**Fusarium Tri4 encodes a multifunctional oxygenase required for trichothecene biosynthesis**


**Abstract:** *Fusarium graminearum* and *Fusarium sporotrichioides* produce the trichothecene mycotoxins 15-acetyldeoxynivalenol and T-2 toxin, respectively. In both species, disruption of the P450 monoxygenase-encoding gene, *Tri4*, blocks production of the mycotoxins and leads to the accumulation of the trichothecene precursor trichodiene. To further characterize its function, the *F. graminearum Tri4* (*FgTri4*) was heterologously expressed in the trichothecene-nonproducing species *Fusarium verticillioides*. Transgenic *F. verticillioides* carrying the *FgTri4* converted exogenous trichodiene to the trichothecene biosynthetic intermediates isotrichodermin and trichothecene. Conversion of trichodiene to isotrichodermin requires seven biochemical steps. The fifth and sixth steps can occur nonenzymatically. Precursor feeding studies done in the current study indicate that wild-type *F. verticillioides* has the enzymatic activity necessary to carry out the seventh step, the C-3 acetylation of isotrichodermin to form isotrichodermin. Together, the results of this study indicate that the *Tri4* protein catalyzes the remaining four steps and is therefore a multifunctional monoxygenase required for trichothecene biosynthesis.

**Key words:** trichothecene, P450 oxygenase, trichodiene, *Tri101, Tri4*, multifunctional oxygenase, monoxygenase.

**Résumé:** *Fusarium graminearum* et *Fusarium sporotrichioides* produisent respectivement les mycotoxines trichothécéniques 15-acétyldeoxynivalenol et la T-2 toxine. Chez les deux espèces, la destruction du gène codant la P450 monoxygénase *Tri4* bloque la production de mycotoxines et mène à l'accumulation du trichodiène, le précurseur des trichothécènes. Afin de caractériser davantage sa fonction, le *Tri4* de *F. graminearum* (*FgTri4*) fut exprimé de manière hétérologue dans l'espèce *Fusarium verticillioides* ne produisant pas de trichothécènes. Le *F. verticillioides* transgénique renfermant le *FgTri4* a converti la trichodiène exogène dans l'intermédiaire biosynthétique des trichothécènes, l'isotrichodermin et le trichothécène. La conversion du trichodiène en isotrichodermin a nécessité sept étapes biochimiques. Les cinquièmes et sixièmes étapes peuvent se produire sans enzyme. Les études d'alimentation avec des précurseurs réalisés dans l'étude présente ont indiqué que *F. verticillioides* possède l'activité enzymatique nécessaire pour accomplir la septième étape, l'acétylation C-3 de l'isotrichodermin pour former l'isotrichodermin. L'ensemble des résultats de cette étude indiquent que le protéine *Tri4* catalyse les quatre étapes restantes et est donc une monoxygénase multifonctionnelle nécessaire à la biosynthèse des trichothécènes.

**Mots clés:** trichothécènes, oxygénase de la P450, trichodiène, *Tri101, Tri4*, oxygénase multifonctionnelle, monoxygénase.

[Traduit par la Rédaction]

**Introduction**

Trichothecenes are a group of sesquiterpene epoxides produced by several species of *Fusarium, Myrothecium,* and *Trichothecium* (Desjardins et al. 1993). The biosynthesis of *Fusarium* trichothecenes involves a complex pathway that begins with cyclization of farnesyl pyrophosphate to form the sesquiterpene hydrocarbon trichodiene, which subsequently undergoes multiple oxygenations, cyclizations, and esterifications (Desjardins et al. 1993). Pulse labeling and feeding experiments with *Fusarium culmorum* (Zamir et al. 1999; Hesketh et al. 1991) and *Fusarium sporotrichioides* (McCormick et al. 1990) have established the initial sequence of steps in *Fusarium* trichothecene biosynthesis (Fig. 1).

The genes required for trichothecene biosynthesis and regulation in *F. sporotrichioides* and *F. graminearum* are located at five loci (Jurgenson et al. 2002; Meek et al. 2003; Brown et al. 2001; Kimura et al. 1998a, 2003a; Alexander et al. 2004). The first locus consists of a 26 kb gene cluster that includes three cytochrome P450 genes, *Tri4, Tri11,* and *Tri13.* *Tri11* and *Tri13* catalyze the hydroxylation of C-15 and C-4, respectively, (Brown et al. 2002; Alexander et al. 1998; McCormick and Hohn 1997), and *Tri4* is at least required for the initial oxygenation of trichodiene. In *F. sporotrichioides Tri4* mutants, generated by gene disruption (Hohn et al. 1995) or UV mutagenesis (Beremand 1987), trichodiene accumulates rather than oxygenated trichothecenes like T-2 toxin.

One of the four other loci identified in *F. sporotrichioides* contains a fourth P450-encoding gene, *Tri1* (Meek et al. 2003), responsible for C-8 hydroxylation in T-2 toxin biosynthesis. The likely homolog of *Tri1* was identified in *F. graminearum* (*FgTri1*) (McCormick et al. 2004). Preliminary
Fig. 1. Pathway for early trichothecene metabolites produced by Fusarium.

data suggest that FgTri1 may control C-7 hydroxylation in addition to C-8 hydroxylation in deoxynivalenol (DON) biosynthesis. Tri4 has not been completely characterized in either F. graminearum or F. sporotrichioides, and since there are three early oxygenation reactions for which genes have not been assigned, it is possible that the Tri4 product catalyzes more than one step.

Some trichothecene biosynthetic genes have been successfully characterized by heterologous expression in the yeast Saccharomyces cerevisiae (McCormick et al. 1999; Alexander et al. 1999; McCormick and Alexander 2002). However, this approach has not been successful with Fusarium P450 genes involved in trichothecene biosynthesis. To more fully characterize Tri4, F. graminearum Tri4 (FgTri4) was expressed in F. verticillioides, a species that does not produce trichothecenes. This approach was taken because F. graminearum is more closely related to F. verticillioides than to S. cerevisiae.

Materials and methods

Strains
The F. graminearum wild-type strain, Z-3639, was isolated from scabby wheat by R. Bowden (Kansas State University) (Bowden and Leslie 1992). Fusarium verticillioides M-3125 is a fumonisins-producing strain (Leslie et al. 1992). Strains GMT301, GMT303, GMT308, GMT311, GMT318, and GMT320 are F. verticillioides transformants that contain the F. graminearum Tri4 gene (FvFgTri4), as described below.

Tri4 expression in F. verticillioides
The Tri4 expression plasmid was constructed by inserting the FgTri4 coding region and 434 bp of 3' flanking sequence behind the FUM8 promoter (Fig. 2), which consisted of the 780 bp immediately upstream of the F. verticillioides FUM8 translation site (Seo et al. 2001). The FUM8 promoter fragment was amplified with primers 484 (GGTTAGATGATTGATTCCAGGGTACTG) and 485 (GATGGGCACTAGTAAGGGCTGACACAAGGT) from genomic DNA of F. verticillioides M-3125 (Fig. 2). The amplification product was cloned into pT7Blue (EMD Biosciences, San Diego, California, USA) to yield plasmid pFvFUM8P. The FgTri4 fragment was amplified using Pfu Turbo enzyme (Stratagene, La Jolla, California) following the manufacturer’s protocol in an MJ Research (Watertown, Massachusetts) PTC-100 thermocycler with primers 1428 (5'-CATACTAGTGCCCATACCATGATTGACAAGATTGATCAAGAG-3') and 1438 (5'-CATGGCGCGCCAATCGCGATGTTCAACTGTGAG-3') from genomic DNA of F. graminearum Z-3639 (Fig. 2). The result-
ing 2175 bp fragment was band purified (UltraClean, MoBio, Solano Beach, California) and cloned into pCR-Blunt II TOPO (Invitrogen, Carlsbad, California). The PCR product was sequenced (ABI Prism BigDye Terminator Cycle Sequencing Kit using a PE Biosystems 3700 automated DNA sequencer (PE Biosystems, Foster City, California)) to ensure the integrity of the sequence. The amplified FgTri4 sequence was inserted behind the FUM8 promoter in pFvFUM8P via SpeI and AscI sites that had been introduced at the 5' and 3' ends, respectively, of the FgTri4 fragment via the PCR primers (see underlined regions of primer sequences). The junction of the FUM8 promoter and the FgTri4 gene coding region was sequenced to ensure integrity. The plasmid was then cut with AscI, and an AscI fragment containing the hygromycin resistance gene HygB was inserted at the 3' terminus of FgTri4. Transformation of F. verticillioides was done as described in Proctor et al. (1999), with selection on hygromycin (300 μg/mL, Sigma Chemical Co., St. Louis, Missouri).

Transformants were analyzed by PCR with a primer in the FUM8 promoter sequence, 484 (sequence shown above), and a primer in the FgTri4 sequence, 1432 (5’-GGTAGGGTTC CAAATGCGCCG-G3’) (Fig. 2). Southern analysis was performed on the genomic DNA, prepared as previously described (Proctor et al. 1999), digested with HindIII and probed with a 550 bp PCR fragment prepared from primers 1433 (5’-AGGGCGAGGTACATCTCAGC-3’) and 1434 (5’-GCTGGT ACTGAGACTACTTC-3’) (Fig. 2) and labeled with β-32P.

RNA was isolated from liquid cultures of M-3125 (wild type) and a FvFgTri4 transformant, GMT301, using TRIZOL following the manufacturer’s protocol (Invitrogen). The RNA was treated with DNaseI (Qiagen, Madison, Wisconsin) and then subjected to reverse transcription PCR (RT-PCR) (Qiagen One-Step Kit), following the manufacturer’s recommendations. Primers 1434 and 1523 (Fig. 2) were used to amplify the cDNA. Genomic DNA was also amplified using these same primers.

Northern analysis was performed by running 10 μg of RNA from the wild type and the transformants on a formaldehyde gel. RNA was transferred to Nytran SuperCharge membrane (Schleicher and Schuell, Keene, New Hampshire) by capillary action in 20x SSC (0.15 mol/L NaCl, 15 mmol/L sodium citrate, pH 7.0). A FgTri4 probe made using primers 1434 and 1523 (Fig. 2) and genomic DNA from Z-3639 was labeled with β-32PdCTP and hybridized (ULTRAhyb, Ambion, Austin, Texas) to the membrane overnight at 42 °C.

### Media and culture conditions

*Fusarium verticillioides* M-3125 and transformants were grown on V-8 juice agar slants or plates (Stevens 1974). For feeding experiments, liquid cultures of *F. verticillioides* were initiated with a plug cut from a V-8 slant inoculated into 20 mL of glucose–yeast extract–asparagine–malic acid medium (GYAM) (Proctor et al. 1999) in 50 mL Erlenmeyer flasks and then grown at 28 °C in the dark at 200 r/min.

### Chemical analyses

Gas chromatography (GC) measurements were made by flame ionization detection with a Hewlett-Packard 5890 Gas Chromatograph fitted with a 30 m fused silica capillary column (DB1; 0.25 μm; J&W Scientific Co., Palo Alto, California). For routine screening of the trichothecene toxin phenotype, the column was held at 120 °C at injection, and then heated to 210 °C at 15 °C/min and held for 1 min, and then heated to 260 °C at 5 °C/min and held for 8 min. Low resolution mass spectra were obtained by GC/MS (Hewlett-Packard 5890 GC and Hewlett Packard 5891 mass selective detector) fitted with a DB-5-MS column (15 m by 0.25 mm film thickness), using the same temperature program as above.

### Whole-cell feeding

GYAM cultures (20 mL in 50 mL Erlenmeyer flasks) of *F. verticillioides* M-3125 and FvFgTri4 transformant strains were initiated with a plug cut from V-8 slants. After 68–70 h, an acetone solution of trichodiene or a trichothecene was added to give a final concentration of 368 μmol/L. The final concentration of acetone in the cultures was less than 1%. Cultures were incubated for an additional 7 days. Aliquots were removed, extracted with ethyl acetate, analyzed by GC and GC/MS at time points up to 7 days, and examined for the appearance of oxygenated and acetylated products. To isolate sufficient quantities of trichothecenes for spectral analysis, we grew larger scale cultures (100 mL in 250 mL Erlenmeyer flasks) and amended them with 10 mg of trichodiene dissolved in 400 μL of acetone.

### Trichothecenes

Isotrichodermol was isolated from cultures of *F. sporotrichioides* Tri101-3D (McCormick et al. 1999) and trichodiene from *F. sporotrichioides* F15 (Hohn et al. 1995). Isotrichodiol was isolated from trichodiene-fed cultures according to Hesketh et al. (1991). The isotrichodermin standard
Fig. 3. Gas chromatograms of ethyl acetate extracts of trichodiene-fed cultures of transformant strain *Fusarium verticillioides* GMT301 over a 7 day period. The y axes indicate relative abundance. Retention times are trichodiene, 6 min, and isotrichodermin, 11.8 min.

was isolated from liquid cultures of *F. sporotrichioides* Allb, a *Tri11* transformant strain (McCormick and Hohn 1997). The trichothecene standard was isolated from liquid cultures of *F. sporotrichioides* 4-4-18, a *Tri4* transformant strain expressing *Myrothecium roridum* *Tri4* (Trapp et al. 1998).

**Results**

**Tri4 expression in *F. verticillioides***

The *Tri4* expression vector consisted of the *FUM8* promoter fused immediately upstream of the *FgTri4* coding region and 3' flanking DNA (Fig. 2). *FUM8* is a fumonisin biosynthetic gene, and in wild-type *F. verticillioides* it is expressed after about 70 h of incubation in liquid GYAM medium (Seo et al. 2001). Following transformation of the *FgTri4* expression vector into *F. verticillioides* M-3125, 21 hygromycin-resistant transformants were recovered and screened for their ability to metabolize trichodiene added to liquid cultures. GC analysis of culture extracts revealed that the trichodiene concentration decreased and two additional metabolites appeared over a 7 day period in cultures of 6 of the 21 transformants. All trichodiene (retention time = 6 min) was metabolized after 4 days in these cultures (Fig. 3). GC/MS analysis indicated that the main metabolite formed was isotrichodermin (retention time = 11.8 min) (for structure see Fig. 1). A small amount of trichothecene (retention time = 8.2 min) was also formed (for structure see Fig. 1). No additional trichodiene metabolites were detected.

To determine whether *F. verticillioides* or the culture medium was responsible for any of the steps in the conversion of trichodiene to isotrichodermin, we incubated uninoculated GYAM and GYAM cultures of untransformed M-3125 with trichodiene, isotrichodiol, isotrichotriol, and isotrichodermol. Trichodiene and isotrichodermol were not metabolized by cultures of untransformed M-3125 or by incubation in GYAM medium. Isotrichodiol and isotrichotriol incubated in GYAM medium alone (pH 2.6) were converted to trichothecene and isotrichodermol, respectively. Incubation of isotrichodermol
**Discussion**

This study used heterologous expression of the *F. graminearum* trichothecene P450 gene *Tri4* in *F. verticillioides*, under the control of the promoter for a fumonisin biosynthesis gene, *FUM8*. *Fusarium verticillioides* is a fumonisin producer and makes no trichothecenes but has P450 reductases that can act as electron donor proteins for trichothecene P450 oxygenases. *Fusarium* trichothecene biosynthesis requires at least six oxygenation steps that utilize molecular oxygen (Desjardins et al. 1986). To date, only four P450 oxygenase genes involved in trichothecene biosynthesis have been identified. In *F. sporotrichioides* and *F. graminearum*, three of the P450 genes are found within a 26 kb gene cluster, *Tri4* (Hohn et al. 1995), *Tril* (Alexander et al. 1998; McCormick and Hohn 1997), and *Tril3* (Brown et al. 2002), although *F. graminearum* strains that produce DON lack a functional *Tril3* (Lee et al. 2002). Recently, an additional trichothecene P450 gene, *Tril*, required for C-7 hydroxylation, was identified in a mini-cluster of two genes in *F. sporotrichioides* (Meek et al. 2003; Brown et al. 2003), and a *Tril* homolog was identified in *F. graminearum* (McCormick et al. 2004). Feeding results with *Tril* transformants suggested that in *F. graminearum*, *Tril* controls both C-7 and C-8 hydroxylation. The discovery of a second locus with structural genes raised the possibility that additional trichothecene P450 genes may reside outside the main cluster. An alternate explanation is
that *Tri4* encodes an enzyme that can complete more than one oxygenation step.

The results of this study showed that trichodiene added to liquid cultures of *F. verticillioides* transformants carrying *FgTri4* converted trichodiene to isotrichodermin and trichothecene. The conversion of trichodiene to isotrichodermin requires seven steps, including an acetylation (Fig. 1). The final acetylation step was found to be unrelated to the introduction of *FgTri4*, since untransformed M-3125 was able to convert isotrichodermin to isotrichodermin. In trichothecene-producing species, such as *F. sporotrichioides* and *F. graminearum*, trichothecene C-3 acetylation is controlled by *Tri101* (Kimura et al. 1998a, 1998b; McCormick et al. 1999). *Tri101* has been identified as a biosynthetic gene as well as a toxin resistance gene, encoding an acetyltransferase that catalyzes the acetylation of isotrichodermol and other trichothecenes (McCormick et al. 1999). Trichothecene C-3 acetylation is not limited to *Fusarium* trichothecene-producing species. A homolog of *Tri101*, *AYT1*, has been identified from *S. cerevisiae* (Alexander et al. 2002). Interestingly, some non-trichothecene-producing Fusaria were also reported to have acetyltransferases that can acetylate the C-3 hydroxyl group of trichothecenes (Kimura et al. 2003b). One possibility is that this acetyltransferase activity acts as a resistance mechanism for species that must co-exist with trichothecene producers. Whether *F. verticillioides* M-3125 has a functional *Tri101* homolog is not known, but it clearly has trichothecene C-3 acetyltransferase activity.

Two of the seven steps required for conversion of trichodiene to trichodiol and cyclization of trichodiol to form isotrichodermol (Fig. 1), have previously been shown to occur nonenzymatically under acidic conditions (McCormick et al. 1990). The pH of the GYAM medium was 2.6, and the results indicate that isotrichodiol isomerized and cyclized to form isotrichodermol when incubated in GYAM. Furthermore, incubation of isotrichodiol in GYAM resulted in the analogous isomerization and cyclization of the diol to form trichothece (Fig. 1). This may account for the small amount of trichothece formed in trichothece-fed cultures of transgenic *F. verticillioides* carrying *FgTri4*.

Two of the seven steps required for conversion of trichodiene to isotrichodermin in transgenic *F. verticillioides* carrying *FgTri4* were due to nonenzymatic isomerizations (McCormick et al. 1990), and a third was due to acetyltransferase activity present in wild-type *F. verticillioides* M-3125. The results suggest that the remaining four steps, trichodiene to 2-hydroxy trichodiene to epoxytrichoene-2-ol to isotrichodiol to trichodiol (Fig. 1), must be due to the expression of *FgTri4*.

Previous work has shown that complementation of the trichodiene-accumulating *F. sporotrichioides* *Tox4* ( = *Tri4*) UV mutant strain MB5493 with *F. sporotrichioides* *Tri4* (*FxTri4*) restored normal T-2 toxin production (Hohn et al. 1995). As part of a study on the trichothece gene cluster in *Myrothecium roridum*, *M. roridum* *Tri4* (*MrTri4*) was inserted into the *F. sporotrichioides* *Tox4* UV mutant strain. This heterologous expression of *MrTri4* into the *F. sporotrichioides* mutant did not restore normal T-2 toxin production but rather resulted in the accumulation of sambucinol and trichothece and only a small amount of T-2 toxin (Trapp et al. 1998). Expression of *MrTri4* in the same *F. verticillioides* system may help to clarify the differences between *Tri4* genes of *Fusarium* and *Myrothecium* species.

Multifunctional oxygenation is not unique in terpene biosynthesis. Two multifunctional oxygenases involved in gibberellin biosynthesis have been described recently in *Gibberella* (Rojas et al. 2001; Tudzynski et al. 2002; Helliwell et al. 2001). One, ent-kaurene oxidase, catalyzed three oxygenation steps; however, these are all at the same carbon, with successive conversions to alcohol, aldehyde, and carboxylic acid. The results from this study indicate that *Tri4* encodes a multifunctional oxygenase required for four separate oxygenation steps at four separate carbons. It is not known if *Tri4* encodes four separate enzymes or if one enzyme processes the substrate. Since some trichothece was found in trichothece-fed cultures, isotrichodiol may be not be tightly bound to the enzyme and may be available for nonenzymatic modifications. Isotrichodiol has been readily isolated from cultures treated with large amounts of trichothece (Hesketh et al. 1991).

In conclusion, *Tri4* encodes a multifunctional oxygenase required for the first four oxygenation steps in 15-ADON (15-acetyldeoxyxovalenol) biosynthesis in *F. graminearum*. This study has also shown that *F. verticillioides* has trichothece C-3 acetyltransferase activity. Finally, we have shown that *F. verticillioides* is an attractive system for heterologous expression of *Fusarium* P450 genes.

**Acknowledgements**

We thank Marcie Moore, Kimberly MacDonald, Judy Blackburn, and Troy Larson for technical assistance and Amy Morgan for sequencing and oligonucleotide synthesis. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

**References**


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