CHARACTERIZATION OF THE *TRI10* GENE FROM *FUSARIUM SPOROTRICHIOIDES*

A Dissertation

by

ANDREW GEORGE TAG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2003

Major Subject: Plant Pathology

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Marian Beremand (Chair of Committee)	James Starr (Member)
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May 2003

Major Subject: Plant Pathology

ABSTRACT

Characterization of the *Tri10* gene from *Fusarium sporotrichioides*. (May 2003)

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The trichothecene mycotoxins are secondary metabolites produced by a variety of fungal genera including *Fusarium, Myrothecium, Trichothecium,* and *Stachybotyris,* that are toxic to humans and animals that ingest them by consumption of contaminated grain. This work details the characterization of a novel regulatory gene from *Fusarium sporotrichioides*, *Tri10*, which is located in the trichothecene gene cluster. Northern analysis of *Tri10* deletion strains, *Tri10* overexpressing strains, and a *Tri6* deletion strain indicated that *Tri10* is required for wild-type trichothecene gene expression and for wild-type expression of a primary metabolic gene, *Fpps*. Analysis of these mutants also provided evidence for a regulatory feedback loop where *Tri10* is required for the expression of *Tri6* and *Tri6* negatively regulates *Tri10*. Furthermore, under certain growth conditions the sensitivity of Δ*Tri10* and Δ*Tri6* strains to T-2 toxin was increased. Analysis of mutants altered in the expression and genomic position of *Tri10* revealed that placing *Tri10* under the control of an exogenous promoter resulted in the overexpression of *Tri10* and the other *Tri* genes whether this construct was located inside or outside of the *Tri* gene cluster.

Work outside of this study has shown that in addition to *Fpps*, three other primary metabolic genes from the isoprenoid pathway feeding into trichothecene biosynthesis (*Acat*, *Mk*, *Hmgs*) are also influenced by the expression of *Tri10* and *Tri6*. In the present study, targeted cDNA microarrays were used in conjunction with multiple mutants to reveal a large group of genes, containing both trichothecene and primary metabolic genes, which were positively influenced by *Tri10* expression. At the same time, a small group of genes negatively influenced by *Tri10* expression was observed. These results were in agreement with observations made outside of this study and validated the use of targeted cDNA microarrays for further studies.

Additional analysis of the regulatory network linking trichothecene secondary metabolism and isoprenoid primary metabolism revealed that in a mutant blocked in the first step of the pathway, and therefore in the absence of trichothecene production, this regulatory link is mediated by *Tri10* and *Tri6*.

DEDICATION

I dedicate this dissertation to my wife, Paula, for her steadfastness during this whole process, and to Dr. H. G. Tag and all of the good science teachers I've had over the years.

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I extend my utmost appreciation to Marian Beremand for giving me the opportunity to work in her laboratory, providing a stimulating and nurturing research environment, and teaching the finer details of writing about and presenting research.

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CHAPTER I

INTRODUCTION

Secondary metabolites are compounds produced by organisms which appear, at least on the surface, to not be necessary for the survival of the organism in the laboratory. However, upon closer investigation it has been revealed that many secondary metabolites do have a function in the well-being of the organism and may in fact increase the fitness of the organism in its natural environment (37). One such activity may be the production of antibiotics or toxins which keep the competition at bay or facilitate infection of a host. Mycotoxins are fungal secondary metabolites which may benefit the fungus in these ways, but were first recognized because they are harmful to humans and animals. Of the great number of mycotoxins being studied, one of the most important groups is the trichothecenes.

Trichothecenes were among the first mycotoxins scientifically documented to be important in human and animal health. In the early 1900's, a disease known as alimentary toxic aleukia (ATA) was widely reported in Siberia which was characterized by a hemorrhagic skin rash, bleeding from the nose, throat and gums, and leucopenia as a result of destruction of the bone marrow.

This dissertation follows the style and format of Applied and Environmental Microbiology.

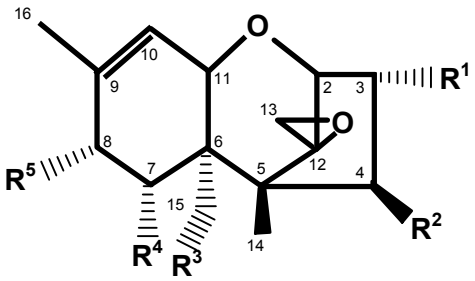
In the following years, the disease reappeared sporadically in other republics of the former Soviet Union until in the early 1940's when a more widespread outbreak of ATA occurred (62). This outbreak coincided with a deteriorating food supply due to World War II and much of the peasant population was forced to harvest grain which had overwintered in the fields covered with snow. At its worst, between 1942 and 1944, it is estimated that the mortality rate approached 60 percent in certain counties and the number of deaths reached into the hundreds of thousands (62, 69).

The health effects observed in both humans and animals that consumed the overwintered cereal grains spurred a tremendous effort to both identify the offending agent and determine its mode of action. Initially it was thought that ATA was due to an infectious epidemic or vitamin deficiency, however, as described below it was eventually determined that the probable cause was due to the consumption of trichothecenes which contaminated overwintered cereal grains (62).

In 1948, Freeman and Morrison isolated a toxic compound from *Trichothecium roseum* and named it trichothecin, the first trichothecene (39). Since then, over 180 different trichothecenes have been identified (43). Trichothecenes are produced by several genera of filamentous fungi including *Fusarium, Stachybotrys, Myrothecium,* and *Trichothecium* (8, 34, 39, 104). The

trichothecenes are tricyclic sesquiterpenoids which possess an epoxide at carbons 12 and 13, and a double bond between carbons 9 and 10 (Fig. 1-1). Type A trichothecenes, which include T-2 toxin and 4, 15-diacetoxyscirpenol (DAS) are defined by the presence of hydrogen, hydroxyl, or acetoxyl at carbons 3, 4, 7, 8, and 15. Type B trichothecenes such as deoxynivalenol (DON) possess a carbonyl group at carbon 8 (Fig. 1-1). Lastly, the macrocyclic trichothecenes are di- and tricyclic esters.

In 1973, Mirocha and Pathre provided evidence implicating T-2 toxin was the probable causal agent of ATA (80). They compared a toxic crude preparation from a Russian ATA outbreak with a T-2 toxin standard by gas chromatography-mass spectrometry, thin-layer chromatography, and infrared spectrometry. Their data suggested that T-2 toxin was more likely the causal agent of ATA than the steroid compounds sporofusarin and poaefusarin which had been previously reported by Olifson (reviewed by (9)). In a crude preparation provided by Olifson, Mirocha and Pathre found that there were no steroid compounds present in the sample but enough T-2 toxin to explain the toxicity found in the rat and rabbit skin tests for the toxic activity (80, 95).



Compound	R^1	R^2	R^3	R^4	R^5
Deoxynivalenol (DON)	OH	$OCOCH_3$	OH	OH	=O
4, 15-Diacetoxyscirpenol (DAS)	OH	$OCOCH_3$	$OCOCH_3$	H	H
T-2 toxin	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$

FIG. 1-1. Selected trichothecene structures.

The mode of action of the trichothecenes has been studied and has, at present, been suggested to function on at least 4 levels in causing disease in animals. In 1968, Ueno, et al. determined that the trichothecenes are extremely effective inhibitors of eukaryotic protein synthesis. As a group, the trichothecenes inhibit eukaryotic protein synthesis by binding to the ribosome (106). However, they can be divided into two groups based on the step of translation that they inhibit. It was suggested by Wei and McLaughlin that compounds with substitutions only at the R2 group (R2=OH) inhibit elongation or termination whereas substitutions at R1 and R3 (R1, R3=OH) promote inhibition of initiation (76, 106, 107) (Fig 1-1). In 1980, Carter, et al showed that two similar trichothecenes, trichodermin and fusarenon-X, inhibit eukaryotic protein synthesis by blocking the peptidyl transferase step (19).

Although trichothecenes are recognized foremost as protein synthesis inhibitors, they also induce apoptosis. The trichothecene T-2 toxin has been shown to induce apoptosis in the thymus (58, 59), spleen, and liver tissue (57) of mice. Other trichothecenes, including the macrocyclic trichothecenes satratoxin G, roridin A, and verrucarin A also induce apoptosis to varying degrees, with the macrocyclic trichothecenes being the most potent inducers of apoptosis (83, 111). This second mode of toxicity appears to be related to the degree to which caspase-3 is induced by the binding of the trichothecene to the ribosome and is

not directly related to the degree with which a given trichothecene inhibits protein synthesis (83, 93).

The trichothecenes are also immunosuppressive, most likely due to their properties as protein synthesis inhibitors and apoptotic agents as discussed above. However, the macrocyclic trichothecenes are also able to stimulate the immune system by increasing the production of cytokines at low concentrations of toxin and suppress the immune system at high concentrations of toxin (14). Lastly, there is some evidence from studies performed on rats that T-2 toxin and deoxynivalenol (DON) may function as neurotoxins by disruption of the blood-brain barrier (105).

Trichothecene production appears to provide an important benefit to the producing organism. All of the fungi reported to produce trichothecenes are plant pathogens. The trichothecenes have been shown to be phytotoxic (16) and play a role in virulence in several specific plant-pathogen interactions. Several species of *Fusarium* cause dry rot diseases on a variety of tuber crops and this was utilized as a model system in experiments to examine what role, if any, the trichothecenes had in the virulence of the pathogen. Mutant strains of both *F. sporotrichioides* and *Gibberella pulicaris* (anamorph=*Fusarium sambucinum*) which are unable to produce trichothecenes, exhibit reduced virulence on parsnip root (*Pastinaca sativa*) (27, 31). However, the ability to produce trichothecenes was unnecessary to achieve wild-type levels of virulence

on potato with *G. pulicaris* (27). In cooler climates, some *Fusarium* species, especially *G. zeae* (anamorph=*F. graminearum*), can cause tremendous crop losses by causing Gibberella ear rot of maize and wheat head scab. Although, in maize and wheat the trichothecenes are not essential for infection of the host plant (7, 45), they do have a role in the virulence of *G. zeae* in Gibberella ear rot of maize and wheat head scab. Studies using trichothecene non-producing mutant strains on maize in the field (45) and on wheat in both the greenhouse and the field (7, 23, 30, 85, 86) have clearly demonstrated the trichothecenes as virulence factors.

Once the chemical structures for the first trichothecenes were determined, efforts were also initiated to determine their biosynthetic origin and biochemical pathways of synthesis. In 1959, Fishman, et al. first established that the trichothecenes are sesquiterpenoid compounds (38). Hanson and Achilladelis subsequently showed that trichothecenes are derived from farnesyl pyrophosphate (reviewed in (98)).

To date, the most complete information for a trichothecene biosynthetic pathway has been obtained from studies on trichothecene production in *F. sporotrichioides*. The trichothecene biosynthetic pathway has been elucidated in *F. sporotrichioides* NRRL 3299 using a variety of approaches including analysis of blocked mutants, isotopic labeling of precursors and cross-feeding experiments. As is true for all trichothecenes, the first step is the cyclization of farnesyl pyrophosphate to form trichodiene (36). The nontoxic compound trichodiene is then converted to the highly toxic T-2 toxin through a series of 14 additional steps involving oxygenation, isomerization, cyclization, esterification, and deacetylation (Fig. 1-2).

While the first 9 steps of the trichothecene biosynthetic pathway appear to be shared across the Fusaria, the predominant trichothecene end product varies from species-to-species and strain-to-strain. The primary end product in *F. sporotrichioides* NRRL 3299 is T-2 toxin, while in many *F. graminearum* strains it is deoxynivalenol (DON) or nivalenol. *F. sambucinum* predominantly produces diacetoxyscirpenol (DAS) but some isolates are known to produce T-2 toxin (69).

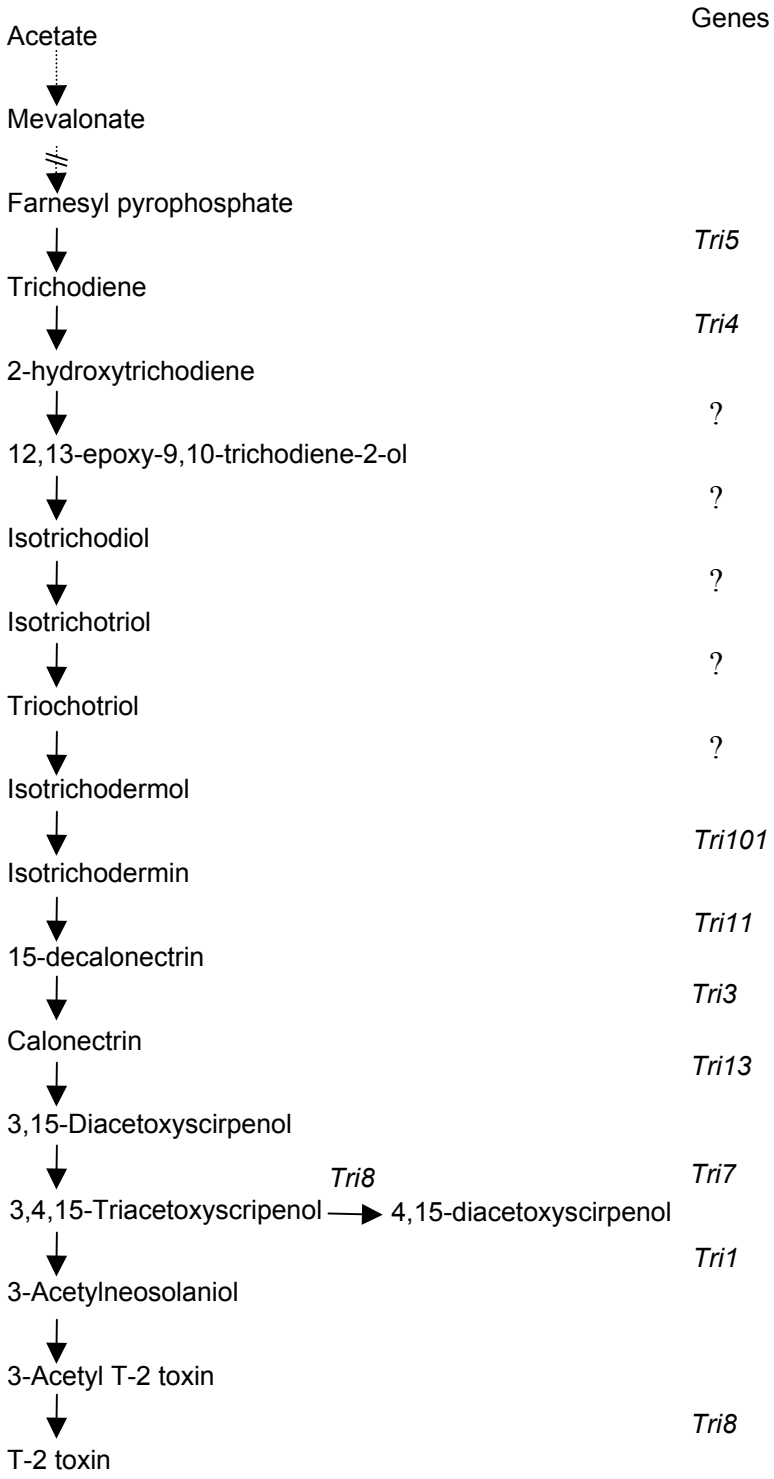


FIG. 1-2. Proposed trichothecene biosynthetic pathway for *Fusarium sporotrichioides* NRRL 3299 leading to the production of T-2 toxin.

The study of the genetics and molecular biology of trichothecene biosynthesis in the Fusaria began in earnest as a multi-pronged approach with the parallel development of *F. sporotrichioides* and *F. sambucinum* (*Gibberella pulicaris*) as model systems. A large scale UV mutagenesis and mutant screen of *F. sporotrichioides* was performed using a monoclonal antibody to T-2 toxin to look for mutants that no longer made T-2 toxin (10). This screen identified the first four trichothecene genetic loci, *Tri1* (formerly *Tox1*), *Tri2* (formerly *Tox2*), *Tri3* (formerly *Tox3*), and *Tri4* (formerly *Tox4*) (10, 74, 75, 84). These UV-induced mutants were instrumental in providing the first critical studies that definitively established the role of trichothecenes as virulence factors (31), in determining the identity and order of many of the intermediates in the T-2 toxin biosynthetic pathway (10, 74, 75, 84), and ultimately in the cloning of several *Tri* genes (50, 73). Of particular interest were strains MB1716 (*Tox1-2*), which accumulates diacetoxyscirpenol (DAS) (10), MB2972 (*Tox3-1*), which accumulates 15-deacetylcalonectrin and 3, 15 dideacetylcalonectrin (84), and MB5493 (*Tox4*-1) which accumulates trichodiene (74). In all three cases, it was possible to feed the strains putative precursors to T-2 toxin which occurred after the block in the pathway and have them converted into T-2 toxin. This provided evidence that an accumulated compound was an intermediate in the biosynthetic pathway and also indicated that the genes necessary for the steps beyond the block were being expressed (75).

At the same time, a genetic system was developed in *Gibberella pulicaris* (*F. sambucinum*) to allow for the mapping of genes by segregation analysis (11, 24). Naturally occurring variants in trichothecene production were initially analyzed. These studies revealed that in *G. pulicaris* the ability to hydroxylate at C-8 segregated as a difference at a single gene locus (*Tox1*) in most crosses between C-8$^+$ and C-8$^-$ strains. However, a few C-8$^-$ strains, when crossed with C-8$^+$ strains, displayed a 3:1 segregation pattern for C-8 hydroxylation (12). Thus this step appears to be controlled by at least two separate genes in *G. pulicaris.* In addition, this study identified at least two loci which affected the quantity of trichothecenes produced (12).

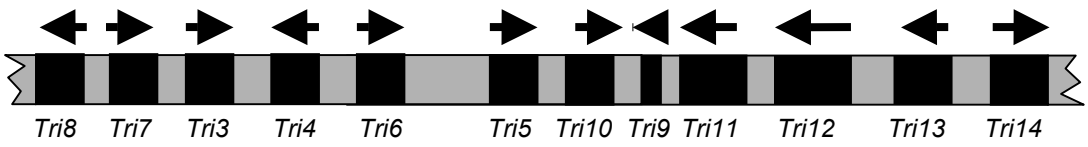
In a third approach, reverse genetics was used to clone the first trichothecene gene, *Tri5* (originally *Tox5*), from *F. sporotrichioides* NRRL 3299 (48). *Tri5* encodes the first enzyme in the trichothecene pathway, trichodiene synthase. *Tri5* was isolated from a λgt11 expression library containing genomic DNA from *F. sporotrichioides* using an antibody generated against purified trichodiene synthase protein. Sequence analysis of additional genomic fragments recovered from the library provided the sequence of the promoter and open reading frame of *Tri5* (48). *Tri5* was disrupted by transforming NRRL 3299 with a vector containing a doubly-truncated fragment of the *Tri5* open reading frame and a hygromycin resistance cassette. Transformants possessing a disrupted *Tri5* gene produced no trichothecenes (49). Furthermore, the feeding

of intermediates beyond trichodiene indicated that the enzymes beyond trichodiene synthase were present and active in the *Tri5* transformants as shown by the conversion of these precursors to T-2 toxin (48, 49). *Tri5* was subsequently cloned and disrupted in *G. pulicaris* and shown to be required for trichothecene production in this fungus (49).

Tri3 and *Tri4* were found to be linked to *Tri5* in *F. sporotrichioides* shortly after a cosmid library was constructed from wild type *F. sporotrichioides* NRRL 3299 genomic DNA and probed with a *Tri5* gene fragment to isolate cosmids harboring *Tri5* and contiguous sequences. When two of these cosmids were used in an attempt to complement the trichothecene mutant strains MB5493 (*tri4*, formerly *Tox4-1*$^{-}$), MB2972 (*tri3*, formerly *Tox3-1*$^{-}$), and MB1716 (*tri1-1*, formerly *Tox1-1*$^{-}$) (28, 51, 54), both cosmids complemented the *tri4* and *tri3* strains, but neither one complemented the *tri1* strain. This provided the first evidence that at least three trichothecene genes were closely linked in a gene cluster in *F. sporotrichioides* (54). Subsequent analysis of the DNA sequence surrounding *Tri5* revealed additional *Tri* genes (Fig. 1-3). Most of the other known core cluster trichothecene genes have been identified and characterized in a uniform fashion by DNA sequencing, transformation-mediated gene disruption and chemical analysis.

The *F. sporotrichioides* NRRL 3299 trichothecene gene cluster spans at least 27 kb and contains 12 identified genes. Among them are: 2 regulatory genes (*Tri10* and *Tri6*) (13, 87), an efflux transporter (*Tri12*) (1, 4), 7 biosynthetic genes (*Tri3, Tri4, Tri5, Tri7, Tri8, Tri11,* and *Tri13*) (2, 3, 17, 48, 50, 67, 71, 73), and two genes of undetermined function (*Tri9* and *Tri14*) (18, 29) (A. W. Peplow, A. G. Tag, G. F. Garifullina, and M. N. Beremand, *in press*) (Fig. 1-3). Similar gene clusters have been found in *Myrothecium* and in other *Fusarium* species (17, 102). Although clustering of genes for secondary metabolism in fungi was once thought to be unusual, it is has been found to be more the rule than the exception. In fact, most genes for secondary metabolism in fungi have been found to be clustered (63).

In addition to the core trichothecene gene cluster, two genes, *Tri101* and *Tri1* have been isolated from *F. sporotrichioides* that are positioned elsewhere in the genome (65, 66).



Tri3 = acetyltransferase
Tri4 = P450 monooxygenase
Tri5 = terpene synthase
Tri6 = transcription factor
Tri7 = acetyltransferase
Tri8 = esterase
Tri9 = unknown
Tri10 = regulatory gene
Tri11 = P450 monooxygenase
Tri12 = transporter
Tri13 = P450 monooxygenase
Tri14 = unknown

FIG. 1-3. The *F. sporotrichioides* trichothecene gene cluster. The gene cluster contains at least 12 genes and spans approximately 27 kb. Black bars represent protein coding sequence and arrows designate direction of transcription.

Tri101 was isolated by two different research groups in screens designed to identify potential trichothecene resistance genes by their ability to convey trichothecene resistance to yeast cells. TRI101 was found to acetylate the C-3 position, converting isotrichodermol to isotrichodermin. In general, the acetylated trichothecenes are less toxic than the corresponding alcohols, thus it represented the resistance conveyed to yeast. However, when *Tri101* was disrupted in *F. sporotrichioides*, it did not appear to play an essential role in the self-protection of the organism from trichothecenes (72). The *Tri1* gene was initially discovered in *F. sporotrichioides* by isolation of T-2 toxin deficient mutants as described above (10), and was recently cloned and characterized (78, 79). The deduced TRI1 sequence indicated that *Tri1* encodes a cytochrome P450 monooxygenase. The disruption of *Tri1* led to the

accumulation of 4, 15-diacetylscirpenol in a similar manner to the UV-induced *tri1* mutant strain MB1716 and it was shown that the introduction of the *Tri1* gene into the UV mutant strain MB1716 complemented the *tri1* mutation (78, 79).

The genetic regulation of trichothecene production has just begun to be understood. *Tri6,* a C_2-H_2 transcription factor located within the core trichothecene gene cluster, is required for production of trichothecenes (87). Transformants in which the *Tri6* gene had been disrupted exhibit a dramatically decreased accumulation of the *Tri4* and *Tri5* transcripts paralleled by the prevention of trichothecene biosynthesis and a slight accumulation of trichodiene (87). Further analysis revealed that TRI6 bound specifically to the sequence 5'-TNAGGCCT-3' which occurs in the promoter region of all of the known trichothecene genes (52). Similarly, other mycotoxin gene clusters also contain pathway-specific regulators. The *aflR* and *aflJ* genes regulate aflatoxin production in *Aspergillus parasiticus* and sterigmatocystin production in *A. nidulans,* and are found within the respective gene clusters for each of these organisms.

Like many secondary metabolites, the production of trichothecenes can be controlled by environmental parameters such as nutrient availability, oxygen, pH, and temperature (104). From this aspect, it is obvious that many genes

exist that can help regulate trichothecene production. Some of these may overlap with other developmental or cellular differentiation processes and therefore may be common to secondary metabolism in general and include genes not in secondary metabolic gene clusters.

In *Aspergillus*, a number of such non-cluster genes have been found that regulate the production of aflatoxin and sterigmatocystin. The production of aflatoxin occurs in acidic media and is inhibited in alkaline media (21). It was demonstrated that PACC, a protein which represses acid-expressed genes under alkaline conditions, bound to the promoter region of the aflatoxin transcription factor *aflR,* and could thereby repress the expression of the aflatoxin genes (35, 100). Global signal transduction pathways are also involved. The *fadA* gene of *A. nidulans* encodes the alpha subunit of a heterotrimeric G-protein and has been shown to regulate sterigmatocystin production and conidiation (47). A constitutively active form of FadA was shown to decrease sterigmatocystin and increase penicillin production in *A. nidulans.* The expression of the same *A. nidulans fadA* allele as described above, in *F. sporotrichioides* resulted in an increase in trichothecene production and a decrease in sporulation (97). Additional genes outside of the trichothecene gene cluster also contribute to the regulation of trichothecene biosynthesis.

The possibility of a regulatory gene downstream of *Tri5* arose with data collected from studies designed to substitute a reporter gene, β-*gal*, for *Tri5*. This involved a two-step process. The first step was the homologous integration of a vector carrying the β-*gal* gene flanked by the *Tri5* promoter and DNA sequence downstream of the *Tri5* ORF. However, comparison of the results of upstream and downstream vector integration revealed two phenotypes (Beremand, unpublished results). Only transformants in which the vector integrated via the *Tri5* downstream flanking sequence, and thus disrupted this sequence, displayed an overproduction of T-2 toxin. This suggested the presence of a possible regulatory gene or region downstream of *Tri5.* Additional studies showed a corresponding overexpression of transcripts from all five *Tri* genes analyzed at that point. Meanwhile, an examination of the genomic DNA sequence from this region revealed the presence of two ORFs downstream of *Tri5* which could be joined into a single ORF by the removal of a putative intron. This ORF was then designated *Tri10* (13). Preliminary Northern analysis showed the presence of a transcript generated from these ORFs which was also overexpressed in the downstream transformants described above (Beremand, unpublished results). It was at this point that I began my investigations into *Tri10*.

Chapter II of this work was published in *Applied and Environmental Microbiology* , 67(11), 5294-5302 and details the initial isolation and disruption of

Tri10 which I and the other authors completed. In that study we conclusively demonstrated that *Tri10* is a positive regulatory gene required for wild-type Tri gene expression. In addition, we also demonstrated that *Tri10* is necessary for wild-type farnesyl pyrophosphate synthetase (*Fpps*) gene expression and set forth a basic model for the regulation of trichothecene biosynthesis. The work presented in Chapter III investigates the effect of overexpressing *Tri10* from positions both within and outside the Tri gene cluster and begins to examine the effects of *Tri10* expression on a larger scale through the use of targeted cDNA microarrays. Finally, I conducted studies which investigated the effects of *Tri10* expression on a key isoprenoid biosynthetic gene in the absence of trichothecene production and establishes the role of *Tri10* in the regulation of the isoprenoid pathway. This work is presented in Chapter IV and extends our knowledge of the regulation of isoprenoid genes by *Tri10*.

CHAPTER II

A NOVEL REGULATORY GENE, *TRI10*, CONTROLS TRICHOTHECENE TOXIN PRODUCTION AND GENE EXPRESSION*

INTRODUCTION

The trichothecenes represent a large family of toxic secondary metabolites produced by a variety of filamentous fungi including *Fusarium*, *Myrothecium*, *Stachybotrys*, *Trichoderma*, and *Trichothecium* (61). They are primarily found as contaminants in food and feedstuffs and consumption of these compounds by humans or livestock results in vomiting, alimentary hemorrhaging, and dermatitis (69). These toxins are potent inhibitors of eukaryotic protein synthesis (76) and induce apoptosis (83). In plants the trichothecenes are also phytotoxic and have been associated with virulence in specific plant-pathogen interactions (26, 31, 45, 85).

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*Reprinted with permission from "A novel regulatory gene, *Tri10*, controls trichothecene toxin production and gene expression" by Tag, Andrew G., G. F. Garifullina, A. W. Peplow, C. Ake, Jr., T. D. Phillips, T. M. Hohn, and M. N. Beremand. 2001. *Applied and Environmental Microbiology*, 67(11):5294-5302.

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 (36). 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 labelling of pathway precursors (10). To date, one regulatory gene, *Tri6*, one transporter gene, *Tri12*, and all but one of the known biosynthetic genes are clustered in *Fusarium sporotrichioides* (17), and similar gene clusters are present in, *F. graminearum* (17), *F. sambucinum* (A.W. Peplow and M.N. Beremand, unpublished data), and in *Myrothecium roridum* (63). *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 (66, 72). In addition to its acetyltransferase activity, the heterologous expression of *Tri101* in yeast cells renders them resistant to trichothecenes (64, 72). However, disruption of *Tri101* does not appear to cause a loss of toxin self-protection in *F. sporotrichioides* (72), suggesting that other genes can provide this function.

The regulation of trichothecene production is equally complex and like many secondary metabolites, can be controlled in liquid culture by the availability of certain nutrients, oxygen, pH, and temperature (104), as well as by

the modulation of signal transduction pathways mediated by a G-protein Gα subunit (97). However, the complete genetic nature of the induction and repression of trichothecene biosynthesis is just beginning to be understood. Recently, TRI6, a zinc-finger DNA-binding protein, was isolated, characterized (52, 87), 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 (87). 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 Peoria, Ill. 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. sporotrichioides* strains MB5493 (*Tri4*$^-$) (10, 75) and NN4 (Δ*Tri6*) (87), and the *Fusarium graminearum* Schwein. (telomorph= *Gibberella zeae* (Schwein.)

Petch) wild-type strains GZ3639 (15) and W-8 (85) were described previously. Fresh stock cultures were routinely established from frozen glycerol stocks of conidia: Transformants were inoculated onto V8 juice agar containing 300 µg/ml hygromycin B and grown in the dark under alternating 12 h 20°C/ 12 h 25°C conditions, while all other strains were inoculated onto V8 juice agar and incubated under alternating 12 h 20°C dark/ 12 h 25°C light conditions (88). 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 (88). The vectors pT7Blue and pCR-Script were purchased from Novagen and Stratagene, respectively. Plasmid pGP53-1 was previously described (49).

RNA extraction and RT-PCR

For RNA isolation, conidia of *F. sporotrichioides* 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 5.0×10^4 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 RT-PCR, the purified 23 h total RNA was treated with RNase-free DNase I (Ambion) and first-strand cDNA was generated from 5 µg 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. 2-1). A portion of the 3' end of the *Tri10* transcript was cloned by pairing primers 516 (5' cttcagcttatcgggttt 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 50-55°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.

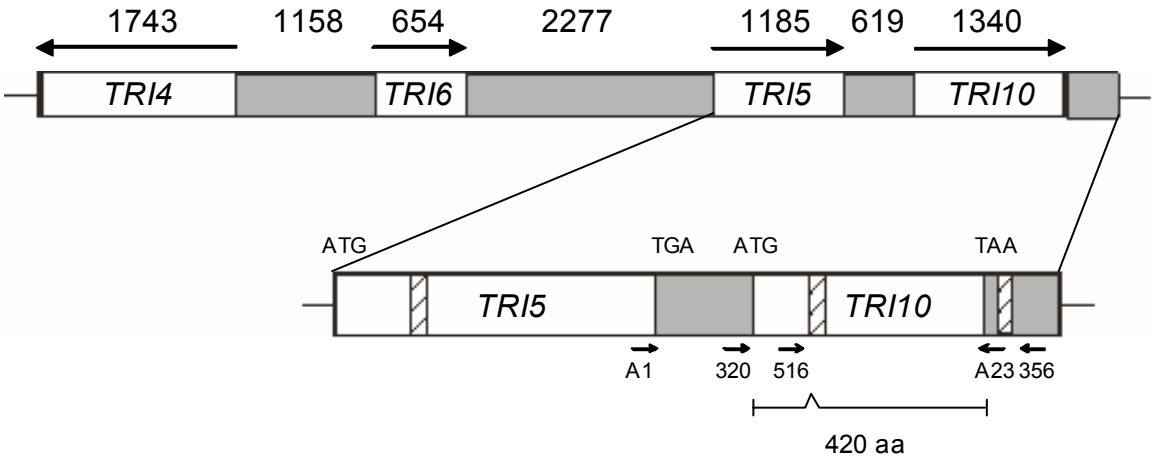

FIG. 2-1. Graphic representation of the location of *Tri10* and position of primers used for RT-PCR and for vector construction. Gray areas indicate intergenic regions while the crosshatched areas represent the relative positions of introns within *Tri5* and *Tri10* in *F. sporotrichioides.* The numbers at the top of the figure indicate base pairs.

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 plasmid pGP53-1, which contains a 4.7-kb *EcoR* I fragment (harboring *Tri5* and downstream sequences) isolated from a λgt11 library (49). *Tri10* was also shown to be present and downstream from *Tri5* in *F. graminearum* GZ3639 by Southern hybridization using the *F. sporotrichioides* NRRL 3299 *Tri10* homolog as a probe. This downstream DNA, which is located on a 3.5 kb *Hin*dIII fragment, was amplified by chromosome crawling (89) via inverse PCR using two primers, B-1 (5'gcgacgctcgataccgcctcc3') and B-2 (5'cgtgtccatcacctgagggtcc3'), corresponding to the *Tri5* gene from *F.*

graminearum strain W-8 (85). All sequencing reactions were performed 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. 2-2), was constructed in two steps. First, the PCR fragment (A1-356) (Fig. 2-1) 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 *BstB* I-*BstE* II fragment and replacing it with the *Cochliobolus heterostrophus* promoter 1 fused to the hygromycin B phosphotransferase coding region (103). For fungal transformation, protoplasts were isolated by the following procedure: Conidia (1 to 5 × 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 µm cellulose nitrate filter, washed once in 25 ml 0.7M NaCl, and resuspended in 20 ml 0.7M NaCl containing 0.1%

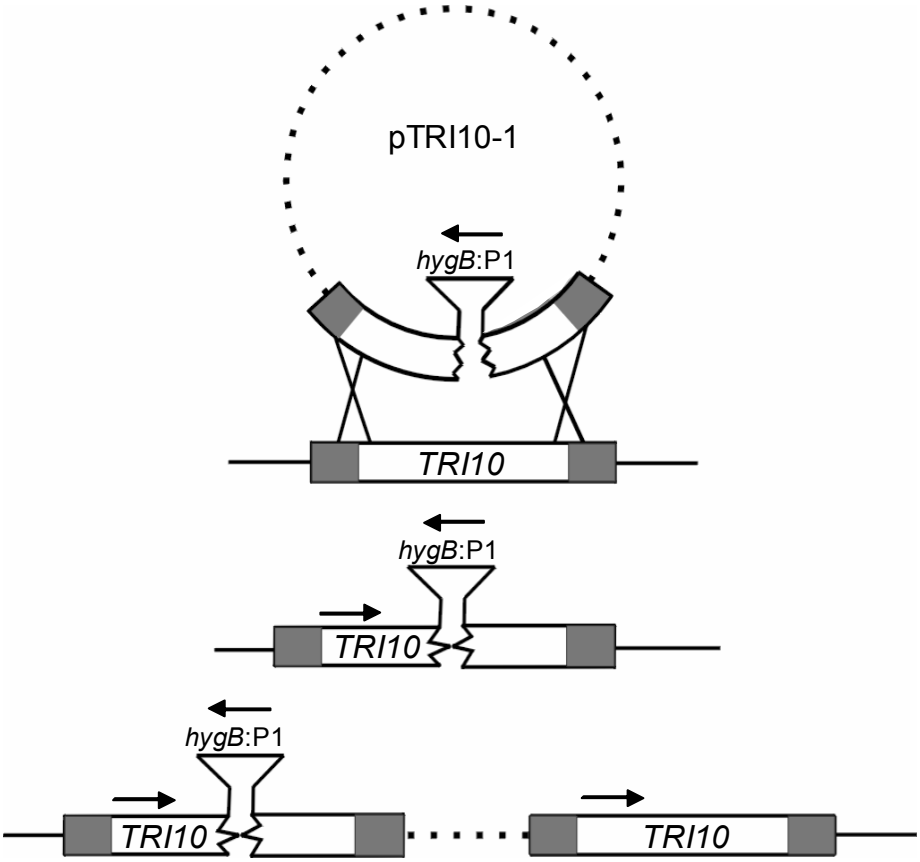

FIG. 2-2. Results of integration of pTri10-1 into the *Tri10* gene. Integration via (a) double and (b) single upstream homologous crossover events.

Novozyme 234 (InterSpex Products), 1% driselase (Sigma) and 0.025% chitinase (Sigma). The germlings were then incubated at 28°C for 20-60 min with gentle shaking (75-90 rpm) until most of the culture had been converted to protoplasts. Protoplasts were collected by centrifugation (1100 x g) for 5 min at room temperature and then washed once in 0.7M NaCl and twice in STC buffer

(1.2M sorbitol, 10mM Tris-HCl, 10mM $CaCl_2$). Transformation was performed according to the procedure described by Salch and Beremand (88).

DNA isolation and analysis

Fungal genomic DNA was isolated as previously described (49). Southern blots were prepared according to standard techniques (89) using DNA digested with *Hin*d III, which cuts the vector pTri10-1 once, and *Bgl* II, which does not cut the vector. The resulting blots were first hybridized with radiolabeled ([^{32}P] dCTP) DNA probes prepared by nick translation (Gibco BRL) and then washed as recommended (Gene Images, USB).

RNA analysis

For RNA blots, 5 µg of total RNA was separated by electrophoresis on formaldehyde-containing 1% agarose gels and transferred to Hybond N^+ (Amersham) nylon membranes (89). Probes for RNA blots were gel-purified PCR fragments radiolabeled with [^{32}P] 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°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 1100 x g for 5 min. Supernatants were removed and diluted with ethyl acetate to various concentrations and 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°C) with an HP 7673 autosampler (2 μl injection volume) into an HP5-MS bonded stationary phase capillary column (30 m X 0.25 μm internal diameter) with a film of 0.25 μm thickness. The oven temperature program began with an initial oven temperature of 90°C for 2.0 min, followed by a ramp (23°C/min) to 275°C for 2.0 min. The temperature was then increased (30°C/min) to 290°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 full spectrum) with an ionization energy of 70 eV and an ion source temperature of 180°C. Identification of T-2 toxin was based on retention times and spectra compared 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 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 μg per 25 μl solvent. A 25-μl sample of each concentration and a solvent control were individually spotted on the surface of a YEPD-2G agar plate (2% agar, 10 ml agar per 100 mm plate) and allowed to evaporate. The plates were immediately inoculated with the desired strain by spreading 100 μl of a freshly prepared conidial suspension containing 1 to 2 x 10^6 conidia/ml and then incubated for 2 days in a growth chamber with an alternating 12 h 25°C light/ 12 h 20°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 μg/ml concentration (4). The third T-2 toxin sensitivity assay was conducted in liquid media. T-2 toxin stock solutions of 40 mg/ml and 20 mg/ml were prepared in ethyl acetate and 25-μl aliquots of the appropriate concentration were added to make 1-ml aliquots of both YEPD-2G and YEPD-5 with final concentrations of 1000 and 500 μg/ml T-2 toxin. Aliquots of both media were also prepared with 25 μl 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 μm filter. All media were transferred to a 96-well microtiter plate (150 μl/well, in duplicate/strain) and inoculated with 7 μl of condia of NRRL 3299, the Δ*Tri6* strain, or the Δ*Tri10* strain to bring the final concentration to 1 X 10^3 conidia/ml. Cultures were grown on a gyratory shaker with vigorous shaking at 28°C for 3 days. The response to T-2 toxin was determined by following optical density at λ=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. graminearium*, and *F. sambucinum* have been submitted to GenBank (accession nos. AF364179, AF365969, and AF386074, respectively).

RESULTS

Identification & 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 they designated *Tri10* (M. N. Beremand, unpublished results) (13) (Fig. 2-1). 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 Figure 2-3. 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 a predicted molecular weight 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 379-455) as well as the excision of a 69-bp intron from the 3' untranslated region (bases 1424-1492) of some of the transcripts. Interestingly, at least one other trichothecene gene, *Tri12*, contains an intron in the 3' untranslated region (4). 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 (44).

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. 2-4). 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 predicted amino acid sequence against multiple protein and DNA databases yielded only a single possible match (tBLASTn score=93, Evalue=$4e^{-17}$) (6) to a pair of nearly identical ESTs (AL113997.1, AL1116861.1) of unknown function from *Botrytis cinera* grown under nitrogen-limiting conditions.

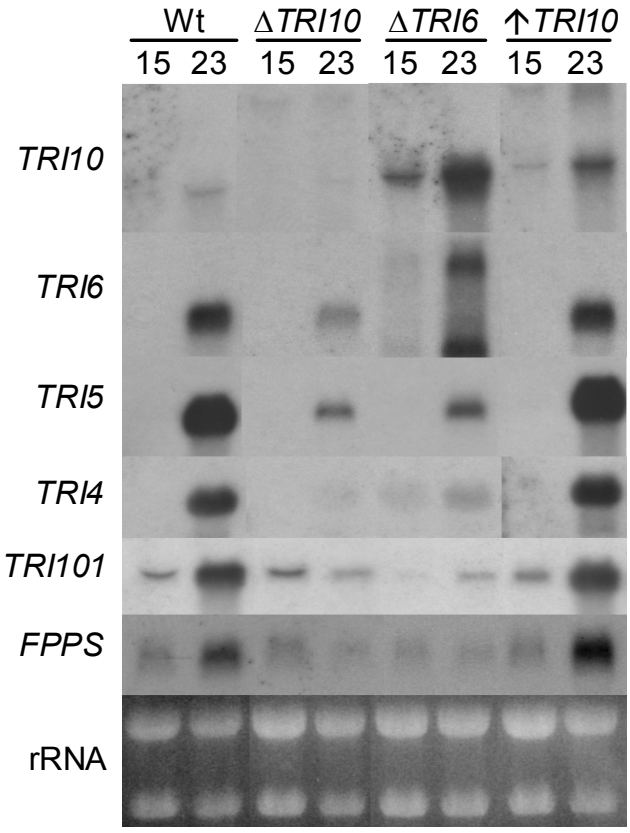

FIG. 2-3. Northern analysis of Δ*Tri10,* Δ*Tri6,* and ↑*Tri10* strains. Northern analyses of *Tri10, Tri6, Tri5, Tri4, Tri101* and *Fpps* in wild-type (NRRL 3299), Δ*Tri10* (FsTri10-1-12), Δ*Tri6* (NN4), and ↑*Tri10* (FsTri10-1-20) strains of *F. sporotrichioides* grown in parallel for 15 and 23 h. 5 μg of total RNA was loaded per lane. Ribosomal RNAs were visualized in the gel by ethidium bromide staining.

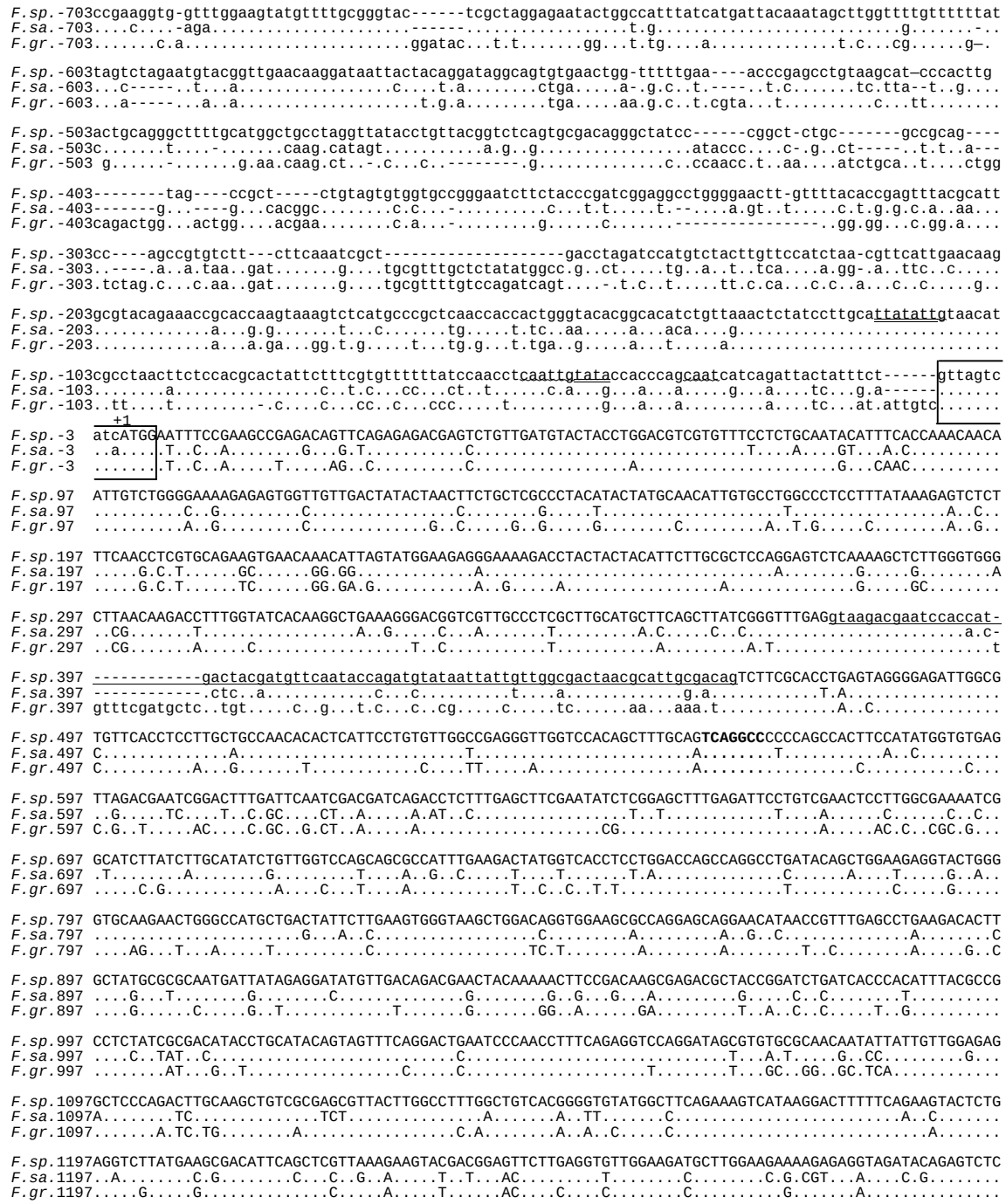

FIG. 2-4. Sequence alignment of *Tri10*. Sequence alignment of *Tri10* from *F. sporotrichioides* (NRRL 3299), *F. sambucinum* (R-6380), and *F. graminearum* (GZ3639) produced using Multiple Sequence Alignment (Baylor College of Medicine [http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/map.html]). Dots indicate conserved bases with *F. sporotrichioides*, dashes indicate gaps between species. Captial letters indicate the putative amino acid coding region. A single underline indicates intronic sequences. A double underline indicates conserved putative TATA elements and a wavy 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.

FIG. 2-4. Continued

Transformation with pTri10-1 leading to the disruption and displacement of *Tri10*

The effect of *Tri10* gene disruption on *Tri* gene expression and toxin production was examined next. 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). Since this vector contained the downstream region of *Tri5* beginning with the last few codons of *Tri5* and extended into what is known 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. 2-2). 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 d and measured the culture filtrate for T-2 toxin using gas chromatography-mass spectrometry (GC-MS). Transformants which contain a single disrupted copy of *Tri10* (Δ*Tri10*) consistently produce no T-2 toxin as seen in Table 2-1 and do not appear to accumulate trichothecene pathway intermediates (data not shown).

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

Group[a]	Strain	Amt of T-2 toxin (μg/ml)[b]
Wild type	NRRL 3299	267±5
Δ*Tri10*	FsTri10-1-12	0
↑*Tri10*	FsTri10-1-8	566±5
	FsTri10-1-16	604±46
	FsTri10-1-20	633±14
	FsTri10-1-23	418±7
	FsTri10-1-24	644±6
	FsTri10-1-25	999±137

[a]Each group was shown to be significantly different by *a priori* contrast.
[b]Values shown represent the mean of duplicate extractions of duplicate cultures ± the standard deviation.

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 in comparison to the wild-type strain (Table 2-1). 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 (↑*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 Δ*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 Δ*Tri10* strain (Fig. 2-3). 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 Δ*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* is expressed at the same level at 15 h in both the wild-type and the Δ*Tri10* strains, the level of *Tri101* transcript increases at 23 h in the wild-type strain but not in the Δ*Tri10* strain (Fig. 2-3). The transcription of *Tri10* is also effectively blocked in the Δ*Tri10* strain. The trace amount of hybridizing material observed in the 23h sample is most likely from a fusion transcript with the 5' end of *Tri10* joined to the P1:*hyg*B sequence inserted in the *Tri10* coding region (Fig. 2-2).

Examination of this same set of *Tri* gene transcripts in the ↑*Tri10* transformant FsTri10-1-20 revealed that all of these transcripts are at least slightly elevated compared to the wild type at 23 h (Fig. 2-3). 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 Figure 2-3. 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 Figure 2-3, *Fpps* transcript levels are reduced in the Δ*Tri10* transformant and slightly

elevated at 23 h in the ↑*Tri10* transformant. It is also noteworthy that the *Fpps* transcripts are reduced in the Δ*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. 2-5) 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 Δ*Tri10* strain clearly displayed reduced growth in the presence of 100 μg and 50 μg 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 simply due to the lack of T-2 toxin production, the ability of a Δ*Tri6* strain and a *Tri4*$^-$ strain to grow in the presence of T-2 toxin was examined. Although both strains fail to make T-2 toxin, the Δ*Tri6* strain does so because it is blocked in the transcription of the known *Tri* cluster genes, and the *Tri4*$^-$ 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*$^-$ strain expresses all of the other pathway enzymes and therefore the genes) (75). As seen in

Figure 2-5, the Δ*Tri6* strain showed an even greater sensitivity to T-2 toxin than did the Δ*Tri10* strain, while the *Tri4*$^-$ 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* (4), 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 Δ*Tri6* or Δ*Tri10* transformants on media containing 100 μg/ml T-2 toxin (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 Figure 2-6. Both the Δ*Tri10* and Δ*Tri6* transformants can be distinguished from the wild-type parent. However, in contrast to the Δ*Tri6* strain which demonstrated no growth at all in the YEPD-2G (1000 μg/ml T-2 toxin) and the spent media, the Δ*Tri10* strain was able to grow under these conditions, albeit in an altered fashion, as measured by optical density.

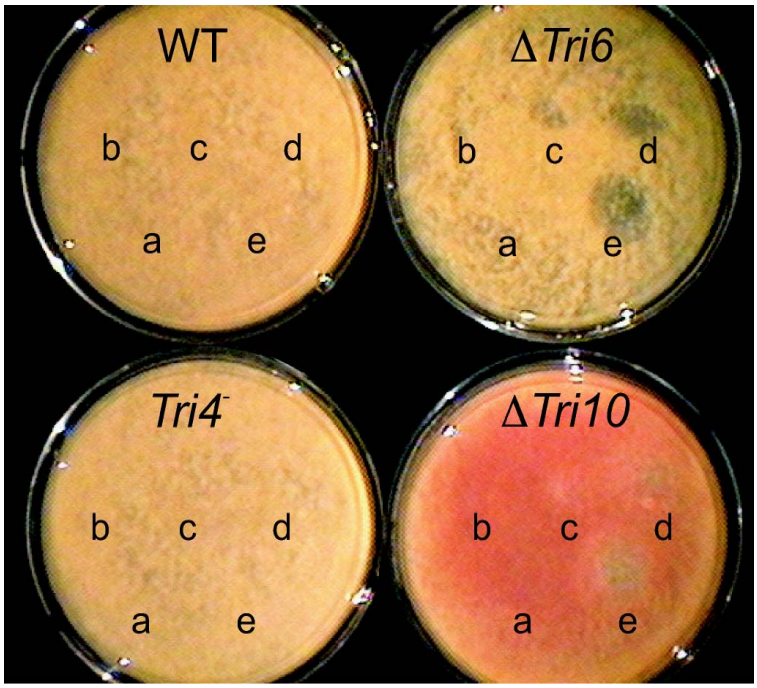

FIG. 2-5. Drop-plate T-2 toxin sensitivity assay. 25μl of ethyl acetate containing (a) 0, (b) 10, (c) 25, (d) 50, or (e) 100 μg T-2 toxin was spotted onto YEPD-2G plates and the ethyl acetate was allowed to evaporate. The plates were then spread with 1 to 2 x 10^5 conidia and allowed to grow 2 days.

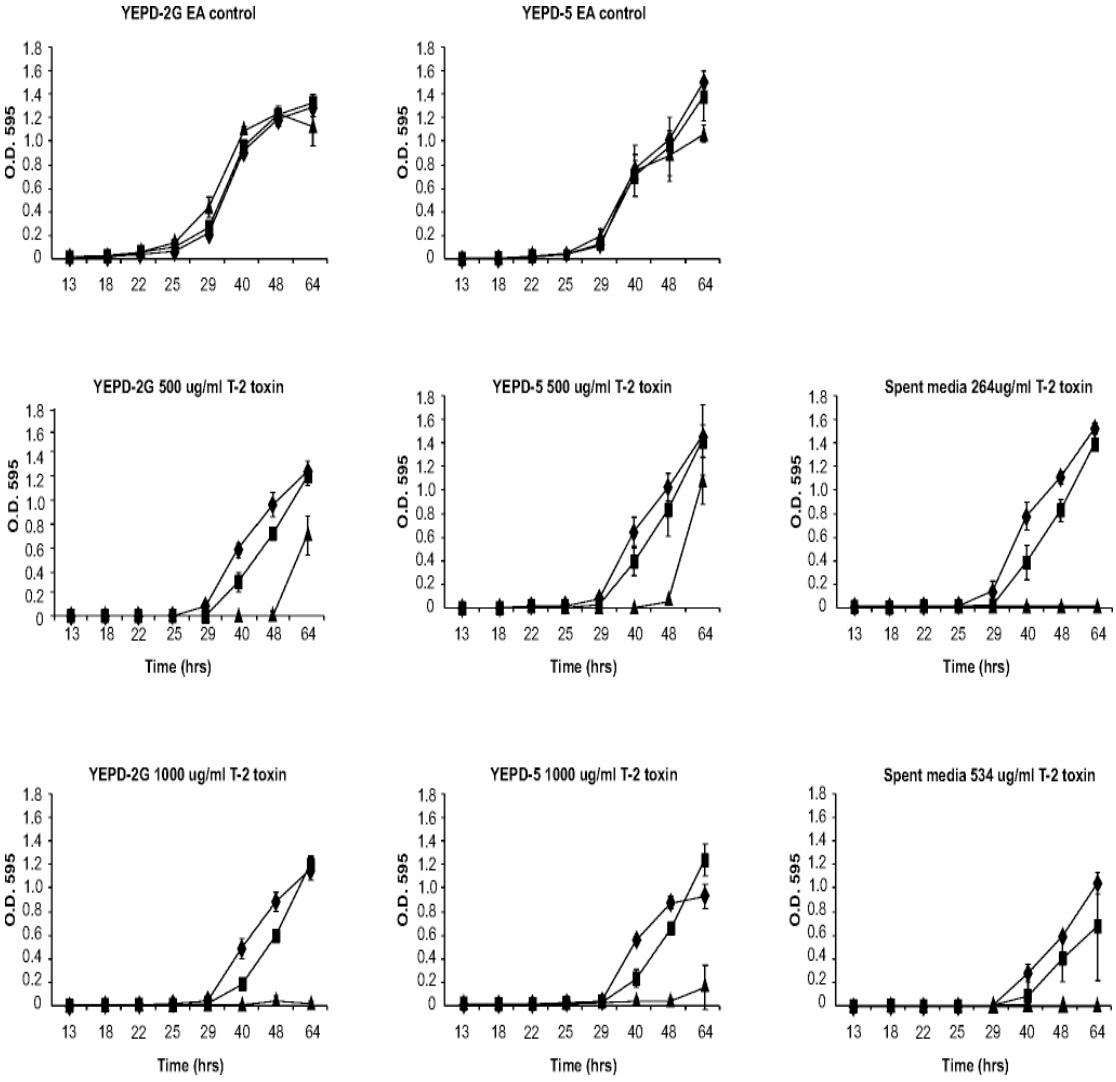

FIG. 2-6. Microtiter plate T-2 toxin sensitivity assay. Wild-type (NRRL 3299)(◆), Δ*Tri10* (■), and Δ*Tri6* (▲) strains were grown in YEPD-2G and YEPD-5 containing either 1000, 500, or 0 μg/ml T-2 toxin. These strains were also grown in spent YEPD-5 media containing 264 or 534 μg/ml T-2 toxin. All strains were grown for 64 h and the optical density was measured at λ=595. Error bars represent the range of readings observed per isolate per time point.

The above experiments indicate that the assay conditions are critical for observing the increased sensitivity of both the Δ*Tri10* and the Δ*Tri6* strains to T-

2 toxin. Overall, the ∆*Tri6* transformant was more sensitive, showing clearly inhibited growth in 2 of the 3 assay formats, while the ∆*Tri10* transformant was intermediate between ∆*Tri6* and wild type, only showing some clearly inhibited growth in the drop plate assay. These results, together with the observation that a low level of the *Tri6* transcript was present in the ∆*Tri10* transformant, imply that *Tri10* via *Tri6* controls the expression of one or more genes that contribute to T-2 toxin self-protection and that the wild-type levels of expression of these genes only become essential under certain growth conditions.

Tri10 **is conserved in other trichothecene-producing** ***Fusaria***

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 the materials and methods and determined that the placement and direction of transcription relative to *Tri5* is 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. 2-4) and approximately 88% amino acid identity. All three species share at least one

putative TATA and CAAT element (Fig. 2-4). 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 (51) (Fig. 2-4). 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. 2-7, 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 only causes a severe reduction of the latter three gene transcripts, each of these genes must also have an additional basal route or mechanism by which their expression is activated. Furthermore, in order to account for the lack of T-2 toxin production by the

Δ*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 Δ*Tri10* strain. These possibilities can be addressed by future experiments.

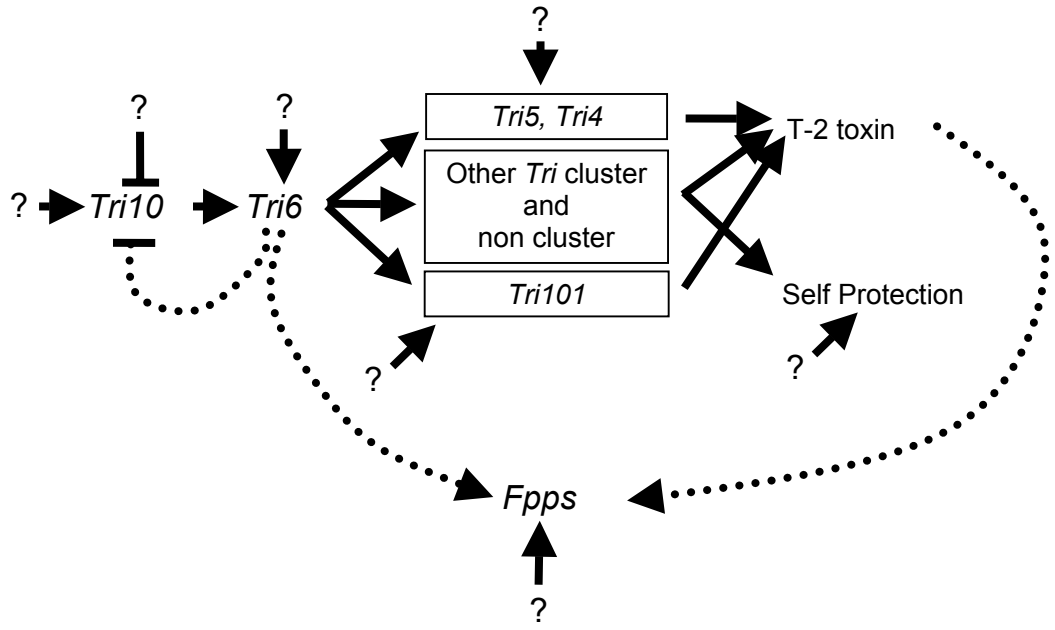

FIG. 2-7. Proposed regulatory model for trichothecene biosynthesis. Solid arrows indicate known positive activators. Dotted arrows indicate possible activation activities. Blocked arrows indicate known inhibitory activities and dotted blocked arrows indicate possible inhibitory activities. Question marks indicate other hypothesized but unknown regulatory signals or factors.

As shown in the model in Fig. 2-7, 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. Beremand, *in press*), 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), 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 controlled 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) (20). 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. 2-7) 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 transcription is conserved in *F. sambucinum* (A.W. Peplow and M.N. Beremand, unpublished) and *F. graminearum* (17). All three species also lack a TRI6 binding site (52) 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 Δ*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* (52), 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 position 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 non-cluster *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 Δ*Tri6* and Δ*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 to otherwise toxin-sensitive yeast cells (64, 72). However, additional genes for self-protection are likely in *F. sporotrichioides* since the deletion of *Tri12* only partially decreased toxin self-protection, and the deletion of *Tri101* had no apparent effect on toxin sensitivity (4, 72).

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 to note that even though *Tri101* displays what appears to be a background constitutive level of transcript accumulation in the Δ*Tri10* and Δ*Tri6* strains, the full expression of both *Tri101* (this study) and presumably *Tri12*, are dependent on the expression of both *Tri6* and *Tri10*. Consequently, the increased sensitivity to T-2 toxin displayed by the Δ*Tri10* and Δ*Tri6* strains could be due to the simultaneous reduction in the expression of both *Tri12* and *Tri101* as well as one or more additional genes that contribute to toxin self-protection.

Finally, we also addressed whether the observed reduction in self-protection in the Δ*Tri10* and Δ*Tri6* strains was due to an inability to make T-2 toxin. Examination of three different T-2 toxin minus mutant strains revealed that only the Δ*Tri10* and Δ*Tri6* strains and not the mutant unable to make a functional trichodiene oxygenase (*Tri4*$^{-}$) 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 between *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 340-360), was identified within TRI10 (Simple Modular Architecture Research Tool) (91, 92). 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*.

CHAPTER III

A FUNCTIONAL GENOMICS APPROACH FOR EXPLORING FUNGAL SECONDARY METABOLISM: TARGETED cDNA MICROARRAYS TO INVESTIGATE GENE EXPRESSION PROFILES ASSOCIATED WITH TRICHOTHECENE BIOSYNTHESIS

INTRODUCTION

The trichothecenes are toxic sesquiterpenoid compounds produced by fungi belonging to the genera *Fusarium, Myrothecium, Trichoderma, Trichothecium* and *Stachybotrys* (8, 60, 104). These fungal secondary metabolites constitute one of the largest and most important groups of mycotoxins both scientifically and economically. Since consumption of trichothecene-contaminated food and feed by humans and livestock can cause alimentary hemorrhage, emesis and immunosuppression (69)and because these mycotoxins can also function as virulence factors in plant-pathogen interactions (26, 85, 86), there is strong interest in obtaining information about the biosynthesis and genetics of trichothecene production in order to identify molecular targets that can be exploited to promote the development of methods which effectively reduce the synthesis of these compounds (81). The identification and study of the regulatory genes and networks that control the

coordinate expression of the trichothecene genes therefore marks an important step in providing additional information and potential targets that can be used to help prevent mycotoxin contamination in crops.

It is well established that trichothecenes are synthesized from the primary metabolite farnesyl pyrophosphate (FPP), a key product of the isoprenoid pathway and the shared precursor for sterol and terpene biosynthesis (Fig. 3-1). During trichothecene biosynthesis, FPP is cyclized to trichodiene (36). Trichodiene is then converted to T-2 toxin, the most predominant trichothecene produced by *Fusarium sporotrichioides*, via a biosynthetic pathway that includes 14 additional steps (53). More than a dozen genes dedicated to trichothecene biosynthesis have been isolated, and most have been found to be organized within a large, coordinately regulated gene cluster (17, 54, 65) (A. W. Peplow, A. G. Tag, G. Garifullina, and M. Beremand, *in press*).

To date, two cluster-encoded regulatory genes, *Tri6* (87) and *Tri10* (96), have been shown to control the expression of trichothecene-specific genes. *Tri6* encodes a Cys_2-His_2 zinc-finger DNA-binding protein which functions as a positive regulator of all the published *Tri* cluster genes (87, 96) (A. W. Peplow, A. G. Tag, G. Garifullina, and M. Beremand, *in press*) except for *Tri10* (96) (Chapter II) (A. W. Peplow, A. G. Tag, G. Garifullina, and M. Beremand, *in press*); it also regulates the non-cluster *Tri* gene *Tri101* (96). Meanwhile, *Tri10*

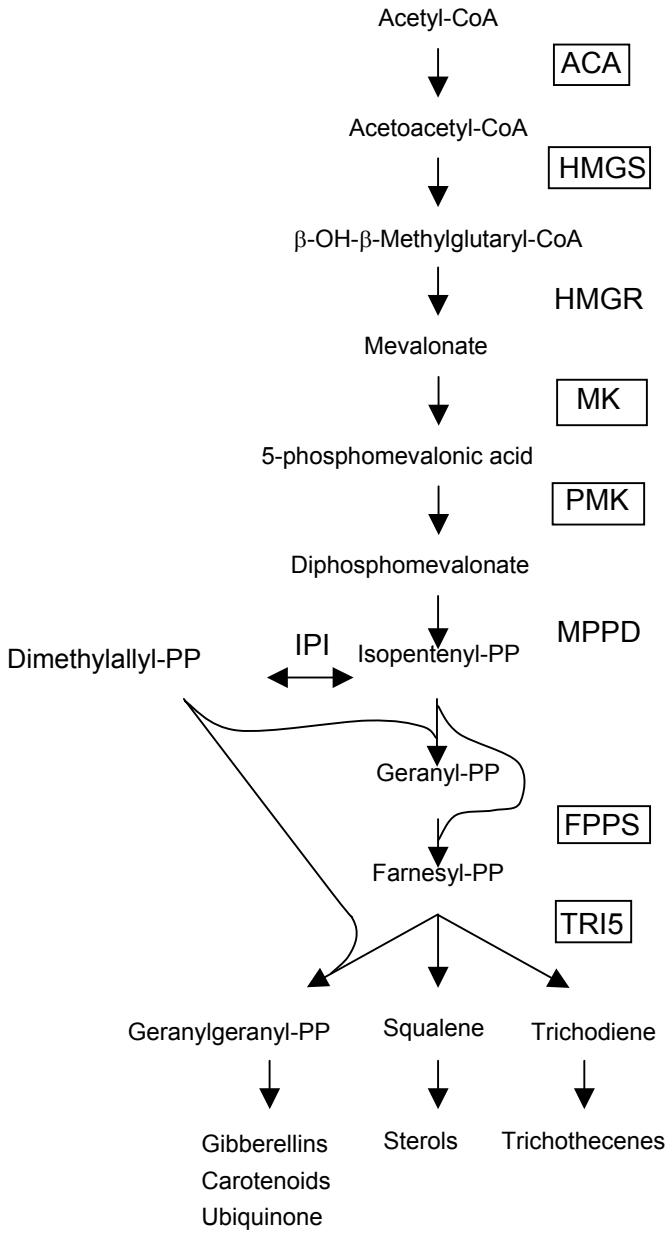

FIG. 3-1. Schematic diagram depicting the relationship of the isoprenoid, trichothecene, and other terpenoid biosynthetic pathways. Enzymes represented by gene sequences on the targeted cDNA microarray are boxed. Abbreviations: ACAT, acetyl-CoA acetyltransferase, HMGS, hydroxymethylglutaryl-CoA synthase, HMGR, hydroxymethylglutaryl-CoA reductase, MK, mevalonate kinase, PMK, phosphomevalonate kinase, MPPD, mevalonate pyrophosphate decarboxylase, IPI, isopentenyl pyrophosphate isomerase, FPPS, farnesyl pyrophosphate synthetase.

encodes a novel protein which acts upstream of *Tri6* by a molecular mechanism which has not yet been defined (96).

The regulation mediated by *Tri10* and *Tri6* also extends to genes involved in primary metabolism. Previous work has shown that the expression of *Tri10* and *Tri6* significantly influence the expression of four genes that encode biosynthetic enzymes in the isoprenoid biosynthetic pathway (acetyl CoA acetyltransferase, *Acat*; hydroxymethylglutaryl-CoA synthase, *Hmgs*; mevalonate kinase, *Mk*; and farnesyl pyrophosphate synthetase, *Fpps*) (96) (A. W. Peplow, A. G. Tag, G. Garifullina, and M. Beremand, *in press*). Transcript levels for these genes are markedly reduced in the *Tri10* and *Tri6* deletion mutants but overexpressed when *Tri10* is overexpressed. Thus, *Tri10* and *Tri6* control a regulatory network linking primary and secondary metabolism, but the full scope of this regulatory network remains to be determined.

Current data also indicate that *Tri10* and *Tri6* form a regulatory loop whereby the expression of *Tri10* positively regulates the expression of *Tri6* and the expression of *Tri6* negatively regulates the expression of *Tri10* (96). In turn, the expression of *Tri10* is modulated by the disruption of DNA sequences upstream of *Tri10* which results in its overexpression (96) (G. Garifullina, A. G. Tag, A. W. Peplow, and M. Beremand, unpublished data) and by a signal transduction pathway associated with the alpha subunit of a heterotrimeric G-

protein (A. G. Tag, M. Beremand, unpublished results). However, little else is known about the parameters that directly affect *Tri10* gene expression.

In this study, cDNA microarrays and additional strains with modified *Tri10* gene constructs were simultaneously employed to (1) gain further knowledge about the regulatory controls that *Tri10* exerts and is subject to during trichothecene production and (2) to better understand the regulatory network(s) connecting the trichothecene-related primary and secondary metabolic pathways. DNA microarray technology affords a global view of gene expression by allowing the rapid, parallel assessment of the relative changes in the expression of large populations of genes or specifically selected groups of genes (22, 32, 33, 56, 90). This ability makes it an exceptionally useful tool for advancing the study of secondary metabolism in filamentous fungi. Thus, one main goal was to produce and evaluate the capabilities of a targeted cDNA microarray with the dual purpose of obtaining new information about the regulation of the expression of genes involved in trichothecene biosynthesis and to serve as a platform for developing microarray technology for use with *Fusarium*.

This study reports the characterization of *F. sporotrichioides* transformants expressing *Tri10* under the control of an exogenous promoter and the development and utilization of a targeted cDNA microarray designed

specifically to monitor the transcription profiles for the primary and secondary metabolic genes associated with T-2 toxin production.

MATERIALS AND METHODS

Strains and culture conditions

The wild-type *F. sporotrichioides* Sherb. strain NRRL 3299 (10), and the derived hygromycin-resistant *Tri10* transformant strains FsTri10-1-12 (Δ*Tri10*) and FsTri10-1-20 (↑*Tri10*) (96) have been described previously. Strains were both maintained on V8-juice agar slants (containing 300 μg/ml hygromycin B for transformants) and preserved at -70°C as conidial glycerol stocks (88). Shake cultures for RNA and T-2 toxin analyses were prepared by inoculating 100 ml or 25 ml, respectively, YEPD-5G medium (5% glucose, 0.1% yeast extract, and 0.1% peptone) to a concentration of 5 X 10^4 conidia/ml from 7-d V8 juice agar plates and were grown at 28°C and 200 rpm. Mycelia were collected by filtration and freeze-dried for RNA isolation (97). Whole culture samples were stored at -20°C for toxin studies. Growth conditions for cultures for DNA isolation have been reported (88, 97).

Construction of pTri10-2 (pTAG15) and transformation of NRRL 3299

The *Tri10* overexpression plasmid pTri10-2 was constructed using pCL5 (110), which contains both a 1.4-kb fragment of the promoter for the glyceraldehyde 3-phosphate dehydrogenase gene from *Trichoderma virens* and a hygromycin B resistance cassette (103). The plasmid pCL5 was digested with *Hin*d III to remove the existing insert downstream of the *Gpd* promoter. The remaining plasmid backbone was gel-purified and ligated to the *Tri10* open reading frame (ORF), which was amplified by PCR from NRRL 3299 genomic DNA using *Pfu* DNA polymerase and the primers A-26 (5' tgaagcttaccacgcaaactcatcaatcgcagtcacaaatcagctacaatggaatttccgaagccg 3') and A-27 (5' gcaagcttcagcacagctggttggatctac 3'). Both primers possess *Hin*d III sites in their 5' termini; the resulting PCR product was digested with *Hin*d III and gel purified prior to ligation to the pCL5 backbone. Primer A-26 also contains a fusion sequence joining the 3' end of the *Gpd* promoter to the *Tri10* ORF. Production and transformation of fungal protoplasts was performed as previously described (88, 97). Transformants were isolated by selection on hygromycin B-containing media (97) and screened by Southern hybridization and PCR using standard protocols (89).

Nucleic acid isolation/manipulation

Fusarium genomic DNA and total RNA were isolated from freeze-dried mycelia according to previously described methods (97). Polyadenylated mRNA was purified from total RNA with the Oligotex mRNA mini kit (Qiagen).

T-2 toxin extraction and quantitation

Cultures for toxin analysis were grown in YEPD-5 for 7 d and extracted with ethyl acetate as previously described (97). Quantitation of T-2 toxin was performed by gas chromatography-mass spectrometry as reported previously (97) except that the mass selective detector was used in either ion-selective mode (for T-2 toxin quantitation) or full-scan mode (to obtain full spectrum). T-2 toxin was identified based on retention times and spectra compared 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 the wild type by ANOVA using the JMP4 statistical software package.

cDNA sequencing and gene identification

cDNA clones were obtained from a cDNA library which had been constructed using RNA isolated from the *Tri10* overproducing strain (↑*Tri10*) grown under conditions favorable for trichothecene gene expression (A. W. Peplow, A. G. Tag, G. Garifullina, and M. Beremand, *in press*). Clones selected for use in microarray analyses were chosen based either on sequence and hybridization data obtained in our laboratory or on sequence data generated for the first publicly released *Fusarium* cDNA sequencing project (http://www.genome.ou.edu/fsporo.html), which was conducted in collaboration with Dr. Bruce Roe and co-workers. Functions assigned to genes other than the published trichothecene genes are based solely on sequence similarity to genes present in GenBank (Table 3-1).

cDNA microarray construction and analysis

DNA microarrays were constructed following procedures provided by the MGuide at http://cmgm.stanford.edu/pbrown/mguide/index.html. PCR products were generated from the cDNA clones listed in Table 3-1 using T3 and T7 primers. A small aliquot of PCR product from each clone was subjected to agarose gel electrophoresis to ensure that only a single band was present and the remainder of the reaction was purified through a Qiaquick column (Qiagen).

20X SSC was added to each purified PCR product to give a final concentration of 3X SSC before being spotted in triplicate on poly-L-lysine-coated microscope slides (Sigma) by an OmniGrid Arrayer (GeneMachines, San Carlos, CA). Printed slides were UV crosslinked with 200 mJ energy in a Stratalinker (Stratagene) and processed in blocking solution (70 mM succinic anhydride, 0.1 M sodium borate, pH 8 in 90% 1-methyl-2-pyrrolidinone). Relative mRNA expression levels were measured via two-color parallel hybridization using Cy-3-dUTP and Cy-5-dUTP labeled cDNA probes. Fluorescently-labeled cDNA probes were prepared from approximately 200 ng poly(A) RNA using an oligo-$dT_{(11\text{-}18)}$ primer

TABLE 3-1. cDNA clones used in targeted cDNA microarray construction.

Gene	Clone ID	BLASTX Score	BLASTX Homology
Tri1	a3e02	311 5e-84	AAK77933.1 p450 monooxygenase [F. sporotric...
Tri3	h1f12	141 6e-48	AY102604 trichothecene 15-O-acetyltransferase
Tri4	a2e03	369 1e-101	AF359360_5 cytochrome P450 [Fusarium sporotrichioides]
Tri5	a2c08	390 1e-107	P13513 Trichodiene synthase
Tri6	pAWP70[a]		AAD11963 trichothecene biosynthesis transcription factor
Tri7	s3b08	246 2e-64	AAK33076.1 Tri7 [Fusarium sporotrichioi...
Tri9	h3d09	89 5e-17	AAK33078.1 Tri9 [Fusarium sporotrichio...
Tri10	r1g06	328 4e-89	AAK53383.1 trichothecene biosynthesis regulator Tri10
Tri11	i2a09	282 4e-75	AAD12755.1 isotrichodermin C-15 hydroxylase
Tri12	t1d12	293 1e-83	AAK33071.1 trichothecene efflux pump
Tri13	c1b10	226 1e-58	AAK15528.1 Tri13 [Fusarium sporotrichio...
Tri14	h1f04	211 5e-82	AAG46054.1 Tri14 [Fusarium sporotrichio
Tri15	j1b02	296 2e-80	AF327521 Tri15 [Fusarium sporotrichioides]
Ibt6	i2d03	177 1e-43	AAC03053.1 lysophospholipase [Neurospora crassa]
Ibt7	o1d10		No BLAST match
Ibt1	e3d06	81 1e-14	BAC00848.1 Aspartic protease
Ibt2	q3d11		No BLAST match
Ibt4	i3c03		No BLAST match
Tri101	m3b07	180 1e-44	AAK77937.1 trichothecene 3-O-acetyltransferase
Pyruvate kinase	a3e11[b]	144 2e-34	P31865 Pyruvate kinase
Dhat	a2g03[b]	104 2e-22	P20285 Dihydrolipoamide acetyltransferase
Acat	s4d11	207 6e-53	NP_596686.1 acetyl-coa acetyltransferase
Hmgs	p1e11	268 4e-71	T49718 probable hydroxymethylglutaryl-CoA synthase
Mk	a4e12[b]	75 1e-12	CAC28692.1 related to mevalonate kinase [Neurosp...
Pmk	a1e02[b]	75 2e-13	S57588 Phosphomevalonate kinase
Fpps	h4c06	192 2e-48	Q92235 Farnesyl pyrophosphate synthetase
Citrate synthetase	a2h08[b]	132 7e-31	AJ296102 mitochondrial citrate synthase
Aconitate hydratase	a3g12[b]	231 9e-61	AF093142 aconitase
Isocitrate dehydrogenase	a1g04[b]	248 1e-65	AY040207 I NADP-dependant isocitrate dehydrogenase
Ibs1	a2d01	160 2e-38	NP_595868.1 putative nadh-dependent flavin oxidoreductase
ADP/ATP carrier protein	d1c01	242 4e-63	P02723 ADP,ATP carrier protein
Cytochrome P450	a1g10[b]	55 2e-7	AAF26280.1 Cytochrome P450 monooxygenase
Alcohol dehydrogenase	i1f08	223 4e-57	Q9P6C8 Alcohol dehydrogenase I
rAsp f9	j2a04	136 3e-31	AJ223327 rAsp f 9
Potassium channel protein	a1e03	200 2e-50	NP_588516.1 putative potassium channel subunit
ABC transporter	e2d03	93 2e-18	NP_013052.1 Yeast bile transporter, similar to mammalian Ybt1p
Transcription factor	r3c10	74 1e-12	CAC14775.1 putative transcription factor [Cladosporium fulvum]
FadA	pJY8[c]		G-protein, alpha subunit *E. nidulans*
Core II protein	d4d09	183 3e-49	CAD21046.1 ubiquinol-cytochrome c reductase complex core pro2
Stress protein	p3d12[b]	78 4e-14	AF352018 mold-specific protein MS95 [Ajellomyces capsulatus]
RNA pol II large subunit	j2a03	174 5e-43	AB017184 RNA polymerase II largest subunit
EF 2	a2e09[b]	263 3e-70	AAK49353.1 Elongation factor 2
Nucleosome assembly	a2g05[b]	87 8e-17	NP_596230.1 Nucleosome assembly protein
Histone H2A subunit	s4c05	89 7e-29	AAL38970.1 histone H2A [Neurospora crassa]
RNase F1/T1	p1f08	167 7e-47	BAA31984.1 ribonuclease F1
Cell wall protein	o2b02	52 5e-6	CAA64974.1 QI74 protein (cell wall protein) [T. harzianum]
Actin	a4e10	289 4e-78	O9UVW9 Actin, gamma
9D4	i4b02		No BLAST match
11K18	ki2f09		No BLAST match
12M24	l2g12		No BLAST match
18I12	r2e06		No BLAST match

BLASTX searches were conducted using the BLASTX 2.2.1 algorhythm (6)and the 18-June-2002 release of the GenBank non-redundant database.

[a]No *Tri6* clones were identified in the cDNA library, thus a plasmid containing the genomic DNA sequence of *Tri6* was used to obtain the *Tri6* amplimer for the microarray. [b]Identified through the *Fusarium* EST sequencing project. All other clones were identified using sequence data obtained in our laboratory. [c]The pJY8 plasmid contains the *fadA*^{G42R} DNA sequence of *FadA* from *E. nidulans*. (97)

and the Superscript Preamplification kit (Life Technologies). Hybridizations were conducted at 62°C for 15 h in 2.5X SSC, 0.2% SDS, and 0.6 μg/μl sheared salmon sperm DNA. After hybridization, the slides were washed once in 2.5X SSC + 0.2% SDS at room temperature for 5 min. and twice in 0.1X SSC at 42°C for 5 min. each. The slides were then scanned using a Scanarray3000 Biochip Analyzer (General Scanning, Watertown, Ma.) scanner at 10 nm per pixel resolution. Scans were acquired as 16-bit TIFF images and were analyzed using the ScanAlyze software version 4.21, publicly available at http://rana.lbl.gov. Cluster analysis of the data was performed using the Cluster (Version 2.11) and TreeView (Version 1.45) software also available at the same web address.

RESULTS

Previous studies demonstrated that the disruption of the region upstream of *Tri10* caused overexpression of *Tri10* and a corresponding increase in *Tri* gene expression and T-2 toxin production (13, 96). However, the disruption did not alter the temporal onset of *Tri* gene expression, including the expression of *Tri10* (96) (M. Beremand, unpublished data). It also raised the possibility that the overexpression phenotype might be dependent upon a disruption in this region of the *Tri* gene cluster and/or the presence of the overexpressed *Tri10* gene in the *Tri* gene cluster. The following experiments were designed to

address these issues and to further investigate how the expression of *Tri10* and its position in the genome, when under the control of a heterologous promoter, mediate the expression of the other *Tri* genes and T-2 toxin production.

Placement of *Tri10* under the control of a heterologous promoter

The required experimental strains were generated by transforming *F. sporotrichioides* strain NRRL 3299 with plasmid pTRI10-2, which contains the *Tri10* coding region fused to the glyceraldehyde 3-phosphate dehydrogenase (*Gpd*) promoter from *Trichoderma virens*. Transformants that arose by either a homologous or ectopic single-site, single-copy integration event were identified by Southern blotting and hybridization (data not shown). While both types of integration events produced transformants that carry a duplication of the *Tri10* gene with one copy regulated by the native *Tri10* promoter and the second copy regulated by the *Gpd* promoter, only homologous integration events placed both copies of *Tri10* in the *Tri* gene cluster (Fig. 3-2).

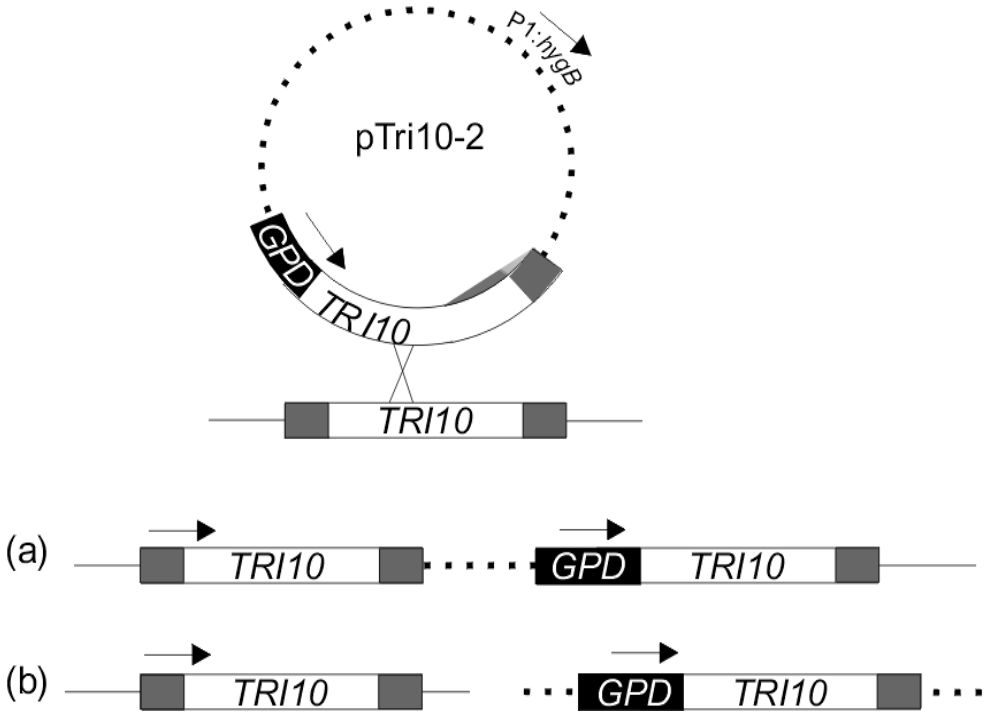

Figure 3-2. Integration of the pTri10-2 plasmid. Schemes of (A) homologous or (B) ectopic integration of the *Gpd*::*Tri10* plasmid pTri10-2. Panel A indicates the tandem arrangement of the two copies of *Tri10* within the *Tri* gene cluster as a result of homologous integration of one copy of pTri10-2. The resulting upstream copy of *Tri10* retains the wild type promoter and the downstream copy of *Tri10* carries the *Gpd* promoter. Panel B indicates the retention of the wild-type copy of *Tri10* within the *Tri* gene cluster and the placement of the second *Gpd* promoter-driven copy of *Tri10* at a separate genomic location.

Several transformants that arose from homologous integrations were obtained, and from these, isolate FsTri10-2-49 (*Gpd::Tri10*^{h}) was chosen as a representative strain. Two representative transformants, FsTri10-2-16 (*Gpd::Tri10*^{e1}) and FsTri10-2-24 (*Gpd::Tri10*^{e2}), which arose by ectopic integration of the pTri10-2 vector, were also selected for further study. Analysis of these three transformants by PCR utilizing primers within the *Gpd* promoter and the 3'-end of the *Tri10* gene confirmed that the Tri10 gene was still physically downstream of the *Gpd* promoter after integration of the pTri10-2 vector (data not shown). Introduction of the heterologous promoter-driven copy of *Tri10* now allowed us to examine what happens when *Tri10* is overexpressed from a cluster-dependent or -independent position under conditions that alter its temporal expression and remove it from the native control imposed by the endogenous *Tri10* promoter.

Expression of *Tri10* via a heterologous promoter results in overexpression of trichothecene genes and overproduction of T-2 toxin

Northern analysis revealed that in comparison to the wild-type strain at 23 h and 48 h, all three *Gpd::Tri10* transformants exhibited increased expression of all the *Tri* cluster (*Tri10, Tri6, Tri5, Tri4, Tri3*), and noncluster (*Tri101*) genes examined (Fig. 3-3). The most dramatic increases were observed for *Tri10* and *Tri3*. Also, unlike the wild-type parent strain, all three transformants

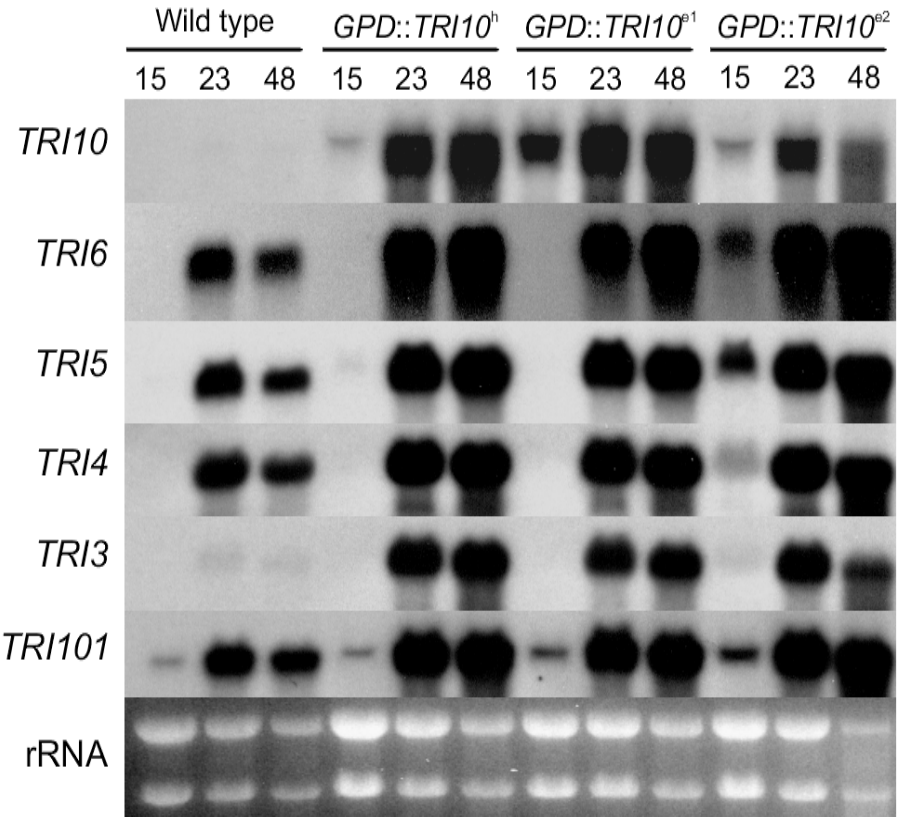

Figure 3-3. Northern analysis of *Gpd::Tri10* transformants. Northern blot analysis of the expression of *Tri10, Tri6, Tri5, Tri4, Tri3,* and *Tri101* transcripts in wild-type (NRRL 3299), *Gpd*::*Tri10*^{h} (FsTri10-2-49), *Gpd*::*Tri10*^{e1} (FsTri10-2-16), and *Gpd*::*Tri10*^{e2} (FsTri10-2-24) strains of *F. sporotrichioides* grown in parallel for 15, 23, and 48 h. 5 μg of total RNA was loaded per lane. ^{32}P-labeled probes for each gene were prepared and hybridized as described in the Material and Methods. Ribosomal RNAs were visualized in the gel by ethidium bromide staining.

expressed the *Tri10* gene at 15 h. Thus, in all three transformants, the placement of *Tri10* under the control of the *Gpd* promoter consistently lead to an overexpression of *Tri10* and the other *Tri* genes at 23 h and 48 h, accompanied by some level of *Tri10* overexpression at 15 h, independent of whether the *Gpd::Tri10* gene construct was positioned inside or outside of the *Tri* gene cluster.

However, it is important to note that each transformant also displayed a unique transcript profile at the 15 h time point. At 15 h, even though the ectopic transformant, *Gpd*::*Tri10*^{e2}, and the homologous transformant, *Gpd*::*Tri10*^{h}, both exhibited a similar *Tri10* transcript accumulation level and the other ectopic transformant, *Gpd*::*Tri10*^{e1}, actually exhibited a much higher *Tri10* transcript level, only the ectopic strain *Gpd::Tri10*^{e2} also simultaneously expressed the other cluster-encoded *Tri* genes; the remaining two transformants both retained an otherwise wild-type parent transcript profile.

An examination of T-2 toxin levels demonstrated that the increased *Tri* gene transcript accumulation observed in the *Gpd::Tri10* transformants was accompanied by an increase in T-2 toxin production. In fact, transformants arising from either homologous or ectopic integration of the *Gpd* promoter-driven copy of *Tri10* displayed a significant increase in T-2 toxin production in

comparison not only to the wild-type strain, but also to the previously described *Tri10* overproducing strain, FsTri10-1-20 (↑*Tri10*) (Table 3-2).

TABLE 3-2. Production of T-2 toxin by pTri10-2 transformants.

Class[a]	Strain	μg/ml T-2 toxin[b]
Gpd	FsTri10-2-16 (*Gpd*::*Tri10*^{e1})	3002±370
Gpd	FsTri10-2-24 (*Gpd*::*Tri10*^{e2})	2899±139
Gpd	FsTri10-2-49 (*Gpd*::*Tri10*^{h})	2440±218
↑*Tri10*	FsTri10-1-20	704±102
Wild-type	NRRL 3299	368±22

[a] The *Gpd* and ↑*Tri10* class transformants were each found to be significantly different from the wild-type strain and from each other regardless of the type of integration by ANOVA.
[b] Values shown represent the mean of individual extractions of triplicate cultures.

Targeted cDNA microarray data parallel Northern data

The following experiment was conducted in order to examine and thereby validate the utility of targeted cDNA microarrays to accurately assess gene transcript profiles. The criteria were the ability of the targeted cDNA microarray to discern the known coordinate regulation of the *Tri* genes by *Tri10* and to determine which, if any, additional genes were also most similarly affected by *Tri10* gene expression. Accordingly, fluorescently labeled cDNA samples derived from 23 h cultures of two *Tri10* transformants, FsTri10-1-12 (Δ*Tri10*) and FsTri10-1-20 (↑*Tri10*), were hybridized to separate targeted cDNA microarrays using labeled wild-type 23 h cDNA as a reference sample, and the resulting data collected from these initial hybridizations were subjected to hierarchical clustering by Cluster software (Vers. 2.11).

As seen in Fig. 3-4A, the above analysis revealed a group of 21 genes that displayed very similar expression patterns with regard to the expression of *Tri10.* When *Tri10* was disrupted these genes were negatively influenced, and when *Tri10* was overexpressed they were slightly overexpressed. Significantly, this group included 14 of the 17 tested *Tri* genes, four genes involved in the isoprenoid pathway leading into trichothecene biosynthesis, one gene involved in the citric acid cycle, and two genes not obviously linked to trichothecene biosynthesis (Fig 3-4A+). These data are consistent with our previous studies

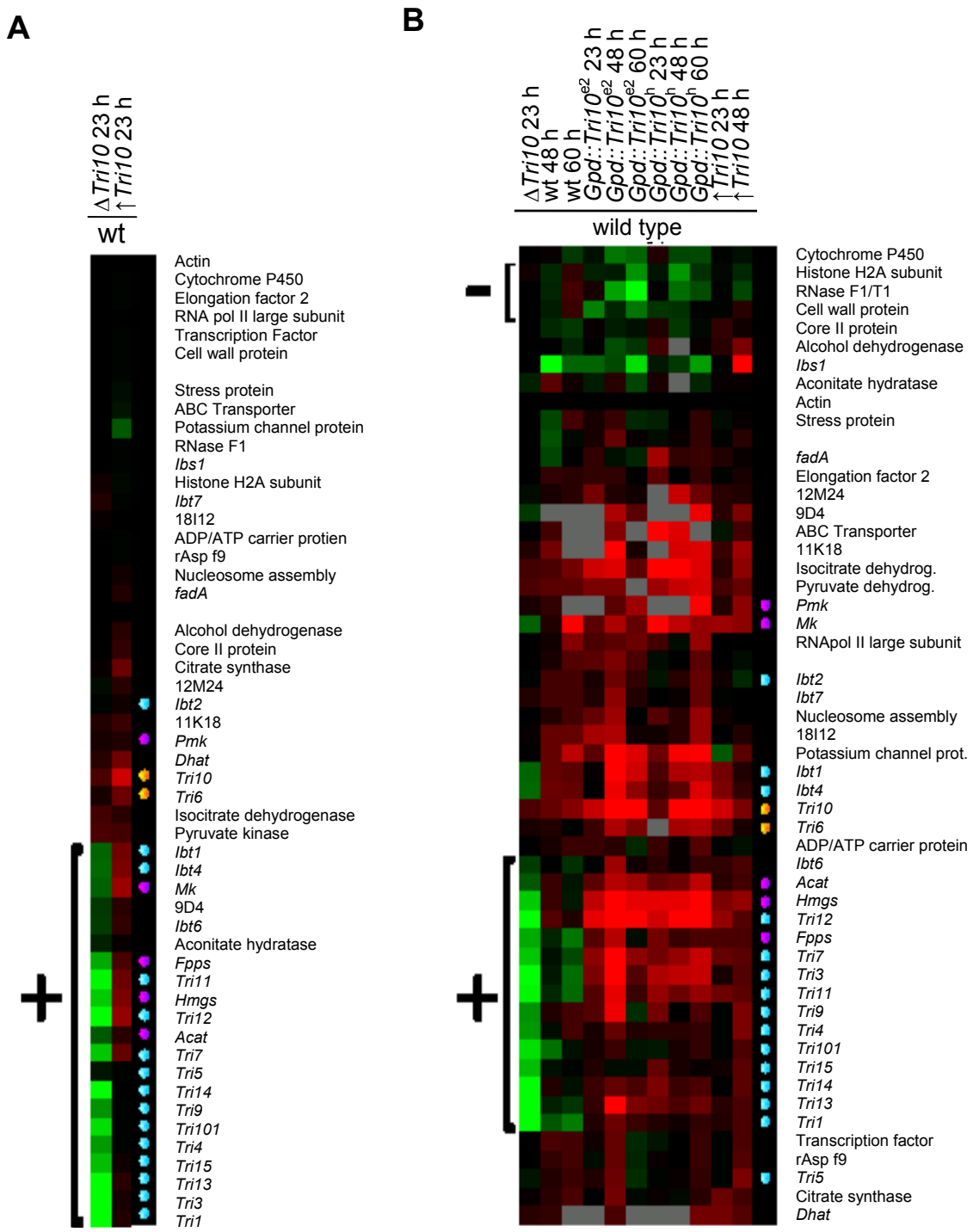

FIG. 3-4. Cluster analysis from targeted cDNA microarrays. Cluster diagrams from targeted microarray analysis of gene expression for wild-type (wt), *Tri10*-deficient (Δ*Tri10*), and *Tri10*-overexpressing (↑*Tri10*) strains, and *Gpd*-driven *Tri10*-overexpressing strains arising from homologous (*Gpd*::*Tri10*^{h}) or ectopic (*Gpd*::*Tri10*^{e1}, *Gpd*::*Tri10*^{e2}) integration of the pTri10-2 vector. Red blocks indicate overexpression in comparison to the wild-type strain at 23 h and green blocks indicate lower expression levels than the wild-type strain at 23 h. Grey blocks indicate missing or poor data. Blue dots represent *Tri* genes, yellow dots represent trichothecene regulators, and magenta dots represent primary metabolic genes in pathways upstream of trichothecene biosynthesis. (A) Cluster analysis of 23-h Δ*Tri10* and ↑*Tri10* strains compared with wild type. (B) Comparison of gene expression profiles for the wild-type strain and *Tri10* transformants over time. Numerical data are provided in Appendix A.

(96) (A. W. Peplow, A. G. Tag, G. Garifullina, and M. Beremand, *in press*) based on Northern and DNA macroarray analyses. Consequently, they not only further substantiate that the expression of *Tri10* coregulates the *Tri* genes and these four isoprenoid genes as determined by the examination of the ∆*Tri10* and ↑*Tri10* strains at 23 h, but they also validate the usefulness and accuracy of the targeted cDNA microarrays.

In addition, the two regulatory genes, *Tri10* and *Tri6*, are clustered together independently of the other *Tri* genes (Fig. 3-4A). This result indicates that these microarrays are also able to separate the trichothecene biosynthetic and regulatory genes into biologically significant groups.

Refinement of hierarchically clustered genes by additional cDNA microarray analysis

In microarray analysis, the power of resolving a large group of genes into informative subgroups can be magnified by meaningfully increasing the number of conditions or experimental parameters. Time and mutation are two such parameters. For several of the *Tri* genes, it has been shown that when the *F. sporotrichioides* NRRL 3299 strain is growing in liquid shake culture, the maximum transcript accumulation level is reached near 23 h post-inoculation and then declines (87, 96). Therefore, in the following experiments the

transcription profiles for the known *Tri* genes were examined over time by comparing 48-h or 60-h RNA to 23-h wild-type RNA. The FsTri10-2 transformant strains were also examined to further assess the influence of *Tri10* on gene expression when *Tri10* is expressed via a heterologous promoter. As seen in Fig. 3-4A, the cDNA microarray experiments included comparisons between *Gpd*::*Tri10*^{h}, *Gpd*::*Tri10*^{e2}, ↑*Tri10* and wild-type strains at selected time points using 23-h wild-type RNA as a reference sample. Hierarchical clustering of the data from these microarrays revealed that 14 of the 21 genes shown to be influenced by *Tri10* in the previous microarray analysis (Fig. 4A), continue to cluster (Fig. 4B+). These 14 genes include 11 *Tri* genes and three isoprenoid biosynthetic genes. The remaining seven genes included one isoprenoid gene (*Mk*) and four *Tri* genes: *Tri5*, the first biosynthetic gene in the trichothecene pathway, and three genes with uncharacterized function, *Ibt1, Ibt2* and *Ibt4*). Of these four genes, only *Ibt1* and *Ibt4* cluster together. Notably, *Tri6* and *Tri10* are still tightly grouped together and again fall outside of any group containing other *Tri* genes. There was also a small group of co-clustered genes whose expression was affected by both time and a general negative influence by *Tri10* overexpression; this group includes a putative histone protein, RNase, and cell wall protein (Fig. 3-4B-). Finally, based on an overall view of the gene transcript profiles as depicted in Fig. 3-4B, it is readily apparent that the *Tri* gene transcripts are present in the three *Tri10* upregulated strains at 48 h and 60 h compared to a corresponding decline in these transcripts in the wild-type strain

at these same time points. This is consistent with all of our Northern analyses conducted for these transformants.

DISCUSSION

This study has demonstrated the successful application of cDNA microarray technology in the investigation of gene regulation specifically related to secondary metabolism in filamentous fungi. The coupling of this technology with the ability to produce genetically engineered mutants altered in trichothecene biosynthesis has provided a fresh approach to unravel the complex details governing the regulation of secondary metabolism. It has further afforded new opportunities to investigate the regulatory networks linking primary and secondary metabolism. In this study the analysis of *Tri10* gene expression directed by an exogenous promoter has expanded our understanding of how *Tri10* gene expression simultaneously affects the expression of the other *Tri* genes, selected isoprenoid genes and T-2 toxin production.

The data presented in this study definitively establish that the overexpression of *Tri10* in an otherwise wild-type background is sufficient to promote the overexpression of *Tri6* and the other *Tri* genes examined. In previous studies, the overproduction of the *Tri10* gene was produced by

disrupting the DNA region upstream of *Tri10* with an inserted DNA fragment, which raised the possibility that either the disruption itself and/or the presence of the overexpressed *Tri10* gene in the *Tri* gene cluster were essential for the resulting phenotype (96) (G. Garifullina, A. G. Tag, A. W. Peplow, and M. Beremand, unpublished data). However, in the present study, the ability of a Gpd-promoter driven copy of *Tri10*-- whether integrated within or outside of the *Tri* gene cluster--to promote the overexpression of *Tri10* and the other *Tri* genes eliminated these possibilities. In fact, the overexpression and overproduction phenotype exhibited by the *Gpd::Tri10* transformants is greater than the phenotype observed in the ↑*Tri10* strain. A comparison of the amount of T-2 toxin accumulated by 7-d cultures revealed that toxin levels for the above *Gpd::Tri10* transformants were four times higher than for the ↑*Tri10* strain and approximately seven-to-eight times higher than for the wild-type parent strain, independent of the integration site of the *Gpd::Tri10* vector.

Since the *Tri* cluster genes are not normally expressed in liquid culture prior to 18 h post-inoculation, the placement of *Tri10* under the control of the *Gpd* promoter was also designed to test (1) if the early expression of *Tri10* was sufficient to activate the early expression of the other *Tri* genes and (2) if the early expression of *Tri10* from a location within or outside the *Tri* gene cluster would influence either the level of *Tri10* gene expression and/or the ability of *Tri10* to activate the early expression of the other *Tri* genes. The current data

did not provide a simple answer. A comparison of the data from the homologous transformant, *Gpd::Tri10*^{h}, and the ectopic transformant, *Gpd::Tri10*^{e1}, alone would indicate that *Tri10* is more highly expressed at 15 h when the *Gpd::Tri10* gene construct is located outside of the *Tri* gene cluster, but that the co-activation of the expression of the other *Tri* cluster genes does not occur at 15 h in either case. On the other hand, a comparison of the data from the homologous transformant with the other ectopic transformant, *Gpd::Tri10*^{e2}, would suggest that the level of expression of *Tri10* from the *Gpd* promoter is the same at 15 h whether the gene construct is inside or outside of the *Tri* gene cluster, but that only an ectopic copy could also stimulate the expression of the other *Tri* genes at 15 h. Thus, the transcription profiles for two of the transformants, *Gpd::Tri10*^{h} and *Gpd::Tri10*^{e1}, suggests that the early increased transcription of *Tri10* is not sufficient to elicit the early transcription of the other *Tri* genes while the transcription profile for the third transformant, *Gpd::Tri10*^{e2}, suggests that it is sufficient. At present, it is not known why the other *Tri* cluster genes were expressed at 15 h only in *Gpd::Tri10*^{e2}. However, the above results do demonstrate that the early expression of *Tri10* does not guarantee the co-activation of the other *Tri* genes, but that it is also clearly possible to express this group of genes earlier than when it occurs in the wild-type parent. Further clarification of the critical factors that allow this temporal change in gene expression will require additional experiments.

It is now well established that DNA microarrays can provide a powerful tool for functional genomic studies by revealing the coordinated patterns of gene expression that mirror the genetic regulatory networks and biosynthetic pathways that direct the cellular metabolism of an organism (32, 33, 56, 82). Data obtained from the targeted cDNA microarrays simultaneously confirmed our previous studies, conducted using both Northern analyses and DNA macroarrays, and validated the application of this technology to our experimental system. As discussed below, the co-clustering of genes, both of known and unknown function, by shared transcription profiles on the targeted cDNA arrays has also extended our previous studies.

Microarray analysis of the ∆*Tri10* and ↑*Tri10* strains, using a single time point of 23 h with the wild-type RNA from 23 h as a reference sample, clustered the two trichothecene regulatory genes, *Tri10* and *Tri6*, apart from a group of genes that are strongly influenced by *Tri10* expression. Not unexpectedly, this latter group includes *Tri* genes from both within and outside of the *Tri* gene cluster. Also present in this group are four primary metabolic genes from the isoprenoid pathway (*Acat, Hmgs, Mk,* and *Fpps*). These data are consistent with previous findings and provides further evidence for a regulatory circuit linking the primary and secondary metabolic pathways involved in trichothecene biosynthesis. Most important, is the presence of two other genes in this group: *Ibt6* (Influenced by ten), which is a possible lysophospholipase, and 9D4, a gene

of unknown function. Significantly, *Ibt6* was previously identified in a differential screen as a gene regulated by *Tri10* (A. W. Peplow, A. G. Tag, G. Garifullina, and M. Beremand, *in press*). The potential relationship of both *Ibt6* and 9D4 to trichothecene production is being investigated. It is also noteworthy that the following three genes, which we might have expected to cluster within this group, failed to do so: two genes recognized as being influence by *Tri10* (*Ibt7* and *Ibt2*) and the isoprenoid biosynthetic pathway gene phosphomevalonate kinase (*Pmk*). This suggests that these three genes are not as closely regulated by *Tri10* as are the other *Tri* genes and the isoprenoid genes.

The above defined groups were further resolved by increasing the number of experimental parameters known to affect *Tri10* gene expression. The *Gpd::Tri10* strains were included in this study because of their much larger effect on *Tri* gene expression and toxin production. Time was also incorporated as a variable because as time progresses from 23 h to 60 h post inoculation *Tri* gene expression generally decreases in the wild-type strain while it is maintained in the *Tri10*-overexpression strains. Despite these increased constraints, three of the isoprenoid pathway genes (*Acat, Hmgs,* and *Fpps*) continued to co-cluster with the majority of the *Tri* genes. Interestingly, these three genes fall at either the beginning or the end of the isoprenoid pathway (Fig. 3-1). While the significance of this clustering remains to be determined, it suggests that the expression of these three isoprenoid genes represent key regulatory points in

relation to trichothecene production. The resolving power of the targeted arrays was additionally illustrated by the fact that the two remaining isoprenoid genes, *Mk* and *Pmk* co-cluster with each other. Notably, these two genes encode the enzymes which catalyze adjacent steps in the isoprenoid biosynthetic pathway (Fig. 3-1). In the same manner, *Tri5*, which previous studies have shown is not as tightly regulated by *Tri10* as are the other *Tri* genes, also clustered separately from the core group of *Tri* genes as did *Ibt1* and *Ibt4*. The co-clustering of these latter two genes further suggests that they share closely related functions; in fact we previously noted that both of these genes might encode pathogenicity factors (A. W. Peplow, A. G. Tag, G. Garifullina, and M. Beremand, *in press*). Lastly, the tight clustering of the trichothecene regulatory genes *Tri10* and *Tri6* demonstrates the ability of these targeted arrays to resolve genes into functionally significant groups. Thus even these small targeted microarrays can be used effectively to detect subtle differences in transcript profiles as well as common trends.

Certainly an analysis of the full genome should reveal a number of genes that are inversely influenced by *Tri10* gene expression. Even within the narrow scope of the genes selected for our targeted microarray, a number of co-clustered genes that were downregulated when the *Tri* genes were upregulated were observed (Fig. 3-4B^{-}). This group includes genes with homology to histone H2A, RNase F1/T, and a cell-wall protein. While the exact function of these

homologs in *Fusarium* is not known, it is tempting to speculate that the expression of the trichothecene genes is coordinated with chromatin remodeling, mediated in part by a reduction in H2A, and with increased RNA stability mediated by a reduction in a specific RNAse. Additionally, increased toxin production also may demand alterations in cell wall composition. Experiments are under way to investigate these possibilities and to determine if these genes may therefore represent important new targets for controlling trichothecene toxin production.

While it is now quite evident that *Tri10* plays a central role in the transcriptional regulation of genes not only for the trichothecene biosynthetic pathway but also for the isoprenoid pathway, its molecular mode of action remains a mystery. However, BLAST searches of data from the first drafts of the *Aspergillus fumigatus* (The Institute for Genomic Research (http://www.tigr.org)) and *Magnaporthe grisea* (Magnaporthe Sequencing Project. Ralph Dean, Fungal Genomics Laboratory at North Carolina State University (http://www.fungalgenomics.ncsu.edu), and Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu)) genome sequencing projects have identified genes with some similarity to *Tri10* in these organisms. Analyses of these two genes may yield critical information that could suggest functional motifs which can be investigated by additional mutational studies. It is also intriguing that the *M. grisea Tri10* ortholog resides adjacent to the only

hydroxymethylglutaryl-coenzyme A reductase (*Hmgr*) gene for the isoprenoid biosynthetic pathway in that organism because we have recently determined that *Tri10* regulates *Hmgr* expression in *F. sporotrichioides* (Chapter IV).

The targeted cDNA microarrays will continue to serve a valuable function in future experiments. At the same time, the ability to interrogate larger sets of genes will provide more information about the regulatory relationships between the primary and secondary metabolic pathways for trichothecene biosynthesis as well as about important regulatory networks involving signal transduction pathways which have been previously shown to affect *Tri* gene expression (97). It will be especially interesting to determine if *Tri10* influences the expression of any of the genes from parallel FPP initiated metabolic pathways, including those for carotenoid, gibberellin, and ergosterol biosynthesis (Fig. 3-1). Thus, information gained from the EST database (http://www.genome.ou.edu/fsporo.html) is being utilized to build expanded targeted arrays containing several hundred genes, and ultimately arrays that represent the unigene set defined by the above EST database will be constructed. These arrays, in conjunction with additional genetically engineered and mutant strains, will provide increased knowledge regarding key aspects of functional genomics as they relate to secondary metabolism. Such arrays will have an added utility in exploring plant-pathogen interactions including further investigation of the role(s) that secondary metabolism plays in the establishment

of important plant diseases. All of these studies have the potential to reveal important new targets for controlling or preventing mycotoxin contamination.

CHAPTER IV

THE ISOPRENOID GENE *HMGR* IS REGULATED BY *TRI10* INDEPENDENTLY OF THE FLOW OF FARNESYL INTO THE TRICHOTHECENE BIOSYNTHETIC PATHWAY

INTRODUCTION

Fusarium species cause scab diseases of small grains. In addition to causing reductions in yield, *Fusarium* species also contaminate the grain with trichothecene mycotoxins. The trichothecenes are potent inhibitors of eukaryotic protein synthesis and cause hemorrhage, emesis, and immunosuppression in humans and animals that consume contaminated grains (69). In addition to causing disease in animals, the trichothecenes have been shown to contribute to the virulence of specific *Fusarium* species on specific plant hosts (25, 27, 30, 31). Thus, efforts to prevent the destruction of small grains by *Fusarium* through preventing trichothecene production by the fungus would have the additional benefit of preventing exposure of humans and animals to trichothecenes.

Two regulators controlling trichothecene biosynthesis have been isolated and characterized. Both reside within the core trichothecene gene cluster. *Tri10* encodes a regulator of unknown mechanism which is required for T-2 toxin

production and wild type expression of the trichothecene genes. *Tri6* encodes a zinc-finger DNA-binding protein also required for trichothecene production and wild type expression of the *Tri* genes. A model of regulation for *Tri* gene expression in which *Tri10* and *Tri6* form a regulatory loop to account for the observation that *Tri10* is required for wild type expression of *Tri6*, and *Tri6* is required to limit the expression of *Tri10* has been proposed (96). The expression of *Tri10* and *Tri6* are also required for the wild type expression of several isoprenoid genes upstream from the trichothecene biosynthetic pathway: farnesyl pyrophosphate synthetase (*Fpps*), 3-hydroxyglutarylmethyl-CoA synthase (*Hmgs*), and acetyl-CoA acetyltransferase (*Acat*) (Chapter II)(A. W. Peplow, A. G. Tag, G. F. Garifullina, and M. N. Beremand, *in press*). However, it was unclear whether the observed regulation of these primary metabolic genes by *Tri10* and *Tri6* was due to the change in the expression of *Tri10* and *Tri6* or due to the resulting changes in metabolite flow and/or T-2 toxin production caused by the disruption of each of these regulatory genes. This study was designed to address these questions in order to better understand the means by which *Tri10* regulates the expression of the genes in the isoprenoid (mevalonate) pathway.

MATERIALS AND METHODS

Strains, culture conditions, and fungal transformation

The *Fusarium sporotrichioides* wild-type strain NRRL 3299 was obtained from the ARS/USDA Culture Collection at the National Center for Agricultural Utilization and Research in Peoria, Ill. The *F. sporotrichioides* strains MB5493 (*tri4*) (10, 75), FsTri10-1-12 (Δ*tri10*) and FsTri10-1-20 (↑*Tri10*) (96), and NN4 (Δ*tri6*) (87), have been described previously. The *tri5* point mutant strain, FsTri5-6, which has a G to T change in codon 30 and is thus a nonsense mutant allele of *Tri5*, was provided by G. Garifullina. Stock cultures were routinely established from frozen glycerol stocks (-80°C) onto V8 juice agar slants either with (for transformants) or without (for the wild-type strain and the UV mutant) the addition of hygromycin B (300 μg/ml). Transformation of FsTri5-6 was performed as described previously (88, 97) using pTri10-2 (Chapter III). Transformants were grown in the dark with an alternating temperature schedule of 12h 25°C/12h 20°C whereas all other strains were grown under conditions of alternating 12h light / 25°C and 12 h dark/20°C. Liquid cultures for DNA, RNA and toxin analysis were inoculated and grown in YEPD-2G medium for DNA isolation and YEPD-5 medium for RNA and toxin analysis as previously described (88, 97).

Nucleic acid isolation and analysis

Fungal genomic DNA isolation was performed as previously described (97). Southern blots were prepared utilizing standard techniques (89). Total RNA was isolated using the Ultraspec II kit (Biotecx, Houston, Tx) according to previously described methods (97). For RNA blots, 5 μg of total RNA were loaded per lane on formaldehyde-containing 1% agarose gels, subjected to electrophoresis, and blotted onto Hybond N+ nylon membranes. Northern hybridization probes were gel purified *Apa* I-*Sma* I restriction fragments of cDNAs corresponding to the genes of interest labeled with ^{32}P-dCTP via nick translation. The *Hmgr* clone (b4g02fs) used in this study was identified through the *Fusarium* cDNA sequencing project (University of Oklahoma, Bruce A. Roe, Qun Ren, Doris Kupfer, HongShing Lai, Marian Beremand, Andrew Peplow and Andrew Tag) by homology to other *Hmgr* genes. Five prime and 3' sequences of this clone have been deposited in GenBank under the accession numbers BI188105 and BI188106 respectively.

Toxin analysis

Cultures were extracted for trichothecenes using previously described procedures and analyzed for T-2 toxin by gas chromatography/mass

spectrometry (97). T-2 toxin was identified by comparison to a commercial standard (Sigma) which was used without further purification.

RESULTS

A premature stop codon in *Tri5* prevents T-2 toxin production

Previous studies have indicated the presence of a regulatory region, extending upstream of *Tri10* into the *Tri5* promoter, that controls *Tri10* gene expression and toxin production (G. Garifullina, A. Tag, A. Peplow, and M. Beremand, unpublished data)(Chapter II)(M. Beremand, unpublished data). Since any attempt to construct a *tri5* mutant by deletion or disruption of the *Tri5* gene would interrupt this sequence and potentially alter the expression pattern of *Tri10*, our approach was to use a *Tri5* point mutant strain that contained a premature stop codon. The introduced stop codon in the *Tri5* gene in strain FsTri5-6 (*tri5-1*) was confirmed by DNA sequence analysis.

The analysis of liquid shake culture extracts by GC-MS revealed that the *tri5-1* strain did not produce any T-2 toxin in comparison to the wild-type strain (Fig 4-1).

Introduction of a *GPD* promoter-driven copy of *Tri10* into FsTri5-6

Strain FsTri5-6 (*tri5-1*) was transformed with pTRI10-2 (Chapter III) which contains the coding region of *Tri10* fused to the glyceraldehyde 3-phosphate dehydrogenase (*Gpd*) promoter from *Trichoderma virens*. Twenty-five hygromycin resistant transformants were recovered and screened by Southern analysis to determine the site of integration. All of the transformants recovered were the result of homologous integration of the pTRI10-2 vector into the *Tri10* locus (data not shown). From these, transformants *tri5-1*/*Gpd*::*Tri10*-1, *tri5-1*/*Gpd*::*Tri10*-3, *tri5-1*/*Gpd*::*Tri10*-8, and *tri5-1*/*Gpd*::*Tri10*-15 were selected for further study. GC-MS analyses of liquid shake culture extracts from these transformants revealed that none produced T-2 toxin (Fig 4-1).

Deletion of *Tri10* and *Tri6*, but not the presence of biochemical blocks early in the trichothecene biosynthetic pathway, reduces *Hmgr* transcript levels

Northern analysis revealed that the deletion of *Tri10* and *Tri6* resulted in a dramatic decrease in *Hmgr* transcript accumulation compared to wild-type in contrast to the *tri5-1* strain which accumulated wild-type levels of *Hmgr* transcript (Fig 4-2). This suggests that *Tri10* and *Tri6* are required for wild-type levels of *Hmgr* transcript accumulation during *Tri* gene expression and that flow of

farnesylpyrophosphate into the trichothecene biosynthetic pathway and T-2 toxin production are not responsible for the observed increased *Hmgr* expression.

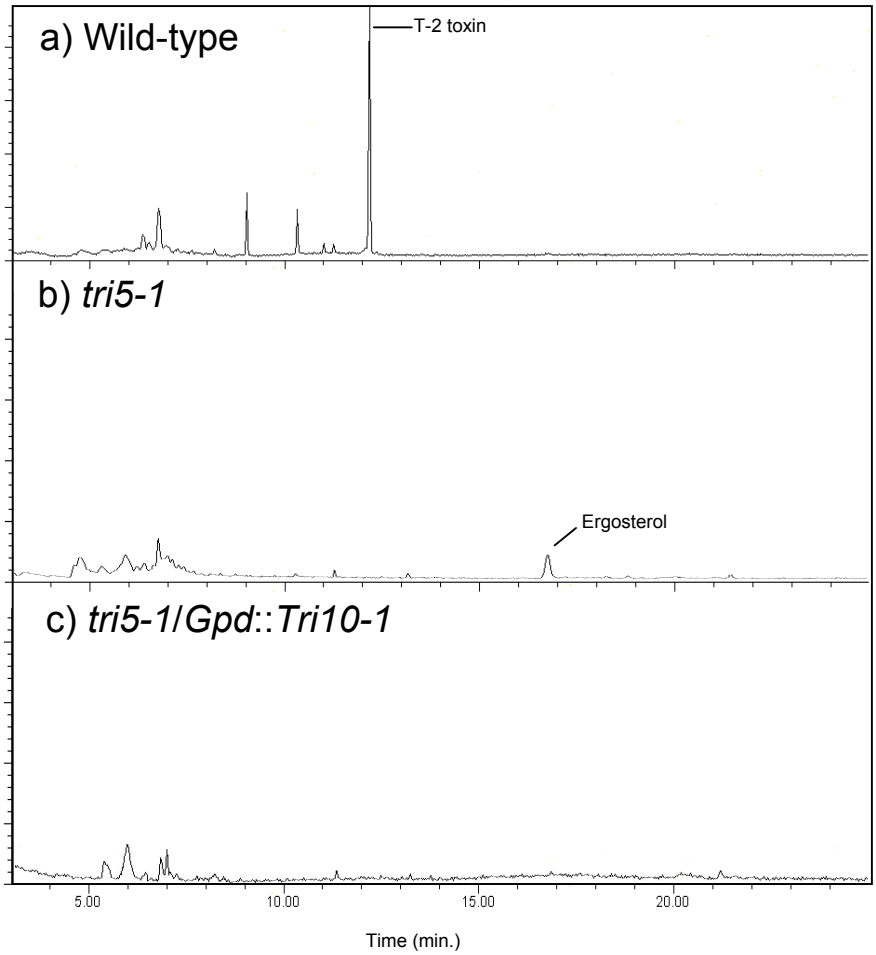

FIG. 4-1. GC-MS analysis of *tri5-1* mutant strains. Chromatograms produced from culture extracts of: (a) Wild-type *F. sporotrichioides* NRRL 3299, (b) the *Tri5* point mutant (*tri5*-1) possessing a premature stop codon FsTri5*-6, and (c) *tri5-1*/*Gpd*::*Tri10-1* which overexpresses *Tri10* in the *tri5-1* mutant background. The small amount of ergosterol seen in the *tri5-1* mutant is most likely mycelial contamination from the extraction process.

Wild type *Hmgr* transcript levels were also observed in the *tri4* mutant, providing further evidence that the lack of T-2 toxin production does not alter the transcription of this gene (Fig 4-3). Similarly, the transcription of *Hmgr* does not appear to be altered by the accumulation of trichodiene since the *tri4* strain is blocked in the second step of the trichothecene pathway and accumulates this pathway intermediate.

Interestingly, there may be a slight increase in the transcript levels for *Tri3, Tri4, Tri6,* and *Tri1* in the *tri5-1* strain (Fig. 4-2). It is conceivable that even the point mutation in the *Tri5* gene sequence produced a slight *Tri10* overexpression phenotype. However, this change did not produce a true *Tri10* overexpression phenotype because the transcript levels for *Tri101* are actually decreased in the *tri5-1* strain compared to the wild type parent strain. Notably, the *Tri101* transcript levels are also slightly reduced in the *tri4* strain, while the transcript levels for *Tri6* and *Tri5* are similar to those in the wild-type parent.

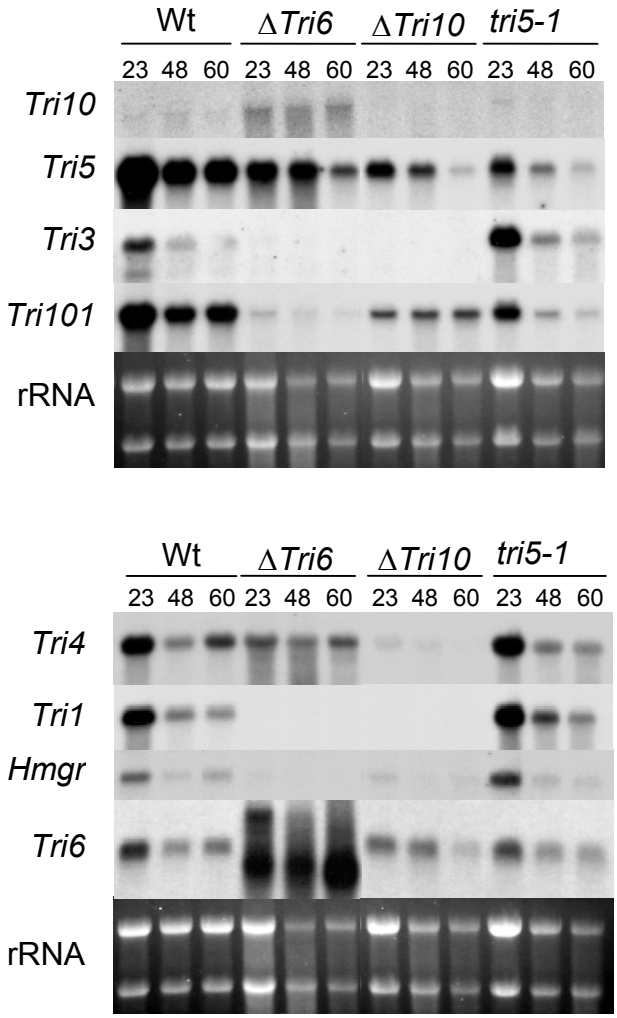

FIG 4-2. Northern analysis of *tri5-1* mutant strains. Northern analysis of *Tri10, Tri5, Tri3, Tri101, Tri4, Tri1, Tri6,* and *Hmgr* in wild-type *F. sporotrichioides* NRRL 3299 and *tri5-1*, which contains a premature stop codon in the *Tri5* gene. Five micrograms of total RNA was loaded per lane. Ribosomal RNAs were visualized by ethidium bromide staining.

Overexpression of *Tri10* in *tri5-1* via a heterologous promoter results in overexpression of trichothecene and isoprenoid pathway genes

An examination of *Tri* gene transcript accumulation in the *tri5-1/Gpd*::*Tri10* strains revealed a dramatic increase in the expression of both regulatory genes, *Tri10* and *Tri6*, and the isoprenoid gene *Hmgr* at 15 h, 23 h and 48 h post-inoculation (Fig. 4-3). The expression of *Tri5* and *Tri101* was also increased in the *tri5-1/Gpd::Tri10* strains at 15 h compared to wild-type. Notably, these strains do not exhibit the same degree of overexpression of *Tri101* transcripts at 23 h and 48 hr as observed for the other genes (with the exception of *Tri5*). However, the evaluation of the levels of the *Tri5* gene transcripts in these transformants must take into account the possible reduced stability of the *Tri5* transcript since it carries a nonsense mutation.

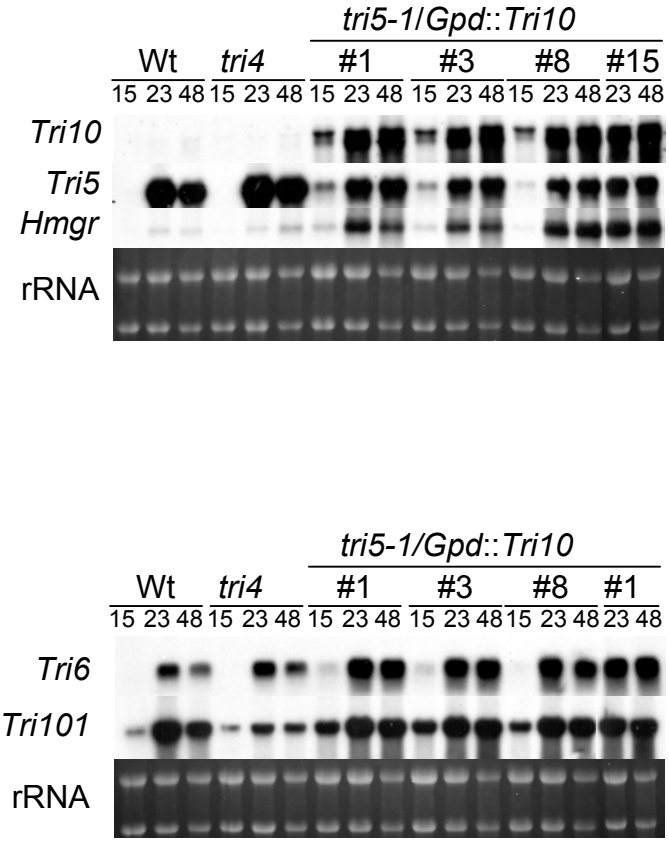

FIG. 4-3. Northern analysis of *tri5-1/Gpd::Tri10* strains. Northern analysis of *Tri10, Tri5, Tri6, Tri101* and *Hmgr* expression in wild-type *F. sporotrichioides*, a *tri4* mutant (MB5493) which is blocked in the second step of the trichothecene pathway, and pTri10-2 transformants of *tri5-1* containing a copy of *Tri10* under the control of the *Gpd* promoter from *T. virens*. Five micrograms of total RNA was loaded per lane.

DISCUSSION

The present study provides compelling evidence that the regulatory link between trichothecene secondary metabolism and isoprenoid primary metabolism in *F. sporotrichioides* is mediated by the expression of *Tri10* and *Tri6*. Previously, studies had shown that 3 isoprenoid genes (*Hmgs, Mk, Fpps*) were influenced by the expression of *Tri10* and *Tri6* (96, 97) (A. W. Peplow, A. G. Tag, G. F. Garifullina, and M. N. Beremand, *in press*)(Chapter III, Fig. 3-4). However, in those studies it was unclear whether the changes observed in isoprenoid gene expression were directly due to the loss of *Tri10* and *Tri6* gene expression, the concomitant decline in the flow of farnesyl into the trichothecene biosynthetic pathway, or the resulting lack of T-2 toxin production. In the present study, the metabolic flow of farnesyl into the trichothecene biosynthetic pathway and T-2 toxin production were prevented while preserving *Tri10* and *Tri6* gene expression. This was accomplished by creating a *Tri5* point mutant and thus only blocking the first dedicated step in the trichothecene pathway: the cyclization of farnesyl pyrophosphate into trichodiene.

A key gene in the regulation of the production of isoprenoid precursors available for terpenoid synthesis in *Saccharomyces cerevisiae* is *Hmgr* (42). Examination of *Hmgr* expression in the Δ*Tri10* and Δ*Tri6* strains revealed reduced transcript accumulation levels, with the lowest levels observed in the Δ*Tri6* strain (Fig. 4-2). *Hmgr* transcript accumulation was not reduced in the *tri5*-

1 strain, implying that the effects on *Hmgr* expression observed in the ∆*Tri10* and ∆*Tri6* strains result from the disruption of *Tri10* and *Tri6* expression and not from a metabolite feedback loop responding to a potential change in FPP pools or T-2 toxin levels. Furthermore, the transcript accumulation of *Hmgr* increased dramatically in the *tri5-1*/*Gpd::Tri10* transformants compared to wild-type at all time points but not in the ∆*Tri6* strain, which also displayed an increase in *Tri10* transcript levels. Together, these data strongly argue that the expression of *Tri10*, through the activity of *Tri6,* is responsible for the enhanced *Hmgr* expression levels observed.

In contrast to the results observed with *Hmgr*, the *tri5-1* mutation, and thus the inability to make trichothecenes, did have an apparent effect on the expression of some of the trichothecene biosynthetic genes. As seen in Fig. 4-2, *Tri1 Tri3,* and *Tri4* appear to accumulate slightly higher transcript levels in the *tri5-1* strain compared to wild-type. However, it is still possible that the point mutation alone may have produced a slight *Tri10* overproduction phenotype. Additional experiments will be needed to determine if this effect is due to the alteration of the DNA sequences upstream of *Tri10*, the possible accumulation of FPP and/or its failure to enter into the trichothecene biosynthetic pathway, and/or the lack of T-2 toxin production or other pathway intermediates.

In addition, the *tri5* and *tri4* mutations had a marked effect on the transcript accumulation of *Tri101*, a trichothecene biosynthetic gene responsible for trichothecene C3-acetylation. *Tri101* exhibited a dramatic reduction in transcript abundance in both the *tri5-1* strain (Fig. 4-2) and the *tri4* strain (Fig. 4-3). These data imply that *Tri101* transcript accumulation is enhanced by the presence of a trichothecene pathway metabolite beyond trichodiene. However, this effect can be partially overcome by overexpressing *Tri10* in a *tri5* mutant background (Fig. 4-3).

The regulation of isoprenoid biosynthesis is complex. Another pathway which shares the same precursors (isoprenoid metabolites) as the trichothecene pathway is the ergosterol biosynthetic pathway. Ergosterol is an essential component of fungal plasma membranes. Regulation of ergosterol synthesis in *S. cerevisiae* hinges on the protein turnover rate of HMGR which has been shown to be controlled by FPP pools and a second unidentified compound believed to be an oxysterol (40, 41). Additionally, transcription of *Hmgr* in yeast is regulated predominantly through a negative regulatory feedback loop in response to sterol. In trichothecene biosynthesis, we have observed increased expression of the *Hmgr* gene coordinated with the expression of trichothecene genes (Chapter III). This would be expected if, as in the yeast model, the sterol concentration in the cell were to drop in response to the increased utilization of FPP by the trichothecene pathway. However, the present study showed that

expression of *Tri10* by either the native *Tri10* promoter or by the *Gpd* promoter was sufficient to increase *Hmgr* gene expression in the absence of the conversion of FPP to trichodiene. Therefore, the expression of the trichothecene regulatory genes, *Tri10* and *Tri6,* is directly tied to the regulation of *Hmgr* in *F. sporotrichioides*. In *Gibberella fujikuroi,* the biosynthesis of another secondary metabolite, the gibberellins, also depends on the activity of HMGR. In this case, it was found that the addition of plant oil to the medium increased the expression of *Hmgr*, however the mechanism of the induction was unknown (109). Curiously, a minimal TRI6 DNA binding sequence (YNAGGCC) is located in the promoter for the *G. fujikuroi Hmgr* gene. As both *F. sporotrichioides* and *G. fujikuroi* are plant pathogens, it is possible that both pathogens share a similar signal transduction pathway to induce *Hmgr* expression. The use of expanded cDNA microarrays coupled with the above mutants will be a powerful tool in identifying additional regulatory factors linking primary and secondary metabolism.

CHAPTER V

CONCLUSION

The body of this work describes the continuing characterization of a novel regulatory gene, *Tri10*, from the trichothecene gene cluster and the discovery of an important regulatory link between primary and secondary metabolism in *F. sporotrichioides* mediated in part by the expression of *Tri10*. The discovery of *Tri10* (13) and its subsequent characterization and disruption (Chapter II) provided critical evidence that *Tri10* was a positive regulator of *Tri* gene expression and allowed the development of a regulatory model for trichothecene production in *F. sporotrichioides*. As depicted in the model shown in Fig. 5-1, the expression of *Tri10* is required for wild-type expression levels of *Tri6,* a cluster-encoded transcription factor (87), and consequently the other cluster-encoded and non cluster-encoded *Tri* genes.

Notably, *Tri10* is the only *Tri* gene that does not depend at least in part on the expression of *Tri6* to activate its transcription. Conversely, *Tri10* is negatively regulated by *Tri6* expression since *Tri10* transcript levels increase in the ∆*Tri6* strain (Fig. 2-3). This provides evidence for a regulatory loop between *Tri10* and *Tri6* which modulates the expression of the *Tri* genes.

Tri10 increases *Tri* gene expression if it is overexpressed in either of two ways. Both disruptions in the continuity of the region upstream from *Tri10*

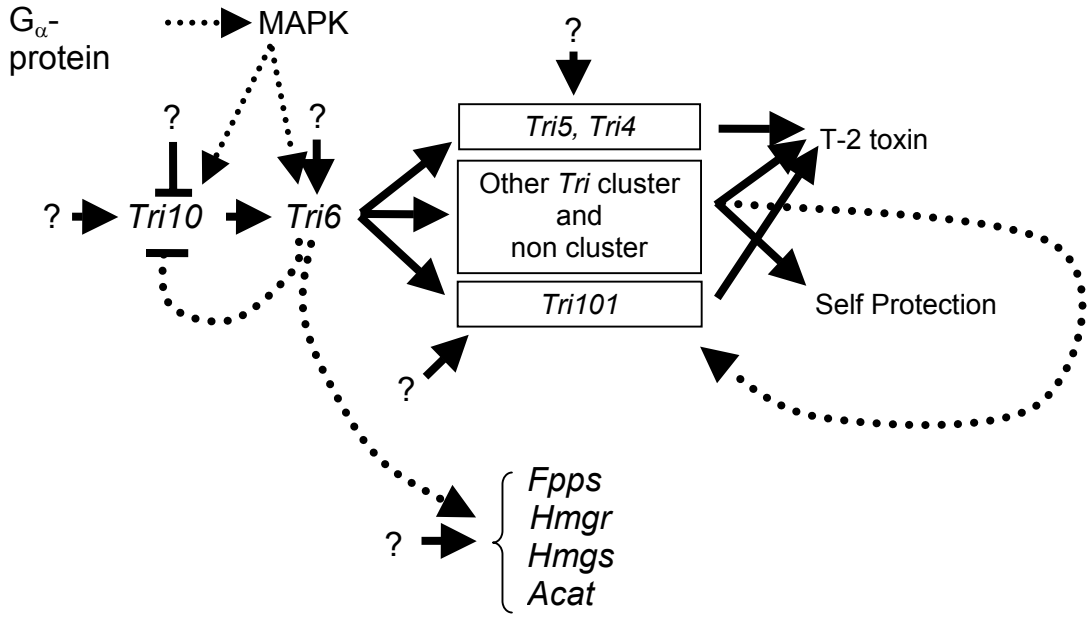

FIG. 5-1. Revised regulatory model for trichothecene production. Solid arrows indicate known positive activities. Dotted arrows indicate possible activation. Blocked arrows indicate known inhibitory activities, and dotted blocked arrows indicate possible inhibitory activities. Question marks indicate other hypothesized but unknown regulatory signals or factors.

and the expression of *Tri10* under the control of the *Gpd* promoter whether located within or outside of the *Tri* gene cluster, also causes overexpression of *Tri10*, the *Tri* genes, and overproduction of T-2 toxin (Chapter III). Thus, while the continuity of the *Tri* gene cluster upstream of *Tri10* is required for wild-type *Tri* gene expression levels, the latter experiments demonstrated that neither the disruption of the *Tri* gene cluster nor the placement of the overexpression copy of *Tri10* within the *Tri* gene cluster are essential requirements for the overexpression phenotype.

During this study, it was discovered that the expression of *Tri10* had an effect on the expression of one of the genes in the isoprenoid pathway; the primary metabolic pathway feeding into trichothecene synthesis. The first isoprenoid gene which we cloned from *F. sporotrichioides* was *Fpps* and thus it was the first isoprenoid gene examined. *Fpps* is responsible for the production of farnesyl pyrophosphate, the immediate precursor for the trichothecene pathway. I examined the expression of *Fpps* in a strain in which *Tri10* had been disrupted, a strain in which *Tri10* was overexpressed, and a strain in which *Tri6* had been disrupted. The disruption of either *Tri10* or *Tri6* led to a decrease in *Fpps* expression while the overexpression of *Tri10* led to the overexpression of *Fpps*. In addition, even though *Tri10* was overexpressed in the Δ*Tri6* strain, *Fpps* was not. Thus both *Tri10* and *Tri6* are required for wild-type levels of *Fpps* expression and the increased transcript level of *Tri10* alone is not sufficient to

increase *Fpps* expression. Taken together, these results indicate that the positive expression of *Fpps* by *Tri10* is mediated via *Tri6*. These results also raised the following two questions. First, were the remaining isoprenoid pathway genes regulated in a similar manner by *Tri10* and *Tri6*? Second, was the expression of *Tri10* and *Tri6* directly responsible for the observed increase in *Fpps* expression or was either the presumed change in the flow of farnesyl pyrophosphate into the trichothecene pathway or the lack of T-2 toxin production responsible for these changes?

Insights into the first of the above two questions were provided by the following experiments. In a differential cDNA macroarray screen for genes influenced by *Tri10* expression, we discovered that the isoprenoid genes *Acat*, *Hmgs*, and *Mk* were positively regulated by *Tri10*. Clones were isolated for these genes and used to generate probes for Northern blots which revealed that these genes were also positively regulated by *Tri6* expression (A. W. Peplow, A. G. Tag, G. Garifullina, M. N. Beremand, *in press*). The clones served as templates for the construction of targeted cDNA microarrays (Chapter III) which were subsequently probed with labeled cDNAs produced from Δ*Tri10* and ↑*Tri10* strains and provided data that confirmed the results obtained from the Northerns. Thus, the expression of *Acat*, *Hmgs*, and *Mk,* like that of *Fpps*, is also reduced in the Δ*Tri10* strain and increased in the ↑*Tri10* strain.

In order to investigate whether *Tri10* and *Tri6* gene expression or either changes in the flow of FPP into the trichothecene pathway or the lack of T-2 toxin production were responsible for changes in isoprenoid gene expression in the Δ*Tri10* and Δ*Tri6* strains, we chose to examine the expression of *Hmgr*, a gene which has been shown to function as a critical control point for isoprenoid synthesis in *S. cerevisiae* (40, 41), in the Δ*Tri10*, Δ*Tri6*, and two strains blocked very early in the trichothecene pathway. The first of these two strains contained a nonsense mutation in *Tri5* (*tri5-1*) that effectively blocked the first step of trichothecene synthesis, and hence, completely prevented the flow of farnesyl pyrophosphate into the trichothecene pathway. The second strain was a *tri4* mutant which is blocked in the second step of the trichothecene pathway and therefore allows the flow of farnesyl pyrophosphate into the trichothecene pathway. At the same time, all of these mutants share in common the failure to make any trichothecenes (Chapter IV). Unlike the Δ*Tri10* and Δ*Tri6* strains, which showed a reduction in *Hmgr* expression, both the *tri5-1* and *tri4* strains exhibited wild type levels of *Hmgr* expression. These data indicate that neither the flow of FPP into the trichothecene pathway nor the production of trichothecenes is necessary for wild-type levels of *Hmgr* expression (Chapter IV). Therefore, *Hmgr* expression is directly related to the expression of *Tri10* and *Tri6*.

Further evidence that *Tri10* expression positively regulates *Hmgr* was provided by the analysis of *tri5-1* transformants which carry a *Tri10* overexpression plasmid (pTri10-2) containing *Tri10* under the control of the *Gpd* promoter (Chapter IV). All of the *tri5-1/Gpd::Tri10* transformants exhibited greatly increased *Hmgr* expression implying that overexpression of *Tri10* was capable of upregulating *Hmgr* in the absence of trichothecene production. However, as was seen with the *Tri* genes, *Tri10* mediates this control through *Tri6* since the Δ*Tri6* strain exhibits reduced *Hmgr* expression and increased *Tri10* expression.

Unlike many of the other *Tri* genes, *Tri101* resides outside of the trichothecene gene cluster (65). During the first study concerning the effect of *Tri10* gene expression on *Tri* gene expression, it became apparent that *Tri101* was under the control of additional regulatory controls which did not appear to respond like the other *Tri* genes (Chapter II). For example, *Tri101* is expressed at 15 h in the wild-type strain, at a time when the other *Tri* genes are not being expressed. This was consistent with previous reports that *Tri101* appeared to be “constitutively” expressed in *F. graminearum* when the *Tri5* and *Tri6* genes were not (65). Therefore, although *Tri10* and *Tri6* play an important role in influencing the expression of *Tri101*, it clearly has other factors that regulate its expression (Chapter IV). Additionally, *Tri101* expression is reduced in both the *tri5* and *tri4* strains suggesting that the production of T-2 toxin or some pathway

intermediate(s) plays a role in increasing *Tri101* expression. In future studies, to determine if the effect is due to T-2 toxin or pathway intermediates, the *tri5* strain could be grown in the presence of the individual trichothecene pathway intermediates from isotrichodermol, the substrate compound for TRI101, to T-2 toxin and, by a process of elimination, the inducing compound should be determined.

Tri10 also affects the expression of one or more genes involved in self protection, however not to the same extent as does *Tri6* (Chapter II). In two different assays, the Δ*tri6* and Δ*tri10* strains exhibited increased sensitivity to T-2 toxin indicating that some of the self-protection genes and/or mechanisms are under the control of *Tri10* and *Tri6*. This control is apparently independent of the inability of the Δ*Tri10* and Δ*Tri6* strains to produce trichothecenes. The trichothecene deficient *tri4* stain exhibited wild-type levels of resistance to T-2 toxin, demonstrating that producing trichothecenes is not a prerequisite for inducing self-protection mechanisms.

Since *Tri10* is a key regulator for *Tri* gene expression and T-2 toxin production, understanding the factors which regulate *Tri10* expression may provide novel methods to prevent trichothecene production.

Tri10 is both positively and negatively regulated by factors which are just beginning to be explored. In filamentous fungi, G proteins have been shown to regulate secondary metabolism (47) and virulence (5, 68) through signal transduction pathways. In a collaborative effort with Keller, et al., we found that a constitutively active allele of the alpha subunit of a heterotrimeric G protein, *fadA*^{G42R} from *Aspergillus nidulans*, simultaneously delayed *Tri* gene expression, including *Tri10* (data not shown), and resulted in increased toxin production in *F. sporotrichioides* (97). This implicates G protein signal transduction pathways in the regulation of *Tri* gene expression and T-2 toxin production. However, it is unclear how the G protein signaling pathway modulates *Tri* gene expression. In *A. nidulans*, it has been shown that the regulation of sterigmatocystin production and conidiation by FadA is partially mediated through an adenylyl cyclase/cAMP/cAMP-dependant protein kinase (94). To that end, a mitogen-activated protein kinase (*MGV1*) has recently been isolated from *F. graminearum* and shown to be required for production of deoxynivalenol, colonization of the wheat head, and female fertility (55). As MAP kinases frequently activate transcription factors, a homologue of *MGV1* in *F. sporotrichioides* might be an intermediate transducer from a G protein to *Tri10* and *Tri6* in the regulatory cascade that induces trichothecene production.

The characterization of *Tri10* has revealed little about its mechanism of action. Database searches have not yielded any conserved functional motifs.

However, recently three fungal genomes have been publicly sequenced. A search of these genomes has revealed that only one gene in each carries a gene which encodes a protein with some similarity to TRI10. However, in each case this similarity is limited: *Aspergillus fumigatus* (BLASTp score = 124, Evalue = 2 e^{-27}), *Neurospora crassa* (*PRO1a*) (BLASTp score = 93, Evalue = 93 e^{-17}), and *Magnaporthe grisea* (BLASTp score = 79, Evalue = 3 e^{-13}). All three proteins potentially contain GAL4-type Zn(II)2Cys(6) binuclear cluster domains (a putative intron would need to be removed from the transcript to add this domain to the *A. fumigatus* protein) in their amino termini which would implicate them in regulatory processes. The *N. crassa PRO1a* gene is the only one of the three to be characterized and it has been shown to be required for perithecial development (70). In a typical member of the GAL4 family, the amino terminus of the protein contains the Zn(II)2Cys6 DNA binding domain and the carboxy terminus beyond the DNA-binding domain frequently contains a coiled-coil dimerization domain used in protein-protein interactions; commonly to form homodimers (101). However, TRI10 does not contain a conserved Zn(II)2Cys6 DNA binding domain and neither TRI10 nor any of the three similar proteins contain a conserved coiled-coil domain. Furthermore, attempts to observe TRI10-TRI10 homodimers and TRI10-TRI6 heterodimers using the GAL4 yeast 2-hybrid system did not indicate these interactions (data not shown). A multiple alignment of TRI10 and the three similar proteins using ClustalW v1.4 (99) revealed that the majority of the conserved residues are nonpolar amino acids

and predominately leucine, although no conserved leucine septad repeats were observed which would indicate a leucine zipper (Fig. 5-2). Experiments which would identify other proteins which interact with TRI10 may provide further understanding of its mechanism of action in the regulation of the *Tri* genes. *Fusarium* species cause devastating diseases of wheat, barley and maize and frequently contaminate the grain with mycotoxins making it unsuitable for food or feed purposes. In the past decade, epidemics of *Fusarium* head blight of wheat have resulted in over 2.6 billion dollars in losses in the United States alone (77, 108). Efforts to control the disease have provided only moderate success since there are only a few sources of natural resistance available while the environmental conditions which favor the disease are common. One potentially promising approach presently being pursued is to produce transgenic plants by incorporating genes for resistance mechanisms to the trichothecene toxins from other organisms, including the trichothecene-producing fungus itself, into plant lines with some naturally occurring resistance. Currently under investigation are genes for an ABC transporter from yeast, a trichothecene 3-o-acetyltransferase (*Tri101*) (81), and a ribosomal protein variant (46). These approaches are centered on countering the presence of toxin (not directly preventing its production) with the goal of providing additional resistance against the toxin to the host and/or as a means to effectively reduce the virulence of the pathogen. An extension of this tactic is the identification of additional fungal genes required

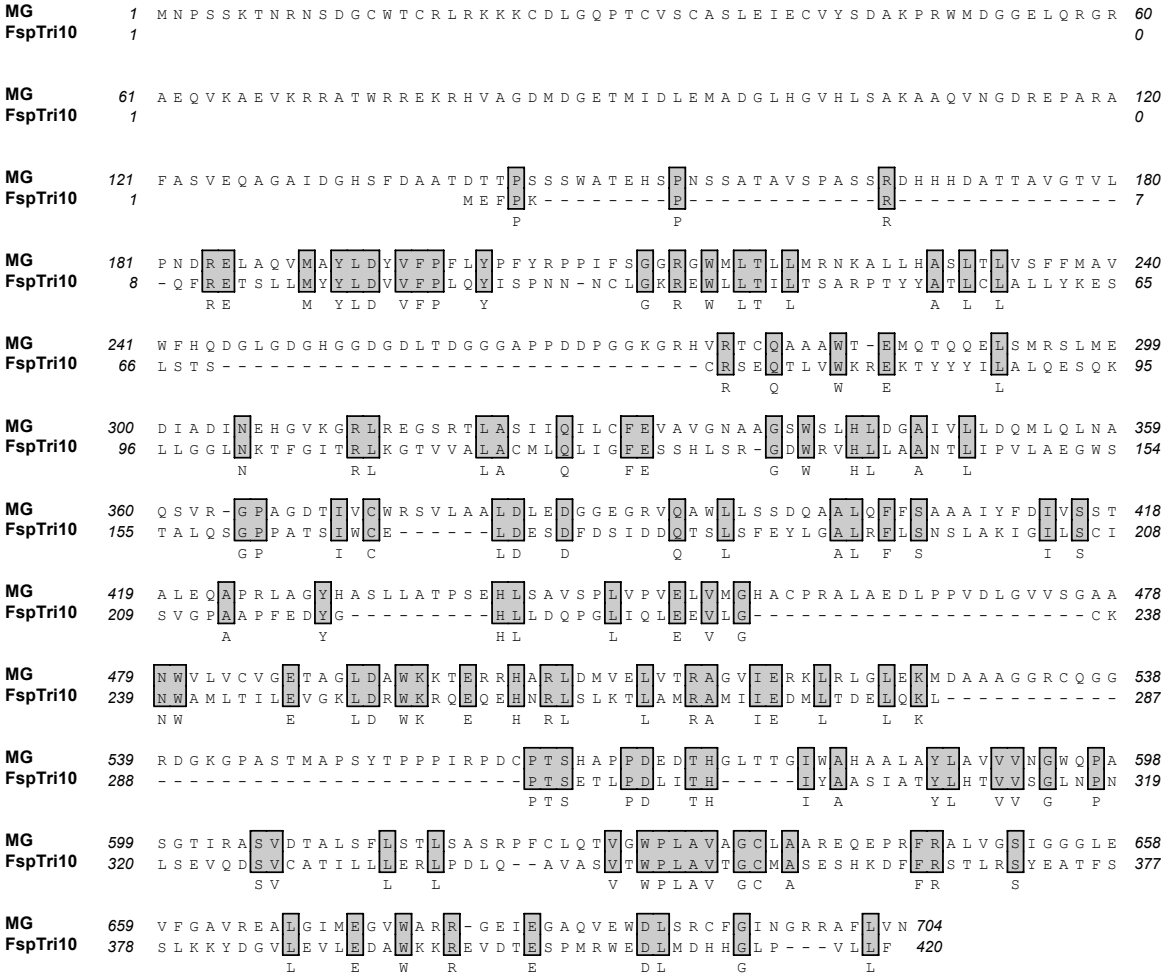

FIG. 5-2. Pairwise alignments of *F. sporotrichioides* TRI10 with similar proteins from *Magnaporthe grisea* (MG), *Aspergillus fumigatus* (Afum), and *Neurospora crassa* PRO1a (NC). Shaded areas indicate conservation. Dots indicate conservative substitutions. Dashes indicate gaps.

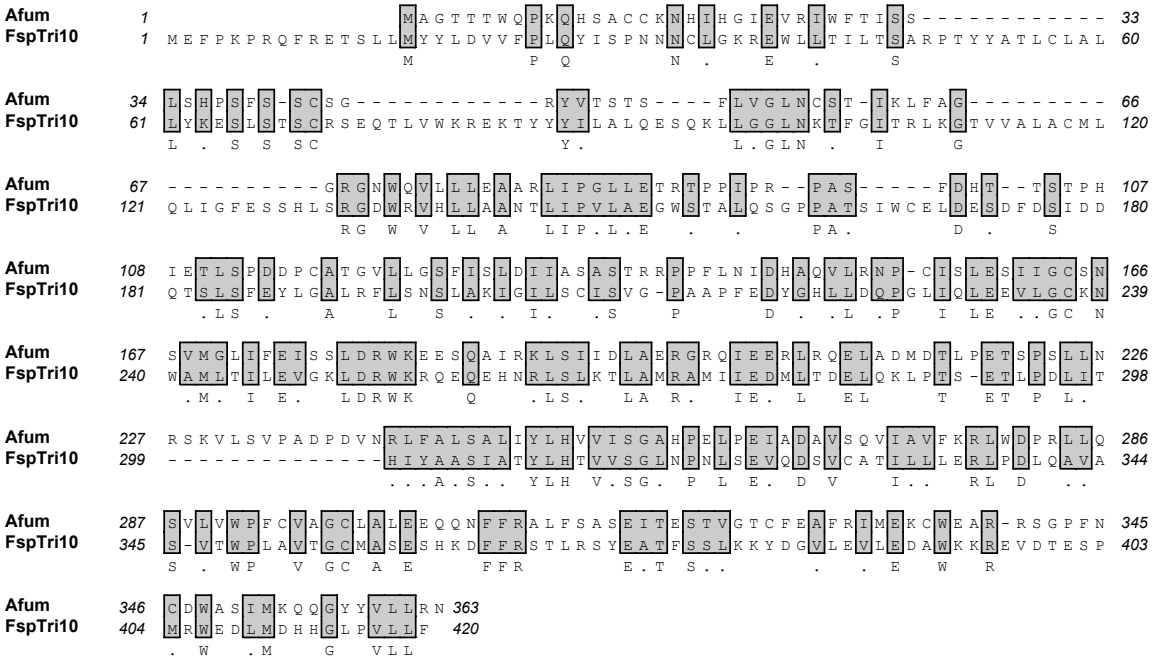

FIG 5-2. Continued

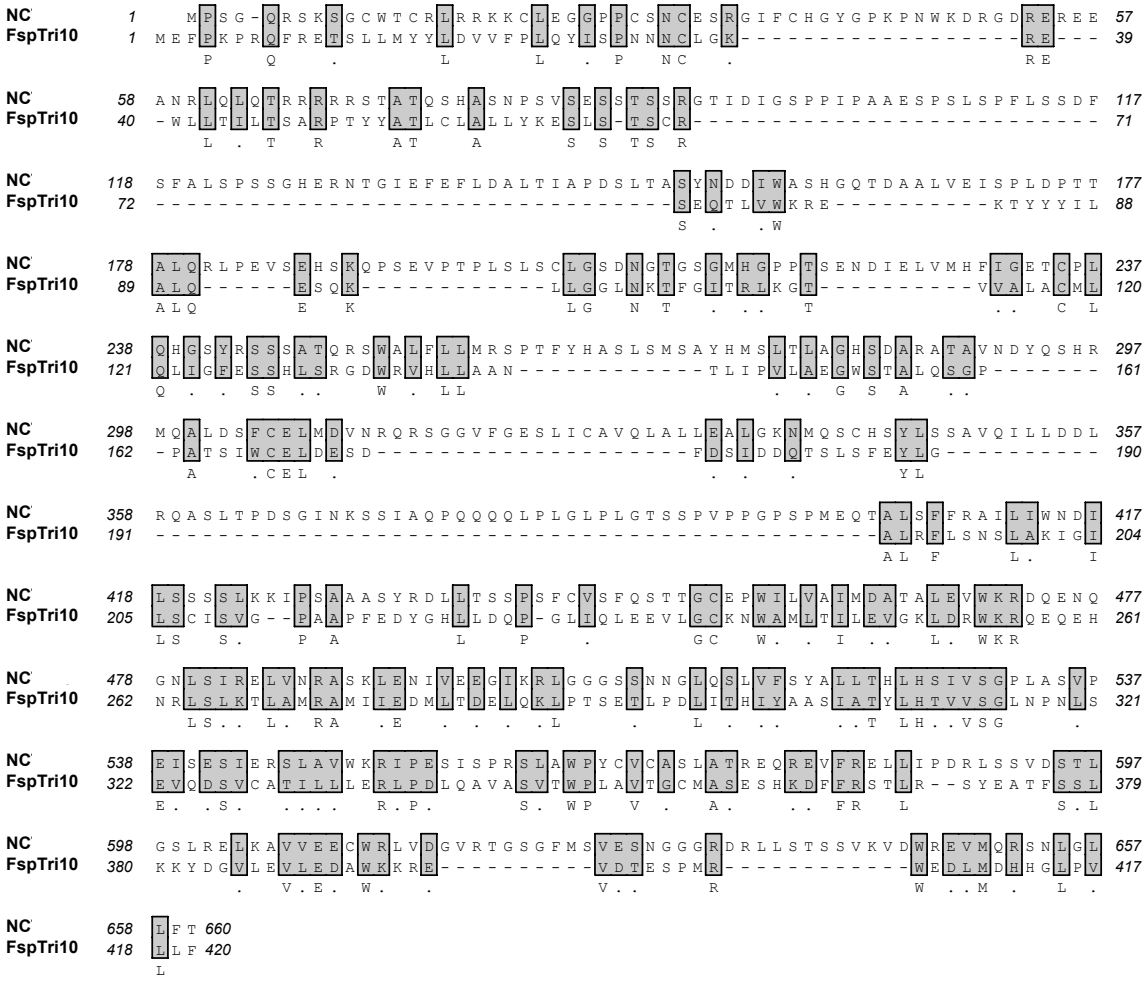

FIG. 5-2. Continued

for toxin production that could be candidate genes for producing resistant transgenic plants.

This research has contributed to the overall understanding of trichothecene gene regulation and has brought to light a novel regulatory link

between secondary and primary metabolism in fungi. Strains and information provided by this work have also be instrumental in the identification of additional genes under the control of *Tri10* (A. W. Peplow, A. G. Tag, G. Garifullina, and M. N. Beremand, *in press*) including additional genes required for T-2 toxin production (78). However, more work is needed to identify those components of the regulatory circuitry that are responsible for turning *Tri10* and *Tri6* on and off. A better understanding of the genetic and environmental factors contributing to the production of trichothecenes may give rise to additional novel strategies to prevent *Fusarium* mycotoxin production in crops.

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APPENDIX A

Supplementary Table 1

Array Identifier	wt/Δ*Tri10* 23h	wt23vswt 48h	wt23vswt 60h	wt23vs#24 23h	wt23vs#24 48h	wt23vs#24 60h
Tri6	1.285	1.360	0.947	1.604	4.141	2.671
Tri4	0.285	0.893	1.748	1.232	2.236	1.341
Tri11	0.135	0.713	0.428	1.964	6.542	1.669
Tri13	0.043	0.754	0.641	1.533	7.789	2.781
Tri1	0.033	0.488	0.356	1.353	2.378	1.683
Tri10	1.826	2.236	2.858	4.901	18.942	15.089
Tri3	0.091	0.808	0.409	2.774	14.233	1.928
Tri7	0.200	0.855	0.538	3.219	9.786	3.036
Tri8	1.003	0.512	0.933	1.277	2.249	1.118
Tri101	0.161	0.396	0.851	1.262	1.650	0.823
Tri15	0.213	0.860	0.959	1.383	0.898	0.89
Tri5	0.836	1.308	1.622	1.313	2.369	0.687
Tri9	0.302	1.325	1.749	1.983	6.761	0.693
Tri12	0.117	1.951	1.310	6.367	24.129	8.554
Tri14	0.046	1.376	0.859	1.603	2.334	1.875
Pyruvate kinase	1.838	2.064	2.057	2.770	2.754	
Dhat	1.400	1.502			1.857	
Acat	0.485	1.635	0.826	2.162	4.452	3.552
Hmgs	0.192	1.592	0.803	4.757	25.718	6.463
Mk	0.452	1.337	7.735	2.166	4.188	2.234
Pmk	1.275	1.508			2.079	3.551
Fpps	0.247	0.745	0.379	1.946	4.329	0.889
Citrate synthase	1.097	1.440	1.816	0.991	2.521	0.909
Aconitate hydratase	0.752	2.133	1.199	0.771	1.408	0.559
Isocitrate dehydrogenase	1.768	1.832	2.609	5.438	6.034	2.169
rAsp f9	1.036	1.566	1.744	0.883	2.257	0.738
Ibt4	0.416	2.458	2.325	0.995	6.887	3.06
Ibt6	0.599	1.032	0.665	0.944	3.517	1.153
Ibs1	0.973	0.122	0.439	0.438	0.549	0.164
Ibt1	0.441	2.318	2.082	1.116	10.406	5.246
ADP/ATP Carrier protein	1.049	1.239	1.590	1.654	1.467	0.717
Potassium Channel protei	0.984	2.326	4.579	2.138	14.793	6.066
RNase F1/T1	0.997	0.738	1.772	1.262	0.266	0.033
Alcohol dehydrogenase	0.979	1.035	0.743	1.022	0.535	0.652
Transcription factor	1.002	1.841	1.757	1.194	2.234	0.702
18I12	1.134	2.281	2.286	3.806	4.023	1.172
15G19	1.334	1.738	1.788	1.334	3.137	1.306
9D4	0.630				3.992	2.07
17H21	0.940	1.758	1.757	1.250	2.482	1.531
12M24	0.906	1.288	1.440	2.607	1.304	1.202
11K18	1.355	2.542			8.362	1.234
Cell wall protein	1.003	0.784	1.592	0.345	0.740	0.388
ABC Transporter	0.992	1.788			2.934	
Histone H2A subunit	1.192	0.714	1.490	0.768	0.794	0.302
Nucleosome assembly	0.974	1.360	1.741	2.250	3.553	1.053
Stress protein	0.996	0.565	1.789	1.278	1.639	0.705
Elongation factor 1 alpha	1.000	1.262	1.988	2.170	2.687	1.918
Elongation factor 2	1.001	1.483	1.431	1.527	1.526	1.029
RNA pol II large subunit	1.001	1.183	2.840	1.817	2.178	2.483
Core II protein	1.057	0.714	0.633	1.133	0.789	0.852
Actin	1.000	1.000	1.000	1.000	1.000	1
Cytochrome P450	1.001	1.086	0.630	0.856	0.382	0.429
fadA	1.000	0.572	0.974	1.523	0.875	0.74

Values shown are Cy5/Cy3 ratios which have been normalized to actin.

Supplementary Table 1 continued

Array Identifier	wt23vs#49 23h	wt23vs#49 48h	wt23vs#49 60h	wtvs↑*Tri10* 23h	wt23vs↑*Tri10* 48h
Tri6		3.803	4.08	2.346	1.268
Tri4	1.399	1.45	1.554	0.962	2.68
Tri11	3.364	2.484	3.256	2.428	2.128
Tri13	2.397	1.832	2.347	1.387	1.907
Tri1	1.344	1.212	1.555	1.332	1.807
Tri10	2.051	10.842	18.226	5.391	2.794
Tri3	4.113	4.587	4.919	1.246	1.988
Tri7	2.493	3.293	4.914	2.263	2.14
Tri8	0.896	1.089	1.538	0.963	1.469
Tri101	0.82	1.252	1.562	0.963	1.851
Tri15	2.283	1.26	1.079	1.207	2.128
Tri5	0.962	1.097	1.543	0.954	2.683
Tri9	2.566	1.052	1.801	0.962	2.684
Tri12	5.227	9.15	13.824	3.357	2.022
Tri14	2.712	1.595	1.85	1.023	2.139
Pyruvate kinase	3.589	5.069	5.644	1.718	2.216
Dhat			2.552	2.519	1.963
Acat	2.251	2.627	4.778	1.458	1.715
Hmgs	6.308	6.586	11.714	2.684	3.416
Mk	7.689	4.683	3.366	3.683	3.06
Pmk			9.178	1.384	3.131
Fpps	2.651	1.635	1.864	1.834	1.9
Citrate synthase	1.124	0.917	1.452	2.492	1.749
Aconitate hydratase	1.278		0.725	1.104	1.121
Isocitrate dehydrogenase	13.48	9.395	6.153	1.406	3.062
rAsp f9	1.1	1.043	1.643	0.966	1.948
Ibt4	1.597	2.127	6.133	2.804	1.51
Ibt6	1.425	1.13	1.509	1.536	1.801
Ibs1	0.817	0.616	0.276	0.963	9.805
Ibt1	1.808	3.956	4.214	2.58	1.321
ADP/ATP Carrier protein	1.642	1.067	2.006	0.96	0.855
Potassium Channel protei	2.44	7.361	9.704	0.48	2.063
RNase F1/T1	1.017	0.403	0.531	0.963	0.56
Alcohol dehydrogenase	1.786		1.114	1.449	2.667
Transcription factor	0.829	1.036	1.579	0.964	1.831
18I12	1.204	1.448	3.446	1.006	1.367
15G19	0.97	1.094	2.256	0.961	0.977
9D4			6.907	1.432	2.687
17H21	0.824	1.448	2.318	1.189	0.719
12M24		5.052	2.342	1.461	1.333
11K18		5.67	6.103	1.681	3.654
Cell wall protein	0.643	0.666	0.954	0.961	1.4
ABC Transporter	10.948	6.172		0.836	1.708
Histone H2A subunit	0.838	0.291	0.659	0.928	0.705
Nucleosome assembly	1.996	1.394	3.279	1.203	1.039
Stress protein	0.768	1.036	1.565	0.906	1.112
Elongation factor 1 alpha	0.886	0.994	2.053	1.014	0.89
Elongation factor 2	2.142	1.065	1.579	0.964	1.241
RNA pol II large subunit	1.715	0.89	3.431	0.984	1.06
Core II protein	1.372	0.628	0.931	1.574	1.204
Actin	1	1	1	1	1
Cytochrome P450	1.356	0.561	0.555	0.964	0.858
fadA	3.757	1.575	1.684	1.318	1.134

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Education

1990-B.S. Southeast Missouri State Univerisity, Major: Biology
1994-M.S. Southeast Missouri State University, Major: Biology
2003-Ph.D. Texas A&M University, Major: Plant Pathology

Professional Experience

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Spring 2001-Fall-2001-Teaching Assistant, Microbiology lab, Biology Dept., Texas A&M University.
Summer 1999-Spring 2000-Teaching Assistant, Honors Microbiology lab, Microbiology Lab, Texas A&M University.
Fall 1994-Spring 1996-Teaching Assistant, Introductory Plant Pathology Lab, Texas A&M University.
Fall 1992-Spring 1994-Teaching Assistant, Human Anatomy and Physiology I & II Laboratory, Southeast Missouri State University.

Research

Fall 1994-Spring 2002-Ph.D. research project, Characterization of the *Tri10* gene from *Fusarium sporotrichioides*.
Spring 1992-Fall 1994-M.S. research project, Construction of a subtraction library from nitrogen-limited mycelia of *Schizophyllum commune.*
Spring 1990-Fall 1990-Undergraduate research project, Dr. Walt Lilly, Department of Biology, Southeast Missouri State University.

Publications

Peplow, A. W., A. G. Tag, G. F. Garifullina, and M. N. Beremand. 2003. Identification of new genes positively regulated by *Tri10* and a regulatory network for trichothecene mycotoxin production. *Appl. Environ. Microbiol.* in press.

Tag, A.G., G. Garifullina, A. W. Peplow, C. Ake, Jr., T. D. Phillips, T. Hohn, and M.N. Beremand. 2001. A novel regulatory gene, *Tri10*, controls trichothecene toxin production and gene expression. *Appl. Environ. Microbiol.* **67**:5294-5302.

Tag, A.G., J. K., Hicks, G. Garifullina, M. N. Beremand, and N.P. Keller. 2000. G-protein signalling mediates differential production of toxic secondary metabolites. *Mol. Microbiol.* **38**:658-667.