Mol Gen Genet (1999) 261: 977–984

ORIGINAL PAPER

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TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides*: gene isolation and expression in yeast

Received: 4 September 1998 / Accepted: 14 April 1999

Abstract Many of the genes involved in trichothecene toxin biosynthesis in *Fusarium sporotrichioides* are present within a gene cluster. Here we report the complete sequence for *TRI12*, a gene encoding a trichothecene efflux pump that is located within the trichothecene gene cluster of *F. sporotrichioides*. *TRI12* encodes a putative polypeptide of 598 residues with sequence similarities to members of the major facilitator superfamily (MFS) and is predicted to contain 14 transmembrane-spanning segments. Disruption of *TRI12* results in both reduced growth on complex media and reduced levels of trichothecene production. Growth of *tri12* mutants on trichothecene-containing media is inhibited, suggesting that TRI12 may play a role in *F. sporotrichioides* self-protection against trichothecenes. Functional analysis of *TRI12* was performed by expressing it in yeast strains that were co-transformed with a gene (*TRI3*) encoding a trichothecene 15-*O*-acetyltransferase. In the presence of the *TRI3* substrate, 15-decalonectrin, cultures of yeast strains carrying *TRI12* and *TRI3* accumulated much higher levels of the acetylated product, calonectrin, than was observed for strains carrying *TRI3* alone. PDR5, a transporter of the ABC superfamily, which is known to mediate trichothecene resistance in yeast, increased calonectrin accumulation in *TRI12*/*TRI3* yeast strains but not in *TRI3* strains. These results confirm the involvement of *TRI12* in the trichothecene efflux associated with toxin biosynthesis, and demonstrate the usefulness of yeast as a host system for studies of MFS-type transporters.

Communicated by C. A. M. J. J. van den Hondel

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Key words *Fusarium sporotrichioides* · Efflux pump · Trichothecenes · Major facilitator superfamily · *TRI12*

Introduction

Trichothecenes are sesquiterpenoid antibiotics produced by diverse genera of fungi including some *Fusarium* species. Several of the *Fusarium* species that produce trichothecene toxins are maize and wheat pathogens. Contamination of grains with trichothecenes substantially reduces their value and has been linked to instances of mycotoxicoses in both animals and humans (Ueno 1980). Trichothecenes are also potent phytotoxins, and production of trichothecenes by *Fusarium* species has been proposed to increase the virulence of these plant pathogens in some hosts (Proctor et al. 1995a; Desjardins et al. 1996). The toxic effects of trichothecenes appear to result from their ability to inhibit protein synthesis in eukaryotes. Based on both in vivo studies (McLaughlin et al. 1977) and the analysis of yeast mutants with increased tolerance to trichothecenes (Fried and Warner 1981), it appears that many trichothecenes specifically inhibit the peptidyltransferase step in protein synthesis.

Biosynthesis of trichothecenes involves a complex pathway that begins with trichodiene and consists of multiple oxygenation, cyclization, and esterification steps (Desjardins et al. 1993). At least ten pathway genes are located in a gene cluster in *F. sporotrichioides* (Hohn et al. 1995; Hohn and McCormick, unpublished). These genes encode several pathway enzymes and a transcription factor, TRI6, that is required for pathway gene expression (Proctor et al. 1995b). Recently, it has been shown that a pathway gene encoding a trichothecene 3-*O*-acetyltransferase is not closely linked to other pathway genes (Hohn et al. 1998; Kimura et al. 1998a). Several lines of evidence indicate that the trichothecene 3-*O*-acetyltransferase (Tri101) in *F. graminearum* plays a role in self-protection against trichothecenes (Kimura

et al. 1998a, 1998b). In *F. sporotrichioides*, disruption of the homologous gene (*FsTRI101*) leads to slightly reduced growth on trichothecene-containing media relative to the parental strain (McCormick et al., submitted for publication). The ability of *Fstri101* mutants to grow in the presence of trichothecenes suggests that, like other antibiotic-producing microorganisms, *Fusarium* species possess multiple mechanisms for self-protection.

Under certain growth conditions, trichothecenes accumulate to high levels in liquid cultures of *F. sporotrichioides* (Hohn et al. 1993) where they are found almost exclusively in culture supernatants. This observation suggests the presence of a trichothecene efflux mechanism. Biosynthesis of microbial antibiotics frequently involves pathway-specific transporters which, in some instances, have been implicated in self-protection (Cundliffe 1989). A fungal toxin transporter, TOXA, has been reported for the HC toxin produced by *Cochliobolus carbonum* (Pitkin et al. 1996). TOXA, which belongs to the major facilitator superfamily (MFS) of transporters (Paulsen et al. 1996), appears to mediate HC toxin efflux and may also function in self-protection. Another fungal toxin transporter from *Cercospora kikuchii* (CFP), which mediates cercosporin transport, has been characterized and shown to contribute to self-protection (Callahan and Upchurch 1995). Many of the previously reported fungal and yeast transporters belong to a multidrug export system powered by a transmembrane proton gradient, and belong within the MFS, having either 12 or 14 transmembrane segments (Marger and Saier 1993; Paulsen et al. 1996; Goffeau et al. 1997).

In this paper, we report on the *TRI12* gene of *F. sporotrichioides*. *TRI12* is located in the trichothecene pathway gene cluster and encodes a protein with structural similarities to MFS transporters. Expression of *TRI12* in yeast suggests that it functions in trichothecene efflux.

Materials and methods

Strains, media, and culture conditions

F. sporotrichioides NRRL 3299 was obtained from the USDA/ARS Culture Collection (NCAUR, Peoria, Ill.) and maintained on V-8 juice agar plates. Transformants of *F. sporotrichioides* were grown on V-8 slants containing 150 μg/ml hygromycin B (Sigma). Conidia were washed from the plates and used to inoculate YD medium (1% yeast extract, 2% peptone, 2% glucose) at a concentration of 5×10^5 spores/ml for DNA isolation, and GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone) for toxin production (Ueno et al. 1975).

Saccharomyces cerevisiae strains RW2802 (*PDR5 leu2 ura3 met5*) and JG436 (*pdr5::Tn5 leu2 ura3 met5*) were generously provided by Dr. J. Golin (Meyers et al. 1992) of The Catholic University of Louvain (Belgium). For yeast transformations, cells were grown on YD plates for 1–3 days; otherwise, cultures were maintained on glucose minimal media with the appropriate nutrient supplements (1.0 g/l leucine, 0.2 g/l uracil, 0.2 g/l methionine).

DNA isolation

Plasmids were prepared from *E. coli* either by an alkaline lysis miniprep method (Sambrook et al. 1989) or using Qiagen columns. Fungal DNA minipreps were prepared as previously described (Alexander et al. 1998). Minipreps of yeast DNA for PCR analysis were prepared from cultures grown in 20 ml of selective medium (glucose minimal medium containing appropriate supplements) for 48 h. Cultures were centrifuged at $3000 \times g$ for 5 min in a Beckman J2-20 rotor and the cell pellet was resuspended in 300 μl of Buffer A (2% Triton X-100, 1% SDS, 1 mM EDTA pH 8.0, 10 mM TRIS-HCl pH 8.0). For cell homogenization, approximately 300 μl of sterile glass beads (0.45–0.55 mm, Braun Melsungen) were added and the mixture vortexed for 2 min. After vortexing, 150 μl of 5 M potassium acetate was added, the mixture was incubated on ice for 5 min and then centrifuged at $6000 \times g$ for 5 min at 4° C in a J2-20 rotor. The resulting supernatant was transferred to a microfuge tube and an equal volume of phenol/chloroform (1:1) was added and mixed by vortexing. Following centrifugation for 2 min in a microfuge, the upper aqueous phase was transferred to a new tube. DNA was precipitated with two volumes of ethanol at room temperature and recovered as a pellet following a 2-min incubation and centrifugation at $12{,}000 \times g$ for 5 min. The pellet was washed with 70% ethanol, dried briefly under vacuum, and resuspended in a small volume of TE buffer (10 mM TRIS-HCl pH 8.0, 0.1 mM EDTA) containing 0.05 mg/ml RNase (Boehringer Mannheim).

Cloning and characterization of *TRI12*

The *TRI12* gene was originally cloned as a 9.5-kb *Sac*I fragment in pFSC3-3 and then more precisely localized on the 3.5-kb internal *Hind*III fragment upstream of *TRI11* (Alexander et al. 1998). This *Hind*III fragment was cloned into pBluescript II KS(-) (Stratagene) to yield plasmid pFSC3-5. Genomic sequencing templates were prepared from plasmids pFSC3-3 and pFSC3-5. To sequence the coding region of the *TRI12* cDNA, it was first amplified using *Pfu* polymerase (Stratagene) and a cDNA library as the template. The cDNA library was constructed in the yeast expression vector pYES2 (Invitrogen) using RNA harvested from a culture of *F. sporotrichioides* grown for 23 h in GYEP (Alexander et al. 1998). The primers for PCR were Nos. 767 (5′-GCCGAATTCGGATCC-CACACGACACC**ATGACTGTCGTAGTTCCAGAG**-3′), which corresponds to the predicted 5′ end of the *TRI12* coding region with additional restriction sites added, and 778 (5′-GCAAGGTTTTGGAAGGCCTAG-3′), which is located 395 bp downstream from the 3′ end of the *TRI12* translational stop codon. The resulting PCR product was extracted from an agarose gel band with Gene Clean (BIO 101) and cloned into pNoTA (5 Prime to 3 Prime) with the Prime PCR Cloner kit (5 Prime to 3 Prime). The 3′ end of the *TRI12* cDNA was amplified by anchored PCR using the cDNA library as a template and primers 777 (5′-CGCGACTGTCCGGCACGC-3′) – specific for the *TRI12* coding sequence, and 513 (5′-GCGTGAATGTAAGCGTGAC-3′) specific for the cDNA cloning vector. The resulting PCR product was cloned into pTA2.1 (Invitrogen). Sequencing reactions covered both strands of the genomic and cDNA copies of *TRI12* and were performed using the DYEdeoxy sequencing kit (Applied Biosystems). The GenBank accession number for *TRI12* is AF011355.

Gene disruption and fungal transformation

Construction of the *TRI12* disruption plasmid began with plasmid pFSC3-5 (Alexander et al. 1998). A *Hpa*I site (underlined) was first introduced into the *TRI12* coding region, using Expand PCR (Boehringer Mannheim) with the divergent primers 660 (5′-GGCGTTAACTGATCTGTAATATCCGTC-3′) and 661 (5′-GGCGTTAACCCTCGCATCGTGGCGTCC-3′), at a site located 118 bp downstream from the start codon. This was followed by the cloning of a chimeric hygromycin B phosphotransferase gene containing promoter1 from *Cochliobolus heterostrophus* (Turgeon et al. 1987) into this site as a blunt-ended fragment. The resulting plasmid, pTri12D-247 (*tri12::hygB*), was used to transform *F. sporotrichioides* by previously described procedures (Hohn et al. 1993). A total of 59 transformants were isolated and, based

on PCR analysis, two of these proved to have gene replacements resulting in *TRI12* disruption. Southern analyses were also performed using *Hind*III-digested genomic DNA and a radioactive probe made using primers 653 (5′-CTTGTACGAGGTCTATGG-3′) and 687 (5′-CGCGCGGATACCGGTTGG-3′) which lie outside of the cloned fragment. Trichothecene production was analyzed as described by McCormick et al. (1996).

TRI12 expression in yeast

Plasmids for *TRI12* expression in yeast were constructed using the cloned cDNA in pTri12-371. The coding region was excised using *Bam*HI and *Xba*I, and cloned into the same sites in the yeast expression vector pYES2 (Invitrogen). The resulting expression cassette containing the *GAL1* promoter, *TRI12* coding sequence, and the *CYC1* termination sequences was amplified using *Pfu* polymerase and primers 861 (5′-GCCCGTACGGCCTCC-TAGGCCACTAGTACGGATTAGAAG-3′) and 860 (5′-GCCC-GTACGCACAGGGTGAGAGCGCCCAATACGCAAACC-3′). Following digestion with *Bsi*WI (sites in primers are underlined), this fragment was cloned into the *Asp*718 site in plasmid pYIplac128 (Gietz and Sugino 1988). The resulting plasmid, carrying the *LEU2* gene as a selectable marker, was designated pTri12Y1-2. Construction of the *TRI3* yeast expression plasmid was accomplished by cloning the *Eco*RI-*Hind*III (filled in) restriction fragment, containing the *TRI3* coding region, from pTri3Exp1 (McCormick et al. 1996) into the *Ecl*136II site of pYES2 to yield pTri3Y1 (*URA3*). Yeast transformations were performed as described (Gietz et al. 1992).

Analysis of yeast transformants

Trichothecene feeding studies were performed with yeast transformants grown on supplemented glucose minimal media (10 ml) for 2 days at 28°C. Cultures were centrifuged and the cells resuspended in YGal (1% yeast extract, 2% peptone, and 2% galactose) to induce gene expression. The TRI3 substrate 15-decalonectrin was added after 2 h and the cultures were harvested at various time points. Following centrifugation, the supernatant and cell pellet fractions were analyzed for 15-decalonectrin and calonectrin by gas-liquid chromatography (GLC) as described in McCormick and Hohn (1997). Cell viability in treated cultures was checked by comparing hemocytometer counts with colony plate counts on complete medium.

Toxin sensitivity was tested by growing the transformed yeast cells in 1 ml of supplemented minimal medium overnight, centrifuging, and resuspending in 1 ml of YGal for 2 h to induce plasmid gene expression. The cells were then centrifuged again, resuspended in 50 µl of YGal, and 5 µl was spotted onto plates containing variable amounts of diacetoxyscirpenol (DAS). Trichothecene toxins were prepared as previously described by McCormick and Hohn (1997) and dissolved in acetone before addition to the media. Crystal violet was purchased from Sigma.

Results

Isolation and nucleotide sequence analysis of *TRI12*

Plasmid pFSC3-3 (Alexander et al. 1998) contains a 9.5-kb *Sac*I fragment that overlaps the cluster of trichothecene pathway genes (Fig. 1). Previously, we had shown that *TRI11* is located within the 2.9-kb *Sac*I-*Hind*III fragment at the 5′ end of FSC3-3. In an effort to identify additional, closely linked pathway genes, we cloned and sequenced the 3.5-kb *Hind*III fragment that lies directly upstream from *TRI11* (pFSC3-5, Fig. 1). Analysis of the FSC3-5 sequence revealed a long ORF

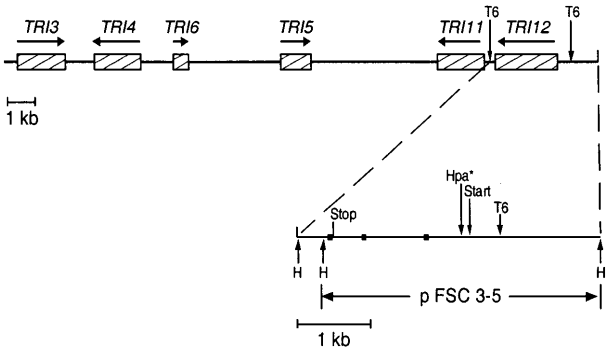

Fig. 1 Map of the trichothecene pathway gene cluster showing the location of *Tri12* relative to other pathway gene coding regions. The direction of transcription is indicated by the *arrows*. *Below* the map is an expanded region showing the *Hin*dIII fragment cloned in pFSC3-5. *Hin*dIII restriction sites (H), translational start and stop codons are indicated; the location of the *Hpa*I site added for construction of the disruption plasmid is shown (Hpa*); *solid boxes* represent the location of introns; the locations of TRI6 binding sites are also indicated (T6)

encoding a portion of the gene that was subsequently designated *TRI12*. Transcription of *TRI12* proceeds in the same direction as in *TRI11*.

The complete sequence of *TRI12* is presented in Fig. 2. Based on the cDNA sequence, *TRI12* predicts a protein of 598 amino acids with a molecular weight of 64.5 kDa. Comparison of the cDNA and genomic sequences revealed the presence of three introns of 56, 49, and 59 bp (Fig. 2). Interestingly, the 59-bp intron occurs in the long 3′ flanking sequence of the *TRI12* transcript that extends 397 bp beyond the translation stop codon. The polyadenylation site for *TRI12* is located only 90 bp upstream from the start of the *TRI11* coding region (Alexander et al. 1998).

A sequence that agrees with the consensus binding sequence (TNAGGACCT, Hohn et al., submitted for publication) for TRI6, a trichothecene pathway transcription factor (Proctor et al. 1995b), was identified 405 bp upstream of the *TRI12* start codon. Two putative TRI6 binding sequences are also located within the 3′ flanking sequence of the *TRI12* transcript at positions 160 and 258 bp upstream from the *TRI11* translational start. Binding of TRI6 to short PCR fragments carrying these sequences was recently demonstrated in vitro (Hohn et al., submitted for publication).

Sequence comparisons

The predicted *TRI12* gene product was compared to proteins in the PIR database using BLAST (Altschul et al. 1990). This comparison indicated that TRI12 is similar to a number of MFS transporters and multidrug resistance proteins from fungi and bacteria (Paulsen et al. 1996). One of the best alignments was observed with SGE1 from yeast, which has been shown to mediate crystal violet resistance in yeast (Ehrenhofer-Murray et al. 1994). The alignment between these two protein

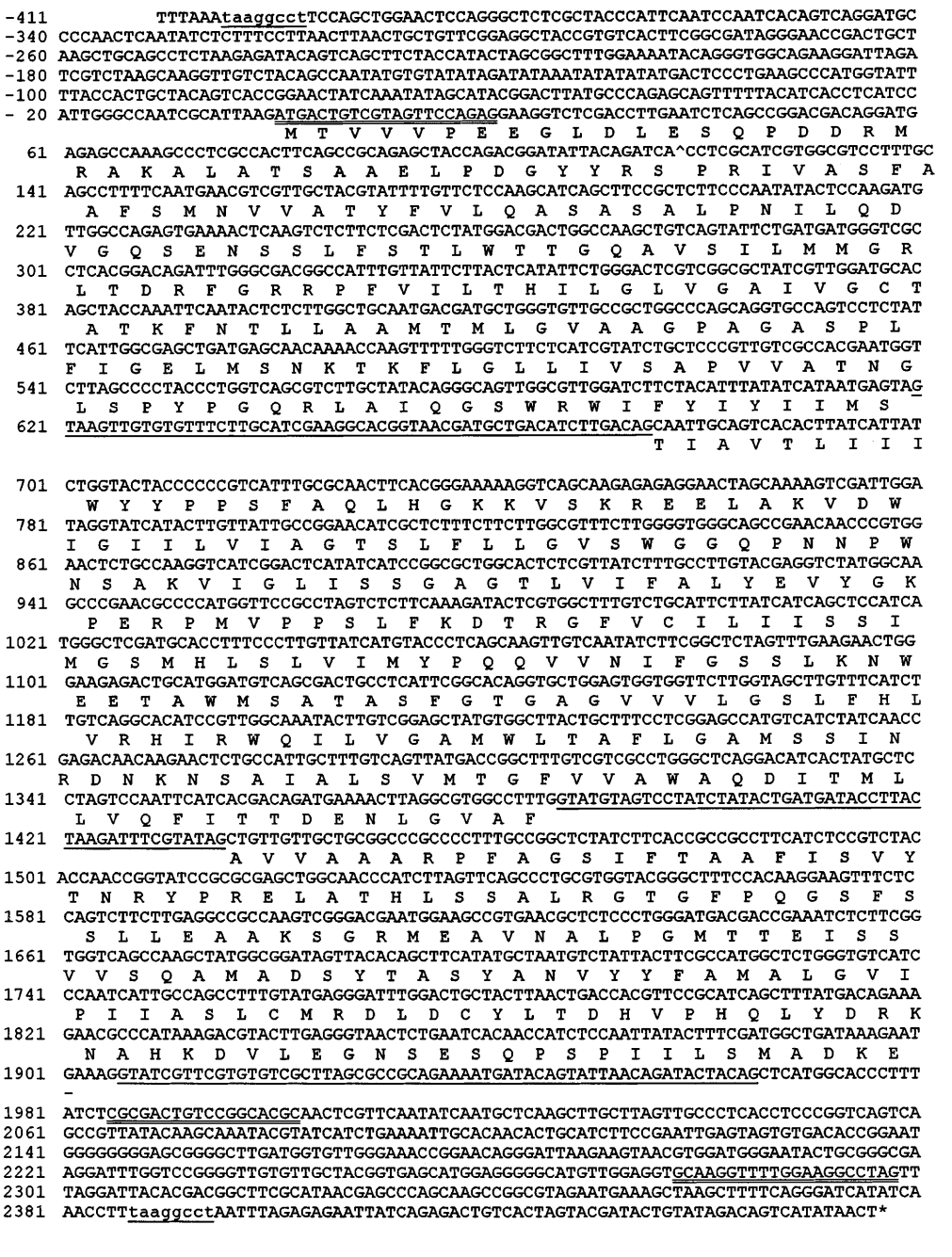

Fig. 2 Nucleotide sequence of *TRI12*. Introns and TRI6 binding sites (*lower case*) are *underlined*. Amino acids are represented by the single-letter code *below* the corresponding codon. The start of the polyA tract is indicated by the *asterisk*. Primers used for cloning are *doubly underlined* (767, 777 and 778, in that order). The site of the gene disruption is indicated by^. GenBank accession number AF011355

sequences revealed a 352-residue overlap with 21% identity.

Hydrophobicity analysis of TRI12 using the TMpred program (Hofmann and Stoffel 1993) predicts 14 transmembrane segments (TMS). Other proteins with sequence similarity were TOXA (*C. carbonum*) and CFP (*Cercospora*), both of which are members of the major facilitator superfamily.

Disruption of *TRI12*

To investigate the role of *TRI12* in trichothecene biosynthesis, *TRI12* mutants were generated by gene disruption. Disruption of *TRI12* was confirmed by PCR using primers corresponding to sequences outside of the cloned *TRI12* gene present in the disruption plasmid. These primers produced a band of approximately 4.8 kb in the wild type (NRRL 3299), whereas a band of approximately 7.2 kb was seen in transformant TRI12D-A28 (Fig. 3). This result is consistent with the presence of the 2.5-kb hygromycin gene disruption fragment in the *TRI12* gene of TRI12D-A28. Southern analysis confirmed the insertion of the disruption fragment within *TRI12* and the absence of other plasmid integration events (data not shown).

Cultures of *F. sporotrichioides* NRRL 3299 grown on GYEP medium for 7 days accumulated primarily T-2

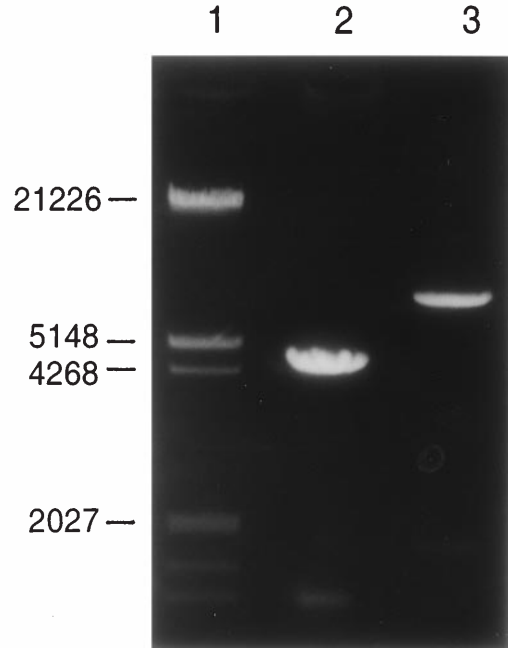

Fig. 3 Analysis of *TRI12* disruption in *F. sporotrichioides* by PCR. DNAs from transformant TRI12D-A28 and the wild-type strain NRRL 3299 were used as templates with primers 849 (5′-AGCGACAAGCCCTTTGACTTTCCTGATG-3′) and 613 (5′-CCACAAGATATGCAAGCC-3′). PCR products were separated on a 1% agarose gel (40 mM Tris-acetate, 1 mM EDTA) and stained with ethidium bromide. Lane 1, λ DNA cut with *Eco*RI and *Hin*dIII; lane 2, wild type NRRL 3299; lane 3, TRI12D-A28

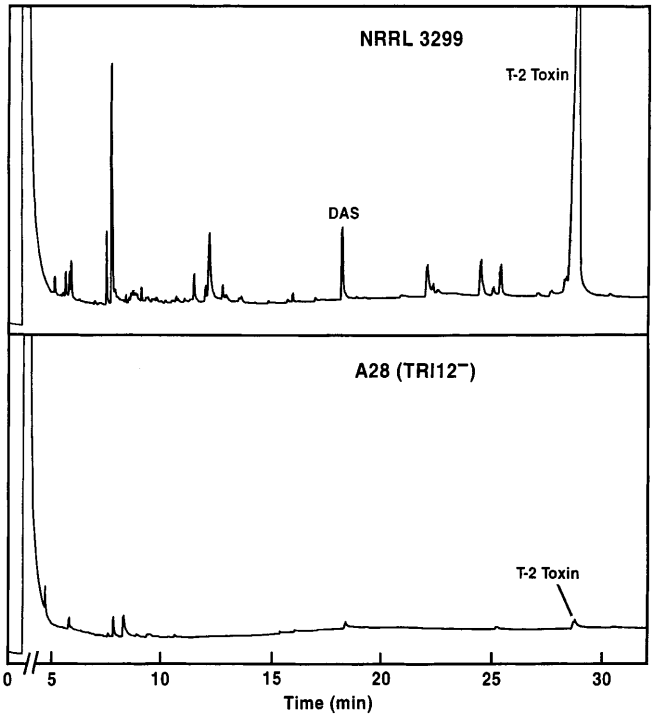

Fig. 4 Gas-liquid chromatography (GLC) of supernatants from 7-day cultures of *F. sporotrichioides* NRRL 3299 and disruption mutant TRI12D-A28, grown in GYEP medium. The positions of DAS and T-2 toxin are indicated

toxin (80%), diacetoxyscirpenol (10%), and neosolaniol (5%). With the exception of small amounts of T-2 toxin representing less than 3% of wild-type production, these trichothecenes were not observed in the *tri12* disruptants grown in GYEP. In addition, no trichothecene pathway intermediates were detected (Fig. 4).

To determine if the *tri12* disruptants, TRI12D-A28 and TRI12D-B9, exhibited increased sensitivity to trichothecene toxins, freshly harvested spores were used to inoculate solid and liquid media containing different concentrations of diacetoxyscirpenol (DAS). As shown in Fig. 5, the two mutants grew more slowly than the wild type in all of the media tested. Increasing concentrations of DAS resulted in higher levels of growth inhibition. This result indicates that TRI12-deficient mutants are sensitive to trichothecene. A low degree of growth inhibition in response to DAS-containing media was also observed for the wild type and a *tri4* mutant (5493). Germination of mutant and wild-type spores occurred at about the same rate in liquid media, with or without DAS (data not shown).

Effects of *TRI12* expression in yeast

In *S. cerevisiae*, *PDR5* encodes a putative ATP-dependent plasma membrane protein that confers resistance to multiple inhibitors (Balzi et al. 1994). Recently it has been shown that PDR5 functions as the primary source of trichothecene resistance in yeast (Adam and Lemmens 1996). Because some antibiotic transporters have been implicated in producer organism self-protection (Cundliffe 1989), we tested whether *TRI12* could protect

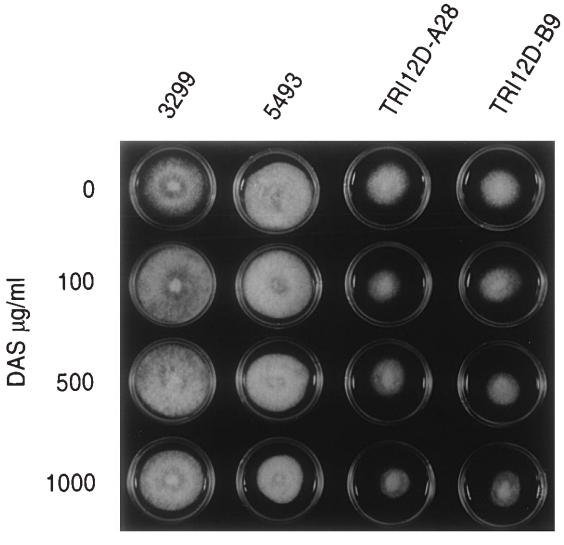

Fig. 5 Growth of *TRI12* disruption mutants TRI12D-A28 and TRI12D-B9 on GYEP medium containing 0, 100, 500, or 1000 µg/ml DAS. 3299, *F. sporotrichioides* NRRL 3299 (wild type); 5493, strain MB 5493 (*TRI4*⁻) a UV mutant derived from *F. sporotrichioides* NRRL 3299

yeast cells against the effects of trichothecenes by introducing *TRI12*, on the plasmid pYES2, into a yeast strain lacking a functional *PDR5* (*pdr5*). The yeast *GAL1* promoter in the pYES2 construct was used to regulate expression of *TRI12*. Transformants carrying *TRI12* did not grow any better than transformants carrying the expression vector alone on YPGAL plates containing 50 mg/ml DAS (Fig. 6). These results indicate that *TRI12* probably plays a relatively minor role in *Fusarium* self-protection from trichothecenes. Because the TRI12 sequence is similar to that of SGE1, which confers resistance to crystal violet (Ehrenhofer-Murray et al. 1994), the transformed strain was also tested for sensitivity to crystal violet (CV, at 5, 10, 25, 50, 100 μg/ml). *TRI12* did not confer resistance to this growth inhibitor.

Analysis of yeast transformed with both *TRI12* and *TRI3*

To determine if *TRI12* functions in some aspect of trichothecene biosynthesis, a yeast strain carrying *TRI12* (*pdr5*) was fed trichothecene pathway intermediates, and the cultures were analyzed for evidence of trichothecene metabolism. No evidence of trichothecene metabolism was detected (data not shown). To rule out the possibility that the pathway intermediates were excluded by the cell wall/cell membrane barrier in these experiments, and to demonstrate that TRI12 functions as a trichothecene transporter, feeding studies were also done on yeast co-transformed with *TRI3* and *TRI12*. *TRI3* encodes a trichothecene 15-*O*-acetyltransferase that converts 15-decalonectrin to calonectrin in the trichothecene pathway (McCormick et al. 1996). The yeast host strain used in these experiments is unable to metabolize either 15-decalonectrin or calonectrin (Fig. 7). Yeast transformants were constructed in both *PDR5* and *pdr5* backgrounds carrying the *TRI3* gene alone or in combination with *TRI12*. The transformants were grown in medium containing 15-decalonectrin, and the supernatants were analyzed for accumulation of the TRI3 reaction product, calonectrin. The degree of onversion of 15-decalonectrin to calonectrin by yeast (*PDR5* or *pdr5*) containing *TRI3* alone was consistently less than 50% after 70 h (Fig. 7). In contrast, 15-decalonectrin conversion by the double transformants (*TRI12*/*TRI3*) was typically 70 to 100% over the same time period. Even greater differences in the amount of 15-decalonectrin conversion due to the presence of *TRI12* were observed at earlier time points. In Fig. 7, the *TRI12*-containing transformants converted between 35 and 80% of 15-decalonectrin by 28 h, while conversion was less than 10% for transformants without *TRI12*. This time point corresponds to the early log phase of growth following the switch to the galactose-containing medium. PDR5 appeared to have no effect on 15-decalonectrin conversion rates in yeast strains carrying only *TRI3*, but significantly increased calonectrin accumulation in the *Tri12*/*Tri3* strains. To ensure that the potentially toxic effects of 15-decalonectrin and its conversion product calonectrin did not affect the results, viability studies were performed under the conditions used for the feeding studies. Data for cell viability were similar for all of the yeast transformants (data not shown).

Discussion

The identification of *TRI12* as a pathway gene located within the trichothecene gene cluster of *F. sporotrichioides* increases to six the number of genes for which a specific role in trichothecene biosynthesis has been clearly defined. *TRI12* is located immediately upstream of *TRI11* and is transcribed in the same direction. Evi-

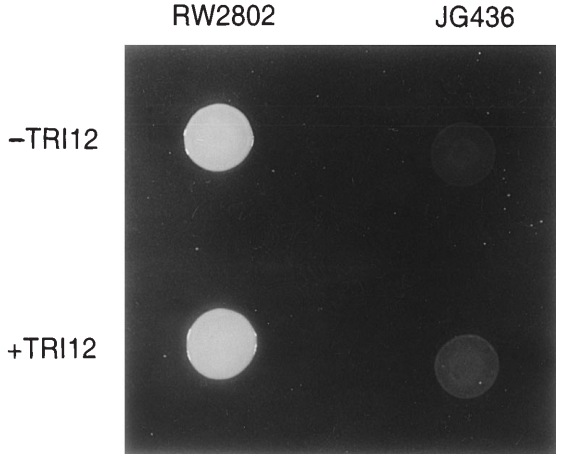

Fig. 6 Growth of yeast transformed with *TRI12* on YGAL medium containing 50 μg/ml of the trichothecene DAS. Yeast strains RW2802 (*PDR5*) and JG436 (*pdr5*) were transformed with a *TRI12* expression plasmid

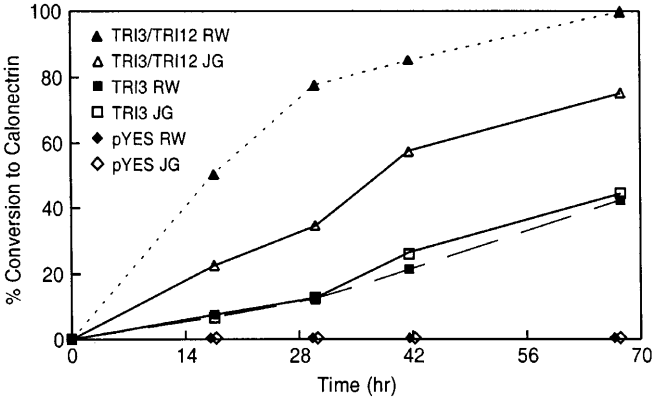

Fig. 7 Conversion of 15-decalonectrin to calonectrin by yeast transformed with both the *TRI12* and *TRI3* genes or with *TRI3* alone. Cultures grown in a supplemented glucose minimal medium were transferred to YGal at time 0. Conversion of the TRI3 substrate, 15-decalonectrin, to calonectrin was monitored by GLC analysis. RW, yeast host strain RW2802 (*PDR5*); JG, yeast host strain JG436 (*pdr5*)

dence that *TRI12* functions in trichothecene efflux includes its sequence similarity to MFS proteins from fungal and bacterial sources (Paulsen et al. 1996). The structure of TRI12 predicts an integral membrane protein consisting of 14 transmembrane segments (TMS); this is also in agreement with the structures of other MFS proteins, and suggests that TRI12 is a member of the 14-TMS group within the MFS of transporters.

The best evidence that *TRI12* encodes a functional trichothecene efflux protein comes from the effects of co-expression of *TRI12* and *TRI3* in yeast, the latter being the gene for trichothecene 15-*O*-acetyltransferase. Expression of *TRI3* alone in yeast results in the slow accumulation of the product of TRI3 activity, calonectrin, in culture supernatants. We reasoned that if the influx of 15-decalonectrin in these transformants is due primarily to passive diffusion processes, then the expression of a trichothecene efflux pump could dramatically increase trichothecene flux in transformed yeast cells. Increased trichothecene flux should, in turn, result in higher rates of 15-decalonectrin acetylation and subsequent increases in the accumulation of calonectrin. Co-expression of *TRI3* and *TRI12* did indeed significantly increase the accumulation of calonectrin in culture supernatants relative to strains expressing only *TRI3* (Fig. 7).

Differences in decalonectrin to calonectrin conversion rates by yeast were observed between *TRI12*/*TRI3* transformants depending on whether or not the yeast host strain carried a functional *PDR5* gene. Because its apparent role in yeast trichothecene resistance implies that PDR5 can function in trichothecene efflux, its presence might be expected to increase the levels of calonectrin accumulation in culture supernatants. Surprisingly, *PDR5* appeared to have no effect on transformants carrying only *TRI3*. Calonectrin accumulation by *TRI3* transformants was the same in both *pdr5* and *PDR5* backgrounds. The reason for the *PDR5* effect in *TRI12*-containing yeast strains is presently unclear, but it is possible that a synergistic interaction takes place between TRI12 and PDR5. Taken together, these results indicate that TRI12 facilitates the efflux of trichothecenes in transformed yeast. The successful expression of *TRI12* in yeast represents the first reported use of yeast as a host system for characterizing MFS proteins.

The levels of trichothecene production and the growth rate on complex media are greatly reduced for *tri12* mutants in *F. sporotrichioides*. Loss of transporter function has also been observed to reduce antibiotic production in some bacteria (Grgurina et al. 1996). Growth of the *tri12* mutants on trichothecene-containing media was reduced compared to growth on a medium without trichothecenes. This result may reflect a contribution by *TRI12* to *Fusarium* self-protection, but the interpretation is complicated by the observation that trichothecene-containing media also cause inhibition of growth of the wild type and a *tri4* mutant. The fact that TRI12 expression fails to protect yeast from the toxic effects of trichothecenes suggests a modest role, at best, for this transporter in *Fusarium* self-protection. In contrast to *TRI12*, the loss of *TOXA* in the HC toxin pathway of *C. carbonum* is lethal. *TOXA* is a pathway-specific transporter that has been proposed to play a role in toxin self-protection (Pitkin et al. 1996).

Microbial resistance to antibiotics can be achieved by several different mechanisms other than the use of antibiotic transporters (Cundliffe 1989). Metabolism of antibiotics and modification of antibiotic target sites are two additional means of protection. Both of these mechanisms have been shown to be effective for trichothecene protection in yeast (Fried and Warner 1981) and *Fusarium* (Kimura et al. 1998a; McCormick et al., submitted). Expression of *TRI101*, which encodes a 3-*O*-acetyltransferase that functions as a component of trichothecene self-protection (Kimura et al 1998a, 1998b, 1998c; McCormick et al., submitted), provides transformed yeast with a high degree of protection against trichothecenes. Construction of *TRI12*/*TRI101* double mutants in *F. sporotrichioides* may help in characterizing the contribution of TRI12 to self-protection.

Several structural features of the *TRI12* gene are noteworthy. The presence of an intron in the relatively long (397 bp) 3′ flanking sequence of the *TRI12* transcript is unusual. Although introns have been reported in the 5′ flanking regions of fungal transcripts, we were unable to find any report of introns located in the 3′ flanking region. It is also interesting that the 3′ flanking sequence of *TRI12* overlaps the promoter region of *TRI11*. Evidence for this comes from the short distance (90 bp) between the polyadenylation site of *TRI12* and the translational start of *TRI11*. In addition, two copies of the consensus binding sequence for the pathway transcription factor TRI6 are found within the region of overlap (Hohn et al., submitted for publication). A copy of the TRI6 binding sequence also occurs 405 bp upstream of the *TRI12* coding region, suggesting that, like other trichothecene pathway genes, *TRI12* expression is positively regulated by TRI6.

TRI12 is the third MFS-type protein identified as part of a fungal toxin pathway. The other two, TOXA (Pitkin et al. 1996) and CFP (Callahan and Upchurch 1995), are involved in pathways for structurally unrelated toxins, one of which is a cyclic peptide (TOXA) and the other a polyketide (CFP). These observations indicate that MFS proteins may be common in fungal toxin biosynthetic pathways. Because fungal toxin pathway genes are frequently organized in gene clusters, the development of molecular approaches for isolating genes encoding MFS proteins could facilitate the identification of closely linked pathway genes.

Acknowledgements We gratefully acknowledge Marcie Moore, Kim McDonald, and Tristan Paige for their excellent technical assistance. We also thank Larry Tjarks (Analytical Chemistry Support, NCAUR) for oligonucleotide preparation and sequencing reactions. The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned. All experiments were performed in accordance with Recombinant DNA Guidelines established by the U.S. Government.

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