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Identification of New Genes Positively Regulated by *Tri10* and a Regulatory Network for Trichothecene Mycotoxin Production

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Tri10*, a regulatory gene in trichothecene mycotoxin-producing *Fusarium* species, is required for trichothecene biosynthesis and the coordinated expression of four trichothecene pathway-specific genes (*Tri4*, *Tri5*, *Tri6*, and *Tri101*) and the isoprenoid biosynthetic gene for farnesyl pyrophosphate synthetase (*FPPS*). We showed that six more trichothecene genes (*Tri3*, *Tri7*, *Tri8*, *Tri9*, *Tri11*, and *Tri12*) are regulated by *Tri10*. We also constructed a cDNA library from a strain of *Fusarium sporotrichioides* that overexpresses *Tri10* (↑ *Tri10*) and used cDNA derived from the ↑ *Tri10* strain and a non-*Tri10*-expressing strain (Δ*Tri10*) to differentially screen macroarrays prepared from the cDNA library. This screen identified 15 additional *Tri10*-regulated transcripts. Four of these transcripts represent *Tri1*, *Tri13*, and *Tri14* and a gene designated *Tri15*. Three other sequences are putative orthologs of genes for isoprenoid biosynthesis, the primary metabolic pathway preceding trichothecene biosynthesis. The remaining eight sequences have been designated *Ibt* (influenced by *Tri10*) genes. Of the 26 transcripts now known to be positively regulated by *Tri10*, 22 are positively coregulated by *Tri6*, a gene that encodes a previously characterized trichothecene pathway-specific transcription factor. These 22 *Tri10*- and *Tri6*-coregulated sequences include all of the known *Tri* genes (except for *Tri10*), the *FPPS* gene, and the other three putative isoprenoid biosynthetic genes. *Tri6* also regulates a transcript that is not regulated by *Tri10*. Thus, *Tri10* and *Tri6* regulate overlapping sets of genes that include a common group of multiple genes for both primary and secondary metabolism.

The trichothecenes are a large group of toxic secondary metabolites produced by a variety of fungi, including *Fusarium*, *Stachybotrys*, and *Myrothecium* (5, 16, 28). These sesquiterpene mycotoxins are strong inhibitors of protein synthesis (29) and can cause toxicoses when humans or animals consume contaminated food or feed (19). They are capable of inducing apoptosis (30) and, in some cases, are influential in plant pathogenesis (4, 11, 24).

The biosynthetic pathway for T-2 toxin, a type A (nonmacrocyclic) trichothecene produced by some *Fusarium* species, has been well characterized. Most of the chemical intermediates in the pathway are known (10), and most of the genes known to be required for T-2 toxin biosynthesis in *Fusarium sporotrichioides* NRRL 3299 are organized in a coordinately regulated gene cluster (15); however, not all of the structural genes assumed to be responsible for these reactions have been described (1, 8, 13, 14, 20, 21, 22). Similarly, several genes for pathway regulation and partial self-protection are known (2, 17, 25, 27), but other genes related to toxin production are presumed to exist.

The expression of three representative *Tri* cluster genes (*Tri6*, *Tri5*, and *Tri4*), the expression of noncluster gene *Tri101*, and T-2 toxin production are controlled by *Tri10*, a regulatory gene in the cluster (27). Disruption of the *Tri10* coding sequence dramatically reduces the transcription of these four *Tri* genes and effectively blocks T-2 toxin production. Disruptions in the region upstream of the *Tri10* coding sequence result in T-2 toxin hyperproduction and coordinated overexpression of *Tri10* and the other four *Tri* genes.

Tri10 is hypothesized to control all other *Tri* genes, both inside and outside the *Tri* gene cluster, in part by positively regulating *Tri6* (27). *Tri6* encodes a previously characterized Cys_2His_2 zinc finger DNA-binding protein that functions as a pathway-specific transcription factor and positively regulates the other *Tri* genes (15, 25). Both *Tri10* and *Tri6* also control transcript levels for the gene encoding farnesyl pyrophosphate synthetase (*FPPS*) (27). Since FPPS catalyzes the last step in the isoprenoid biosynthetic pathway, the primary metabolic pathway leading to trichothecene biosynthesis, we further hypothesized that *Tri10* and/or *Tri6* regulate the expression of additional isoprenoid biosynthetic genes.

Our objective in this study was to identify other genes from *F. sporotrichioides* involved with or related to T-2 toxin biosynthesis, including those that might be located outside the *Tri* gene cluster. We used radiolabeled cDNAs prepared from Δ*Tri10* (*Tri10* knocked out) and ↑ *Tri10* (*Tri10* upregulated) strains to screen high-density cDNA macroarrays printed from a ↑ *Tri10* cDNA library (i.e., a library enriched for genes positively regulated by *Tri10*). We also further examined the regulatory relationships of *Tri10* and *Tri6* by determining whether the genes regulated by *Tri10* were regulated similarly by *Tri6*.

MATERIALS AND METHODS

Strains, media, and culture conditions. Wild-type strains *F. sporotrichioides* NRRL 3299, *Gibberella pulicaris* (*F. sambucinum*) R-6380, and *G. zeae* (*F. graminearum*) GZ3639 and transformed strains FsTri10-1-12 (Δ*Tri10*), FsTri10-1-20 (↑ *Tri10*), and NN4 (Δ*Tri6*) were described previously (7, 25, 27). The wild-type strains were maintained on V8 juice agar (200 ml of V8 juice, 3 g of $CaCO_3$, and 20 g of agar per liter), and the transformed strains were maintained on V8 juice

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agar plus 300 μg of hygromycin B (Calbiochem, La Jolla, Calif.)/ml. Conidia for all strains were stored as frozen (−80°C) glycerol-water (15:85) stocks. Cultures were grown for 23 h at 28°C and 200 rpm in YEPD-5G medium (5% glucose, 0.1% yeast extract, 0.1% peptone) for RNA extraction or YEPD-2G medium (2% glucose, 0.3% yeast extract, 1% peptone) for DNA extraction. Individual bacterial colonies from the cDNA library were maintained on Luria-Bertani agar plates at 4°C and stored at −80°C in 384-well plates (Nalge Nunc International, Naperville, Ill.) containing multiwell plate freezer medium, which is 9 volumes of Luria-Bertani medium combined with 1 volume of 10× freezer medium (containing, per liter, 63 g of K_2HPO_4, 18 g of KH_2PO_4, 5 g of sodium citrate, 1 g of $MgSO_4 \cdot 7H_2O$, 9 g of $(NH_4)_2SO_4$, and 440 ml of glycerol). All bacterial culture media were supplemented with ampicillin at 100 μg/ml.

Nucleic acid treatments. Procedures for the isolation of genomic DNA and mRNA were described previously (12, 27). Plasmid DNA was isolated by using a QIAprep spin miniprep kit (Qiagen, Valencia, Calif.). All RNA samples for cDNA library construction, library screening, and Northern analyses were isolated from cultures grown for 23 h. Both wild-type *F. sporotrichioides* strain NRRL 3299 and the ↑ *Tri10* strain show maximum expression of trichothecene genes at about 23 h in liquid cultures, although expression is elevated and persists for an extended period of time in the ↑ *Tri10* strain (27).

PCR was performed either with Platinum *Pfx* DNA polymerase (GIBCO BRL, Rockville, Md.) or with *Taq* DNA polymerase (Promega, Madison, Wis.) according to the manufacturers' instructions. Probes for trichothecene genes were made from three different *Fusarium* species.

Primer names and sequences for each gene were as follows: *Tri3*, A-41 (5′-GATGAGCGCTTCATCCTCCTC-3′) and A-42 (5′-CCTAAAGTCGGAAGGCAAGC-3′); *Tri4*, A-37 (5′-GGAAAGATGGTTGATCAAGACTGG-3′) and A-38 (5′-GCCACTGAAGCTTACAAAGC-3′); *Tri5*, 60 (5′-CGGATCCATGGAGAACTTTCCCAC-3′) and 59 (5′-CCCATGGTGGATAAGCCCACTC-3′); *Tri6*, 292 (5′-GCTCTAGATGATTTACATGGCGTCCG-3′) and 293 (5′-GCCTCGAGTCAACACTTGTGTATCCG-3′); *Tri7*, A-54 (5′-ATGGATATCGCATCGAAAGTGGAAGG-3′) and A-55 (5′-ACTATTGTGGTACAAATACCAGGGCG-3′); *Tri8*, A-56 (5′-ATGGCTCTTAATCGTTTGGTGTTTTC-3′) and A-57 (5′-TTACCAGGCTGCCGACCAG-3′); *Tri9*, A-49 (5′-GAATTCAGGCCTTCTACTGACG-3′) and A-50 (5′-CGATTGTAAGCCGCGTCTTATC-3′); *Tri10*, 320 (5′-CCACCCAGCAATCATCAG-3′) and 356 (5′-GTACCTCGTTTCATGCC-3′); and *Tri11*, A-43 (5′-GGGCTTGCATATCTTGTGGTAG-3′) and A-44 (5′-AGTTCCTTTAGATTTCAGCCC-3′)—amplified from genomic DNA from *F. sporotrichioides* NRRL 3299; *Tri12*, 46MB (5′-GGGTTGCCATAAACCTCG-3′) and 47MB (5′-CAGGCCTTCTCCCTCAAC-3′)—amplified from genomic DNA from *G. pulicaris* R-6380; and *Tri101*, B-3 (5′-ATGGCTTTCAAGATACAGCTCG-3′) and B-4 (5′-CTAACCAACGTACTGCGCATAC-3′)—amplified from genomic DNA from *G. zeae* GZ3639.

Inserts of selected clones from the cDNA library in the pBluescript SK phagemid were excised by using the restriction enzyme pair *Apa*I and *Sma*I or *Sac*I and *Kpn*I (New England Biolabs, Beverly, Mass.). Restriction fragments and PCR products were run on 1.2% agarose and 1% agarose (Boehringer Mannheim, Indianapolis, Ind.) gels, respectively, and purified with a QIAquick gel extraction kit (Qiagen). Purified PCR products were cloned into pCR-Script (Stratagene, La Jolla, Calif.). For RNA electrophoresis, 1% agarose gels with 1.1% formaldehyde were used, and 5 μg of total RNA was run for each sample (26). Individual radioactively labeled DNA probes were prepared by using [α-^{32}P]dCTP (Amersham, Piscataway, N.J.) and a nick translation system (GIBCO BRL). All Northern and Southern blots, as well as bacterial colony filters, were made on Hybond-N+ nylon membranes (Amersham) and hybridized, washed, and stripped under high-stringency conditions as specified in the Gene Images nonisotopic nucleic acid detection kit (United States Biochemical Corp., Cleveland, Ohio). Autoradiographic images were produced on BIOMAX MR or X-OMAT LS imaging film (Kodak, Rochester, N.Y.).

cDNA library and macroarray construction. A cDNA library enriched for genes positively regulated by *Tri10* was constructed in Uni-ZAP XR (Stratagene) with mRNA isolated from the ↑ *Tri10* strain. The library was excised, and 7,680 pBluescript SK phagemid colonies were picked and grown individually in 1 of 20 384-well plates containing freezer medium. High-density cDNA macroarrays were prepared by transferring samples from these plates to nylon membrane filters with a Biomek 2000 workstation (Beckman Coulter, Fullerton, Calif.) such that each clone was represented in duplicate and DNAs from colonies on four plates were combined on a single filter. Thus, each filter contained 1,536 clones in duplicate. Low-density macroarrays of a small sample of the cDNA library also were prepared. Samples from 3 of the 20 plates were transferred to individual filters such that each filter held 384 clones. Candidate genes were identified with both types of arrays, but the majority of the candidate genes came from the high-density arrays.

cDNA library screen. The arrayed portion of the cDNA library was subjected to several parallel screenings. One set of cDNA macroarrays was differentially screened by first hybridizing the filters with radiolabeled cDNA derived from the non-T-2 toxin-producing Δ*Tri10* strain and then, after stripping them, hybridizing them with radiolabeled cDNA derived from the T-2 toxin-hyperproducing ↑ *Tri10* strain. Each cDNA sample was prepared from poly(A)$^+$ mRNA isolated from total RNA samples by using an Oligotex mRNA mini kit (Qiagen). Radiolabeled first-strand cDNA was synthesized from poly(A)$^+$ mRNA by using an adaptation of the Superscript preamplification system (GIBCO BRL). Specifically, the RNA mixture contained 1.5 μg of poly(A)$^+$ mRNA and oligo(dT) as the primer, and [α-^{32}P]dCTP was used in place of dCTP in the deoxynucleoside triphosphate mixture. After incubation with RNase H, the labeled probe was denatured in a boiling water bath for 5 min, chilled on ice for 2 min, and hybridized to the macroarrays as described above. Additional hybridizations with gene inserts from selected cDNA clones and, if necessary, with additional sets of cDNA macroarrays were conducted as needed to identify the entire group of cDNA clones that represented each of the newly identified *Tri10*-regulated genes. In a confirmatory screen, these gene inserts also were used as probes to hybridize Northern blots containing RNAs from strain NRRL 3299 and the Δ*Tri10* and ↑ *Tri10* strains. Northern analyses for the resulting group of genes were repeated at least once, and genes showing any inconsistencies were excluded. Ultimately, only clones representing new sequences clearly regulated by *Tri10* were evaluated further.

Additional identical sets of cDNA macroarrays were probed with radiolabeled gene-specific PCR products prepared from available *Fusarium* trichothecene gene sequences (*Tri3*, *Tri4*, *Tri5*, *Tri6*, *Tri7*, *Tri8*, *Tri9*, *Tri10*, *Tri11*, *Tri12*, and *Tri101*).

A final set of *Tri* genes and *Tri10*-regulated sequences was used as a probe to hybridize Northern blots containing RNAs from strain NRRL 3299 and the Δ*Tri10*, ↑ *Tri10*, and Δ*Tri6* strains. All of these Northern analyses were repeated at least once.

DNA sequencing and sequence homology searches. Inserts isolated from plasmid DNA preparations of cDNA or genomic DNA clones were sequenced by using either an ABI Prism Dye Terminator cycle sequencing core kit or a BigDye Terminator cycle sequencing core kit (Perkin-Elmer, Boston, Mass.). All reactions were run on a model 373 or 377 DNA sequencer (Applied Biosystems, Foster City, Calif.) at the Gene Technologies Laboratory at Texas A&M University. Nucleotide sequences and their putative corresponding amino acid sequences were compared to the National Center for Biotechnology Information nr database (GenBank+EMBL+DDBJ+PDB sequences) by using the blastn and blastx algorithms (3).

RESULTS

Identification of cDNA clones for the first 11 cloned *Tri* genes. cDNA clones for *Tri101* and 10 *Tri* cluster genes (*Tri3*, *Tri4*, *Tri5*, *Tri6*, *Tri7*, *Tri8*, *Tri9*, *Tri10*, *Tri11*, and *Tri12*) were identified by hybridization of the macroarrays with radiolabeled gene-specific PCR products. The arrayed portion of the cDNA library contains clones for all of these genes except *Tri6* (Table 1). In general, there is a positive correlation between the percentage of clones for each of the *Tri* genes on the macroarrays and the corresponding transcript signals seen in Northern hybridizations (see Discussion for exceptions). Collectively, these 11 *Tri* genes represent 4 to 5% of the clones in the cDNA library.

Identification of *Tri10*-regulated genes by the differential screen. The differential screen of the high-density macroarrays identified 228 clones for genes that appeared to be overexpressed in the toxin-hyperproducing strain relative to the non-toxin-producing strain. Of these 228 clones, 131 represented 5 of the above 11 *Tri* genes: *Tri4* (49 copies), *Tri5* (47 copies), *Tri101* (28 copies), *Tri8* (6 copies), and *Tri3* (1 copy). These five genes are among the most highly represented trichothecene genes in the cDNA library in general (Tables 1 and 2). Clones for the other six *Tri* genes, most of which are expressed at lower levels, were not identified by the differential screen.

TABLE 1. *Tri* gene cDNA clones identified by hybridization to *F. sporotrichioides* gene-specific probes

Gene	% of cDNA library[a]	Reference
Tri4	1.61	14
Tri5	1.07	13
Tri101	0.79	21
Tri8	0.53	20
Tri3	0.23	22
Tri11	0.13	1
Tri12	0.08	2
Tri7	0.07	8
Tri9	0.07	15
Tri10	0.01	27
Tri6	0.00	25
Total	4.59	

[a] Based on an analysis of 7,680 clones.

The remaining 97 clones, plus a few additional clones from the differentially screened low-density arrays, were characterized further to identify all of the clones for each sequence to permit the selection of one representative clone for each validated *Tri10*-regulated sequence. This process resulted in the identification of 15 additional sequences under the positive control of *Tri10* (Table 2 and Fig. 1). These 15 sequences include three putative orthologs of isoprenoid biosynthetic genes, designated acetyl-coenzyme A (CoA) acetyltransferase (*ACAT*), hydroxymethylglutaryl-CoA synthase (*HMGS*), and mevalonate kinase (*MK*) genes. Two sequences represent *Tri13* and *Tri14*, which reside in the *Tri* cluster (9, 18); the former encodes a cytochrome P450 monooxygenase that catalyzes C-4 hydroxylation (9, 18), and the function of the latter is unknown (9). Another sequence represents *Tri1*, a previously identified but uncloned *Tri* gene (23). TRI1 and TRI13 have been assigned to the families CYP68C1 and CYP526A1, respectively, by the Committee for Standardized Cytochrome P450 Nomenclature (David Nelson, personal communication). We have designated another *Tri10*-regulated sequence *Tri15*. The remaining eight new *Tri10*-regulated sequences (Table 2) have been designated *Ibt* (influenced by *Tri10*).

***Tri10* regulation of known *Tri* genes.** Northern blot analysis confirmed that, like those for the other identified *Tri* genes, the transcript levels for *Tri3*, *Tri7*, *Tri8*, *Tri9*, *Tri11*, and *Tri12* were increased in the ↑*Tri10* strain and decreased in the Δ*Tri10* strain (Fig. 1). However, unlike the transcripts for the 4 previously examined *Tri* genes (*Tri4*, *Tri5*, *Tri6*, and *Tri101*), there were no detectable transcripts in the Δ*Tri10* strain for 9 (*Tri1*, *Tri3*, *Tri7*, *Tri8*, *Tri9*, *Tri11*, *Tri12*, *Tri13*, and *Tri14*) of the 10 other *Tri* genes. Only *Tri15* displayed a low transcript level in the Δ*Tri10* strain (Fig. 1).

***Tri6* regulation of *Tri10*-regulated sequences.** We compared the expression of the 15 *Tri10*-regulated sequences and the remaining *Tri* genes to the expression of *Tri4*, *Tri5*, and *Tri101* in the Δ*Tri6* strain. The deletion of *Tri6* markedly reduced the transcript levels for 21 of the 24 *Tri10*-regulated sequences (Fig. 1). There were three broad categories of expression within this group. Six genes (*Tri1*, *Tri3*, *Tri7*, *Tri9*, *Tri13*, and *Tri14*) were under the tight control of both *Tri10* and *Tri6*; that is, the lack of either *Tri10* or *Tri6* effectively blocked detectable transcript accumulation for these genes. Three genes (*Tri8*, *Tri11*, and *Tri12*) were under the tight control of *Tri10* but not *Tri6*. Ten genes (*Tri4*, *Tri5*, *Tri101*, *Tri15*, *Ibt1*, *Ibt4*, *Ibt5*, *ACAT*, *HMGS*, and *MK*) were not under the tight control of either *Tri10* or *Tri6*. Although *Ibt2* and *Ibt3* belonged in one of the two latter categories, we could not assign them to either category with confidence due to their low levels of expression.

Four genes had a pronounced differential pattern of gene expression with regard to *Tri10* and *Tri6* (Fig. 1B). Transcript levels for three sequences (*Ibt6*, *Ibt7*, and *Ibt8*) whose normal levels of expression at 23 h were equal to or lower than those in the ↑*Tri10* strain were reduced in the Δ*Tri10* strain but not in the Δ*Tri6* strain. The level of expression of *Ibt8* actually increased when *Tri6* was knocked out. The size of the *Ibt8* transcript also was increased in the Δ*Tri6* strain. The fourth transcript showed little or no dependence on *Tri10* but was

TABLE 2. cDNA clones isolated as positively regulated by *Tri10* and/or *Tri6*

Gene[a]	% of cDNA library	GenBank accession no.	DNA sequence length (kb)	Similar sequence(s) found by blastx or blastn search
Tri1	0.31	AY040587	1.86	Cytochrome P450 monooxygenase
Tri13	0.29	AF330109	1.88	Cytochrome P450 monooxygenase
Tri14	0.48	AF326571	1.18	No significant match
Tri15	0.07	AF327521	0.96	Putative zinc finger protein
ACAT	0.25	BQ789674, BQ789675	0.72	Acetyl-CoA acetyltransferase
HMGS	0.39	BQ789676, BQ789677	0.82	Hydroxymethylglutaryl-CoA synthase
MK	0.01	BQ789678, BQ789679	0.80	Mevalonate kinase
Ibt1	0.04	BQ789666, BQ789667	0.74	Aspartyl protease
Ibt2	0.01	BQ789668	0.28	No significant match
Ibt3	0.14	BQ789669, BQ789670	0.80	Fumarate reductase
Ibt4	0.01	BQ789671, BQ789672	0.72	Cellulase, endoglucanase
Ibt5	NA[b]	BQ789673	0.34	Cytochrome b_5
Ibt6	0.14	BQ789680, BQ789681	0.89	Lysophospholipase, phospholipase B
Ibt7	0.05	BQ789682	0.23	No significant match
Ibt8	0.05	BQ789683, BQ789684	0.76	No significant match
Ibs1	0.33	BQ789685, BQ789686	0.87	NADH-dependent flavin oxidoreductase

[a] *Ibt* genes are regulated by *Tri10*; *Ibs* gene is regulated by *Tri6* but not by *Tri10.*
[b] NA, not available.

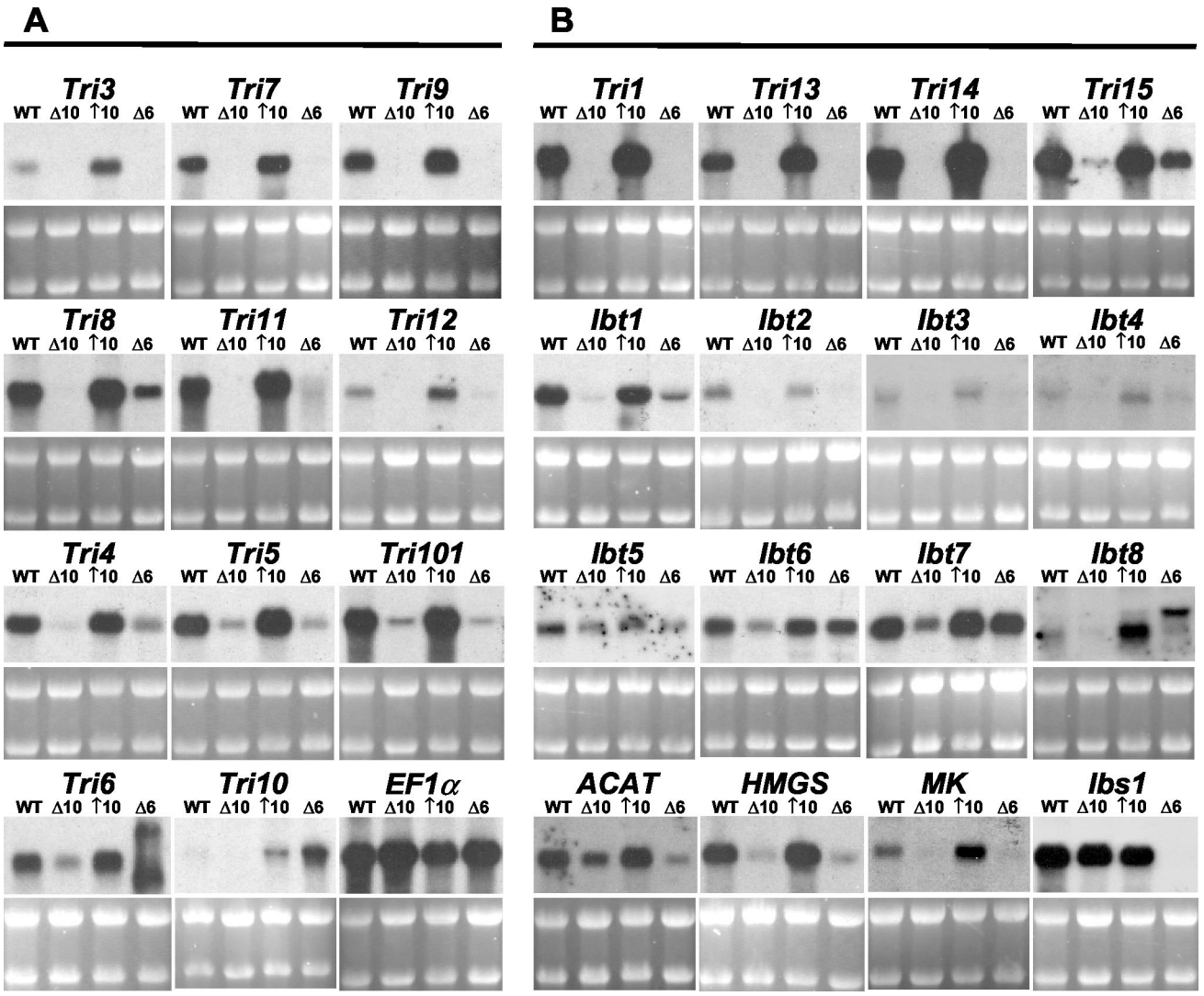

FIG. 1. Northern analyses of *Tri10*- and/or *Tri6*-regulated genes. (A) Genes listed in Table 1. (B) Genes listed in Table 2. Wild-type (WT), Δ*Tri10* (Δ10), ↑ *Tri10* (↑ 10), and Δ*Tri6* (Δ6) strains of *F. sporotrichioides* were grown in liquid cultures for 23 h. Five micrograms of total RNA was loaded in each lane. Panels below the Northern analyses depict the corresponding ethidium bromide staining of rRNA in the gels. The transcriptional profile of translation elongation factor 1α (EF1α) is shown as a constitutive control.

strongly dependent on *Tri6* and was designated *Ibs1* (influenced by *Tri6*).

DISCUSSION

The results from this study support the hypotheses that *Tri10* and *Tri6* regulate the expression of (i) all of the *Tri* genes, both within and outside the core *Tri* gene cluster, and (ii) additional genes for isoprenoid biosynthesis. Tag et al. previously showed that two *Tri* cluster genes (*Tri4* and *Tri5*), one noncluster gene (*Tri101*), and one isoprenoid biosynthetic gene (*FPPS*) depend on *Tri10* and *Tri6* for full expression and are overexpressed in a ↑ *Tri10* strain (27). Here we establish that the other known *Tri* genes, including *Tri1* and the eight remaining *Tri* cluster genes (*Tri3*, *Tri7*, *Tri8*, *Tri9*, *Tri11*, *Tri12*, *Tri13*, and *Tri14*), and three more putative isoprenoid biosynthetic genes (*ACAT*, *HMGS*, and *MK*) also depend on *Tri10* and *Tri6* for full expression and are overexpressed in the ↑ *Tri10* strain.

Five additional genes are similarly regulated. One, *Ibt2*, has no significant similarities to known sequences. Four encode several different putative proteins, including aspartyl protease (*Ibt1*), reductase (*Ibt3*), cellulase (*Ibt4*), and cytochrome b_5 (*Ibt5*). The cellular functions of these genes are currently unknown, although they could have a role in trichothecene production. However, it is unlikely that all of these genes are directly involved in either trichothecene or isoprenoid biosynthesis, suggesting that the regulatory control exerted by *Tri10* and *Tri6* extends beyond genes for isoprenoid and trichothecene biosynthesis.

Although *Tri10* and *Tri6* coregulate a fairly large set of transcripts (at least 22), they also regulate some genes (at least four) independently of each other. This regulatory pattern was

not unexpected, since the Δ*Tri6* and Δ*Tri10* strains have some distinct phenotypic differences (27). One gene, *Ibs1*, is markedly dependent on *Tri6* and has no apparent dependence on *Tri10*. Three transcripts (*Ibt6*, *Ibt7*, and *Ibt8*) are downregulated in the Δ*Tri10* strain but not in the Δ*Tri6* strain and appear to be exceptions to the general hypothesis that *Tri10*-regulated genes are regulated by *Tri6*. However, *Ibt8* is still influenced by *Tri6*. *Ibt8*, like *Tri10* (27), is overexpressed with the loss of *Tri6*. Moreover, the loss of *Tri6* also increases the size of the *Ibt8* transcript; this result could be due to the use of a different transcriptional start site or different posttranscriptional processing activities. Thus, *Tri10* and *Tri6* control slightly different sets of genes and, in some instances, may regulate the same gene in different ways.

Meanwhile, *Tri6* regulation of most of the *Tri10*-regulated genes is consistent with the previously reported regulation of some *Tri* genes (*Tri5*, *Tri4*, *Tri3*, and *Tri101*) by *Tri6* (22, 25, 27) and with the observation that all of the previously known *Tri* genes (except for *Tri10*) appear to have TRI6-binding sites in their promoters (15).

One of our main objectives was to identify additional *Tri* genes both inside and outside the *Tri* gene cluster by obtaining *Tri10*-regulated genes. Three of the *Tri10*-regulated sequences that we identified by using the differential screen represent recognized *Tri* genes (*Tri1*, *Tri13*, and *Tri14*). *Tri1* had been identified on the basis of a UV-induced mutation, but no DNA sequence had previously been available for this gene (6, 23). Both Brown et al. (9) and Lee et al. (18) cloned *Tri13* and *Tri14*. Brown et al. (9) cloned these genes from *F. sporotrichioides* NRRL 3299 by sequencing a cosmid region downstream of *Tri12* in the *Tri* gene cluster. Lee et al. (18) cloned *Tri13* and *Tri14* orthologs from *G. zeae* 88-1 (GenBank accession number AF336365) by PCR with our *Tri13* and *Tri14* gene sequences and genomic positions from *F. sporotrichioides*. We named a new *Tri10*-regulated sequence *Tri15* based on preliminary evidence indicating that disruption of this gene alters *Tri* gene expression and toxin production (A. W. Peplow and M. N. Beremand, unpublished data). *Tri15* does not have high sequence similarity with known genes, but it does have two putative Cys_2His_2 zinc finger DNA-binding domains, consistent with a regulatory function. By using Southern analysis, we detected *Tri15*, *Tri14*, and *Tri13* orthologs in *G. pulicaris* R-6380 and *G. zeae* GZ3639 and a *Tri1* ortholog in R-6380 but not GZ3639.

The transcription profiles for the *Tri* genes and the isoprenoid genes are correlated with the order of the 15 steps that define the T-2 toxin biosynthetic pathway in *F. sporotrichioides* NRRL 3299 (10). Genes that function before step 9 (*Tri6*, *Tri5*, *Tri4*, *Tri101*, and the isoprenoid pathway genes *ACAT*, *HMGS*, *MK*, and *FPPS*) are expressed at low levels in both Δ*Tri10* and Δ*Tri6* strains and thus are not under the tight control of either *Tri10* or *Tri6*. The low levels of expression of these early genes may be related to their use in shared pathways. By Southern analysis, it appears that the isoprenoid pathway genes *ACAT*, *HMGS*, *MK*, and *FPPS* are present in single copies in the genome, and it seems unlikely that trichothecene biosynthesis recruits specific gene paralogs of isoprenoid synthesis that are distinct from those normally used by the cell. Early trichothecene biosynthetic intermediates also are precursors for other metabolites, including apotrichothecenes (14). Genes that most likely function in conjunction with step 9 (*Tri11*, *Tri12*, and *Tri8*) appear to be under the tight control of *Tri10* but not *Tri6*, while genes that function after step 9 (*Tri3*, *Tri13*, *Tri7*, and *Tri1*) are tightly regulated by both *Tri10* and *Tri6*. These results are consistent with prior observations that neither the Δ*Tri10* strain nor the Δ*Tri6* strain makes any T-2 toxin, even though both strains express low levels of *Tri5* (25, 27). They also provide experimental evidence supporting the hypothesis that the lack of T-2 toxin production in these strains is due to a lack of transcripts for later pathway-specific genes. Moreover, the loose versus tight division in *Tri* gene expression mediated by *Tri10* and/or *Tri6* may define a previously unrecognized important regulatory control point for trichothecene biosynthesis.

The coregulation of *FPPS* by *Tri10* and *Tri6* demonstrates a regulatory link between the isoprenoid and trichothecene biosynthetic pathways (27). The coordinated expression of four isoprenoid biosynthetic pathway genes by both *Tri10* and *Tri6* identifies a regulatory network that links these primary and secondary metabolic pathways. While the regulation of the *Tri* genes by *Tri10* is mediated by *Tri6*, neither the extent nor the means by which *Tri10* and *Tri6* control the expression of the isoprenoid genes is known. It will be interesting to determine whether TRI6-binding sites occur in the promoters of the isoprenoid genes and whether *Tri10* and *Tri6* regulate the expression of additional isoprenoid genes.

It is unlikely that we have identified all of the genes positively controlled by *Tri10*. While the differential screening of the cDNA macroarrays was fruitful, we did not detect some of the known *Tri* genes that are expressed at lower levels (e.g., *Tri11*, *Tri12*, *Tri7*, and *Tri10*) or that have short sequences (e.g., *Tri6* and *Tri9*). Therefore, if other *Tri10*-regulated genes with a low transcript abundance or with a small transcript size exist, they also might have been missed. Thus, the number of genes regulated by *Tri10* identified in this study is probably an underestimate, and it is likely that other *Tri10*-regulated genes, including additional isoprenoid and trichothecene genes, remain to be identified.

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