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A Trichothecene Efflux Pump Encoded by *Tri102* in the Biosynthetic Gene Cluster of *Fusarium graminearum*

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Trichothecenes are toxic secondary metabolites produced by fungi such as *Fusarium*, *Myrothecium*, and *Trichothecium* species. These mycotoxins inhibit protein synthesis in eukaryotes by binding to the 60S ribosomal subunit[1]. We have previously found that 3-*O*-acetylation is a resistance mechanism in the producing organism and proposed to classify trichothecenes by the presence (t-type) or absence (d-type) of the 3-hydroxyl group[2]. They could also be reasonably divided into two classes by the presence or absence of a keto group at the C-8 position (type A; *e.g.*, T-2 toxin produced by *Fusarium sporotrichioides*, and type B; *e.g.*, deoxynivalenol (DON) produced by *F. graminearum*)[1].

The biosynthetic mechanism of the trichothecenes has been studied mainly in *Fusarium* species by both chemical and genetic approaches[3]. Genes involved in the biosynthesis proved to be clustered in the genome of the producer[4], and so far, three biosynthetic genes (*Tri3*, *Tri4*, and *Tri11*) and one regulatory gene (*Tri6*) have been identified on cosmids containing *Tri5*[5], which codes trichodiene synthase. Here we report the isolation and analysis of *Tri102*, a novel gene for the toxin transport, in the biosynthetic gene cluster of *F. graminearum*.

Results and Discussion

While sequencing pCosTr032[2], which includes a part of the gene cluster for trichothecene biosynthesis, we found a region with a significant similarity to the major facilitator superfamily (MFS)[6] which consists of some groups that confer resistance to antibiotics. This region appeared to be transcribed in the fungus throughout the vegetative stage as revealed by reverse transcription (RT)-PCR analysis (data not shown). Comparison of the genomic and cDNA sequences identified a novel gene, *Tri102*, 642 base pairs upstream of *Tri11*[5]. *Tri102* was located in the same orientation of *Tri11*, encoded a membrane protein of 598 amino acid residues, and was interrupted by two introns (see accession no. AB024617). The predicted protein sequence showed significant similarities to SGE1 from *Saccharomyces cerevisiae*[7] and TOXA from *Cochliobolus carbonum*[8] (Fig. 1A), which have been identified as drug resistance efflux pumps lacking the ATP-binding cassette. Hydrophobicity analysis using the Kyte-Doolittle algorithm[9] predicted that TRI102 has 14 membrane-spanning regions, which is characteristic of the drug efflux family of MFS (Fig. 1B) conferring resistance to antibiotics.

Tri102 appeared to be present as a single copy in the genomes of *F. graminearum* and other t-type trichothecene producers. No homologs were detected by Southern blotting of genomic DNA of the d-type producer *Trichothecium roseum* and the non-producer *Fusarium* species using *Tri102* as a probe (Fig.2A). These results suggest that *Tri102* is involved in extrusion of trichothecenes across the fungal plasma membrane and so work as a self-defense mechanism for the producer. The presence of exogenous trichothecene in the culture medium of *F. graminearum* is known to induce expression of *Tri101*, which is responsible for 3-*O*-acetyltransferase of the trichothecenes[10]. However, we found that expression of *Tri102* was not influenced (or induced only to a limited extent) by DON added to the culture (Fig. 2B). This suggests that *Tri102* may not serve as the primary self-defense mechanism for *F. graminearum*.

To confirm whether or not *Tri102* functions as a drug efflux pump, we cloned the *Tri102* gene into *Schizosaccharomyces pombe ura4⁻294 h⁻* (ATCC38436) and examined the phenotype of *Tri102* in the heterologous host. In the presence of various concentrations of antibiotics, the growth of the wild-type and the *Tri102* transformant of *S. pombe* were monitored and MIC was determined essentially as described by ARIOKA *et al.*[11] As shown in Table 1, *S. pombe* cells carrying the *Tri102* expression vector were more resistant to trichothecenes than the parent strain. In contrast, the transformant did not show resistance to blasticidin S, aureobasidin A, and actinomycin D. These results indicate that *Tri102* is not a multidrug resistance protein but is likely to be a trichothecene-specific efflux pump.

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Fig. 1. Predicted amino acid sequence of TRI102.

(A) Amino acid sequence alignment of TRI102 with *S. cerevisiae* SGE1 and *C. carbonum* TOXA. Sequences were aligned using the CLUSTAL program[14]. TRI102 showed 39% and 42% of similarity to SGE1 and TOXA, respectively.

A

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Consensus  M..T..... ..........  .....A. .LP....SL ..I...LFS      50
Tri102/    MTATVHEKGV DLESQPDDRL RAQALATTAD ELPEGYYTSP RVIASFAGFS  50
TOXA/      MDEQIVSASS NVKDGVEKQP VKDREDVDAN VVPPHSTPSL PKISLISLFS  50
Sge1/      MKSTLSLTLC VI-------- ---------S LLLTLFLAAL DIVIVVTLYD  33

Consensus  I........F .G...A---- --.P.L.Q.. ....A..... A...L..G..  100
Tri102/    INVCATYFVL QASASA---- --LPNILQDI GQSENQGLFS TLWTMGQAVS  94
TOXA/      IVMSLGAAAF LGALDATVVA VLTPTLAQEF HSVDAVAWYG AIYLLMSGTT  100
Sge1/      TI-GIKFHDF -GNIG----- ----WLVTGY ----ALS--N AVFMLLWGRL  66

Consensus  ....G.L..E F....L.I.. .IV...GSLV .ALA....TL I.GR.VAG.G  150
Tri102/    ILVMGRLTDR FGRRPFVIAT HIIGLVGAIV GCTAKEFNTL LAAMTLLGVA  144
TOXA/      QPLFGKLYNE FSPKWLFITC LIVLQLGSLV CALARNSPTF IVGRAVAGIG  150
Sge1/      AEILGT--KE C----LMIS- VIVFEIGSLI SALSNSMATL ISGRVVAGFG  109

Consensus  AGGI.S.AL. VG.LIV...H R....T...I ...IA...GP .IGGA.....  200
Tri102/    AGPAGSSPLF VGELMSNKTK FLGLLT-VTI PTII-MSAGP YFGQSLSIQG  192
TOXA/      AGGILSGALN IVALIVPLHH RAAFTGMIGA LECVALIIGP IIGGAIADNI  200
Sge1/      GSGIESLAFV VGTSIVRENH RGIMITALAI SYVIAEGVGP FIGGAFNEHL  159

Consensus  .WRWCFYINL PIGAAV.AIL .F-------- --.W.PP..- ---.Y.....  250
Tri102/    NWRWIFYIYI -ISSAVAALL IV-------- --VWYHPP-- ---SFRQLHG  226
TOXA/      GWRWCFWINL PIGAAVCAIL LF-------- --FFHPPRS- ---TYSASGV  236
Sge1/      SWRWCFYINL PIGAFAFIIL AFCNTSGEPH QKMWLPSKIK KIMNYDYGEL  209

Consensus  .KAS..---- E.L.-KLD.I G-----AG-- .....L.--- ----------  300
Tri102/    KKARKR---- DELA-KLDWI G--------- IFLVTAG--- ----------  249
TOXA/      PR-SYS---- EILG-NLDYI G-----AG-- MIISSL---- ----------  259
Sge1/      LKASFWKNTF EVLVFKLDMV GIILSSAGFT LLMLGLSFGG NNFPWNSGII  259

Consensus  VCLF------ ---------- --SL.GL.WG GK..KP---W ------G--.  350
Tri102/    VSLF------ ---------- ---LLGVSWG GKPNNP---W ------N--S  269
TOXA/      VCL------- ---------- --SL-ALQWG GTKYK----W ------G--D  277
Sge1/      ICFFTVGPIL LLLFCAYDFH FLSLSGLHYD NKRIKPLLTW NIASNCGIFT  309

Consensus  G.I.GLL.-- ---------- -VFG...Q.. ..V.LF.--- ------L...  400
Tri102/    GKIIGLMTSG LGSLVVFALY EVFGKPVQPI IPPVLFKDTR GFVCILLISS  319
TOXA/      GRVVALLV-- ---------- -VFG------ --V-LF---- ------L---  292
Sge1/      SSITGFLS-- ---------- -CFAYELQSA YLVQLYQ--- ------LVFK  337

Consensus  .....----- ---------- --SS...W.- ...AM.AT.- ------..G.  450
Tri102/    IMGAMNLCLT ILYPQQVINI FGSSLKNWQ- ETAWMTATA- ------SFGT  361
TOXA/      ---SA----- ---------- --SGHQYWK- GEKALFPTR- ---------L  311
Sge1/      KKPTL----- ---------- --ASIHLWEL SIPAMIATMA IAYLNSKYGI  370

Consensus  .......--- -----.G.F. LI-----..I ..SIL.G..F G..F.AAL..  500
Tri102/    WAGVMIL--- -----GNVFH LI-----RHI RWQILVGAMW LTAFLGAMSS  398
TOXA/      LRQ------- -----RG-FL L--------- --SLFNGLCF GGVQYAALYY  337
Sge1/      IKPAIVFGVL CGIVGSGLFT LINGELSQSI GYSILPGIAF GSIFQATLL-  419

Consensus  .-------. .....A.IQ. ....G----- -.V.G..DF T.....FI..  550
Tri102/    V--------N RDNKNAAIAL SFFSG----- -FVVGWAQDI TMLMVQFITT  434
TOXA/      LPTWFQAIKG ETRVGAGIQM LPIVGAIIGV NIVAGITISF TGRLAPFIVI  387
Sge1/      ---------- ----SSQVQ- ---------- --ITSDDPDF QNK---FIEV  439

Consensus  ...L.....- -.Y.F.---- ..R..... Y....G.... .AF.G-----  600
Tri102/    DEDLGVAFC- -MYPYL---- YGRDVSNNNQ YPKEIGSHLT SAMRG-----  473
TOXA/      ATVLASVGSG LLYTFTPTKS QARIIGYQLI YGAGSGAGVQ QAFIGAQAAL  437
Sge1/      T--------- ---AF----- ---------- ---------- NSF-------  445

Consensus  --.D...AS. ..LL.....G .I..------ -.......T. ....V.K..L  650
Tri102/    --TDIPQASF PSLLEAAKTG RIDA------ -IKALPGMTN STATVVSQAM  514
TOXA/      DPADVTYASA SVLLMNSMSG VITLCVCQNL FTNRINALTE VLPGVTKETL  487
Sge1/      ---------- ---------- ---------- ---------- -----AK-SL  449

Consensus  ..GFAF---- ------..L. .......LGV --...I.TL- -...----..  700
Tri102/    ADSYTA---- ------SYAN VYYFAMALGV --IPIIASL- -CMR----DF  546
TOXA/      QSGFAF---- --------LR STLTPAEFGV ----AIQT-- -FNS----AI  514
Sge1/      --GFAFGGNM GAMIFTASLK NQMRSSQLNI PQFTSVETLL AYSTEHYDGP  497

Consensus  Q..L..F... AI.DV..CA. --.VL...F. ...SSK...- ..K...  746
Tri102/    DRYLTDHVPH QIYDRKKADK --DVLEGDSD TPSSSTIHS- TIEVKA  589
TOXA/      QDA---FL-V AI--VLSCA- --SVLGWPFL SWASVKGQK- --KMNK  548
Sge1/      QSSLSKFINT AIHDVFYCAL GCYALSFFFG IFTSSKKTTI SAKKQQ  543
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Fig. 1. (Continued)

(B) Hydropathy plot of TRI102. Bold lines indicated the predicted transmembrane domains.

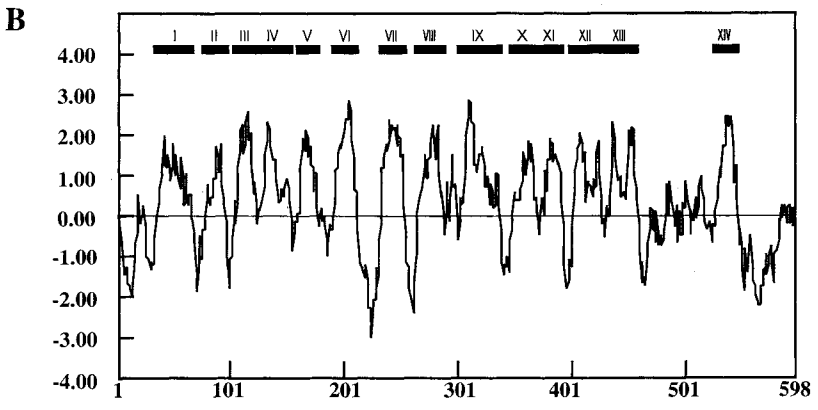

Fig. 2. DNA and RNA hybridization analyses of *Tri102*.

The probe was prepared by the PCR-DIG system with primers FgTr102-U1 (5′-TCCAAAATGACTGCT-ACAGTT-3′) and FgTr102-D4 (5′-TGGCAATTACCTTATGCCTTAACC-3′). (A) Southern analysis. Genomic DNA of each fungus digested with *Hin*dIII was hybridized to the *Tri102* probe. *F. graminearum* (lane 1), *F. croockwellense* (lane 2), *F. sporotrichioides* (lane 3) are t-type trichothecene producers. *T. roseum* (lane 4) is a d-type trichothecene producer. *F. moniliforme* (lane 5) and *F. oxysporum* (lane 6) are the non-producers. (B) Northern analysis. *F. graminearum* was incubated with or without 100 μg/ml of DON on YEL medium (0.5% yeast extract, 3% glucose) for 6 hours. Total RNA samples (20 μg) were run on a 0.8% agarose gel (stained with ethidium bromide; left panel), and then hybridized with the *Tri102* probe after transfer to the nylon membrane(right panel).

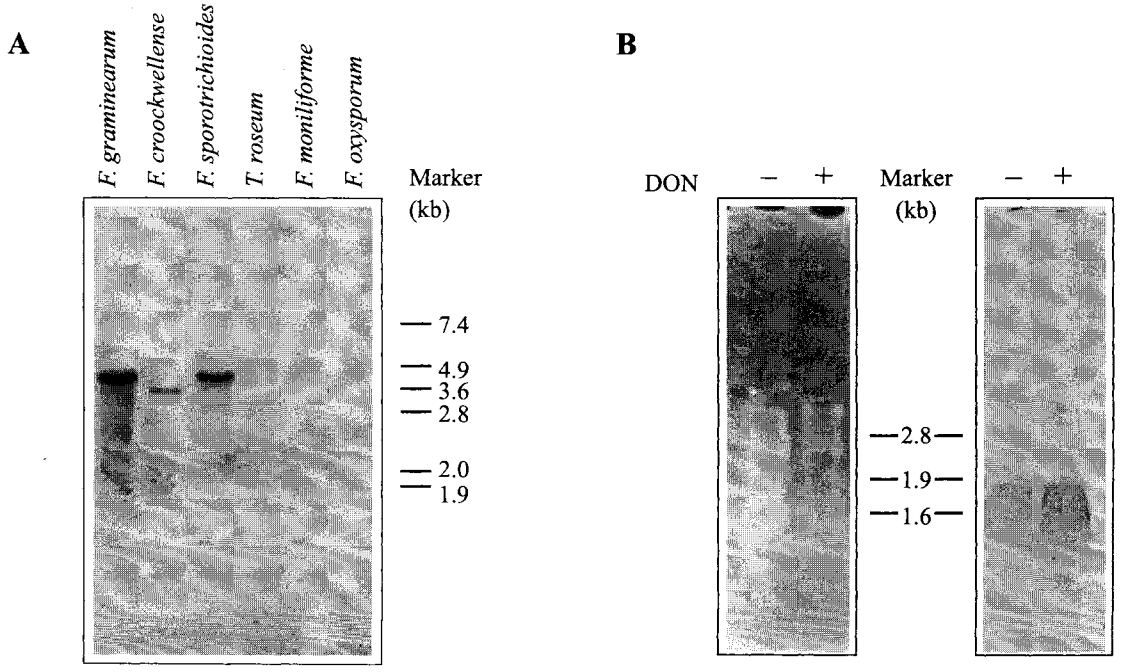

Table 1. Comparison of minimum inhibitory concentration for several drugs in *S. pombe* cells carring *Tri102* gene.

Strain	Drugs (μg/ml)						
	T2-Toxin	DON	Fusarenon X	Trichothecin	Blastisidin S	Aureobasidin A	Actinomycin D
Wild type	0.2	10	100	0.06	5	0.1	5
pcDTr102Fg	0.6	>100	>100	0.15	5	0.1	5

Cells were grown on a YEL medium containing 0.006 % SDS at 25°C for two days. MIC at which growth of cells (OD_{660}) was less than 10 % of that in the absence of drugs were determined.

Notably, DON appeared to be most effectively transported into the extracellular media by TRI102; *i.e.*, the MIC for the *Tri102* transformant was more than ten times higher than that for the parent strain. It might be interesting to examine if the substrate specificity of TRI102 has evolved to effectively extrude the toxin of the fungus possessing the efflux pump. A search of the NCBI database showed *Tri102* to be identical to *Tri12*, a putative efflux pump gene of *F. sporotrichioides* (see accession no. AF011355), but the function of *Tri12* has not yet been demonstrated. The specificity of the trichothecene efflux pumps from the *Fusarium* species that produce other types of trichothecene is now under study.

Experimental

The *Bam*HI, *Bgl*II, *Eco*RI, *Eco*T14I, *Hin*dIII, *Sal*I, and *Xba*I fragments of pCosTr032 were randomly sequenced and mapped to the cosmid clone. The DNA sequences were searched for similarities with BLASTX 2.0.3 program[12] at NCBI. The expression vector for *S. pombe*, pcDSP21-Tri102, was constructed by insertion of the *Tri102* cDNA into the *Not* I site of pcDSP21[13]. Transformants were selected by complementation of uracil auxotrophy as described previously[13].

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Note added in proof

While this manuscript was in the reviewing process, ALEXANDER *et al.* have published an original article which described about the same trichothecene efflux pump from *F. sporotrichioides* (ALEXANDER, N. J.; S. P. MCCORMICK & T. M. HOHN: Mol. Gen. Genet. 261: 977~984, 1999)

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