Regulation of Trichodiene Synthase in Fusarium sporotrichioides and Gibberella pulicaris (Fusarium sambucinum)

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The regulation of trichodiene synthase (TS) and its relationship to trichothecene biosynthesis was investigated in Fusarium sporotrichioides NRRL 3299 and Gibberella pulicaris R-6380. Cultures were analyzed for the presence of TS activity, trichothecenes, and immunodetectable TS polypeptide over a time period of 144 h. Enzyme activity increased from barely detectable to maximum levels over a period of 3 h for F. sporotrichioides, while in G. pulicaris, a steady increase was observed over 144 h. Increases in TS activity of 50-fold for F. sporotrichioides and 10-fold for G. pulicaris R-6380 preceded by several hours the detection of trichothecenes. Immunoblot analysis employing polyclonal serum specific for the enzyme from F. sporotrichioides showed that increases in the levels of TS polypeptide corresponded to the observed changes in enzyme activity for both organisms. These data indicate that the regulation of TS activity is accomplished through increases in its cellular concentration and that TS may serve as a useful indicator of trichothecene biosynthetic activity.

The trichothecene family of bicyclic sesquiterpenes is produced by several genera of fungi. Many of the naturally occurring trichothecenes are potent inhibitors of protein synthesis, and their presence in grains has been associated with mycotoxicoses (14). The trichothecenes have also been shown to play a role in a plant disease caused by Fusarium sporotrichioides (A. E. Desjardins, G. F. Spencer, R. D. Plattner, and M. N. Beremand, Phytopathology, in press).

Little is known about the regulation of enzymes involved in fungal secondary metabolism. This is partly due to difficulties in isolating and studying many of these enzymes. Recently, as part of our investigations of trichothecene biosynthesis, we described the isolation of trichodiene synthase (TS) from F. sporotrichioides NRRL 3299 (9). TS catalyzes the isomerization-cyclization of farnesyl pyrophosphate (FPP), resulting in formation of the bicyclic olefin, trichodiene (5). This reaction is the first unique step in trichothecene biosynthesis and a potential site for pathway regulation. The identification of other enzymes in the pathway has not been reported.

In this study, we chose to investigate the regulation of TS in F. sporotrichioides NRRL 3299 and Gibberella pulicaris R-6380 (Fusarium sambucinum). Each of these organisms possesses unique advantages as a model system for the study of trichothecene biosynthesis. F. sporotrichioides NRRL 3299 has been widely studied since it was first shown to produce T-2 toxin (1). Several mutants, each blocked at a different step in the trichothecene biosynthetic pathway, have been isolated from F. sporotrichioides NRRL 3299 (2; M. N. Beremand and R. D. Plattner, unpublished data). Some strains of G. pulicaris, a heterothallic ascomycete, are documented producers of trichothecenes, and the potential for applying a classical genetic approach to the study of trichothecene biosynthesis was recently demonstrated (3, 8).

We report here on changes in the levels of TS activity and polypeptide in cultures of F. sporotrichioides NRRL 3299 and G. pulicaris R-6380 grown under conditions which permit trichothecene production.

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MATERIALS AND METHODS

Organisms and growth conditions. F. sporotrichioides NRRL 3299 was obtained from the culture collection at the Northern Regional Research Center. G. pulicaris (F. sambucinum) strains R-6380, R-5455, and R-5389 were obtained from P. E. Nelson (Fusarium Research Center, Pennsylvania State University). Single-spore isolates of these cultures were maintained on V-8 agar (13) slants at 4°C. Plates of V-8 agar were inoculated with 2-mm squares of agar from a stock slant and incubated for 5 to 8 days on an alternating 12-h 25°C light–12-h 20°C dark schedule. Plates were flooded with sterile water and rubbed with a bent glass rod. The conidial suspension (microconidia and macroconidia) was counted by means of a hemacytometer. Erlenmeyer flasks (250 ml) containing 125 ml of GYE medium (15), 5% glucose (autoclaved separately), 0.1% yeast extract (Difco Laboratories, Detroit, Mich.), and 0.1% peptone (Difco) were inoculated to a final concentration of 5 × 10⁴ conidia per ml. These flasks were incubated for up to 8 days at 28°C and at 180 rpm on a gyratory shaker.

Preparation of cell homogenates. Cultures were harvested by filtration on Whatman no. 4 paper, and the filtrate was stored at −20°C for further analysis. The mycelial mat was washed with 0.1 mM phenylmethylsulfonyl fluoride, immediately frozen in liquid N₂, and stored at −75°C. Cell breakage was accomplished by grinding the mycelia in a precooled mortar with liquid N₂, immediately followed by suspension in 8 to 10 ml of breakage buffer (10 mM Tris [pH 7.8], 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 15% glycerol). Cell homogenates were centrifuged at 8,500 × g and 4°C for 10 min, and the supernatants were saved. Immediately after decanting the supernatants, a 0.3-ml volume was removed, diluted (1/2) with (2×) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and incubated in a boiling water bath for 3 min to be used for immunoblotting.

Analysis of culture filtrates for glucose and trichothecenes. Glucose concentrations were quantitated by a coupled enzymatic assay utilizing hexokinase and glucose-6-phosphate dehydrogenase (Glucose Assay Kit no. 16-UV; Sigma
Chemical Co., St. Louis, Mo.). The levels of T-2 toxin and
diacetoxyscirpenol were determined by gas chromatography
of ethyl acetate extracts which had been passed over char-
col columns (Romer Labs, Washington, Mo.). The trime-
ethylsilyl derivatives were prepared by reaction with Tri-Sil
TBT (Pierce Chemical Co., Rockford, Ill.) and measured on
a Spectra Physics gas chromatograph (model SP7100) by
capillary column chromatography and flame ionization de-
tection as previously described (8).

**Determination of dry weights.** Dry weights were deter-
mined from 5- or 10-ml samples of culture by collecting
the mycelia on dried and preweighed GF/C filters (Whatman,
Inc., Clifton, N.J.). Filters were stored at ~20°C, dried for
22 h at 80°C, and immediately weighed.

**Preparation of substrate.** The preparation of trans,trans-
[1-3H]farnesyl pyrophosphate (1-3H]FPP) employed a mod-
ification of the procedure of Cane et al. (6) and has been
described previously (9). The radiospecific activity of [1-
3H]FPP was 192 Ci/mmol.

**TS assay.** TS was assayed by the method previously
described (9). The reaction mixture consisted of 10 mM
HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic
acid [pH 7.5]), 3 mM MgCl₂, 1 mM dithiothreitol, and 10 μM
[1-3H]FPP in a total volume of 150 μl. Enzyme preparations
(supernatants from centrifugation at 8,500 x g) were added
in 5- to 10-μl volumes to the assay. Incubation was for 10
min at 30°C. Less than 10% of the substrate was converted
to trichodienone over the course of the reaction.

**Protein determinations.** Protein concentrations were deter-
mined by the method of Bradford (4) with the Bio-Rad
protein assay (Bio-Rad Laboratories, Richmond, Calif.)
and bovine gamma globulin as the standard.

**Immunodetection of proteins.** SDS-PAGE was performed
by the method of Laemmli (10). Immunoblotting was per-
duced by the electrophoretic transfer of proteins from
polyacrylamide slab gels to nitrocellulose (pore size, 0.2 μM)
in a buffer consisting of 20 mM Tris base, 150 mM
glycine, and 20% methanol. Proteins were transferred overnight
by the Transphor (Hoefer Scientific Instruments, San
Francisco, Calif.) at a current of 0.25 A (50 V) and a constant
temperature of 15°C. After the blocking step, blots were
incubated for 2 h at 22°C in antisera diluted 1/2,000.
Immunodetection was accomplished with goat anti-rabbit
alkaline phosphatase conjugate (Sigma) as the second anti-
body. The protocol for substrate preparation (5-bromo-
4-chloro-3-indolyl phosphate and Nitro Blue Tetr bazolium)
and color reaction were as described in the Protoblot
(Promega Biotech, Madison, Wis.) technical manual.

**Preparation of antiserum.** Antiserum was raised in New
Zealand White rabbits against a preparation of TS purified as
described previously (9). The initial injection (day 1) and the
booster injection (day 21) both consisted of 50 to 75 μg of
protein emulsified in Ribi adjuvant (Ribi ImmunoChem
Research, Inc., Hamilton, Mont.). Injections were made at five
or six sites on the back, and the serum was collected (day 41)
from the ear vein. The serum was allowed to clot, and the
resulting supernatant was used without further treatment.

**Product identification.** Trichodienone was identified by gas
chromatography-mass spectrometry analysis as described
previously (9). Hexane extraction of reaction mixtures em-
ploying supernatant from centrifugation at 8,500 × g from
either *F. sporotrichioides* NRRL 3299 or *G. pulicaris* R-6380
as the enzyme source and [1-3H]FPP as the substrate yielded
trichodienone as the primary (>90%) 3H-labeled compound.

**RESULTS**

**Relationship of TS activity to trichothecene production and
growth.** The regulation of TS was investigated in cultures
grown on GYE medium. Cultures were analyzed for tricho-
theclones, growth, and TS activity. The choice of GYE
medium was based on previous results indicating that this
medium supported high levels of trichothecene production in
liquid shake culture for *F. sporotrichioides* and *G. pulicaris
(8, 15).

Culture filtrates were analyzed for the major trichothecene
product of each organism. A preliminary experiment in
which culture filtrates, whole cultures, and mycelia were
analyzed at each time point revealed that greater than 99% of
trichothecenes were present in culture filtrates. Under the
growth conditions used, *F. sporotrichioides* produced pri-
marily T-2 toxin (≈80% of total trichothecenes) and *G.
pulicaris* R-6380 produced primarily diacetoxyscirpenol
(≥90% of total trichothecenes). A nearly linear increase in
trichothecenes was observed starting at between 24 and 27 h
for *F. sporotrichioides* and between 30 and 42 h for *G.
pulicaris* (Fig. 1).

Growth was monitored by measuring changes in dry
weight (Fig. 1). The dry weight continued to increase over a
period of 144 h. A steady decrease in culture pH from a
starting value of 6.5 to 4.5 and a decrease in glucose
concentration from 5.0% to 3.0% accompanied the growth of
both organisms. The concentration of glucose in the culture
medium was 4.5% at the time trichothecenes were first
detected.

In both organisms, a change in the level of TS activity
occurred several hours prior to the initial detection of
trichothecenes. In *F. sporotrichioides*, a 50-fold increase in
TS activity was observed between 18 and 21 h, while in *G.
pulicaris* a 10-fold increase in activity was observed between
15 and 25 h. The patterns of changes in TS activity over the time course of trichothecene production were different for the two organisms. Activity increased to nearly maximum levels within 3 h for *F. sporotrichioides* but increased gradually over 144 h for *G. pulicaris*. An apparent transient decrease in TS activity, as measured by specific activity in crude homogenates, was observed for *F. sporotrichioides* between 30 and 75 h.

**Immunodetection of TS polypeptide from *F. sporotrichioides* and *G. pulicaris***. In order to utilize immunoblotting as a means of monitoring changes in the levels of TS polypeptide, antiserum was prepared against a purified preparation of TS from *F. sporotrichioides*. The specificity of the antiserum was determined by immunoblot analysis of crude homogenates. The resulting blot (Fig. 2A) showed a strong signal for a single band which comigrated with TS. This band was not observed when preimmune serum was used as a probe (Fig. 2B), indicating that the antiserum was highly specific for TS.

Immunoblot analysis was performed on crude cell homogenates from three strains of *G. pulicaris* to determine if the enzyme from this organism could be detected with antiserum raised against TS from *F. sporotrichioides* (Fig. 2A). The cultures were grown on GYEP medium under the same conditions described for *F. sporotrichioides*. For two of the strains examined, R-6380 and R-5389, a strong signal was observed for a single band which appeared to comigrate with the TS from *F. sporotrichioides* (*M*ₐ, 45,000). This band was not detected when preimmune serum was used as a probe (Fig. 2B).

The two *G. pulicaris* strains in which the 45,000-*M*ₐ polypeptide was observed produced moderate levels of trichotheccenes on GYEP medium. The third strain, R-5455, in which there was no immunodetectable 45,000-*M*ₐ polypeptide, produces barely detectable amounts of trichotheccenes under these growth conditions (8). These results are consistent with an interpretation of the immunodetectable 45,000-*M*ₐ polypeptide observed in *G. pulicaris* crude homogenates as TS.

**Appearance of TS polypeptide and its relationship to TS activity**. The changes observed in TS activity described above could result from a number of factors, such as the modification of preexisting enzyme, changes in the concentration of specific inhibitors or activators, or changes in enzyme concentration. In an effort to better understand which, if any, of these mechanisms are important to the regulation of TS, the samples collected at each time point were analyzed for immunodetectable TS polypeptide (Fig. 3). Changes in the relative amount of TS polypeptide were found to correspond with the changes observed in enzyme activity shown in Fig. 1. In *F. sporotrichioides* cultures, the amount of TS polypeptide increased from undetectable to near maximum levels between 15 and 21 h, while in *G. pulicaris* R-6380 cultures it showed a steady increase for up to 144 h.

It should be noted that in *F. sporotrichioides*, purified preparations of TS have been observed to consist primarily of two polypeptides of different molecular weights (9). During the course of this study, it was found that decreasing the time involved in the manipulation of samples resulted in significantly less polypeptide heterogeneity. This and the additional observation that the amounts of lower-molecular-weight polypeptides increased at later harvest times suggest that these lower-molecular-weight polypeptides are of artificial origin. The gene for TS has been recently isolated and Southern blotting analysis indicates that it exists as a single copy in the *F. sporotrichioides* genome (8a).

**DISCUSSION**

The results obtained for trichotheccene production and growth are similar to those previously reported for *F. sporotrichioides* (15). They clearly demonstrate that the initiation of trichotheccene biosynthesis occurs with a high concentration of glucose remaining in the culture medium. This is in contrast to the results reported for *Fusarium graminearum* grown in GYEP medium which indicated that the initiation of trichotheccene biosynthesis was dependent on the reduction of glucose in the medium to low levels (12).

The significance of the differences observed between *F. sporotrichioides* and *G. pulicaris* with respect to changes in
TS activity levels over time is unknown. It is possible that these differences may reflect different regulatory roles for this enzyme in *F. sporotrichioides* and *G. pulicaris*. However, both organisms were similar in that the appearance of TS preceded by several hours the initiation of trichothecene biosynthesis. This lag between the appearance of TS and the initial detection of trichothecenes could be the result of TS expression occurring prior to that of other pathway enzymes. It may also be due to the fact that TS catalyzes the first step in the pathway and that the trichothecenes analyzed are pathway end products. In addition, TS is localized in the cytosol (7), while the other enzymes in the pathway are likely to be membrane associated. The lag period could represent the time required for the accumulation of sufficient trichodiene in the appropriate membrane compartment. Microbial sesquiterpene biosynthesis has also been studied in *Streptomyces arenace* (11). In this organism, changes in the levels of pentalenene synthase activity are correlated with pentalenolactone biosynthesis. The time interval between the appearance of pentalenene synthase activity and the detection of pathway end products such as pentalenolactone was not reported.

The transient decrease in *F. sporotrichioides* TS activity between 30 and 75 h was also observed in the two other experiments performed and did not appear to affect the rate of trichothecene production. This decrease in enzyme-specific activity may not represent a change in the level of TS activity but may instead result from an increase in other cellular proteins relative to TS. In *F. graminearum*, a transient 50% increase in protein nitrogen has been observed under similar growth conditions (12).

Analysis of immunodetectable TS polypeptide indicated that TS activity is regulated, at least in part, by changes in the cellular concentration of enzyme. Increases in the levels of TS polypeptide were observed to parallel increases in TS activity. Since no obvious polypeptide precursor was detected, the increases in enzyme concentration probably resulted from de novo synthesis. However, other interpretations, such as a change in the rate at which TS turnover occurs, cannot be ruled out. Studies are in progress to further define the mechanisms involved in the regulation of TS.

These results suggest a possible means for the investigation of trichothecene biosynthesis in complex substrates. Currently, such studies are solely dependent on substrate analysis for the presence of trichothecenes. With this approach, the sites of trichothecene biosynthetic activity are difficult to identify because of trichothecene diffusion. TS is a useful marker for trichothecene biosynthesis in *F. sporotrichioides* and *G. pulicaris* because it appears at or close to the initiation of trichothecene production. Thus, TS antiserum could be employed as a probe for investigating both the production of trichothecenes in agricultural products and the role of trichothecenes in plant diseases. The cross-reactivity observed between TS from *F. sporotrichioides* and *G. pulicaris* suggests that the antiserum raised against the *F. sporoti-

trichodioides* enzyme may also detect TS from other trichothecene-producing fungi.

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**LITERATURE CITED**


