Expression of Tri15 in Fusarium sporotrichioides

Abstract In the fungus Fusarium sporotrichioides, biosynthesis of trichothecene mycotoxins requires at least three genetic loci: a core 12-gene cluster, a smaller two-gene cluster, and a single-gene locus. Here, we describe the Tri15 gene, which represents a fourth locus involved in trichothecene biosynthesis. Tri15 is predicted to encode a Cys2-His2 zinc finger protein and is expressed in a manner similar to genes in the core trichothecene gene cluster. However, disruption of F. sporotrichioides Tri15 does not affect production of T-2 toxin, the major trichothecene produced by this fungus. This result suggests that Tri15 is not necessary for the production of T-2 toxin. Cultures with exogenously added T-2 toxin have high levels of Tri15 expression and no detectable expression of the trichothecene biosynthetic genes Tri5 and Tri6. The expression analysis is consistent with Tri15 being a negative regulator of at least some of the trichothecene biosynthetic genes. In F. graminearum, Tri15 has been mapped to linkage group 2 and is therefore unlinked to the main trichothecene biosynthetic gene cluster.

Keywords Mycotoxin · Deoxynivalenol · T-2 toxin Zinc finger protein · Feedback inhibition · Negative control

Introduction

The genus Fusarium contains several species that infect economically important agricultural crops, causing millions of dollars of damage each year. In addition to the losses in crop yield and quality that result from Fusarium infection, many species produce a family of mycotoxins known as trichothecenes that are toxic to plants and/or animals (Desjardins et al. 1993).

The biosynthesis of trichothecenes involves a complex pathway that begins with the sesquiterpene hydrocarbon trihodiene and consists of multiple oxygenation, cyclization, and esterification steps (Desjardins et al. 1993). Many of the genes involved in trichothecene biosynthesis in Fusarium have been identified within a 26-kb segment of DNA (Brown et al. 2001). This cluster includes three P450 oxygenase genes [Tri4 (Hohn et al. 1995), Tri11 (McCormick and Hohn 1997; Alexander et al. 1998), Tri13 (Brown et al. 2002; Lee et al. 2002)], a sesquiterpene cyclase gene [Tri5 (Hohn and Van Middendorf 1986)], an esterase gene, Tri8 (McCormick and Alexander 2002), and two acyltransferase genes [Tri3 (McCormick et al. 1996), Tri7 (Brown et al. 2001; Lee et al. 2002)]. Also within the cluster is a transport pump gene [Tri12 (Alexander et al. 1999)] that functions in transporting trichothecenes out of the cell. Extension of the sequence of the cluster from both ends identified genes that appear not to be involved with toxin biosynthesis (Kimura et al. 2003). Since the trichothecene genes identified to date do not account for all the steps in the biosynthetic pathway, there are likely to be other genes involved with toxin biosynthesis that are not located within the cluster.

Indeed, one gene [Tri101, encoding a trichothecene O-3 acetyltransferase (Kimura et al. 1998a; McCormick et al. 1999)] is located outside the cluster. Another locus involved with trichothecene biosynthesis was recently described in F. sporotrichioides (Meek et al. 2003) and includes an acyltransferase gene (Tri16) and a P450 oxygenase gene (Tri7). Adjacent to these genes is a
Gal4-like transcription activator that appears not to be required for toxin production.

To date, only two genes \([Tri6 \text{ (Proctor et al. 1995a)}, Tri10 \text{ (Tag et al. 2001; Peplow et al. 2003)}]\) have been identified as positive regulators of the trichothecene pathway genes. Both genes are located within the main trichothecene gene cluster. The exact nature and the interaction of these two genes on the control of the other biosynthetic pathway genes has still to be elucidated, but it appears that \(Tri10\) regulates the transcription of \(Tri6\) (Tag et al. 2001) while the \(Tri6\) gene product binds to the promoter regions of trichothecene biosynthetic genes, inducing their expression (Hohn et al. 1999). In this study, we characterized \(Tri15\), a gene that represents a fourth trichothecene locus. Due to the rapid induction of \(Tri15\) expression by T-2 toxin, we propose that \(Tri15\) may have a negative control on the expression of the toxin biosynthetic genes.

**Methods and materials**

**Strains**

\(F. \text{ sporotrichioides NNRL3299}\) was obtained from the USDA Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research (Peoria, Ill.) and was maintained on V-8 juice agar slants (Stevens 1974). \(F. \text{ sporotrichioides FsTri15D lines}\) were transformants carrying the disrupted \(Tri15\) plasmid sequence. Strain NN4 (\(Tri6\) disruption mutant) and \(F. \text{ graminearum Z3639}\) (teleomorph, \(Gibberella zeae\)) were described by Proctor et al. (1995a) and Bowden and Leslie (1992), respectively.

**Media and culture conditions**

All non-transformed cultures were grown on V-8 juice agar plates while hygromycin-resistant transformants were maintained on V-8 juice agar supplemented with 300 \(\mu\)g hygromycin B/ml (Sigma, St. Louis, Mo.). Liquid cultures were grown on GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone; 20 ml in a 50-ml Erlenmeyer flask). For toxin production, liquid cultures of \(F. \text{ sporotrichioides}\) were inoculated with spores (10^5/ml) washed from 1-week-old cultures grown on V-8 juice agar. All liquid cultures were grown at 28 °C in the dark at 200 rpm.

For studies on gene expression, 50 ml of GYEP were inoculated with 1x10^8 spores/ml and the cultures grown at 200 rpm. After 16 h of growth, T-2 toxin dissolved in acetone was added for a final concentration of 270 \(\mu\)g/ml. An equal volume of acetone (< 1%) was added to the control cultures. Mycelia were harvested through Whatman number 4 qualitative filters at various time points and flash-frozen in liquid nitrogen for the RNA to be processed.

**Gene disruption and transformations**

Vector construction used a truncated \(Tri5\) coding sequence to facilitate gene disruption via a single recombination event between the \(Tri5\) sequence of the vector and the homologous sequence in \(F. \text{ sporotrichioides}\) (Proctor et al. 1995a). Genes disrupted in this manner consist of two copies of the coding region, but each copy is truncated and therefore should not produce a functional protein. Primers 1412 (\(\text{S'-GGCACTGCAGCCATGACCAC-3}'\); located 33 bp downstream of the AGT start) and 1399 (\(\text{S'-GTCGCTTC-TCCGGTCCTGGTC-3}'\); located approximately 205 bp upstream of the termination codon) were used to amplify a truncated \(F. \text{ sporotrichioides Tri5 (FsTri5)}\) coding region. PCR employed \(Pfu\) Turbo (Stratagene, La Jolla, Calif.), following the manufacturer’s protocol and using a PTC-100 thermocycler (MJ Research, Watertown, Mass.) with a template of genomic DNA from \(F. \text{ sporotrichioides}\). The resulting 720-bp fragment was band-purified (UltraClean; MoBio, Solana Beach, Calif.) and cloned into the pCR-Blunt II TOPO vector (Invitrogen, Carlsbad, Calif.). The resulting vector, pFsTri15, was cut with \(NdeI\), treated with CIP (New England Biolabs, Beverly, Mass.), and ligated with promoter I from \(Cochliobolus heterostrophus\) containing the chimeric hygromycin B phosphotransferase gene (\(\text{hygB, Turgeon et al. 1987}\)) to form pTri15D-I (Fig. 1A).

\(F. \text{ sporotrichioides NNRL3299}\) protoplasts were transformed with the disruption plasmids as described by Proctor et al. (1995a).

**Nucleic acid manipulation**

Plasmids were isolated using MoBio UltraClean kits or Qiagen kits (Madison, Wis.). In Southern analyses (Sambrook and Russell 2001), genomic DNA of \(F. \text{ sporotrichioides NNRL3299}\) and the transformant strains was digested with \(HindIII\), blotted onto a Nytran SuperCHarge membrane (Schleicher & Schuell, Keene, N.H.), and hybridized to a \(^32\)P-labeled probe [Prime-A-Gene (Promega, Madison, Wis.) or RediPrime II (Amersham)]

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**Fig. 1A-C**

**Tri15. A** Map of \(Tri15\) disruption vector pTri15D-1, showing restriction sites and orientation of primers. **B** Map of \(Tri15\) in wild-type strain NNRL3299. **C** Structure of \(Tri5\) that results from a single homologous recombination event between pTri15D-1 and the 3' end of the \(Tri15\) coding region. \(H \text{ HindIII, N NdeI. Arrows indicate primers and their respective numbers}\).
BioSciences, Piscataway, N.J.), consisting of 540 bp of coding region from FsTri5. The DNA fragment used to prepare the probe was amplified by PCR with primers 1345 (5'-ATGCTGGGAGCAAACACTG-3') and 1388 (5'-GATAAGTCTTCCGGATGGG-3') from a template of cloned FsTri5.

For Northern analyses, mycelia were ground in liquid nitrogen and extracted with TRizol reagent (Invitrogen). Then, 10-20 μg of the resulting total RNA were run on denaturing agarose gels (Sambrook and Russell 2001). The RNA was blotted onto NyTran SuPer Charge membrane and probed with gel-purified PCR fragments labeled with 32P-dCTP.

A F. graminearum cosmid library (Brown et al. 2001) was screened by Southern hybridization, using a probe made from F. sporotrichioides genomic DNA. A cosmid was selected and sequenced using Gene Jumper (Invitrogen). Sequence data were obtained using the ABI Prism BigDye terminator cycle sequencing kit (PE Biosystems, Foster City, Calif.) on a PE Biosystems 3700 automated DNA sequencer. Sequences were analyzed using DNAman (Lynnon Software, Canada).

Chemical analyses

Liquid cultures of wild-type and transformed cultures of F. sporotrichioides were harvested after 7 days, extracted with ethyl acetate, and analyzed by gas chromatography (GC), as described by McCormick and Hohn (1997).

Virulence assays

F. sporotrichioides has been shown to be virulent on parsnip roots (Pastinaca sativa; Desjardins et al. 1989). Plugs of mycelium grown on V8 media were made using an inverted 20-μl micropipette tip and placed on sterilized slices of parsnip root, following the protocol of Desjardins et al. (1989). The weight of the rotted tissue was measured. All assays were done in triplicate.

Mapping analysis

Southern analysis of 100 progeny and the two parentals used to do mapping studies of the F. graminearum genome (Jurgenson et al. 2002) was performed using the restriction enzyme MspI and a labeled fragment of FsTri5. Data analysis was performed using Map Manager QTX11 (http://mapmgr.roswellpark.org; Manly et al. 2001).

Sequence data

Sequence data from this article have been deposited with the GenBank data library under accession number AY347604.

Results

Tri5 sequence analysis

The F. sporotrichioides Tri5 (FsTri5) sequence deposited in GenBank (accession number AF327521) contains only the protein coding sequence. We wished to compare the Tri5 sequences from F. sporotrichioides and F. graminearum, to determine the sequence homology between the two species. Using primers from F. sporotrichioides, we were able to identify a clone in our F. graminearum cosmid library. The F. graminearum Tri5 (FgTri5) sequence (accession number AY347604) has 81% identity to the sequence from F. sporotrichioides, with a resulting protein identity of 82%. The FsTri5 coding region is comprised of 957 bp and forms a predicted protein of 318 amino acids (aa), 35,533 kDa, whereas the FgTri5 coding region is comprised of 975 bp and forms a predicted protein of 328 aa, 36,669 kDa. The greater size of the FgTri5 coding region is due to two inserts. Beginning 647 bp downstream from the start site in FgTri5, there is a 6-bp insert separated by 9 bp from a 12-bp insert. Sequence analysis of the F. graminearum cosmid clone revealed a TATA box approximately 60 bp upstream of the ATG site in F. graminearum. There is no apparent Tr6 (YNAGGCCCT) binding site upstream of FgTri5, although there is a truncated site (TGAGGCC) within the coding region. A tRNA gene for Ile (isoleucine) is located approximately 1,100 bp upstream of the start site in F. graminearum. There are two Cys2Hys2 zinc finger motifs located near the amino-terminal end of the predicted protein in both species. The first motif is at residues 54–75 and the second at residues 106–128.

Tri5 linkage analysis

An established linkage map of F. graminearum (Jurgenson et al. 2002) was used to locate the FgTri5 gene. Using the two parental strains from the mapping study, we were able to detect a RFLP (restriction fragment length polymorphism) that contained FgTri5. Comparison of the data from the Tri5 analysis of the parentals and over 100 progeny with the data from the linkage studies showed that Tri5 mapped to linkage group 2.

Tri5 disruption analysis

We chose to study the function of TRI5 in F. sporotrichioides, as the analysis for toxin in liquid culture is much easier with F. sporotrichioides than with F. graminearum. Eighty-three transformants were selected following transformation of wild-type F. sporotrichioides strain NRRL3299 with the disruption vector pTri15D-1. Southern analysis indicated that two of these showed a disrupted Tri5. In NRRL3299 genomic DNA, HindIII cuts on either side of the Tri5 coding region (Fig. 1B) to yield a 3-kb band in Southern analysis (Fig. 2, lane 1). Most transformants yielded a 3-kb band and other, larger bands. However, HindIII digestion of transformants FsTri15D#45 and FsTri15D#89 yielded a hybridization pattern consisting of three bands (Fig. 2, lane 2), which is consistent with integration of the vector by a single homologous recombination event resulting in disruption of Tri5 (see Fig. 1C).

Trichothecene production assays revealed that the two Tri5 disruption mutants did not differ from the wild-type progenitor strain in their ability to produce T-2
Toxin. Also, disruption of Tri15 did not affect the ability of the mutants to cause rot of parsnip roots, an assay for virulence (described in Methods and materials).

**Tri15 expression analysis**

Northern analysis of strain NRRL3299 showed that Tri15 was more strongly expressed at 24 h and 26 h than at 18 h and 22 h post-inoculation (Fig. 3). The expression of Tri15 followed the same time-course as expression of Tri5, which encodes the trichothecene biosynthetic enzyme trichodiene synthase, in that it was more highly expressed at the later time-points. However, Tri15 expression was detected at earlier time-points (i.e. 18 h, 22 h) when Tri5 expression was not detected (Fig. 3). It should be noted that, although the onset of Tri5 expression varies from culture to culture, it is not constitutively expressed.

To determine whether Tri15 was under the positive control of Tri6, we assayed Tri15 expression in a Tri6 disruption mutant NN4 (Proctor et al. 1995b). In the Tri6 mutant, Tri15 expression was low at 18 h but was high at 22 h and beyond (Fig. 3). In contrast, previous studies (Proctor et al. 1995b) showed no or little expression of the trichothecene biosynthetic genes Tri5 and Tri4 in the Tri6 mutant.

The wild-type T-2 toxin production in FsTri15 disruption mutants indicates that the TRI15 protein is not a positive regulator of other trichothecene biosynthetic genes. To determine whether Tri15 is a negative regulator, the expression of Tri15, Tri5, Tri6, Tri10 and Tri101 was analyzed in the wild-type strain NRRL3299 and the Tri15 disruption mutant FsTri15D#45 with and without the addition of exogenous T-2 toxin. The T-2 toxin was added to a concentration of 270 μg/ml GYEP medium at 16 h post-inoculation. At this time-point, toxin was not yet detectable in wild-type cultures. A level of 270 μg T-2 toxin/ml is usually seen in 7-day-old cultures of NRRL3299. At 1 h after addition of T-2 toxin, Tri15 expression was high in NRRL3299, as seen in a 15-min exposure to film (Fig. 4). An increase in expression in the

Fig. 2 Southern hybridization analysis using genomic DNA restricted with HindIII. Lane 1 (wt) Wild-type strain NRRL3299, lane 2 Tri15 disruption mutant FsTri15D#45

Fig. 3 Northern analysis of wild-type strain NRRL3299 (WT) for Tri15 and Tri5 expression and of the Tri6 disruption mutant, NN4, for Tri15 expression at various time-points (h) post-inoculation. rRNA was visualized in the gel by ethidium bromide-staining

Fig. 4 Northern analysis of wild-type strain NRRL3299 and Tri15 disruption mutant FsTri15D#45 for expression of Tri15, Tri5, Tri10, Tri101, Tri6, and β(B)-tubulin at 1 h post-addition of either acetone (−) or 270 μg T-2 toxin/ml in acetone (+). Tri15 expression from 15 min exposure to film; all others from 24 h exposure to film. rRNA was visualized in the gel by ethidium bromide-staining
Discussion

Tril5 was identified in an expressed sequence tag (EST) library (http://www.genome.ou.edu/fsporo.html) prepared from a *F. sporotrichioides* Tril5 overexpressing strain (†Tril5; Tag et al. 2001). In the current study, we characterized the Tril5 structure and proposed a function for the gene.

The predicted amino acid sequence of the Tril5 protein (TRI15) suggests it is a C$_2$H$_2$ zinc finger protein. Both *F. sporotrichioides* and *F. graminearum* have two conserved sequences of -C-X$_2$-C-X$_{12}$-H-X$_{4.5}$-H- (Table 1), which are characteristic of transcription factors (Coleman 1992). Tril6, a characterized positive transcription factor involved in trichothecene biosynthesis in *F. sporotrichioides*, has three zinc finger motifs (Proctor et al. 1995b).

Tril5 does not appear to be under the control of Tril6, because Tril5 is expressed in the Tril6 disruption mutant (this study; Peplow et al. 2003). There are also no Tril6 binding sites in the promoter region of FgTril5. Tril5 may be under the control of Tril6, as transcripts of Tril5 are found in an over-expressed mutant of FsTril0 (EST library; Peplow et al. 2003), although an EST library from a wild-type culture has not been examined. The Tril5 transcript is also abundant in an EST library of *F. graminearum*-infected wheat (Muehlbauer, personal communication), suggesting that Tril5 may be a relatively abundant transcript under certain conditions. As shown here, Tril5 transcripts are induced at the later stages of development and especially by T-2 toxin.

Northern analysis indicated that Tril5 was expressed in a manner similar to trichothecene biosynthetic genes, such as Tri5. However, disruption analysis indicated that Tril5 is not required for trichothecene biosynthesis in *F. sporotrichioides*. In addition, Tril5 does not appear to be a positive regulator of trichothecene biosynthetic genes, because its disruption does not affect the expression of other trichothecene genes under culture conditions conducive to trichothecene production. Therefore, we propose that Tril5 is a negative regulator of at least some of the trichothecene biosynthetic genes. Such a negative regulatory role is supported by the absence of Tri5 and Tril6 transcripts in cultures with high levels of Tril5 expression in the presence of T-2 toxin and by the presence of transcripts for those two genes in the Tril5 mutant. Toxin itself does not inhibit the transcription process, as Tril5, Tril01, Tril0, and β-tubulin (a housekeeping gene not involved with toxin biosynthesis) are all transcribed in the presence of toxin. We only used RNA levels in this investigation as a measurement of gene expression. It is possible that RNA degradation could explain the lower levels of RNA seen for some genes. However, there is no visible degradation in the ribosomal RNA levels nor is there apparent degradation (smearing of the bands in the blot) of the other genes being analyzed.

The current model for the regulation of trichothecene biosynthetic genes proposes that Tril0, presumably turned on by some unknown factor, regulates Tril6 (Tag et al. 2001). Tril6, which has been shown to be necessary to turn on the transcription of several toxin biosynthetic genes (Hohn et al. 1999), then turns on Tril5, which encodes the trichodiene synthase used for the first committed step in the pathway. To extend this model, we propose that Tril5 is turned on when toxin begins to build up in the cell or surrounding media. TRI15, as a negative transcription factor, then turns off other pathway or regulatory genes, presumably by binding to regions of the gene, thereby stopping transcription.

It is possible that Tril5 upregulates genes that are involved in self-protection or toxin degradation. And, as can be seen, Tril01 is upregulated in the presence of toxin (this study; Kimura et al. 1998b). However, Tril01 is also turned on in the Tril5 disruption mutant, suggesting that Tril01 is not regulated by Tril5.

The linkage analysis mapped FgTril5 to *F. graminearum* linkage group 2. This indicates that FgTril5 is not located near any known trichothecene biosynthetic genes. The 26-kb segment carrying the main cluster is located on linkage group 1 (Jurgenson et al. 2002); and FgTril01 is located outside the main cluster on linkage group 3. Also, FgTril5 does not map near the TOXI locus (linkage group 4), which affects the levels of trichothecenes produced by *F. graminearum* (Jurgenson et al. 2002). With the recent release of the *F. graminearum* genomic sequence (http://www-genome.wi.mit.edu), we were able to locate Tril5 on contig 1.457, Tri5 on contig 1.159, and Tril01 on contig 1.321.

Together, the data presented here indicate that Tril5 encodes a DNA-binding protein with two zinc finger domains. This gene is induced during the later stages of

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Table 1 Amino acid residue comparison of zinc finger regions in *Fusarium sporotrichioides* Tril6 and Tril15

<table>
<thead>
<tr>
<th>Region</th>
<th>Amino acids</th>
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<tbody>
<tr>
<td>FsTril6</td>
<td>CPFPN–CKSTTIFESGRDFRR—HYRQ–H</td>
</tr>
<tr>
<td>FsTril6</td>
<td>CRYPD–CSQSTQDIEVGTGFAITRKD</td>
</tr>
<tr>
<td>FsTril6</td>
<td>RARHESK–H</td>
</tr>
<tr>
<td>FsTril5</td>
<td>CPWHDOEQGQQLRFSVRDVMRD—HYRRI H</td>
</tr>
<tr>
<td>FsTril5</td>
<td>CLF–CITISSTLESVM–HMQKA H</td>
</tr>
<tr>
<td>FsTril5</td>
<td>CHL–CGTSRSTQAVQQ–HMTGKG H</td>
</tr>
<tr>
<td>FsTril5</td>
<td>CLF–CITISSSTLESVM–HMQKS H</td>
</tr>
<tr>
<td>FsTril5</td>
<td>CLF–CETSRSTQAVQQ–HMTGKG H</td>
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growth or in the presence of T-2 toxin and may negatively control other trichothecene biosynthetic genes. However, unlike some trichothecene biosynthetic genes, $Tri15$ is not controlled by $Tri6$ and is not necessary for toxin production. The gene is physically located on a linkage group separate from the main trichothecene biosynthetic cluster. Further work is underway to more fully characterize the exact function of $Tri15$.

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