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The mystery of the trichothecene 3-*O*-acetyltransferase gene

Analysis of the region around *Tri101* and characterization of its homologue from *Fusarium sporotrichioides*

Makoto Kimura*, Gentaro Matsumoto, Yoshinori Shingu, Katsuyoshi Yoneyama[1], Isamu Yamaguchi

Microbial Toxicology Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako, Saitama 351-0198, Japan

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Abstract **The trichothecene 3-*O*-acetyltransferase gene, *Tri101*, plays a pivotal role for the well-being of the type B trichothecene producer *Fusarium graminearum*. We have analyzed the cosmids containing *Tri101* and found that this resistance gene is not in the biosynthetic gene cluster reported so far. It was located between the UTP-ammonia ligase gene and the phosphate permease gene which are not related to trichothecene biosynthesis. These two 'house-keeping' genes were also linked in *Fusarium* species that do not produce trichothecenes. The result suggests that the isolated occurrence of *Tri101* is attributed to horizontal gene transfer and not to the reciprocal translocation of the chromosome containing the gene cluster. Interestingly, 3-*O*-acetylation was not always a primary self-defensive strategy for all the t-type trichothecene producers; i.e. the type A trichothecene producer *Fusarium sporotrichioides* did not acetylate T-2 toxin in vivo although the fungus possessed a functional 3-*O*-acetyltransferase gene. Thus *Tri101* appears to be a defense option which the producers have independently acquired in addition to their original resistance mechanisms.**

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Key words: Trichothecene mycotoxin; Antibiotic resistance; Biosynthetic gene cluster; Acetyltransferase; Independent evolution; *Fusarium graminearum*

1. Introduction

Trichothecenes are the secondary metabolites of a group of fungi, including genera *Fusarium*, *Trichothecium*, and *Myrothecium* species [1]. The individual metabolites differ in the modification pattern of the 12,13-epoxytrichothec-9-ene (trichothecene) ring to form a variety of derivatives coupled with ketones, alcohols, and/or short-chain esters. They are chemically divided into two groups based on the presence (i.e. type B trichothecenes) or absence (i.e. type A trichothecenes) of a keto group at the C-8 position [2]. The in vitro toxicity of trichothecenes proved to be inhibition of protein synthesis in eukaryotes [2,3], and their presence in agricultural products often causes incidents of mycotoxicoses.

The production of trichothecenes by fungi is determined by both genetic and environmental factors. The trichothecene biosynthetic mechanism has been studied at the molecular level by both chemical and biological approaches [4]. An enzyme that catalyzes the first unique step in the trichothecene pathway, the isomerization-cyclization of farnesyl pyrophosphate, was purified from *Fusarium sporotrichioides* [5], and *Tri5* (formerly *Tox5*) encoding this enzyme has been identified and characterized [6,7]. Other biosynthetic genes including *Tri3* [8], *Tri4* [9], and *Tri11* [10], and a regulatory gene *Tri6* [11], have been isolated from the *Tri5*-containing cosmids that cover a part of the gene cluster [12].

We found that 3-*O*-acetylation of the trichothecene ring leads to its inactivation and confers resistance to the deoxynivalenol (3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one) producer *Fusarium graminearum* [13]. Thus this acetylation step on the biosynthesis (see [13]) was considered to serve as a self-defensive strategy for fungi that produce t-type trichothecenes (i.e. 3-hydroxy or 3-*O*-acetyl trichothecenes). Based on this finding, the responsible biosynthetic gene, *Tri101*, was isolated and characterized. Since the previously cloned biosynthetic genes [6,9,11] were not located near this resistance gene, the *Tri101*-containing cosmids were expected to cover other parts of the large biosynthetic gene cluster [13]. Here we have analyzed the region around *Tri101* and obtained unexpected results.

2. Materials and methods

2.1. Strains and media

F. graminearum F15 is a deoxynivalenol (type B trichothecene) producing strain from which *Tri101* was originally isolated [13]. This strain was used for characterization of the *Tri101*-flanking regions. *F. sporotrichioides* M-1-1 (IFO 9955; previously misidentified as *Fusarium solani*) [14] is a producer of T-2 toxin and neosolaniol (type A trichothecenes) and was used for analysis of *FsTri101*, a homologue of *Tri101*. *Fusarium oxysporum* Nara ichigo-3 (ATCC 60843) [15] was used to determine whether the adjacent genes are also linked in non-producing strains. Fungal cultures were maintained on potato dextrose agar (Nissui, Tokyo) and were inoculated in liquid YG medium (0.5% yeast extract, 2% glucose) for preparation of nucleic acids.

2.2. Nucleic acid hybridizations

Total DNA and RNA was isolated from the mycelia ground under liquid N_2 using the total DNA isolation kit and the SNAP total RNA isolation kit (Invitrogen, San Diego, CA, USA), respectively. DNA probes were generated by labeling fragments with digoxigenin using the PCR DIG probe synthesis kit (Boehringer Mannheim, Mannheim, Germany). RNA probes were prepared from the PCR product of the desired region cloned in pGEM-TEasy (Promega) using the DIG RNA labeling kit (SP6/T7) (Boehringer Mannheim). Standard hybridization techniques were used for Southern and Northern analysis [16].

*Corresponding author. Fax: (81) (48) 462-4676.
E-mail: mkimura@postman.riken.go.jp

[1]Permanent address: Faculty of Agriculture, Meiji University, Kawasaki 214-8571, Japan.

Abbreviations: Fgpho5, phosphate permease gene from *F. graminearum*; *Fgura7*, UTP-ammonia ligase gene from *F. graminearum*; *FsTri101*, trichothecene 3-*O*-acetyltransferase gene from *F. sporotrichioides*; RT-PCR, reverse-transcription PCR

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2.3. Sequence analysis of the Tri101-containing cosmids

The cosmid inserts of pCosTr135 and pCosTr137 [13] were separated from the vector by *Not*I digestion and purified by agarose gel electrophoresis using the Geneclean spin kit (BIO101, La Jolla, CA, USA). They were partially digested with *Alu*I or *Sau*3AI, and the fractions containing fragments between 0.5∼1.5 kb were subcloned into pBluescript vector. The ligation mixtures were transformed into *Escherichia coli* DH5α and the recovered plasmids were randomly sequenced using the Thermo Sequenase sequencing kit (Amersham, Buckinghamshire, UK). The nucleotide sequences were translated in all six reading frames and compared to the protein data bases by the BLASTX 2.0.3 program [17] (Swiss-Prot and GenBank) at NCBI. The DNA fragments that showed significant similarity with previously described ORFs were further analyzed; the sequences were determined on both strands, their expression confirmed by Northern analysis, and the locations on the cosmids determined by Southern analysis.

2.4. Polymerase chain reaction (PCR)

The candidates of ORFs identified in the *Tri101*-containing cosmids were analyzed by reverse-transcription PCR (RT-PCR) using the RNA PCR kit (Takara Shuzo, Shiga). The single-stranded cDNA was synthesized with avian reverse transcriptase and the oligo-dT adaptor supplied in the kit. The cDNAs were amplified by PCR with primers as follows: (i) FAD-U1 (5′-GATCTCGACATGGCCTTTGTCCCC-3′) and FAD-D1 (5′-GAACAGGTGGTGAATGACGTGCTTC-3′) for the putative fatty acid desaturase gene (partial); (ii) PHO-U1 (5′-ACTCTACAAGATGGCCCAAGAAGC-3′) and PHO-D1 (5′-CACCAAAGTGACACTCTCCTAAAC-3′) for *Fgpho5* (complete); (iii) URA-U1 (5′-ACGTAATGGCAGAAATTTGTCGTCC-3′) and URA-D1 (5′-CTCATCCATTCTAAAAGTTGGTGAC-3′) for *Fgura7* (complete); (iv) ADH-U1 (5′-CCAAGGGCGAGACCTACAAGGCTG-3′) and ADH-D1 (5′-GCGATGAGGACCTGAGCAAGGGTG-3′) for the putative dehydrogenase gene (partial).

The region between the phosphate permease gene and the UTP-ammonia ligase gene of *F. sporotrichioides* was amplified by a low-error-rate PCR system using the LA-PCR kit (Takara Shuzo). Specific amplification of this region was performed with primers PHO-U2 (5′-TTGACCATCATGCTCGGCATCGTCTAC-3′) and URA-U2 (5′-CTTCCTGAGCATCAGCGTTTCTTATGT-3′), which are derived from the coding sequence of these genes from *F. graminearum*.

2.5. Expression of FsTri101 in E. coli

Primers Fs101-U1 (5′-CACATCATCCATATGGTCGCAACG-3′) and Fs101-D1 (5′-CTCTGATCAATGGCTACTAGCTTC-3′) were used to amplify the ORF of *FsTri101* by PCR (mismatched bases are underlined). The amplified gene was cloned into pCR2.1 (Invitrogen) and confirmed by nucleotide sequencing. The *Nde*I (partial)-*Hin*dIII fragment of *FsTri101* in this plasmid was then transferred to the expression vector pET-23a (Novagen, Madison, WI, USA) and the resulting plasmid, pET-23a-*FsTri101*, was used for overproduction of the protein in *E. coli* HMS174 (DE3) (Novagen). Induction of the gene and preparation of the crude extract were exactly the same as described for recombinant TRI101 of *F. graminearum* [13].

2.6. Acetyltransferase assays

Trichothecene 3-*O*-acetyltransferase activity was assayed in vivo by germinating the fungal spores in the presence of 100 μg/ml T-2 toxin. The activity of recombinant FsTRI101 was examined in vitro using the crude extract of the IPTG induced bacteria. The reaction mixture comprised 250 μg/ml trichothecene, 1 mM acetyl-CoA, and the crude enzyme fraction in 10 mM Tris-HCl (pH 7.5). The samples were developed on a TLC plate (Merck F_{254} silica TLC) using ethyl acetate/toluene (3:1) as the solvent. Trichothecenes on TLC were detected as described previously [13].

3. Results

3.1. Sequence analysis of the Tri101-containing cosmids

While we were sequencing the 3-kb genomic DNA fragment containing *Tri101*, the 3′ end of the UTP-ammonia ligase gene that obviously is not related to trichothecene biosynthesis was identified upstream of this biosynthetic gene [18]. To examine whether *Tri101* defines either end of the trichothecene biosynthetic gene cluster, we analyzed the previously isolated cosmids as described in Section 2. Four candidates of

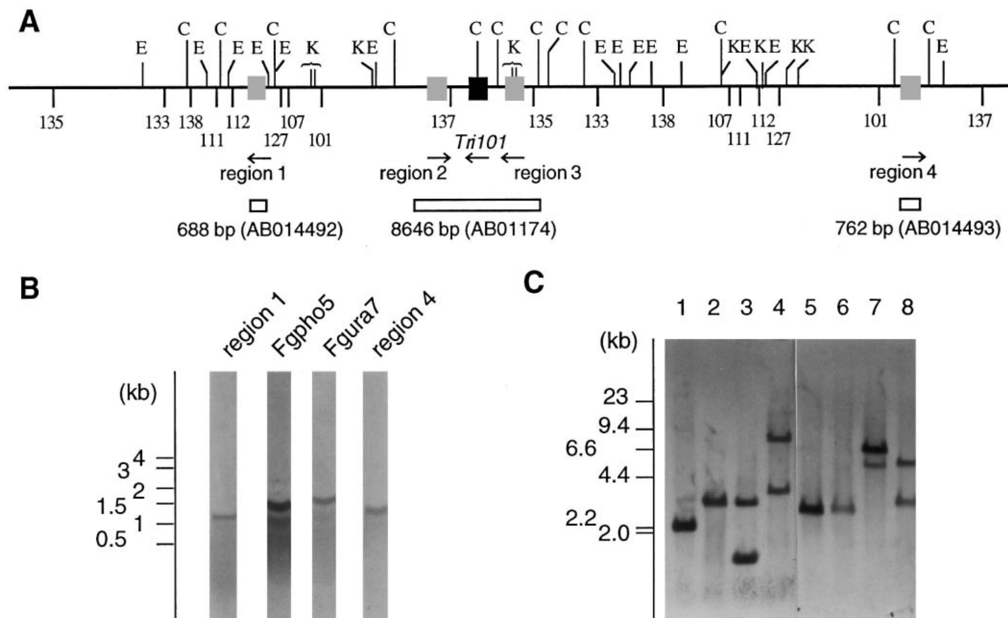

Fig. 1. Structural organization of the genomic DNA region on both sides of *Tri101*. A: Physical map of the cosmids. Both ends of each *Tri101*-containing cosmid are indicated by the cosmid number (e.g. 135 represents pCosTr135) on the map. The sequenced regions are shown by boxes with their accession numbers. C: *Cla*I; E: *Eco*RI; K: *Kpn*I. B: RNA blots hybridized to the probe indicated above each at 54 h after inoculation. Transcripts of *Tri4*, *Tri5*, and *Tri6* were never detected under the culture conditions of this experiment. C: Southern blots hybridized to *Fgpho5* (lanes 1–4) and *Fgura7* (lanes 5–8). The genomic DNA was digested with the following restriction enzymes. Lane 1: *Dra*I; lane 2: *Ssp*I; lane 3: *Bsp*1286I; lane 4: *Bst*XI; lane 5: *Cla*I; lane 6: *Sna*BI; lane 7: *Eco*RV; lane 8: *Nru*I.

A

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FgPHO5    -----------MAQEARPVAKTSGGNNAFHNFHNDFAHIADPNERRRLALAEIDKAPFGWYHVRACIVAGVGFFTDSYDIFCVSMLTIMLGIVYYPGKGK-LPTSSDNAIKLSTSAGTVIGQFGFGMLADIVGRKRMYGLELIVTIFATL  138
PHO5_Nc   --------------MSTP-QKTAGGNNAYHNFYNDFLHIKDPNERRRLALAEVDRAPFGWYHVRAVAVAGVGFFTDSYDIFTVSLLTLMLGIVYFQAKARCLQPPTQPSSSQHRPARSLGRSASAPLLMSLVVRA--------CTDWNCC  127
PHO84_Sc  MSSVNKDTIHVAERSLHKEHLTEGGNMAFHNHLNDFAHIEDPLERRRLALESIDDEGFGWQQVKTISIAGVGFLTDSYDIFAINLGITMMSYVYWHGSMP--GPSQTLL-KVSTSVGTVIGQFGFGTLADIVGRKRIYGMELIIMIVCTI  147
                                                                     TM1                                    TM2                 TM3

FgPHO5    AQALTAGSPSTSLVGLIIFWRV--IMGVGIGGDYP--LSSI--ITSEFATTKWRGAMMAAVFAMQGIGQLVAALVMMFLTLGFKSSLEQAADTKSCTGDCQIAVDKMWRTLVGFGAVPACIALYYRLTIPETPRYTFDVARDVEQADEDV  282
PHO5_Nc   SSSLPLSLRPWLRVTFHQHHRYHYLLACSYGGRYRWRLSSFQYITSEFATTKWRGAMMGAVFAMQGLGQLAAAFVMLFVTLGFKKSLEAAPTLASCTGDCAVAVDKMWRTVIGVGAVPGCIALYYRLTIPETPRYTFDVKRDVEQASDDI  277
PHO84_Sc  LQTTVAHSPAINFVAVLTFYRI--VMGIGIGGDYP--LSSI--ITSEFATTKWRGAIMGAVFANQAWGQISGGIIALILVAAYKGELEYANSGAECDARCQKACDQMWRILIGLGTVLGLACLYFRLTIPESPRYQLDVNAKLELAAAAQ  291
             TM4                               TM5                                        TM6

FgPHO5    KAYINGKSEGNTDEVTRAQNLQSAKTNLEV-----PKASWRDFFQHYSKWKNASLLLGTAGSWFCLDVAFYGLSLNNGTILKVIGYSTKDATNVYEFLHNTAVGNIIIVLAGAVPGYWVSVATIDTLGRKTIQLGGFIILTILFIVMGFA  427
PHO5_Nc   EAFKTGKPKGQPDEATRIVAKQEAEKEMEI-----PKASWGDFFRHYSRKKNAMLLAGTALSWFCLDIAYYGVSLNNATILNVIGYSSTGAKNTYEILYNTAVGNLIIVLAGAVPGYWVTVFTVDTVGRKPIQFMGFGILTILFVVMGFA  422
PHO84_Sc  EQDGEKKIHDTSDEDMAINGLERASTAVESLDNHPPKASFKDFCRHFGQWKYGKILLGTAGSWFTLDVAFYGLSLNSAVILQTIGYA--GSKNVYKKLYDTAVGNLILICAGSLPGYWVSVFTVDIIGRKPIQLAGFIILTALFCVIGFA  439
                                                           TM7                                    TM8                      TM9

Fgpho5    YNHIPSNGLLAIYVLAQFFFNFGPNTTTFIVPGEVFPTRYRSTSHGISAASGKIGSIIGQGAISILRTHGATDK--NEAPWMDHVLEIYALFMLLGIPTSLLIPETARKTLEELSGEDDYANHDHALESETQASHDKARRTSV-----  568
PHO5_Nc   YKHLSPHALLAIFVLAQFFFNFGPNATTFIVPGEVFPTRYRSTSHGLSAAMGKIGSIIGQGAIAPLRTRGAVKGG-NPNPWMNHVLEIYALFMLLGVGTTFLIPETKRKTLEELSGEFDMSGEEEAQRDTTLTEHKTEAPTSSAAVNA  569
PHO84_Sc  YHKLGDHGLLALYVICQFFQNFGPNTTTFIVPGECFPTRYRSTAHGISAASGKVGAIIAQTALGTLIDHNCARDGKPTNCWLPHVMEIFALFMLLGIFTTLLIPETKRKTLEEINELYHDEIDPATLNFRNKNNDIESSSPSQLQHEA  587
                  TM10                       TM11                             TM12
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B

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FgURA7    MKVVLVSGGVISGVGKGIIASSAGLLLKTLGLRVTAIKTDPYINTDAGLLNPLEHGECFVLDDGGETDLDLGNYERYLGIQLSRDSNITTGKIYKQVIEKERRGDYLGKTVQVVPHITDAIQDWIERVAKIPVDASGEAPDVCIIELGGT  150
URA7_Sc   MKYVVVSGGVISGIGKGVLASSTGMLMKTLGLKVTSIKIDPYMNIDAGTMSPLEHGECFVLDDGGETDLDLGNYERYLGVTLTKDHNITTGKIYSHVIAKERKGDYLGKTVQIVPHLTNAIQDWIERVAKIPVDDTGMEPDVCIIELGGT  150
URA8_Sc   MKYVVVSGGVISGIGKGVLASSTGMLLKTLGLKVTSIKIDPYMNIDAGTMSPLEHGECFVLDDGGETDLDLGNYERYLGITLSRDHNITTGKIYSQLISRERRGDYLGKTVQIVPHLTNAIQDWIQRVSKIPVDDTGLEPDVCIIELGGT  150

FgURA7    IGDLESGPFVEALSQLRHRLGRDNFLSISVSYVPIINGEEKTKPTQHAIRQVRSAGLIPDVIACRCERELDQATITKIARSCQVEDEQAIGVRNMDTIYQVPLLLEQEGLLKLLQKGLALDKCQVTPPMAQKGQALWDLWKKTVVP-DRH  299
URA7_Sc   VGDIESAPFVEALRQFQFKVGKENFALIHVSLVPVIHGEQKTKPTQAAIKGLRSLGLVPDMIACRCSETLDKPTIDKIAMFCHVGPEQVVNVHDVNSTYHVPLLLLEQKMIDYLHARLKLDEISLTEEEKQRGLELLSKWKATTGNFDES  300
URA8_Sc   VGDIESAPFVEALRQFQFEVGRENFALIHVSLVPVIHGEQKTKPTQAAIKDLRLLGLIPDMIACRCSEELNRSTIDKIAMFCHVGPEQVVNVHDVNSTYHVPLLLLKQHMIDYLHSRLKLGEVPLTLEDKERGSQLLTNWENMTKNLDDS  300

FgURA7    LEPVNIILVGKYVSLDDSYLSVHKALEHSAMRCNRKLNLVSVDSEHLEPEMQEKDPRKFHEAWAHVVRAQGIIVPGGFGTRGIQGMVDVAKWARERKLPYLGICLGMQTAVIEYARNVMGLKGATSEEFSAT----AEHRVVIFMPEGSK  445
URA7_Sc   METVKIALVGKYTNLKDSYLSVIKALEHSSMKCRRKLDIKWVEATDLEPEAQESNKTKFHEAWNMVSTADGILIPGGFGVRGTEGMVLAARWARENHIPFLGVCLGLQIATIEFTRSVLGRKDSHSAEFYPD--IDEKNHVVVFMPEIDK  448
URA8_Sc   DDVVKIALVGKYTNLKDSYLSVTKSLEHASMKCRRQLEILWVEASNLEPETQEVDKNKFHDSWNKLSSADGILVPGGFGTRGIEGMILAAKWARESGVPFLGVCLGLQVAAIEFARNVIGRPNSSSTEFLDETLLAPEDPSSHIHAEIDK  450

FgURA7    EQMGGTMRLGSRTSHFKPGTEWSKLRGLYGGVDVVEERHRHRYEVNPDYIEDLEKAGLSLTSMDDQGVRVETIELKDHPFFVGLQAHPEYKSKTLAPAPSLLGLVAASSGCLDEIIEAAHKKQSSSNGVSDVTNF      580
URA7_Sc   ETMGGSMRLGLRPTFFQNETEWSQIKKLYGDVSEVHERHRHRYEINPKMVDELENNGLIFVGKDDTGKRCEILELKNHPYYIATQYHPEYTSKVLDPSKPFLGLVAASAGILQDVIEGKYDLEAGENKFNF----      579
URA8_Sc   EHMGGTMRLGLRPTIFQPNSEWSNIRKLYGEVNEVHERHRHRYEINPKIVNDMESRGFIFVGKDETGQRCEIFELKGHPYYVGTQYHPEYTSKVLEPSRPFWGLVAQ----LRHTCEVIKDINLSEGNENE----      577
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Fig. 2. The deduced amino acid sequence of *Fgpho5* (A) and *Fgura7* (B). These sequences were compared to their homologues in the data base. Identical and similar amino acids are indicated on top of the sequence by asterisks (*) and colons (:), respectively. A: The deduced ORF of *Fgpho5* was interrupted by three introns and coded for a protein of 568 amino acids with a calculated MW of 61 843 Da. The protein data base search indicated that this protein sequence has a maximum identitiy of 60% with the high-affinity phosphate permease (PHO5) of *Neurospora crassa*. An alignment of FgPHO5 with the well characterized PHO84 of yeast (Swiss-Prot accession no. P25297) revealed nine highly conserved transmembrane domains (twelve TM domains of PHO84 are shown by bold underlines), but the sequence lacked the rest of the three transmembrane domains (TM2, TM3, and TM4 of PHO84). B: The deduced ORF of *Fgura7* was interrupted by 17 introns and coded for a protein of 580 amino acids with a calculated MW of 63 837 Da. FgURA7 showed a high degree of identity, 58% and 56%, with the yeast CTP-ammonia ligases encoded by *URA7* and *URA8*, respectively [34]. Conserved sequences in the glutamine amide transfer domain are boxed.

coding region were obtained and mapped to either upstream or downstream of *Tri101* (see Fig. 1A): the BLASTX 2.0.3 program [17] generated a statistically significant alignment of region 1 (688 bp, AB014492), 2 (1488 bp), 3 (1177 bp), and 4 (762 bp, AB014493) to the fatty acid desaturase gene (L43920), the phosphate permease gene (L36127), the UTP-ammonia ligase gene (X53995), and the aldehyde reductase gene (J04794) at an *E* value [17] of $4.5\times e^{-21}$, $2.0\times e^{-28}$, $2.0\times e^{-94}$, and $1.2\times e^{-45}$, respectively.

Based on the above result, we have determined the nucleotide sequence of the region (8646 bp, AB01174) between the phosphate permease gene (*Fgpho5*) and the UTP-ammonia ligase gene (*Fgura7*). The complete coding region of *Fgpho5* (see Fig. 2A and the legend), *Tri101*, and *Fgura7* (see Fig. 2B and the legend) were found in this region, but no other ORFs were identified. Therefore, *Tri101* appears to be the only trichothecene biosynthetic gene located on the cosmids. Northern (Fig. 1B) and RT-PCR analysis indicated that transcripts of these four (putative) genes (except that of the putative fatty acid desaturase gene which became detectable 48 h after the fungal germination) are present in cultures at all time points (data not shown), further supporting the sequence data that these adjacent genes are not related to trichothecene biosynthesis.

Using *Fgpho5* or *Fgura7* (the complete cDNA) as a probe, we performed Southern analysis (Fig. 1C). When the genomic DNA was digested with an enzyme whose recognition site is not present within the coding region, a single band of the expected size was detected (2302-bp *Dra*I fragment and 3186-bp *Ssp*I fragment for *Fgpho5*, and 2740-bp *Cla*I fragment and 2856-bp *Sna*BI fragment for *Fgura7*; lanes 1, 2, 5, 6). When digested with enzymes that cut once in the coding region (*Bsp*1286I and *Bst*XI for *Fgpho5*, and *Eco*RV and *Nru*I for *Fgura7*; lanes 3, 4, 7, 8), two hybridization signals were detected. We could not observe any other bands that are inconsistent with the sequence data. These results indicate that both *Fgpho5* and *Fgura7* are single copy genes and that no homologues exist in *F. graminearum*.

3.2. Analysis of the trichothecene non-producing strain

Since *Fgpho5* and *Fgura7* were single copy genes and appeared to be transcribed constitutively, they are most likely to be the 'house-keeping' genes whose homologues also exist in other *Fusarium* species. To determine whether the phosphate permease gene and the UTP-ammonia ligase gene are also linked in the trichothecene non-producing strain, we examined the genome of *F. oxysporum*. This strain possesses neither *Tri101* nor other trichothecene biosynthetic genes. The genomic DNA digested with various restriction enzymes were separated by CHEF electrophoresis (BioRad) in 0.5× TBE buffer [16] and subjected to Southern analysis. When probed with *Fgpho5* and *Fgura7* (the complete cDNAs), hybridization signals of exactly the same size were detected from each blot of genomic DNA digested with several restriction enzymes, i.e. *Bam*HI, *Cpo*I, *Eco*RI, *Nhe*I, *Not*I, *Nru*I, *Sma*I, and *Sna*BI (marked by arrowheads in Fig. 3). Therefore, (at least a part of) both genes are considered to be contained in each restriction DNA fragment marked on the blot. Similar banding patterns were also observed in genomic blots of another non-producer *Fusarium equiseti* 72 (IFO 31095); i.e. hybrid-

ization signals of exactly the same size were obtained by digestion with *Bam*HI, *Bln*I, *Cpo*I, *Nru*I, *Sma*I, *Sna*BI, *Spe*I, and *Xba*I (data not shown). These results are strongly suggestive of a close linkage of the phosphate permease gene and the UTP-ammonia ligase gene in the non-producing strain.

3.3. Analysis of the type A trichothecene-producing strain

We next examined whether *Tri101* is also located between the phosphate permease gene and the UTP-ammonia ligase gene in *F. sporotrichioides* which produces type A trichothecenes [14]. Primers PHO-U2 and URA-U2 worked well for specific amplification of this region (7 kb) and the subsequent restriction and sequence analyses revealed a homologue of *Tri101* (referred to as *FsTri101*) on this PCR product.

Based on the above results, the nucleotide sequence of *FsTri101* was carefully determined: a series of overlapping fragments were directly amplified from the genomic DNA of this strain, and three independent clones were sequenced for each fragment to eliminate any possible errors in PCR. The combined nucleotide sequence of these fragments (1713 bp, AB014491) contained an ORF (1377 bp) with a highly conserved A at −3, as in the case of *Tri101* [13]. This sequence was compared to that of the cDNA obtained by RT-PCR with primers Fs101-U1 and Fs101-D1. As expected, it contained no introns and specified a protein of 459 amino acids with a calculated MW of 50 327 Da (Fig. 4).

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FsTRI101  M-----VATSSTSS-QSFDIE-LDLIGQQPPLLMIYTQISLLYPVSDPSQYPTIVSTLEQ  53
TRI101    M---------------A.K.Q-..TL..LPG..S.......................F..  44
YLL063c   MFRVKIISQKR.K.V.MLENDQ..IL....S.YKL....CSI.R.P...AHDH..N..TR  60

GLKRLSQTFPWVAGQVKTEGVSEGNTGISKIIPYEETPPLVVKDLRDDSSAPTIEGLRKAGFPIEMFDEN  123
...F.EAV........A...I......T.F.V.F.DV.RV........P.......M....Y.MA.....  114
..ET.AKN.Q.L..N.VN..AD.....TYR.V.SDKI.-.I.Q...E.L....MDS.E.AD...Y.L..K  129

VVAPKKTL-AIGPGNG-PNDPKPVLLLQLNFIKGGLILTINGQHGAMDMTGQDAIIRLLSKACRNESFTE  191
II..R...-P....T.-.D......I.............V.........V....V..........DP...  182
TF..CM.INPP.NTI.MAAKSG..FAV.A..IS...V...V...NI..I...ES..N..N.S.HQKP.SD  199

EEVSAMNLERKMVVP-FLENYKVGPELDHQIVKPA--PAGVAPP-A-PAKASWGFFSFTPKALSELKDVA  256
..MT....D..TI..-Y....TI...V.......D--V..GDAVLT-.VS...A..K.S...M.....A.  248
..LLIG.IDKSKSI.L.D.TWEPDTT.V.E..ETSRNTS.EEKEQSCSSNST.AYVE.SAIS.QN.RIL.  269

TKTLDASTKFVSTDDALSAFIWQSASRVRLARLDASTPTEFCRAVDMRGPMGVSSTYPGLLQNMTYHNST  326
......................K.......E.I.G.A.........A.PA....NN..............  318
MQ.CTSG........IVT....K.V..A..S..KPE.KSNLG....V.KRL.LPE......V...FNTGS  339

VSEIANEPLGATASRLRSELNSD--RLRRRTQALATYMHDLPDKSSISLTADADPSSGIMLSSWAKVGCW  394
IG.....S............DPA--SM.Q..RG....L.NN....NV.........TSV.........L.  386
LKSLDHKS..VL..QI.RK.DPKVFD.AYN.C....LLSRQ...TKV.IPQPI.TL..I.V......SLY  409

EYDFGFGLGKPESVRRPRFDPFESLVYLMPKKPDGEFTASISLRDEDMERLKADEEWTKYAQYIG      459
D...........T....I.E.V...M.F.........C.AL.......D.....K........V.      451
DV..NL.....K.......ISL...I.F..RSSR..MVVALQ...K.W.C.N..K...N..TH..      474
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Fig. 4. Alignment of the deduced amino acid sequences of FsTRI101 (upper), TRI101 (middle), and ORF YLL063c (lower). Dots indicate identical amino acids with those of FsTRI101. Common amino acids among three proteins are boxed.

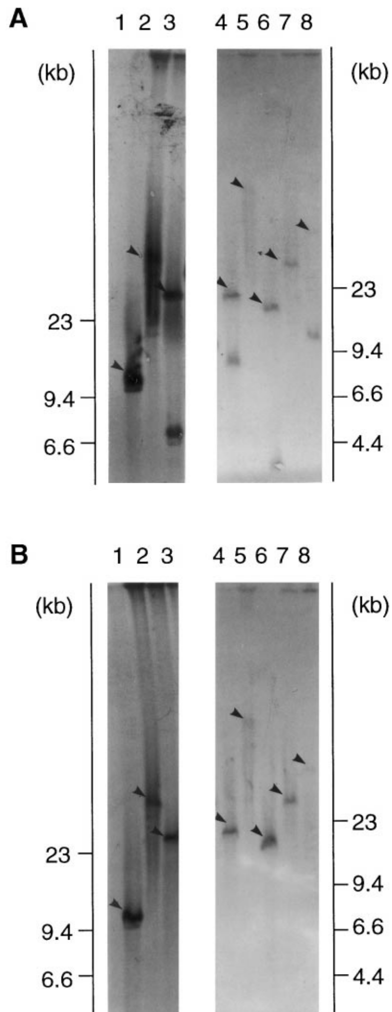

Fig. 3. Southern blots of genomic DNA of the non-producer fungus probed with *Fgpho5* (A) and *Fgura7* (B). Genomic DNA of *F. oxysporum* was digested with various restriction enzymes and separated by CHEF electrophoresis. The separation condition was 1% agarose, 120°, 6 V/cm, 0.22 s initial switching time, and 8.53 s final switching time for 15.3 h. The hybridization signals that showed exactly the same size when probed with *Fgpho5* and *Fgura7* are indicated by arrowheads. Lane 1: *Bam*HI; lane 2: *Cpo*I; lane 3: *Eco*RI; lane 4: *Nhe*I; lane 5: *Not*I; lane 6: *Nru*I; lane 7: *Sma*I; lane 8: *Sna*BI.

Transcripts of *FsTri101* were detected from the fungal culture grown either in the absence or presence of 100 μg/ml T-2 toxin (Fig. 5A). Expression of the resistance gene was not strongly induced by sublethal levels of T-2 toxin added to the culture. This feature is quite different from that observed in *F. graminearum*, in which transcripts of *Tri101* dramatically increased upon addition of the toxin [18]. To examine if *FsTri101* codes for a functional enzyme, the resistance gene was overexpressed in *E. coli* (Fig. 5B) and the crude recombinant enzyme was used for in vitro acetylation assay. As shown in Fig. 5C, recombinant FsTRI101 could acetylate the C-3 hydroxyl group of T-2 toxin in the presence of acetyl-CoA (lane 2). However, T-2 toxin was not acetylated in vivo by *F. sporotrichioides* (lane 3) whereas the 3-*O*-specific in vivo acetylation was observed in *F. graminearum* (lane 4).

4. Discussion

Many antibiotic producing organisms have resistance genes that are responsible for inactivation of their autogenous antibiotics by means of modification. These resistance genes participate in antibiotic biosyntheses, lie within the gene cluster, and are co-ordinately regulated with other biosynthetic genes of the antibiotic. Examples include the streptomycin 6-phosphotransferase gene (*aphD*) of *Streptomyces griseus* [19], the puromycin *N*-acetyltransferase gene (*pac*) of *Streptomyces alboniger* [20], the phosphomycin phosphotransferase gene (*fomA*) of *Streptomyces wedmorensis* [21], and the demethylphosphinothricin *N*-acetyltransferase gene (*bar*) of *Streptomyces hygroscopicus* [22]. These organisms carry the target molecules that are sensitive to their antibiotics and thus require the enzyme activities for metabolic shielding of the biologically active intermediates [23]. Here we have analyzed the large genomic regions on both sides of *Tri101* and found that this resistance gene is not in the cluster of the trichothe-

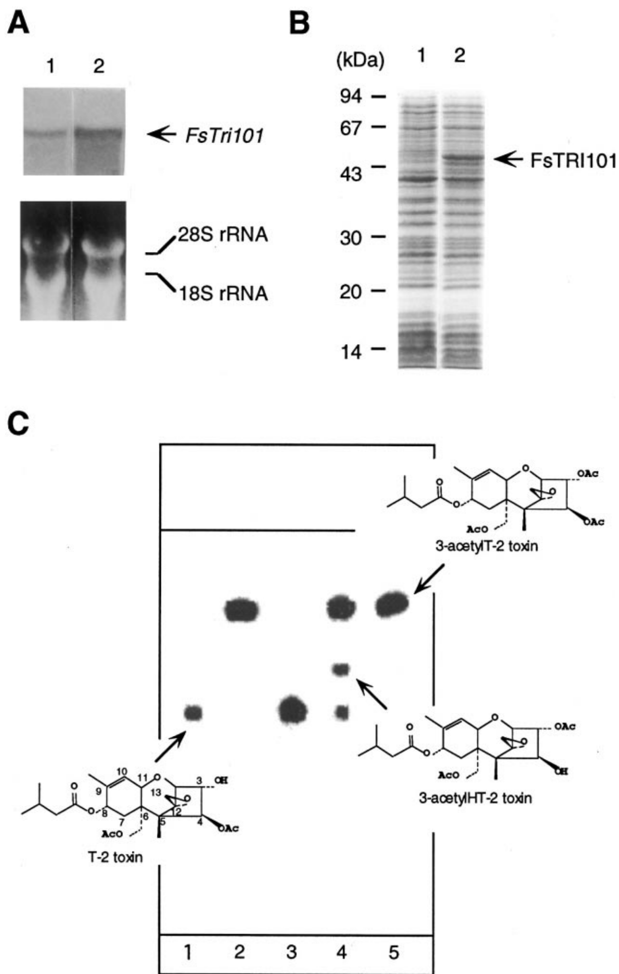

Fig. 5. In vitro and in vivo 3-*O*-acetyltransferase assay of T-2 toxin by *F. sporotrichioides*. A: Northern blotting. The RNA probe of *FsTri101* was hybridized to total RNA of the fungus germinated either in the absence (lane 1) or presence (lane 2) of T-2 toxin. B: Overexpression of recombinant *FsTri101* in *E. coli*. Total proteins were analyzed by 15% SDS-PAGE after heat treatment in denaturation buffer. The gel was stained with Coomassie Brilliant Blue. Lane 1: Total proteins from uninduced cells harboring pET-23a; lane 2: total proteins from induced cells harboring pET-23a-*FsTri101*. C: Schematic diagram of TLC from in vitro and in vivo 3-*O*-acetylation assay. Lane 1: T-2 toxin standard; lane 2: T-2 toxin incubated with crude extract of recombinant FsTRI101 and acetyl-CoA; lane 3: T-2 toxin incubated with *F. sporotrichioides*; lane 4: T-2 toxin incubated with *F. graminearum*; lane 5: 3-acetyl T-2 toxin standard.

cene biosynthetic genes. It was located between the phosphate permease gene and the UTP-ammonia ligase gene which appeared to play pivotal roles for fungal growth and viability, but not for trichothecene biosynthesis (supported by Northern and Southern analyses in Fig. 1). There has been no precedence so far that only one antibiotic biosynthetic gene is separated from others in the gene cluster [23].

4.1. *Tri101 evolved independently of other trichothecene biosynthetic genes in the cluster*

One possible interpretation for the above unexpected result might be that the isolated occurrence of *Tri101* is attributed to the breakup of the original trichothecene biosynthetic gene cluster. This model is based on the hypothesis that *Tri101* coevolved with other biosynthetic genes and was originally in the gene cluster. If *Tri101* is located at either end of the gene cluster and the reciprocal translocation breaks the chromosome between *Tri101* and its adjacent biosynthetic gene, *Tri101* would be separated from all the other biosynthetic genes. In fact, the breakup of the biosynthetic gene cluster was observed in the fungus *Cochliobolus heterostrophus* that produce the polyketide T-toxin [24].

This possibility was investigated by analyzing the non-producing *Fusarium* strains. These strains also carried the phosphate permease gene and the UTP-ammonia ligase gene at the same locus on the chromosome (Fig. 3), suggesting that the translocation event is not responsible for the linkage of these genes in the trichothecene producing strains. We are left with an alternative model that the evolutionary origin of *Tri101* is distinctively different from that of other biosynthetic genes in the cluster. This hypothesis appears to be supported by the facts that expression of *Tri101* is independent of the transcriptional regulator *Tri6* [18] and that its homologues were found in *Saccharomyces cerevisiae* (ORF YLL063c; Z73168) and *Schizosaccharomyces pombe* (SPCC338.19; AL023781), which are non-producers and sensitive to trichothecenes. Thus it is feasible that the trichothecene producers have independently acquired the gene through horizontal gene transfer from an as yet unidentified organism.

4.2. *3-O-Acetylation is not always a primary self-defensive strategy for all the t-type trichothecene producers*

The t-type trichothecene-producing *Fusarium* species are phylogenetically resolved into two monophyletic groups and this classification correlates well with the structure of their secondary metabolites (i.e. either type A or type B trichothecene) [25]. Resistance in the type B producers would be achieved mainly via 3-*O*-acetylation of trichothecenes, since expression of *Tri101* is triggered by sublethal levels of the toxin added to the culture [18]. Besides, their ribosomes appear not to be fully resistant to trichothecenes [13,26], which is in further support of this possibility.

The type A trichothecene producer *F. sporotrichioides*, as in the case of the type B producer, also carried the functional 3-O-acetyltransferase gene between the phosphate permease gene and the UTP-ammonia ligase gene. However, expression of *FsTri101* was not strongly induced by T-2 toxin (Fig. 5A), and the 3-*O*-acetyltransferase activity was not detected in vivo from the fungus (Fig. 5C). Indeed, the type A producer has not been previously described to produce a trichothecene with an acetyl group at the C-3 position, although the type B producers possessed such an activity [27–29]. Despite these facts, 15-deacetylcalonectrin (3α-acetoxy-15-hydroxy-12,13-epoxytrichothec-9-ene) was isolated as a pathway intermediate from a blocked mutant of *F. sporotrichioides* [30]. Further, isotrichodermin (3α-acetoxytrichothecene) proved to be a biosynthetic precursor of T-2 toxin [31].

In view of the above apparently conflicting observations, a likely scenario is that the biosynthetic gene cluster contains an original trichothecene 3-*O*-acetyltransferase gene under the control of *Tri6* (and it might not show a nucleotide sequence similarity to *Tri101*). This *Tri6*-dependent 3-*O*-acetyltransferase gene would play a role in both self-defense and toxin biosynthesis, which is a common functional feature of the antibiotic resistance gene [19–22], and might not be expressed by trichothecenes added to the culture. Thus it would be a serious problem for the producer considering the instability of

the 3-*O*-acetyl group of trichothecenes [13,26]; i.e. if the antibiotic is not effectively released into the media or penetrates back into the cell after the secretion, the 3-hydroxylated end product would exert a deleterious effect. To avoid self-intoxication against their own products, the fungus would have evolved options of resistance mechanisms.

We speculate that *Tri101* is such as option, which the trichothecene producers have acquired through horizontal gene transfer in addition to their original resistance mechanisms. In the type A trichothecene producer, however, the primary self-defensive strategies are other resistance mechanisms dispensable with 3-*O*-acetylation. These possible mechanisms might include modification or replacement of the drug target ribosome [32], efficient efflux of the antibiotic by the membrane transporter [33], and/or restriction of the membrane permeability against substances in the medium [23], which appear to be substantially defective in the type B trichothecene producers.

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