

## A Novel Regulatory Gene, *Tri10*, Controls Trichothecene Toxin Production and Gene Expression

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We report here the characterization of *Tri10*, a novel regulatory gene within the trichothecene gene cluster. Comparison of *Tri10* genomic and mRNA sequences revealed that removal of a single 77-bp intron provided a 1,260-bp open reading frame, encoding a 420-amino-acid protein. Disruption of *Tri10* in *Fusarium sporotrichioides* abolished T-2 toxin production and dramatically decreased the transcript accumulation for four trichothecene genes (*Tri4*, *Tri5*, *Tri6*, and *Tri101*) and an apparent farnesyl pyrophosphate synthetase (*Fpps*) gene. Conversely, homologous integration of a disruption vector by a single upstream crossover event significantly increased T-2 toxin production and elevated the transcript accumulation of the trichothecene genes and *Fpps*. Further analysis revealed that disruption of *Tri10*, and to a greater extent the disruption of *Tri6*, increased sensitivity to T-2 toxin under certain growth conditions. Although *Tri10* is conserved in *Fusarium graminearum* and *Fusarium sambucinum* and clearly plays a central role in regulating trichothecene gene expression, it does not show any significant matches to proteins of known or predicted function or to motifs except a single transmembrane domain. We suggest a model in which *Tri10* acts upstream of the cluster-encoded transcription factor TRI6 and is necessary for full expression of both the other trichothecene genes and the genes for the primary metabolic pathway that precedes the trichothecene biosynthetic pathway, as well as for wild-type levels of trichothecene self-protection. We further suggest the presence of a regulatory loop where *Tri6* is not required for the transcription of *Tri10* but is required to limit the expression of *Tri10*.

The trichothecenes represent a large family of toxic secondary metabolites produced by a variety of filamentous fungi, including *Fusarium*, *Myrothecium*, *Stachybotrys*, *Trichoderma*, and *Trichothecium* (16). They are primarily found as contaminants in food and animal feed, and consumption of these compounds by humans or livestock results in vomiting, alimentary hemorrhaging, and dermatitis (20). These toxins are potent inhibitors of eukaryotic protein synthesis (23) and induce apoptosis (24). In plants the trichothecenes are also phytotoxic and have been associated with virulence in specific plant-pathogen interactions (8, 9, 12, 25).

Significant progress has been made towards determining the trichothecene biosynthetic pathway and trichothecene gene organization and function. Trichothecenes are derived from farnesyl pyrophosphate, which is cyclized to form trichodiene (10). The biosynthetic sequence of events proceeding from trichodiene to complex trichothecenes, such as T-2 toxin, has been established by a combination of feeding experiments utilizing blocked mutants and heavy-isotope labeling of pathway precursors (3). To date, one regulatory gene, *Tri6*, one transporter gene, *Tri12*, and all but one of the known biosynthetic genes are clustered in *Fusarium sporotrichioides* (6), and similar gene clusters are present in *Fusarium graminearum* (6), *Fusarium sambucinum* (A. W. Peelow and M. N. Beremand, unpublished data), and *Myrothecium roridum* (17). *Tri101*,

which encodes isotrichodermol 3-*o*-acetyltransferase, has been identified in both *F. sporotrichioides* and *F. graminearum* and appears to reside outside of the trichothecene gene cluster (19, 21). In addition to its acetyltransferase activity, the heterologous expression of *Tri101* in yeast cells renders them resistant to trichothecenes (18, 21). However, disruption of *Tri101* does not appear to cause a loss of toxin self-protection in *F. sporotrichioides* (21), suggesting that other genes can provide this function.

The regulation of trichothecene production is equally complex, and as with many secondary metabolites it can be controlled in liquid culture by the availability of certain nutrients, oxygen, pH, and temperature (33), as well as by the modulation of signal transduction pathways mediated by a G-protein G $\alpha$  subunit (31). However, the complete genetic nature of the induction and repression of trichothecene biosynthesis is just beginning to be understood. Recently, the gene for TRI6, a zinc-finger DNA-binding protein, was isolated, characterized (15, 26), and shown to be required for induction of two trichothecene genes (*Tri5* and *Tri4*), which encode, respectively, the enzymes for the first two steps in the T-2 toxin biosynthetic pathway (26). Here, we report the isolation and characterization of a second regulatory gene from within the trichothecene gene cluster, designated *Tri10*, which is required for trichothecene biosynthesis in *F. sporotrichioides*.

### MATERIALS AND METHODS

**Strains, plasmids, media, and culture conditions.** The *F. sporotrichioides* Sherb. wild-type strain NRRL 3299 was obtained from the ARS/USDA Culture Collection at the National Center for Agricultural Utilization Research in Peo-

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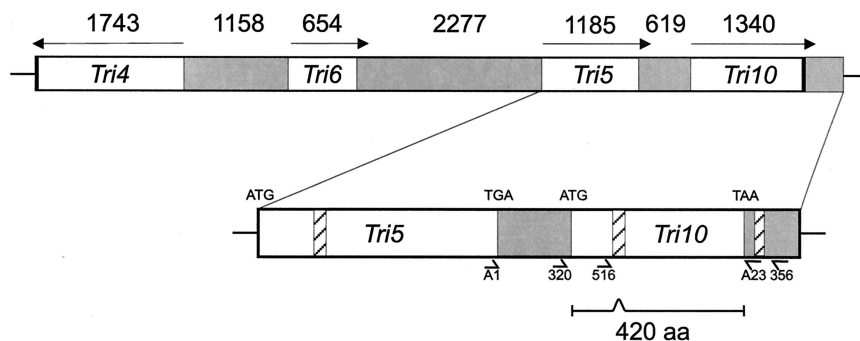


FIG. 1. Graphic representation of the location of *Tri10* and position of primers used for RT-PCR and for vector construction. Gray areas indicate intergenic regions, and the crosshatched areas represent the relative positions of introns within *Tri5* and *Tri10* in *F. sporotrichoides*. The numbers at the top of the figure indicate base pairs.

ria, III. The *Fusarium sambucinum* Fuckel (telomorph = *Gibberella pulicaris* [Fr.] Sacc.) wild-type strain R-6380 was obtained from the Fusarium Research Center, Pennsylvania State University. *F. sporotrichoides* strains MB5493 (*Tri4* mutant) (3, 22) and NN4 ( $\Delta$ *Tri6*) (26) and the *Fusarium graminearum* Schwein. (telomorph = *Gibberella zeae* [Schwein.] Petch) wild-type strains GZ3639 (5) and W-8 (25) were described previously. Fresh stock cultures were routinely established from frozen glycerol stocks of conidia: transformants were inoculated onto V8 juice agar containing 300  $\mu$ g of hygromycin B/ml and grown in the dark under alternating 12 h at 20°C and 12 h at 25°C conditions, while all other strains were inoculated onto V8 juice agar and incubated under alternating 12 h at 20°C dark, 12 h at 25°C light conditions (27). Strains were grown in liquid YEPD-2G medium (2% glucose, 0.3% yeast extract, 1% peptone) for DNA isolation and in liquid YEPD-5 medium (5% glucose, 0.1% yeast extract, and 0.1% peptone) for trichothecene analysis and RNA isolation (27). The vectors pT7Blue and pCR-Script were purchased from Novagen and Stratagene, respectively. The plasmid pGP53-1 was previously described (13).

**RNA extraction and RT-PCR.** For RNA isolation, conidia of *F. sporotrichoides* were washed from 7-day V8 juice agar cultures, filtered through sterile muslin, and inoculated into 100 ml of YEPD-5 to give a final concentration of  $5.0 \times 10^4$  conidia per ml. Parallel cultures were grown for 15 h and 23 h (28°C and 200 rpm) and harvested by vacuum filtration through P-8 qualitative filter paper (Fisher). The mycelia were immediately frozen in liquid nitrogen, lyophilized, and stored at -80°C. Samples were pulverized in liquid nitrogen immediately prior to extraction. Total RNA was isolated using the Ultraspec II kit (Biotecx) according to the manufacturer's protocol with the addition of an acid phenol-chloroform-isoamyl alcohol (25:24:1, pH 4.2) extraction following the initial extraction. For reverse transcription (RT)-PCR, the purified 23-h total RNA was treated with RNase-free DNase I (Ambion), and first-strand cDNA was generated from 5  $\mu$ g of total RNA and an oligo(dT) primer using the Superscript Preamplification kit (Gibco-BRL). The *Tri10* cDNA was amplified from the first-strand cDNA reaction by PCR using oligonucleotides 320 (5' CCACCCAGCAATCATCAG 3') and A23 (5' CTGTTGTCAATAGGCGAGTG 3'), which lie 22 bp upstream and 19 bp downstream of the putative start and stop codons, respectively (Fig. 1). A portion of the 3' end of the *Tri10* transcript was cloned by pairing primers 516 (5' CTTAGCTTATCGGGTTT 3') and 356 (5' GTACCTCGTTTCATGCC 3'), which lie 357 bp downstream of the putative start codon and 192 bp downstream of the putative stop codon, respectively. The PCR amplifications consisted of a single cycle of 5 min at 95°C; 25 cycles of 1 min at 95°C, 1 min at 50 to 55°C, and 1.5 min at 72°C; and 1 cycle of 10 min at 72°C. The amplified products were run on a 1% agarose gel and purified using the Qiaquick gel extraction kit (Qiagen). The purified products were then cloned into pCR-Script (Stratagene) and sequenced.

**DNA sequencing.** Cloned RT-PCR products and genomic DNA clones were sequenced using specifically designed primers. *Tri10* of *F. sambucinum* R-6380 was sequenced from the plasmid pGP53-1, which contains a 4.7-kb *EcoRI* fragment (harboring *Tri5* and downstream sequences) isolated from a  $\lambda$ gt11 library (13). *Tri10* was also shown to be present and downstream from *Tri5* in *F. graminearum* GZ3639 by Southern hybridization using the *F. sporotrichoides* NRRL 3299 *Tri10* homolog as a probe. This downstream DNA, which is located on a 3.5-kb *HindIII* fragment, was amplified by chromosome crawling (28) via inverse PCR using two primers, B-1 (5' GCGACGCTCGATACCGCTCC 3') and B-2 (5' CGTGTCATCACTGAGGGTCC 3'), corresponding to the *Tri5* gene from *F. graminearum* strain W-8 (25). All sequencing reactions were per-

formed using the ABI PRISM Dye Terminator Cycle Sequencing Core Kit or the BigDye Terminator Cycle Sequencing Core Kit (Perkin-Elmer Corporation). Reactions were run on a Model 373A or Model 377 DNA Sequencer (Applied Biosystems) by the Gene Technologies Laboratory at Texas A&M University.

**Plasmid construction and fungal transformation.** The *Tri10* gene disruption plasmid, pTri10-1 (Fig. 2), was constructed in two steps. First, the PCR fragment (A1-356) (Fig. 1) containing the *Tri10* coding region and flanking sequences was cloned into pT7-Blue. Then, the *Tri10* amino acid coding region was disrupted by removing a 101-bp *BstBI*-*BstEII* fragment and replacing it with the *Cochliobolus heterostrophus* promoter 1 fused to the hygromycin B phosphotransferase coding region (32).

For fungal transformation, protoplasts were isolated by the following procedure: conidia ( $1 \times 10^8$  to  $5 \times 10^8$ ) were inoculated into 100 ml of YEPD-2G broth and incubated for 7 h at 28°C with vigorous shaking (200 rpm). Germlings were harvested by filtration on a 0.2- $\mu$ m-pore-size cellulose nitrate filter, washed once in 25 ml of 0.7 M NaCl, and resuspended in 20 ml of 0.7 M NaCl containing 0.1% Novozyme 234 (InterSpex Products), 1% driselase (Sigma), and 0.025% chitinase (Sigma). The germlings were then incubated at 28°C for 20 to 60 min with gentle shaking (75 to 90 rpm) until most of the culture had been converted to protoplasts. Protoplasts were collected by centrifugation ( $1,100 \times g$ ) for 5 min at room temperature and then washed once in 0.7 M NaCl and twice in STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, 10 mM CaCl<sub>2</sub>). Transformation was performed according to the procedure described by Salch and Beremand (27).

**DNA isolation and analysis.** Fungal genomic DNA was isolated as previously described (13). Southern blots were prepared according to standard techniques (28) using DNA digested with *HindIII*, which cuts the vector pTri10-1 once, and

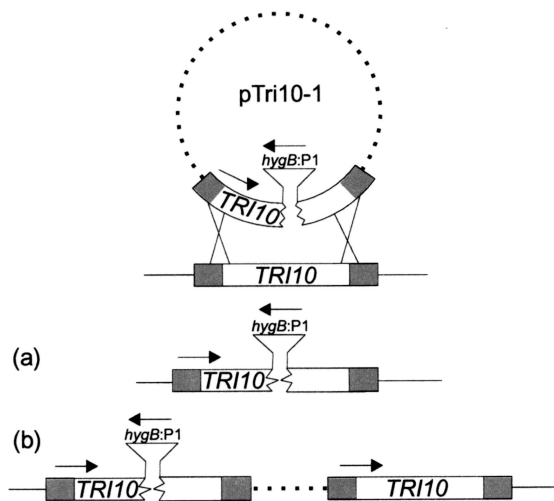


FIG. 2. Results of integration of pTri10-1 into the *Tri10* gene via double (a) and single (b) upstream homologous crossover events.

*Bg*/II, which does not cut the vector. The resulting blots were first hybridized with radiolabeled ( $[^{32}\text{P}]\text{dCTP}$ ) DNA probes prepared by nick translation (Gibco BRL) and then washed as recommended (Gene Images; USB).

**RNA analysis.** For RNA blots, 5  $\mu\text{g}$  of total RNA was separated by electrophoresis on formaldehyde-containing 1% agarose gels and transferred to Hybond N<sup>+</sup> (Amersham) nylon membranes (28). Probes for RNA blots were gel-purified PCR fragments radiolabeled with  $[^{32}\text{P}]\text{dCTP}$  by nick translation (Gibco BRL).

**T-2 toxin analysis.** Duplicate YEPD-5 liquid cultures (25 ml) were inoculated and grown in parallel as described above for RNA extraction. After 7 days of growth, a 5-ml aliquot of whole culture material was removed and frozen at  $-20^\circ\text{C}$  until analyzed. T-2 toxin was extracted by vortexing 2 ml of whole culture material with 2 ml of ethyl acetate for 90 s. The phases were separated by centrifugation at  $1,100 \times g$  for 5 min. Supernatants were removed and diluted with ethyl acetate to various concentrations and then analyzed by gas chromatography-mass spectrometry (GC-MS) on a Hewlett Packard 5890 GC equipped with an HP 5972 MS engine. Samples were introduced via a heated injection port ( $260^\circ\text{C}$ ) with an HP 7673 autosampler (2- $\mu\text{l}$  injection volume) into an HP5-MS bonded stationary phase capillary column (internal diameter, 30 m by 0.25  $\mu\text{m}$ ) with a film of 0.25- $\mu\text{m}$  thickness. The oven temperature program began with an initial oven temperature of  $90^\circ\text{C}$  for 2.0 min, followed by a ramp ( $23^\circ\text{C}/\text{min}$ ) to  $275^\circ\text{C}$  for 2.0 min. The temperature was then increased ( $30^\circ\text{C}/\text{min}$ ) to  $290^\circ\text{C}$  and held for 4.7 min. Helium was used as the carrier gas at a constant flow rate of 0.75 ml/min. The mass selective detector was used in either ion-selective mode (T-2 toxin quantitation) or full-scan mode (to obtain a full spectrum) with an ionization energy of 70 eV and an ion source temperature of  $180^\circ\text{C}$ . Identification of T-2 toxin was based on comparison of retention times and spectra to those of standards. T-2 toxin was purchased from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification. Toxin levels of transformants were compared to that of the wild type by a priori contrast using the JMP4 statistical software package.

**T-2 toxin sensitivity assays.** *F. sporotrichioides* strains were tested for resistance or sensitivity to T-2 toxin by using three different assays. In the first assay, designated the drop-plate assay, T-2 toxin solutions were prepared in ethyl acetate to yield a concentration of 10, 25, 50, or 100  $\mu\text{g}$  per 25  $\mu\text{l}$  of solvent. A 25- $\mu\text{l}$  sample of each concentration and a solvent control were individually spotted on the surface of a YEPD-2G agar plate (2% agar, 10 ml of agar per 100-mm-diameter plate) and allowed to evaporate. The plates were immediately inoculated with the desired strain by spreading 100  $\mu\text{l}$  of a freshly prepared conidial suspension containing  $1 \times 10^6$  to  $2 \times 10^6$  conidia/ml and then incubated for 2 days in a growth chamber with an alternating 12 h at  $25^\circ\text{C}$  light, 12 h at  $20^\circ\text{C}$  dark cycle. The second assay was conducted on GYEP agar (=YEPD-5 in this study) as previously described with the exceptions that plugs of mycelia were used for inoculation and that T-2 toxin was used instead of diacetoxyscirpenol and only at a 100- $\mu\text{g}/\text{ml}$  concentration (1). The third T-2 toxin sensitivity assay was conducted in liquid media. T-2 toxin stock solutions of 40 and 20 mg/ml were prepared in ethyl acetate, and 25- $\mu\text{l}$  aliquots of the appropriate concentration were added to make 1-ml aliquots of both YEPD-2G and YEPD-5, with final concentrations of 1,000 and 500  $\mu\text{g}$  of T-2 toxin/ml. Aliquots of both media were also prepared with 25  $\mu\text{l}$  of ethyl acetate as controls. Additionally, spent YEPD-5 media from 3-day cultures of NRRL 3299 and FsTri10-1-25 (this study) were analyzed by GC-MS to quantitate the amount of T-2 toxin present, and aliquots were sterilized through a nylon 0.2- $\mu\text{m}$ -pore-size filter. All media were transferred to a 96-well microtiter plate (150  $\mu\text{l}/\text{well}$ , in duplicate/strain) and inoculated with 7  $\mu\text{l}$  of conidia of NRRL 3299, the  $\Delta\text{Tri}6$  strain, or the  $\Delta\text{Tri}10$  strain to bring the final concentration to  $10^3$  conidia/ml. Cultures were grown on a gyratory shaker with vigorous shaking at  $28^\circ\text{C}$  for 3 days. The response to T-2 toxin was determined by following optical density at  $\lambda = 595$  at selected time points. A complete lack of increase in optical density corresponded to a complete lack of growth, as confirmed by visual examination of the cultures.

**Nucleotide sequence accession numbers.** The nucleotide sequence and the predicted polypeptide sequence for *Tri10* from *F. sporotrichioides*, *F. graminearum*, and *F. sambucinum* have been submitted to GenBank (accession no. AF364179, AF365969, and AF386074, respectively).

## RESULTS

**Identification and sequence analysis of *Tri10*.** Using mutant analysis and genomic DNA sequence from the trichothecene pathway gene cluster in *F. sporotrichioides*, Beremand and Hohn previously identified a potential 1,260-bp open reading frame (ORF) 619 bp downstream of the *Tri5* stop codon which

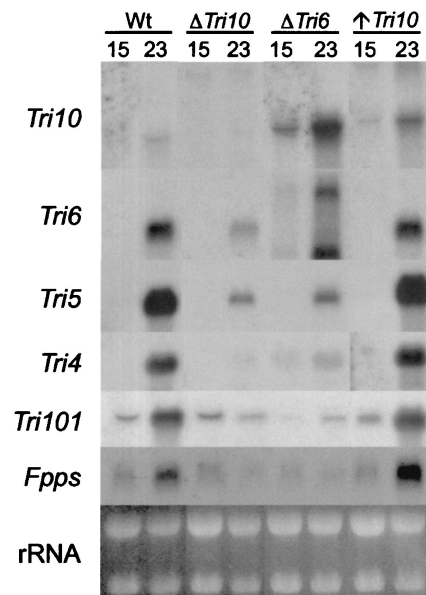


FIG. 3. Northern analyses of *Tri10*, *Tri6*, *Tri5*, *Tri4*, *Tri101*, and *Fpps* in wild-type (Wt) (NRRL 3299),  $\Delta\text{Tri}10$  (FsTri10-1-12),  $\Delta\text{Tri}6$  (NN4), and  $\uparrow\text{Tri}10$  (FsTri10-1-20) strains of *F. sporotrichioides* grown in parallel for 15 and 23 h. Five micrograms of total RNA was loaded per lane. Ribosomal RNAs were visualized in the gel by ethidium bromide staining.

they designated *Tri10* (4; M. N. Beremand, unpublished results) (Fig. 1). RNA blots probed with labeled DNA fragments from this region revealed the low-level production of a transcript from the predicted ORF, as seen in Fig. 3. In this study, the *F. sporotrichioides* *Tri10* transcript was amplified by RT-PCR, cloned, and sequenced, confirming a 1,260-bp ORF encoding a putative protein of 420 amino acids and having a predicted molecular mass of 47,427 Da. Comparison of the genomic and cDNA nucleotide sequences indicated the excision of the predicted 77-bp intron from the coding region of all transcripts (bases 379 to 455) as well as the excision of a 69-bp intron from the 3' untranslated region (bases 1424 to 1492) of some of the transcripts. Interestingly, at least one other trichothecene gene, *Tri12*, contains an intron in the 3' untranslated region (1). Both *Tri10* introns contain the consensus splicing signal sequence GT::AG for filamentous fungi, but only the 77-bp intron contains a consensus branch point (11).

*Tri10* is transcribed in the same direction as *Tri5*. Two putative TATA boxes are present at positions  $-44$  and  $-111$  from the predicted translational start codon (Fig. 4). Five putative CAAT consensus sequences are located at positions  $-32$ ,  $-48$ ,  $-50$ ,  $-107$ , and  $-207$ . A consensus eukaryotic ribosome-binding site exists beginning at position  $-6$ . No polyadenylation signals were found downstream of the stop codon. Comparisons of both the nucleotide sequence and the predicted amino acid sequence with multiple protein and DNA databases yielded only a single possible match (tBLASTn score = 93; Evalue =  $4e^{-17}$ ) (2) to a pair of nearly identical expressed sequence tags (ESTs) (AL113997.1 and AL1116861.1) of unknown function from *Botrytis cinerea* grown under nitrogen-limiting conditions.

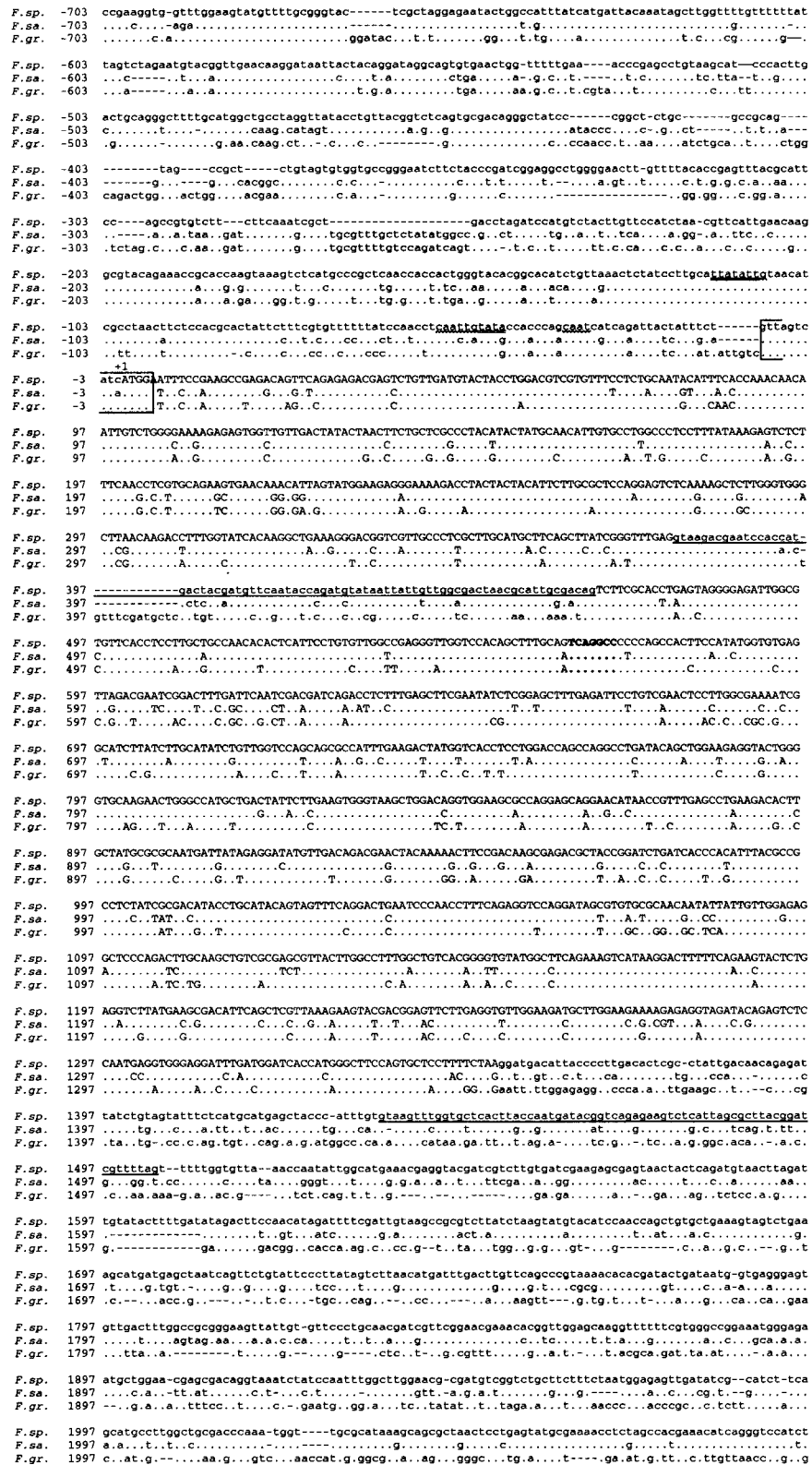


FIG. 4. Sequence alignment of *Tri10* from *F. sporotrichoides* (F. sp.) (NRRL 3299), *F. sambucinum* (F. sa.) (R-6380), and *F. graminearum* (F. gr.) (GZ3639) produced using Multiple Sequence Alignment (Baylor College of Medicine [http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/map.html]). Dots indicate conserved bases with *F. sporotrichoides*, and dashes indicate gaps between species. Capital letters indicate the putative amino acid coding region. A single underline indicates intron sequences. A double underline indicates conserved putative TATA elements, and a way underline represents conserved putative CAAT elements. The boxed region is the conserved ribosome binding site. The conserved TRI6 binding site is indicated by boldface type.

TABLE 1. Production of T-2 toxin by *Tri10* transformants

Group <sup>a</sup>	Strain	Amt of T-2 toxin ( $\mu\text{g/ml}$ ) <sup>b</sup>
Wild type	NRRL 3299	267 $\pm$ 5
$\Delta$ <i>Tri10</i>	FsTri10-1-12	0
$\uparrow$ <i>Tri10</i>	FsTri10-1-8	566 $\pm$ 5
	FsTri10-1-16	604 $\pm$ 46
	FsTri10-1-20	633 $\pm$ 14
	FsTri10-1-23	418 $\pm$ 7
	FsTri10-1-24	644 $\pm$ 6
	FsTri10-1-25	999 $\pm$ 137

<sup>a</sup> Each group was shown to be significantly different by a priori contrast.

<sup>b</sup> Values shown represent the means of duplicate extractions of duplicate cultures  $\pm$  the standard deviations.

**Transformation with pTri10-1 leading to the disruption and displacement of *Tri10*.** We wanted to examine the effect of *Tri10* gene disruption on *Tri* gene expression and toxin production. However, in previous work the homologous integration of a *Tri5* gene replacement vector into the *Tri5* downstream sequence resulted in an increase in trichothecene gene expression and toxin production (M. N. Beremand, unpublished data). Since this vector contained the downstream region of *Tri5* beginning with the last few codons of *Tri5* and extended into what we now know to be the middle of the *Tri10* gene, we designed the pTri10-1 vector so that it could either disrupt *Tri10* as a result of homologous integration via a double crossover event or essentially duplicate the homologous integration event described above via a single crossover event (Fig. 2). Accordingly, the pTri10-1 vector carried a 2.1-kb DNA fragment which began at the end of the *Tri5* amino acid coding region and included the entire *Tri10* gene except for a 101-bp amino acid coding segment which was replaced by a hygromycin resistance cassette. Southern blot analysis (data not shown) of the hygromycin-resistant transformants obtained following transformation of the wild-type *F. sporotrichioides* NRRL 3299 strain with pTri10-1 revealed that both classes of transformants were produced.

***Tri10* plays a major role in controlling toxin production and the transcription of known trichothecene cluster genes and *Tri101*.** To investigate if disruption of *Tri10* had an effect on T-2 toxin production, we grew YEPD-5 liquid shake cultures for 7 days and measured the culture filtrate for T-2 toxin using GC-MS. Transformants which contain a single disrupted copy of *Tri10* ( $\Delta$ *Tri10*) consistently produced no T-2 toxin, as seen in Table 1, and do not appear to accumulate trichothecene pathway intermediates (data not shown).

In contrast, transformants resulting from a single homologous integration event in the upstream portion of *Tri10* displayed a significant ( $P = 0.0003$ ) increase in T-2 toxin production over that of the wild-type strain (Table 1). The observation that T-2 toxin production is substantially elevated in these transformants parallels our previous and subsequent observations with integration events in this region of the gene cluster. Thus, we have designated this group of pTri10-1 transformants as *Tri10* overproducers ( $\uparrow$  *Tri10*).

RNA blot analysis was employed to examine the effect of the disruption of *Tri10* on *Tri* gene expression. Comparison of transcript levels of representative *Tri* cluster genes between the  $\Delta$ *Tri10* transformant (FsTri10-1-12) and the wild type showed

that the expression of the trichothecene biosynthetic genes *Tri5* and *Tri4* and the regulatory gene *Tri6* was greatly reduced in the  $\Delta$ *Tri10* strain (Fig. 3). The *Tri5*, *Tri4*, and *Tri6* gene transcripts are not detectable in the wild-type strain at 15 h but reach a high level of accumulation by 23 h. In contrast, only low levels of these gene transcripts appeared in the  $\Delta$ *Tri10* strain. Full expression of *Tri101*, a *Tri* gene which putatively lies outside of the *Tri* gene cluster, was also dependent upon *Tri10*. While *Tri101* was expressed at the same level at 15 h in both the wild-type and the  $\Delta$ *Tri10* strains, the level of *Tri101* transcript increased at 23 h in the wild-type strain but not in the  $\Delta$ *Tri10* strain (Fig. 3). The transcription of *Tri10* is also effectively blocked in the  $\Delta$ *Tri10* strain. The trace amount of hybridizing material observed in the 23-h sample is most likely from a fusion transcript with the 5' end of *Tri10* joined to the P1:*hygB* sequence inserted in the *Tri10* coding region (Fig. 2).

Examination of this same set of *Tri* gene transcripts in the  $\uparrow$  *Tri10* transformant FsTri10-1-20 revealed that all of these transcripts are at least slightly elevated over wild-type levels at 23 h (Fig. 3). Notably, the *Tri10* transcript is obviously elevated. Collectively, these data further demonstrate that T-2 toxin production parallels *Tri* gene expression and support the hypothesis that *Tri10* plays a major regulatory role in coordinately regulating *Tri* gene expression and T-2 toxin production.

***Tri6* is not required for transcription of *Tri10*.** Interestingly, *Tri10* appears to be overexpressed at both time points in the *Tri6* disruption mutant NN4 relative to the wild-type strain, as seen in Fig. 3. This is in sharp contrast to the other *Tri* genes, which show a marked decrease in the amount of transcript in response to the disruption of *Tri6*. These data suggest that the transcription of *Tri10* is not positively regulated by *TRI6* but instead may be negatively regulated either directly or indirectly when *TRI6* is present.

***Tri10* gene expression indirectly controls *Fpps* gene expression.** To determine if *Tri10* gene expression affects the expression of genes in the primary metabolic pathway that directly feeds into the trichothecene biosynthetic pathway, we examined the expression of *Fpps*. *FPPS* catalyzes the formation of farnesyl pyrophosphate, the last intermediate in the isoprenoid primary metabolic pathway and the immediate precursor to trichodiene, the first intermediate of the trichothecene biosynthetic pathway. As seen in Fig. 3, *Fpps* transcript levels are reduced in the  $\Delta$ *Tri10* transformant and slightly elevated at 23 h in the  $\uparrow$  *Tri10* transformant. It is also noteworthy that the *Fpps* transcripts are reduced in the  $\Delta$ *Tri6* transformant even though *Tri10* transcripts are elevated. This suggests that *Tri10* does not directly control *Fpps* transcript levels but rather does so indirectly via its control of the expression of *Tri6*, another gene(s), or trichothecene toxin levels.

***Tri10* expression affects self-protection from T-2 toxin.** The results shown in the drop plate assay (Fig. 5) indicate that the deletion of *Tri10* reduces the level of T-2 toxin self-protection and suggest that the expression of *Tri10* may be required for wild-type levels of T-2 toxin self-protection. Unlike the wild-type parent, the  $\Delta$ *Tri10* strain clearly displayed reduced growth in the presence of 100 and 50  $\mu\text{g}$  of T-2 toxin. To determine if this response was mediated through the lack of *Tri6* gene expression and hence the lack of expression of a gene(s) under the control of *Tri6* or was simply due to the lack of T-2 toxin production, the abilities of a  $\Delta$ *Tri6* strain and a *Tri4* mutant

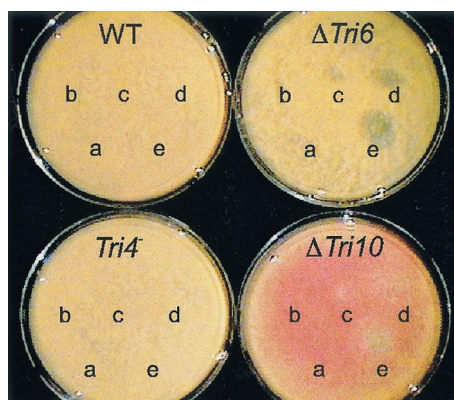


FIG. 5. Drop-plate T-2 toxin sensitivity assay. Twenty-five microliters of ethyl acetate containing 0 (a), 10 (b), 25 (c), 50 (d), or 100 (e)  $\mu$ g of T-2 toxin was spotted onto YEPD-2G plates, and the ethyl acetate was allowed to evaporate. The plates were then spread with  $1 \times 10^5$  to  $2 \times 10^5$  conidia and allowed to grow for 2 days.

strain to grow in the presence of T-2 toxin were examined. Although both strains fail to make T-2 toxin, the  $\Delta Tri6$  strain does so because it is blocked in the transcription of the known *Tri* cluster genes, and the *Tri4* mutant strain does so because it does not make an active trichodiene oxygenase enzyme and is therefore blocked in the second enzymatic step (feeding experiments have shown that this *Tri4* mutant strain expresses all of the other pathway enzymes and therefore the genes) (22). As seen in Fig. 5, the  $\Delta Tri6$  strain showed an even greater sensitivity to T-2 toxin than did the  $\Delta Tri10$  strain, while the *Tri4* mutant strain looked like the wild-type parent. We also examined T-2 toxin sensitivity using the YEPD-5 plate assay described by Alexander et al. (1) with the exceptions that T-2 toxin and mycelial plugs were used. Under these test conditions we saw no difference between the growth of the wild-type strain and either the  $\Delta Tri6$  or  $\Delta Tri10$  transformants on media containing 100  $\mu$ g of T-2 toxin/ml (data not shown). Because we had observed a discernible difference between these strains and the wild-type strain in the drop-plate assay, we performed a third assay utilizing both YEPD-2G and YEPD-5 liquid media as well as spent YEPD-5 media containing known amounts of T-2 toxin. The results of this assay are shown in Fig. 6. Both the  $\Delta Tri10$  and  $\Delta Tri6$  transformants can be distinguished from the wild-type parent. However, in contrast to the  $\Delta Tri6$  strain, which demonstrated no growth at all in the YEPD-2G (1,000  $\mu$ g of T-2 toxin/ml) and the spent media, the  $\Delta Tri10$  strain was able to grow under these conditions, albeit in an altered fashion, as measured by optical density.

The above-described experiments indicate that the assay conditions are critical for observing the increased sensitivities of both the  $\Delta Tri10$  and the  $\Delta Tri6$  strains to T-2 toxin. Overall, the  $\Delta Tri6$  transformant was more sensitive, showing clearly inhibited growth in two of the three assay formats, while the  $\Delta Tri10$  transformant was intermediate between  $\Delta Tri6$  and the wild type, showing some clearly inhibited growth only in the drop plate assay. These results, together with the observation that a low level of the *Tri6* transcript was present in the  $\Delta Tri10$  transformant, imply that *Tri10* via *Tri6* controls the expression of one or more genes that contribute to T-2 toxin self-protec-

tion and that the wild-type levels of expression of these genes become essential only under certain growth conditions.

**Tri10 is conserved in other trichothecene-producing *Fusarium* species.** To determine if *Tri10* was present in other trichothecene-producing species and also to ask what motifs within *Tri10* might be conserved, we examined the genomes of *F. sambucinum* R-6380 (*G. pulicaris*), which produces primarily diacetoxyscirpenol, and *F. graminearum* GZ3639 (*Gibberella zeae*), which produces primarily deoxynivalenol. We isolated and sequenced *Tri10* from both of these strains as described in Materials and Methods and determined that the placement and direction of transcription relative to *Tri5* are conserved. Likewise the amino acid coding sequence of *Tri10* in *F. sambucinum* and *F. graminearum* is of identical length to that found in *F. sporotrichioides*, and all three species share >85% nucleotide identity (Fig. 4) and approximately 88% amino acid identity. All three species share at least one putative TATA and CAAT element (Fig. 4). They also share the conserved eukaryotic ribosome-binding site (with one nucleotide substitution), conserved 5' and 3' splice sites surrounding the intron within the coding region, and the conserved branch point within the intron (14) (Fig. 4). However, the removal of this intron has not been confirmed by cDNA sequencing in *F. sambucinum* or *F. graminearum*. In addition, *F. sambucinum* and *F. graminearum* appear to lack the consensus splice sites for the intron in the 3' untranslated region.

## DISCUSSION

In this study we define a key regulatory gene, *Tri10*, which controls trichothecene production and related gene expression. As depicted in the model in Fig. 7, the data suggest that *Tri10* exerts this control, at least in part, by regulating the transcription of *Tri6*. *Tri10* appears to be a positive regulator of *Tri6*, since *Tri10* transcripts appear before *Tri6* transcripts in the *Tri10* overproducing strain and deletion of *Tri10* severely inhibits trichothecene gene expression and blocks trichothecene production. However, because disruption of *Tri10* does not cause a complete loss of *Tri6*, *Tri5*, *Tri4*, and *Tri101* gene transcripts and the disruption of *Tri6* likewise causes a severe reduction only of the last three gene transcripts, each of these genes must also have an additional basal route or mechanism by which its expression is activated. Furthermore, in order to account for the lack of T-2 toxin production by the  $\Delta Tri10$  strain, the loss of *Tri10* must completely block the transcription of some additional gene(s) required for T-2 toxin production. Alternatively, a combined marked reduction in both the *Tri* gene transcript levels and the farnesyl pyrophosphate pool, as a result of the parallel inhibition of *Fpps* and other isoprenoid genes (described below), could be sufficient to prevent T-2 toxin production in the  $\Delta Tri10$  strain. These possibilities can be addressed by future experiments.

As shown in the model in Fig. 7, this study provides evidence for a regulatory circuit that links the primary and secondary metabolic pathways involved in trichothecene production by the coordinate regulation of transcript levels for these pathway enzymes. A deletion of either *Tri10* or *Tri6* causes a severe reduction in *Fpps* transcripts, while overexpression of *Tri10* causes an increase. Based on additional work with our cDNA library (A. W. Peplow, A. G. Tag, G. Garifullina, and M.

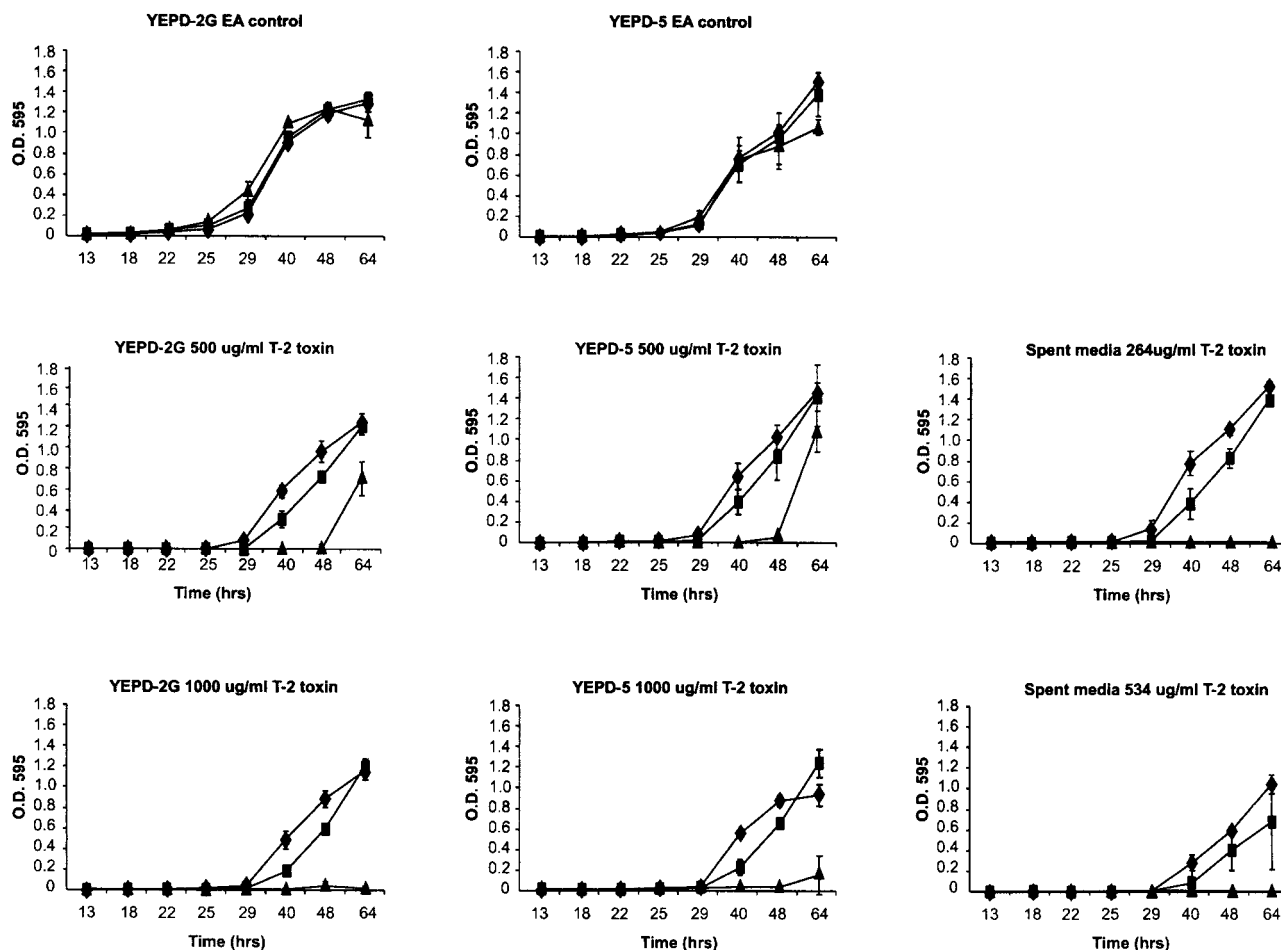


FIG. 6. Microtiter plate T-2 toxin sensitivity assay. Wild-type (NRRL 3299) (◆),  $\Delta Tri10$  (■), and  $\Delta Tri6$  (▲) strains were grown in YEPE-2G and YEPE-5 containing either 1,000, 500, or 0  $\mu\text{g}$  of T-2 toxin/ml. These strains were also grown in spent YEPE-5 media containing 264 or 534  $\mu\text{g}$  of T-2 toxin/ml. All strains were grown for 64 h, and the optical density was measured at  $\lambda = 595$  (O.D. 595). Error bars represent the range of readings observed per isolate per time point.

Beremand, unpublished data), EST database (*Fusarium sporotrichioides* Sequencing Project; B. A. Roe, Q. Ren, D. Kupfer, Hong-Shing Lai, M. Beremand, A. Peplow, and A. Tag [http://www.genome.ou.edu/fsporo.html]), and cDNA microarray studies (A. G. Tag, A. W. Peplow, T.-F. Hsieh, T. L. Thomas, and M. N. Beremand, unpublished data) of members of our group, we have determined that this regulation extends to other genes in the isoprenoid biosynthetic pathway. We are currently investigating the underlying regulatory mechanisms that mediate this control.

The regulatory relationship between *Tri10* and *Tri6* appears to be further linked. The elevation of *Tri10* transcripts in the *Tri6* deletion strain compared to the wild-type strain raises the interesting possibility of a regulatory loop whereby activation of *Tri10* upregulates *Tri6* transcription and the activation of *Tri6* in turn directly or indirectly downregulates *Tri10* transcription. However, there must also be an independent mechanism which turns off *Tri10* gene expression; otherwise, the *Tri10* transcript levels would be constitutive in the *Tri6* deletion strain.

The regulation of *Tri10* and toxin production is also con-

trolled by a regulatory region upstream of *Tri10*. In this study we show that homologous integration of pTri10-1 upstream of *Tri10* increased trichothecene toxin production and *Tri10*, *Tri6*, *Tri5*, *Tri4*, *Tri101*, and *Fpps* gene expression. This integration event produced a break in the *Tri* gene cluster approximately 700 bp upstream of *Tri10* at the very end of the *Tri5* amino acid-coding region. We have conducted additional experiments which have revealed that this coordinate increase in toxin production and gene expression is defined by a regulatory region which extends upstream of the *Tri10* coding region to the *Tri5* promoter region and that overproduction can be caused by the disruption of this contiguous sequence (G. Garifullina, A. Tag, A. Peplow, and M. Beremand, unpublished data) (7). These data suggest that the overproduction phenotype is due to the interruption of a cluster regulatory region that normally downregulates *Tri10* gene expression. This downregulation could be mediated in part through the *Tri10-Tri6* regulatory loop proposed in the model above (Fig. 7) and further discussed below.

Interestingly, both regulatory genes *Tri6* and *Tri10* flank the gene for the first biosynthetic step of the trichothecene pathway, *Tri5*, and this topography as well as the direction of

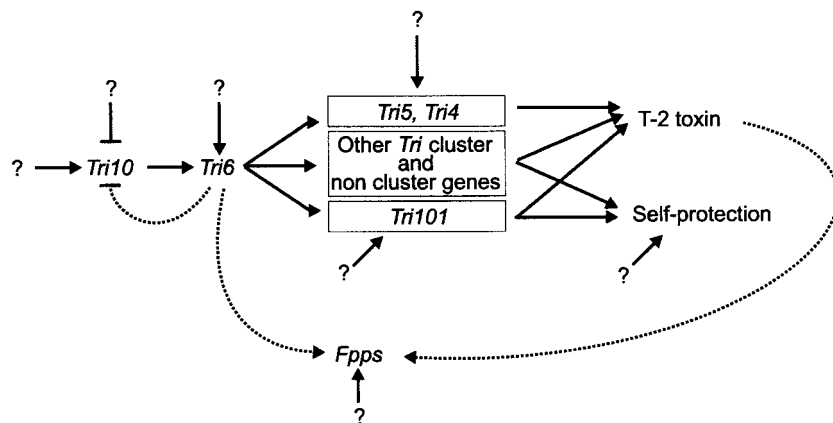


FIG. 7. Proposed regulatory model for trichothecene biosynthesis. Solid arrows indicate known positive activators. Dotted arrows indicate possible activation. Blocked arrows indicate known inhibitory activities, and dotted blocked arrows indicate possible inhibitory activities. Question marks indicate other hypothesized but unknown regulatory signals or factors.

transcription are conserved in *F. sambucinum* (A. W. Peplow and M. N. Beremand, unpublished data) and *F. graminearum* (6). All three species also lack a TRI6 binding site (15) immediately upstream from *Tri10*, which is consistent with our data and the observation that the transcription of *Tri10* is not dependent on TRI6 (this study). Conversely, *Tri10* is overexpressed in the  $\Delta Tri6$  strain, and notably, it contains a TRI6 binding site motif in the middle of its amino-acid coding region which could potentially be instrumental in mediating a reduction in *Tri10* gene expression. Although this internal binding site has not been shown to bind TRI6 in vitro (15), it could be utilized in vivo by TRI6 alone or associated with another inhibitory factor(s) to effectively block *Tri10* gene expression. It is also possible that the *Tri6*-dependent downregulation of *Tri10* is directly associated with the downregulation of *Tri10* exerted by the regulatory region upstream of *Tri10*. For example, the enhanced transcription of *Tri5* by *Tri6* may interfere with the transcription of *Tri10*. Chromatin remodeling could also be involved, independently or in conjunction with *Tri6* activity. However, regardless of the underlying mechanism(s) involved, the dependency of the wild-type regulation of *Tri10* gene expression and toxin production on the DNA sequence extending upstream of *Tri10* to in front of the *Tri5* ORF suggests that the relative positions of *Tri5* and *Tri10* may impose some evolutionary constraints on this region of the cluster, such that it may be more stable than other regions within the cluster.

The regulatory controls exerted by *Tri10* and imposed on *Tri10* are complex and intimately intertwined, with *Tri6* and cluster topography playing important roles. As discussed above, these controls extend beyond the *Tri* gene cluster to a noncluster *Tri* gene (*Tri101*) and to genes for primary metabolism. As discussed below, the expression of *Tri10* and *Tri6* also impact some aspects of trichothecene self-protection.

While both *Tri6* and to a lesser extent *Tri10* are required for wild-type levels of T-2 toxin self-protection, the loss of self-protection in the  $\Delta Tri6$  and  $\Delta Tri10$  strains is partial and dependent upon the culture conditions. Thus, *Tri6* and *Tri10* appear to play roles in self-protection which only become critical under certain conditions. *Tri6*, and *Tri10* via *Tri6*, may help

to mediate the expression of one or more self-protection genes. To date, two genes that potentially contribute to trichothecene self-protection have been identified: *Tri12*, which is a major facilitator superfamily transporter gene located within the *Tri* gene cluster, and *Tri101*, which is a 3-*o*-acetyltransferase gene located outside of the *Tri* gene cluster. *Tri101* was first identified as a potential self-protection gene as it conferred trichothecene toxin resistance on otherwise toxin-sensitive yeast cells (18, 21). However, additional genes for self-protection are likely to be present in *F. sporotrichioides*, since the deletion of *Tri12* only partially decreased toxin self-protection and the deletion of *Tri101* had no apparent effect on sensitivity to toxin (1, 21). Nonetheless, it is possible that *Tri101* may contribute to self-protection under certain conditions, including those when other self-protection genes are repressed. In this regard, it is interesting that even though *Tri101* displays what appears to be a background constitutive level of transcript accumulation in the  $\Delta Tri10$  and  $\Delta Tri6$  strains, the full expression of both *Tri101* (this study) and presumably *Tri12* is dependent on the expression of both *Tri6* and *Tri10*. Consequently, the increased sensitivity to T-2 toxin displayed by the  $\Delta Tri10$  and  $\Delta Tri6$  strains could be due to the simultaneous reduction in the expression of both *Tri12* and *Tri101* as well as of one or more additional genes that contribute to toxin self-protection.

Finally, we also addressed whether the observed reduction in self-protection in the  $\Delta Tri10$  and  $\Delta Tri6$  strains was due to an inability to make T-2 toxin. Examination of three different T-2 toxin mutant strains revealed that only the  $\Delta Tri10$  and  $\Delta Tri6$  strains and not the mutant unable to make a functional trichodiene oxygenase (*Tri4* mutant) displayed an increased sensitivity to T-2 toxin. Thus, the lack of T-2 toxin production itself does not lead to the reduction in self-protection, and consequently the expression of self-protection in *F. sporotrichioides* is not dependent upon the self-production of T-2 toxin.

The features of *Tri10* strongly suggest a role as a regulatory gene in trichothecene biosynthesis. The mRNA expression pattern of *Tri10* is consistent with that of a regulatory gene. The level of *Tri10* transcript accumulation is low in wild-type cultures, and the transcripts appear before *Tri6* transcripts in the *Tri10* overproducing strain. Although *Tri10* is highly conserved



among *F. sporotrichioides*, *F. graminearum*, and *F. sambucinum*, only one other gene (an EST, function unknown) in GenBank had similarity to *Tri10*. Likewise, a single motif, a putative transmembrane domain (amino acids 340 to 360), was identified within TRI10 (Simple Modular Architecture Research Tool) (29, 30). Curiously, TRI10 is predicted to contain 16% leucine, but it does not contain any conserved motifs consistent with leucine repeats or zipper structures. While the precise mode of action of TRI10 remains a mystery, its function is clear as an essential control point in trichothecene production and gene expression. We are currently utilizing other molecular approaches in parallel with DNA microarrays to elucidate the function of *Tri10* and further investigate the extent of the regulatory circuits defined by *Tri10*.

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