accumulation of a metabolite derived from the expression of a foreign sesquiterpene cyclase in tobacco cell-suspension cultures offers a unique opportunity to study plant isoprenoid metabolism following the elicitation of sesquiterpene phytoalexin biosynthesis.

Trichodiene and 15-hydroxytrichodiene are present at relatively low levels in untreated transformant cell cultures. This observation is consistent with an earlier report of low-level trichodiene accumulation in whole transgenic tobacco plants expressing trichodiene synthase (Hohn and Ohlrogge, 1991). Reasons for the low-level accumulation of trichodiene and 15-hydroxytrichodiene are unclear. Trichodiene and 15-hydroxytrichodiene may be turned over at a higher rate than that of elicitor-treated cells, or there may be a lower concentration of "available" FPP for trichodiene synthase in untreated cells relative to elicitor-treated cells due to subcellular compartmentalization and/or the activity of other isoprenoid enzymes (e.g. squalene synthase). Elicitation of tobacco cell cultures has been reported to sharply decrease the activity of squalene synthase (Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988).

Following treatment with cellulase, however, there is a large increase in both the accumulation of 15-hydroxytrichodiene (Fig. 4) and the rate of biosynthesis of trichodiene and 15-hydroxytrichodiene (Figs. 5 and 6). Because the levels of trichodiene synthase are similar in untreated and elicitor-treated cell cultures (Fig. 2), the increase in the rate of biosynthesis of 15-hydroxytrichodiene most likely reflects an increase in the levels of FPP available to trichodiene synthase after elicitor treatment. Elicitor treatment of tobacco cell cultures has been reported to induce an increase in the enzyme activities of several isoprenoid pathway enzymes between the acetyl-CoA and FPP steps in the pathway (Vögeli and Chappell, 1988; Hanley et al., 1991).

The production of 15-hydroxytrichodiene by transgenic cell cultures implies that trichodiene is metabolized by a tobacco hydroxylase. Since there appears to be a transient increase in the biosynthetic rate for trichodiene accompanying a similar increase in the biosynthetic rate for 15-hydroxytrichodiene after elicitor treatment (Fig. 5), there is no clear evidence for an elicitor-induced increase in the

activity of a trichodiene hydroxylase. However, it is possible that elicitor treatment does induce an increase in the activity of a nonspecific sesquiterpene hydroxylase(s). It is tempting to speculate that one of the hydroxylases involved in capsidiol biosynthesis (Fig. 1) may also catalyze the conversion of trichodiene to 15-hydroxytrichodiene. Two different hydroxylase activities have been demonstrated using 5-epi-aristolochene as a substrate in cell-free extracts from tobacco cell-suspension cultures (Whitehead et al., 1989). One of these, a C3 hydroxylase, is not present in extracts from unelicited cultures, whereas the other, a C1 hydroxylase, is present in extracts from both elicited and unelicited cultures. Alternatively, the hydroxylation of trichodiene could also be catalyzed by enzymes involved in unrelated pathways or by enzymes specific for terpenoid catabolism if such enzymes exist in tobacco. The turnover of tobacco sesquiterpenoids such as capsidiol is well documented (Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988), but few details are known concerning the mechanisms by which this occurs.

The accumulation of trichodiene in transformant cell-suspension cultures provides insight into the regulation of sesquiterpenoid biosynthesis in tobacco. Data presented in the current report demonstrate that a foreign sesquiterpene cyclase has access to FPP produced following elicitation in transgenic tobacco. In addition, biosynthesis of the foreign sesquiterpenoid product appears to occur at the expense of the native sesquiterpene synthase; i.e. the expression of trichodiene synthase appears to decrease the biosynthetic rate of capsidiol (Fig. 6). This could be due to competition between trichodiene synthase and 5-epi-aristolochene synthase for the available FPP.

It has been suggested that access to FPP might be tightly regulated through the formation of specific enzyme complexes (Choi et al., 1994; Chappell, 1995). This theory of isoprenoid regulation proposes that the pool of FPP destined for sesquiterpene biosynthesis is compartmentalized or separated from pools of FPP destined for the biosynthesis of other isoprenoids, such as sterols. This physical separation or "channeling" of isoprenoid intermediates is thought to be achieved by the formation of hypothetical enzyme complexes that allow for efficient transfer of intermediates between enzymes of the complex. For instance, a particular isozyme of FPP synthase might couple to squalene synthase. If such a system of isoprenoid pathway enzyme coupling does exist in tobacco, one would expect little or no "free" FPP. Thus, the expression of a foreign sesquiterpene synthase would presumably not yield a product.

On the other hand, it is possible that a foreign sesquiterpene synthase, such as trichodiene synthase, may fortuitously participate in the formation of a specific enzyme complex. However, this possibility seems unlikely given the apparent lack of relatedness between trichodiene synthase and 5-epi-aristolochene synthase (Colby et al., 1994). It is therefore difficult to imagine how trichodiene synthase could participate in the formation of enzyme complexes such as those that have been proposed for the channeling of isoprenoid pathway intermediates. Although the in vivo

production of trichodiene and 15-hydroxytrichodiene in transformants tends to argue against the existence of tightly coupled enzyme complexes in plant isoprenoid biosynthesis, the results of the current study do not entirely exclude the possibility of their existence. It is possible that the isoprenoid pathway enzymes that are devoted to sesquiterpene biosynthesis in tobacco are "loosely" organized in the cytosol/ER rather than organized in a tightly coupled enzyme complex. This type of organization could allow for access of a foreign sesquiterpene cyclase to available pools of FPP, but the organization and/or subcellular compartmentalization would still allow for separate regulation and synthesis of sesquiterpenes apart from other classes of isoprenoids.

The ability to alter plant sesquiterpenoid production through the introduction of foreign biosynthetic enzymes will facilitate studies of plant isoprenoid metabolism. It also suggests possible approaches for introducing traits into plants that will improve resistance to disease and pests or modify plant flavor and fragrance properties.

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