

Communication

Expression of a Fungal Sesquiterpene Cyclase Gene in Transgenic Tobacco¹

Thomas M. Hohn* and John B. Ohlrogge

Mycotoxin Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois 61604 (T.M.H.), and Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824–1312 (J.B.O.)

ABSTRACT

The complete coding sequence for the trichodiene synthase gene from *Fusarium sporotrichioides* was introduced into tobacco (*Nicotiana tabacum*) under the regulation of the cauliflower mosaic virus 35S promoter. Expression of trichodiene synthase was demonstrated in the leaves of transformed plants. Leaf homogenates incubated with [³H]farnesyl pyrophosphate produced trichodiene as a major product. Trichodiene was detected in the leaves of a transformed plant at a level of 5 to 10 nanograms per gram fresh weight. The introduction of a fungal sesquiterpene cyclase gene into tobacco has resulted in the expression of an active enzyme and the accumulation of low levels of its sesquiterpenoid product.

Sesquiterpene cyclases participate in the biosynthesis of most sesquiterpenoids. This group of enzymes converts the isoprenoid pathway intermediate, FPP,² into an estimated 100 to 200 different cyclic sesquiterpenoids (2). Information concerning the properties of sesquiterpene cyclases has been reported for isolated or partially purified enzyme preparations from plants (4, 13), fungi (9, 11), and a streptomycete (1). Comparisons among these enzymes indicate that they are mechanistically similar but differ with respect to properties such as molecular weight and subunit composition.

Plants, fungi, insects, and marine invertebrates all produce large numbers of structurally distinct sesquiterpenoids. In many cases, distantly related organisms accumulate the same sesquiterpene cyclase product or produce sesquiterpenoids derived from the same parent compound. This means that genes coding for any particular sesquiterpene cyclase might be available from several sources.

The modification of plant sesquiterpenoid biosynthesis could result in altered resistance to disease and insect pests. Potential targets of such efforts include the production of sesquiterpenoid phytoalexins within the Solanaceae (16) and allelopathic agents produced by a variety of plants (5). Additional targets could include sesquiterpenoids that regulate

insect behavior. A number of insect hormones and pheromones have been identified as sesquiterpenoids. Tobacco produces at least eight different sesquiterpenoids in response to invading pathogens (6). One of these, capsidiol, accumulates to high levels in tobacco cell cultures that have been treated with an elicitor (21). Studies in which cell cultures were used have resulted in the identification of (+)5-epi-aristolochene as the parent compound of capsidiol and debneyol (22), and the purification of the sesquiterpene cyclase, 5-epi-aristolochene synthase (19).

Trichodiene is the parent compound for the trichothecene family of toxic sesquiterpenoids. Trichothecenes have been implicated in outbreaks of mycotoxicoses resulting from contaminated agricultural products (17). Both trichodiene and trichothecene biosynthesis appear to be limited to several genera of fungi and possibly some *Baccharis* species (12). Trichodiene synthase is the enzyme involved in trichodiene biosynthesis. A gene encoding trichodiene synthase has been isolated from *Fusarium sporotrichioides* (8) and expressed in *Escherichia coli*. Expression of trichodiene synthase in *E. coli* resulted in the production of active enzyme and in the accumulation of trichodiene (10).

The expression of foreign sesquiterpene cyclase genes in plants represents one possible approach for altering sesquiterpenoid biosynthesis. To determine the feasibility of this approach, we introduced the trichodiene synthase gene into tobacco. Expression in tobacco may provide insights into both the regulation of sesquiterpenoid biosynthesis and the role of these compounds in disease resistance.

MATERIALS AND METHODS

Transformation of Tobacco

The *Bam*HI fragment from pTS37–4Δ(470–529), containing the complete coding sequence for the trichodiene synthase gene of *Fusarium sporotrichioides* (10), was inserted into the plasmid, pBI221 (Clontech³), from which the β-glucuronidase-coding sequence had been deleted (*Sma*I–*Sst*I fragment). This vector contains a *Hind*III–*Eco*RI fragment upon which resides an expression cassette consisting of the cauliflower

¹ This work was partially supported by a competitive grant from the U.S. Department of Agriculture to T.M.H.

² Abbreviation: FPP, farnesyl pyrophosphate.

³ The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

mosaic virus 35S promoter, a unique *Bam*HI site and the nopaline synthase transcription terminator. The *Hind*III-*Eco*RI fragment from the newly constructed expression unit, containing the trichodiene synthase-coding region in the correct orientation behind the 35S promoter, was cloned into the binary expression vector, pBI121 (Clontech), to yield pTH70-1. This construct was subsequently used to transform tobacco leaf discs (*Nicotiana tabacum*, cv Petite Havana) from which kanamycin-resistant plants were regenerated (15).

Measurements of Trichodiene Synthase in Transgenic Tobacco

Transformed tobacco (*N. tabacum*) plants were grown in a greenhouse under ambient lighting conditions. After the transformants were transferred to potting soil (20–40 d), leaves were collected, frozen in liquid N₂, and stored at –70°C. Leaf tissue (0.2 g) was homogenized in 2 mL of 80 mM potassium phosphate buffer (pH 7.0), 20% glycerol, 10 mM sodium bisulfite, 10 mM sodium ascorbate, 15 mM magnesium chloride, 5 mM DTT containing 0.10 g polyvinylpyrrolidone (18). After 1 g of Amberlite XAD-4 was added, the mixture was incubated in an ice bath for 5 min and then centrifuged at 10,000g, at 4°C, for 10 min. The supernatant was recovered and centrifuged at 130,000g, at 4°C, for 60 min. The resulting supernatant was assayed for sesquiterpene cyclase activity as previously described (11). Trichodiene was identified as a major product of the reaction by GC/MS analysis (9). The protein concentration of homogenates was determined by a modified Coomassie blue G dye-binding assay (14) with bovine γ -globulin as the standard. Leaf tissue (100 mg) was macerated in microfuge tubes with 100 μ L of SDS-polyacrylamide gel sample buffer and then incubated at 100°C for 3 min. Samples were run on an SDS-polyacrylamide gel (10% acrylamide) and immunoblotted as previously described (7).

Analysis of Transformed Plants for Trichodiene

The extraction of trichodiene from leaf tissue (0.5 g) was accomplished by homogenization in 2.5 mL of hexane:ether (49:1). After the supernatant was decanted, homogenization was continued in an additional 2.0 mL of hexane:ether. The supernatants were pooled, and a 1.0-mL sample was applied to a Prepsep silica gel column (Fisher, 1.0 g silica gel). Elution was continued with hexane:ether until the yellow-orange carotenoid band reached the column bed support. All of the material eluted from the column was collected (2.0 mL), and the volume was reduced to 50 μ L under a gentle stream of N₂ at 23°C. The sample was analyzed for trichodiene by GC/MS as described (4).

RESULTS AND DISCUSSION

Expression of Trichodiene Synthase in Transgenic Plants

Transformed plants appeared normal with respect to growth and gross morphology. To confirm the expression of trichodiene synthase in transformants, leaf homogenates were analyzed by immunoblotting (Fig. 1). Leaves from three different transformed plants were found to contain a unique immunodetectable polypeptide that comigrated with the tri-

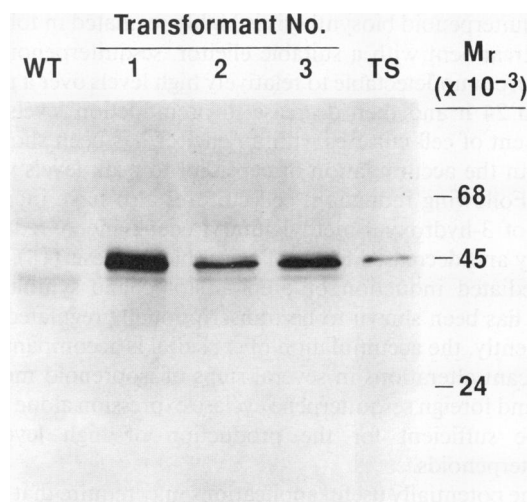


Figure 1. Immunoblot analysis of leaf homogenates from transformed tobacco plants. The blot was incubated with antiserum against purified trichodiene synthase. WT, untransformed plant; TS, purified trichodiene synthase. The migration of protein standards is indicated.

chodiene synthase of *F. sporotrichioides* (M_r 45,000). The trichodiene synthase antiserum used does not cross-react with 5-epi-aristolochene synthase (T. M. Hohn and J. Chappell, unpublished).

Leaf homogenates were assayed for sesquiterpene cyclase activity. Transformed plants had enzyme activity levels in the range of 2 to 3 nmol $\cdot$ mg protein $\cdot$ h. These levels are similar to those reported for induced cell cultures of tobacco (18). Sesquiterpene cyclase activity was undetectable in homogenates from an untransformed plant. Although the level of sesquiterpene cyclase activity in tobacco plants has not been reported previously, this result is consistent with the observation that the accumulation of sesquiterpenoids occurs in response to specific elicitors and that uninduced tobacco cell cultures have undetectable levels of sesquiterpene cyclase activity (18). Analysis of the sesquiterpene cyclase reaction products by GC/MS revealed that trichodiene accounted for at least 30% of the labeled product.

Analysis of Transformed Plants for Trichodiene

The above results demonstrate that plants transformed with a fungal sesquiterpene cyclase gene express that enzyme in an active form. The successful expression of trichodiene synthase in tobacco raises the question of whether its product, trichodiene, is synthesized. Leaf extracts from transformant TH1 were analyzed for trichodiene by GC/MS. Trichodiene was detected at a level of approximately 5 to 10 ng/g fresh weight of tissue. Trichodiene recoveries for the method used were determined by adding 57 ng of [³H]trichodiene (420,000 dpm) to a homogenized leaf sample and analyzing fractions after each step in the procedure. Extracted trichodiene (39%) was recovered in the samples analyzed by GC/MS analysis. To determine whether interference by contaminating materials had occurred, GC/MS analysis was performed in the presence and absence of nanogram amounts of unlabeled trichodiene.

Sesquiterpenoid biosynthesis is highly regulated in tobacco. After treatment with a suitable elicitor, sesquiterpenoids increase from undetectable to relatively high levels over a period of 6 to 24 h and then decrease to preinduction levels. The treatment of cell cultures with an elicitor has been shown to result in the accumulation of capsidiol to peak levels within 15 h. Following induction, cell cultures also have increased levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and decreased levels of sterol biosynthesis (18). Elicitor-mediated induction of 5-epi-aristolochene synthase recently has been shown to be transcriptionally regulated (20). Apparently, the accumulation of capsidiol is accompanied by significant alterations in several steps of isoprenoid metabolism, and foreign sesquiterpene cyclase expression alone might not be sufficient for the production of high levels of sesquiterpenoids.

Some potentially useful applications may require that transgenic plants constitutively produce low levels of novel sesquiterpenoids. These include the production of sesquiterpenoids that alter insect behaviors, many of which are active at very low levels. Foreign sesquiterpene cyclase expression could result in low levels of novel sesquiterpenoids being produced, because FPP is an essential metabolite that participates in the biosynthesis of a number of isoprenoids. Both sesquiterpene cyclases and FPP synthetase are located in the cytosol of plant and fungal cells (2, 3), suggesting that cytoplasmic pools of FPP may be accessible to foreign sesquiterpene cyclases. However, factors such as the sequestration and/or channeling of FPP to specific enzymes and competition with other enzymes could limit the amount of FPP available to these enzymes. In addition, the levels of novel sesquiterpenoids produced will depend on the extent to which these compounds are further metabolized. The low levels of trichodiene observed could be due to its metabolism in transgenic plants. Although native sesquiterpenoids are efficiently metabolized, the fate of trichodiene synthesized by tobacco is unknown.

In future investigations of trichodiene metabolism in the transgenic plants, tissues treated with an elicitor should be used. Induced tissues are likely to present the most favorable conditions for trichodiene synthesis. Expression of trichodiene synthase may perturb sesquiterpenoid biosynthesis in transgenic plants even if trichodiene metabolites do not accumulate. If this occurs, then transgenic plants could provide a unique opportunity for studying the role of these compounds in disease resistance.

ACKNOWLEDGMENTS

We thank Dusty Post-Beittenmiller for carrying out the transformation of tobacco with pTH70-1 and R. D. Plattner for the GC/MS analysis of trichodiene and acknowledge the excellent technical assistance of R. W. Reeser. We also thank Dr. Rodney Croteau for advice on the isolation of trichodiene and Dr. Jan Miernyk for critically reading the manuscript.

LITERATURE CITED

1. Cane DE, Pargellis C (1987) Partial purification and characterization of pentalene synthase. *Arch Biochem Biophys* **254**: 421-429
2. Croteau R, Cane DE (1985) Monoterpene and sesquiterpene cyclases. *Methods Enzymol* **110**: 383-405
3. Croteau R, Purkett PT (1989) Geranyl pyrophosphate synthetase: characterization of the enzyme and evidence that this chain length specific prenyltransferase is associated with monoterpene biosynthesis in sage (*Salvia officinalis*). *Arch Biochem Biophys* **271**: 524-535
4. Dehal SS, Croteau R (1988) Partial purification and characterization of two sesquiterpene cyclases from sage (*Salvia officinalis*) which catalyze the respective conversion of farnesyl pyrophosphate to humulene and caryophyllene. *Arch Biochem Biophys* **261**: 346-356
5. Elakovich SD (1987) Sesquiterpenes as phytoalexins and allelopathic agents. In G Fuller, WD Nes, eds, *Ecology and Metabolism of Plant Lipids*. American Chemical Society, Washington, DC, pp 93-108
6. Guedes MEM, Kuc J, Hammerschmidt R, Bostock R (1982) Accumulation of six sesquiterpenoid phytoalexins in tobacco leaves infiltrated with *Pseudomonas lachrymans*. *Phytochemistry* **21**: 2987-2988
7. Hohn TM, Beremand MN (1989) Regulation of trichodiene synthase in *Fusarium sporotrichioides* and *Gibberella pulicaris* (*Fusarium sambucinum*). *Appl Environ Microbiol* **55**: 1500-1503
8. Hohn TM, Beremand PD (1989) Isolation and nucleotide sequence of a sesquiterpene cyclase gene from the trichothecene-producing fungus *Fusarium sporotrichioides*. *Gene* **79**: 131-138
9. Hohn TM, Plattner RD (1989) Purification and characterization of the sesquiterpene cyclase aristolochene synthase from *Penicillium roqueforti*. *Arch Biochem Biophys* **272**: 137-143
10. Hohn TM, Plattner RD (1989) Expression of the trichodiene synthase gene from *Fusarium sporotrichioides* in *Escherichia coli* results in sesquiterpene production. *Arch Biochem Biophys* **275**: 92-97
11. Hohn TM, VanMiddlesworth FL (1986) Purification and characterization of the sesquiterpene cyclase trichodiene synthetase from *Fusarium sporotrichioides*. *Arch Biochem Biophys* **251**: 756-761
12. Jarvis BB, Midiwo JO, Bean GA, Aboul-Nasr MB, Barros CS (1988) The mystery of trichothecene antibiotics in *Baccharis* species. *J Nat Prod* **51**: 736-744
13. Munck SL, Croteau R (1990) Purification and characterization of the sesquiterpene cyclase patuol synthase from *Pogostemon cablin*. *Arch Biochem Biophys* **282**: 58-64
14. Read SM, Northcote DH (1981) Minimization of variation in the response to different proteins of the Coomassie blue G dye binding assay. *Anal Biochem* **116**: 53-64
15. Rogers SG, Horsch RB, Fraley RT (1986) Gene transfer in plants: production of transformed plants using Ti plasmid vectors. *Methods Enzymol* **118**: 627-640
16. Stoessl A, Stothers JB, Ward EWB (1976) Sesquiterpenoid stress compounds of the Solanaceae. *Phytochemistry* **15**: 855-872
17. Toxicology and Environmental Health Hazards Board, National Research Council (1983) Protection against Trichothecene Mycotoxins. National Academy Press, Washington, DC, pp 17-44
18. Vogeli U, Chappell J (1988) Induction of sesquiterpenoid cyclase and suppression of squalene synthetase activities in plant cell cultures treated with fungal elicitor. *Plant Physiol* **88**: 1291-1296
19. Vogeli U, Chappell J (1990) Purification and characterization of an inducible sesquiterpene cyclase from elicitor-treated tobacco cell suspension cultures. *Plant Physiol* **93**: 182-187
20. Vogeli U, Chappell J (1990) Regulation of a sesquiterpene cyclase in cellulase-treated tobacco cell suspension cultures. *Plant Physiol* **94**: 1860-1866
21. Watson DG, Rycroft DS, Freer IM, Brooks CJW (1985) Sesquiterpenoid phytoalexins from suspended callus cultures of *Nicotiana tabacum*. *Phytochemistry* **24**: 2195-2200
22. Whitehead IM, Threlfall DR, Ewing DF (1989) 5-EPI-Aristolochene is a common precursor of the sesquiterpenoid phytoalexins capsidiol and debneyol. *Phytochemistry* **28**: 775-779