Trichothecene 3-O-Acetyltransferase Protects Both the Producing Organism and Transformed Yeast from Related Mycotoxins

CLONING AND CHARACTERIZATION OF Tri101*

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Trichothecene mycotoxins such as deoxynivalenol, 4,15-diacetoxyscirpenol, and T-2 toxin, are potent protein synthesis inhibitors for eukaryotic organisms. The 3-O-acetyl derivatives of these toxins were shown to reduce their in vitro activity significantly as assessed by assays using a rabbit reticulocyte translation system. The results suggested that the introduction of an O-acetyl group at the C-3 position in the biosynthetic pathway works as a resistance mechanism for Fusarium species that produce t-type trichothecenes (trichothecenes synthesized via the precursor trichothriol).

A gene responsible for the 3-O-acetylation reaction, Tri101, has been successfully cloned from a Fusarium graminearum cDNA library that was designed to be expressed in Schizosaccharomyces pombe. Fission yeast transformants were selected for their ability to grow in the presence of T-2 toxin, and this strategy allowed isolation of 25 resistant clones, all of which contained a cDNA for Tri101. This is the first drug-inactivating O-acetyltransferase gene derived from antibiotic-producing organisms. The open reading frame of Tri101 codes for a polypeptide of 451 amino acid residues, which shows no similarity to any other proteins reported so far. Tri101 from recombinant Escherichia coli catalyzes O-acetylation of the trichothecene ring specifically at the C-3 position in an acetyl-CoA-dependent manner. By using the Tri101 cDNA as a probe, two least overlapping cosmids clones that cover a region of 70 kilobase pairs have been isolated from the genome of F. graminearum. Other trichothecene biosynthetic genes, Tri4, Tri5, and Tri6, were not clustered in the region covered by these cosmids clones. These new cosmids clones are considered to be located in other parts of the large biosynthetic gene cluster and might be useful for the study of trichothecene biosynthesis.

Trichothecenes belong to a family of sesquiterpenoid secondary metabolites produced by Fusarium species and other molds

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB000874.

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1 The abbreviations used are: DON, deoxynivalenol; BHK, baby hamster kidney; kb, kilobase pair(s); PCR, polymerase chain reaction; DAS,
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Trichothecenes in the cultured broth were extracted three times with an equal volume of ethyl acetate and concentrated in vacuo. The samples were analyzed on TLC by using ethyl acetate/toluene (3:1) as the solvent. Trichothecenes on silica gel layers were made by spraying with 1% 4-(4-nitrobenzyl)pyridine and then heated in an oven at 30 °C for 20 min, and the products were quantitated by measuring absorbance at 254 nm. The samples were resolved on precoated TLC plates of Kieselgel 60 F254 (Merck). 4-(4-Nitrobenzyl)pyridine and tetraethylenepentamine were purchased from Wako.

Effects of Trichothecenes on Protein Synthesis—Effects of trichothecenes on protein synthesis in cultured mammalian cells were determined with virus-infected cells. Monolayer cultures of BHK-21 cells in 96-well titer plates were infected with Newcastle disease virus, and trichothecenes at various concentrations were added at the time of infection. Virus glycoprotein synthesis was quantified by determining hemagglutination units as described previously (13). Effects of trichothecenes on protein synthesis in a cell-free system were investigated by using the rabbit reticulocyte lysate system (Promega). The translation reaction was initiated by adding components of the system and trichothecenes at designated concentrations. Proteins were synthesized at 30 °C for 20 min, and the products were quantitated by measuring luciferase activity using a Luciferase assay system kit (PicaGene PGK-L100, Toyo Ink Co., Tokyo).

Trichothecene Analysis—Trichothecenes in the cultured broth were extracted three times with an equal volume of ethyl acetate and concentrated in vacuo. The samples were analyzed on TLC by using ethyl acetate/toluene (3:1) as the solvent. The developed TLC plate was sprayed with 1% 4-(4-nitrobenzyl)pyridine and then heated in an oven for 30 min at 150 °C. Trichothecenes on silica gel layers were made visible by treatment with 10% tetraethylenepentamine (14). Structures of trichothecenes acetylated by the crude recombinant enzyme were determined by 1H NMR spectra obtained with a Joel JNM A400 spectrometer in CDCl3, using CHCl3 (7.26 ppm) as an internal standard.

Construction of a F. graminearum cDNA Library—Poly(A)-rich mRNA was isolated from young mycelia germinated in the presence of T-2 toxin by using a Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). The Superscript cDNA synthesis system (Life Technologies, Inc.) was used for construction of a directional library (cDNA with a NotI site at the poly(A) proximal end and a SalI site at the opposite end). After double digestion with NotI and SalI, the synthesized cDNA was ligated into the expression cloning vector pCDSP21 (15) and transformed to E. coli DH5.

Construction of a F. graminearum Cosmid Library—A cosmid library was constructed by using the SuperCos1 cosmid vector (Stratagene, La Jolla, USA). Genomic DNA was partially digested with Sau3AI and checked by electrophoresis on a 0.3% agarose gel. The partial digest that showed the most appropriate size distribution (30–45 kb) was ligated between the arms of the SuperCos1 vector after dephosphorylation. Packaging of DNA was performed by using Gigapack III gold packaging extract (Stratagene) as described in the manufacturer instructions.

Cloning of the cDNA for Tri101 and Screening of the Tri5- and Tri101-containing Cosmid Clones—Screening of the cDNA library was carried out either directly or indirectly by selecting an aliquot of S. pombe-transforming mixture with 20 μg/ml T-2 toxin. The molecular cloning techniques of S. pombe were the same as described previously (16). For screening of the cosmid library, the digoxigenin system (Boehringer Mannheim) was used. The digoxigenin-labeled probes were prepared from the 1.2-kb PCR product for Tri5 or the 1.4-kb PCR product for Tri101 (see the last section of “Experimental Procedures”). Colony hybridization and purification of positive colonies were carried out as described in the manufacturer instructions.

Expression of Tri101 in E. coli and Cell-free Acetyltransferase Assays—The coding region of Tri101 was amplified by PCR with primers EU101(5'-CACCACCCATATGGCTTTC-3') and ED101(5’-GTCCTAGGATCCATACTCTAAC-3'). The amplification consisted of 25 cycles under the following conditions: template denaturation for 30 s at 94 °C, primer annealing for 1 min at 55 °C, and primer extension for 2 min at 72 °C (5 min at last cycle). The amplified product was cloned into the expression vector pET-19b (Novagen) after double digestion with

FIG. 1. A proposed trichothecene biosynthetic pathway of Fusarium and Trichothecium species. t-type trichothecene is formed through a series of enzymatic reactions beginning with the cyclization of farnesyl pyrophosphate to trichodiene by trichodiene synthase (Tri5). Subsequent biosynthetic steps involve oxygenations, isomerization, and the second cyclization to give isotrichodermol, the first pathway intermediate that has a toxic trichothecene ring (4). The specific O-acetylation at the C-3 position occurs before oxygenations and esterifications at the C-4, C-7, C-8, or C-15 position of the trichothecene ring (8, 37), leading to a later accumulation of the deacetylated end products, 3-hydroxy trichothecenes, in the media (35, 39).
37°C for 1 h. After the ethyl acetate extraction and concentration, 1/50 obtain the supernatant (crude enzyme fraction). Acetyltransferase assays were initiated by the addition of 100 μl of the crude enzyme fraction to a reaction mixture containing 3.66 ml of 10 mM Tris-HCl (pH 7.5), 200 μl of trichothecenes in 50% ethanol (5 mg/ml), and 40 μl of acetyl-CoA in water (100 μM). The reaction mixture was incubated at 37°C for 1 h. After the ethyl acetate extraction and concentration, 1/50 of the products was analyzed by TLC, and the rest was used to confirm their structure by 1H NMR.

Mapping of the Cosmid Clones Containing Tri101—Cosmid mapping was performed by using FLASH nonradioactive mapping kit (Stratagene). Each cosmid clone containing Tri101 was completely digested with NotI, an enzyme that cut off the insert DNA from the vector, and then partially digested with EcoRI, ClaI, and KpnI. The partial digests were separated by electrophoresis on a 0.4% agarose gel and transferred to a Nytran N membrane (Schleicher & Schuell). The membrane was first hybridized with the T3 probe, washed, and exposed to a film as described in the supplier instructions and then re-hybridized with the T7 probe after stripping off the T3 probe. Based on the size of hybridization signals that appeared on the film, a restriction map was constructed for the large genomic DNA region that extends for 70 kb around Tri101.

Analysis of the Trichothecene Biosynthetic Gene Cluster by PCR—PCR was performed to check if a portion of Tri4 (17), Tri5 (18), and Tri6 (19) could be amplified from the cosmid clones containing Tri101. The conditions for PCR were as described above, and the following primers were used for amplification: U4 (5'-TCCGACGTCATGCAATTCATTGAGGGGG-3') for Tri4, U5 (5'-GATGGTAATGTACTTCTTGGAGGGG-3') and D5 (5'-GCTTACACCGCCTCATCGCTCTC-3') for Tri5, U6 (5'-CGTCCAGGACTTGCACTTG-3') and D6 (5'-CTCGCCCAAGGCCCGCAGGACCTCATTG-3') for Tri6, and U101 (5'-ATGGTCTCAAGATACAGCT GCATCTACATC-3') and D101 (5'-TCAACCTCAAGGCTCATCGCTTTG-3') for PCR. PCR products were cloned directly into TA cloning vector pCRII (Invitrogen), which were confirmed to contain the corresponding genes by partial nucleotide sequence analyses.

RESULTS

Difference between In Vivo and In Vitro Protein Synthesis Inhibition by 3-O-Acetyl Trichothecenes—The effect of the 3-O-acetyl group attached to the trichotheccene ring on protein synthesis was examined by quantitating hemagglutination units in Newcastle disease virus-infected BHK cells. Acetylation at the C-3 position reduced the inhibitory actions of trichotheccenes to about one-fourth of their 3-hydroxy derivatives, i.e., the concentration required to reduce virus hemagglutinin biosynthesis by 50% was estimated to be 300 ng/ml for DON, 2000 ng/ml for 3-acetyldeoxynivalenol, 12 ng/ml for 4,15-diace
toxyyscirpenol (DAS), 35 ng/ml for 3,4,15-triace
toxyyscirpenol (3-ADAS), 3 mg/ml for T-2 toxin, and 15 ng/ml for 3-acetylT-2 toxin (3-AT-2 toxin) (Fig. 2A). This unexpected slight decrease in the activity of 3-O-acetyl trichotheccene was predicted to be caused by deacetylation. To evaluate this possibility, trichotheccenes were extracted after incubation with BHK cells and analyzed by TLC. Intense blue spots corresponding to 3-hydroxytrichotheccenes were detected with cultures treated with 3-AT-2 toxin (3-O-AT-2 toxin) (Fig. 2B). This result indicates that 3-O-acetyl trichotheccenes do not exert inhibitory actions on in vitro protein synthesis in contrast to the high toxicity on animals and cultured mammalian cells.

Cloning Strategy for Tri101, the Trichotheccene 3-O-Acetyltransferase Gene—The new finding raised the possibility that the biosynthetic gene involved in 3-O-acetylation may be responsible for the resistance mechanism in producing strains. The biosynthetic pathway involves 3-O-acetylation of isor
tochromol, the first proposed tricyclic intermediate that inhibits growth of the fungi, to produce a nontoxic derivative isor
tochromol (11). After isor
tochromol, the route proceeds along a series of 3-O-acetylated intermediates that are devoid of biological ac
tivity, followed by the conversion to 3-deacetylated products at the last step (Fig. 1). Such metabolic shielding is reminiscent of the self-defense mechanism for the antibiotic-producing bacteria (20) from which the corresponding genes were isolated by using drug-sensitive organisms as a heterologous host.

Based on this consideration, we searched for an appropriate host organism to establish an efficient cloning strategy for the 3-O-acetyltransferase gene, which we designated Tri101. Several different trichotheccenes were tested for their ability to inhibit growth of yeasts, and as a consequence, S. pombe was found to be very sensitive to trichotheccenes. Above all, T-2 toxin inhibited its growth quite effectively. We thus examined whether the DON-producing strain of F. graminearum F15 has an ability to convert T-2 toxin to an 3-O-acetyl derivative. Spore germination was not profoundly affected by 100 μg/ml T-2 toxin in YPD medium, and analysis of the biotransformed products revealed production of HT-2 toxin (a 4-hydroxy derivative of T-2 toxin), 3-acetylHT-2 toxin, and
3-AT-2 toxin. The inhibitory effects of these toxins on growth of *S. pombe* were then investigated as shown in Table I. In sharp contrast with the severe toxicity to cultured mammalian cells, complete loss of biological activity was observed by 3-O-acetylation of the trichothecene ring. These results suggested that Tri101 would be isolated by T-2 toxin selection of fission yeast transformants carrying an expression library of *F. graminearum*.

**Isolation and Analysis of the T-2 Toxin-resistant Plasmid Cloned in Fission Yeast**—A cDNA expression library consisting of 2.0 $\times$ 10^6 independent *E. coli* colonies was constructed and recovered as a pool of plasmids to transform *S. pombe*. Although as many as 21 positive clones were obtained by replica-plotting the 4.2 $\times$ 10^4 Ura^+ transformants to the T-2 toxin-containing medium, only 4 positive clones were identified by direct selection with the drug. All these colonies showed a stable resistance phenotype after transfer to YEA medium containing 100 $\mu$g/ml T-2 toxin. Southern analysis revealed that sequences homologous to the 1.6-kb cDNA insert for Tri101 (see below) were contained in DNA from all the 25 resistant colonies (data not shown).

A resistant plasmid was recovered in *E. coli* from one of the positive clones and subjected to further analysis. As shown in Fig. 3, the original structure of the library was identified on the 6.9-kb *NotI* fragment of pTR19–1 in which the 1.6-kb cDNA insert was correctly positioned between the *SalI* and *NotI* cloning sites of pcDSP21 (pTR19-1-4 in Fig. 3). Both plasmids pTR19–1 and pTR19-1-4 conferred resistance to T-2 toxin when transformed in *S. pombe*. Since pcDSP21 was constructed so that the initiator AUG codon was located between the 16S splicing (21) donor and acceptor sequences upstream of the cloning site (15), we could not rule out the possibility that an incomplete cDNA lacking an initiation codon might have been rescued by producing a functionally active fusion protein. To examine whether the positive clone contained a full-length cDNA, plasmid pUCSVTri101 was constructed. It placed the cDNA insert under the direct control of the SV40 early promoter and was transduced into *S. pombe* by cotransformation with pAU9 (16). The transformed cells showed enough resistance to T-2 toxin, which strongly suggests that the cloned cDNA insert contained a complete open reading frame of the

| Trichothecene       | Amount of trichothecene on a paper disc | Diameter of inhibition zone |
|---------------------|----------------------------------------|----------------------------|
| T-2 toxin          | 2.5                                    | 25                         |
| 3-acetylT-2 toxin   | 25                                     | 0                          |
| HT-2 toxin         | 25                                     | 13                         |
| Trichothecin       | 25                                     | 22                         |

**TABLE I**

Inhibitory effects of trichothecenes on growth of *S. pombe*

Each trichothecene was applied onto an 8-mm paper disk. The discs were placed on plates seeded with *S. pombe* in the upper layer of YPD agar.

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**FIG. 3.** Construction of pUCSVTri101 carrying Tri101 under the direct control of the SV40 early promoter. The structure of pTR19–1 presumably resulted from duplication of the library followed by subsequent deletion or rearrangement of a portion, which are the intrinsic recombinational properties of *S. pombe*. As shown in the figure, pTR19-1-4 retained the original structure of pcDSP21; the directionally synthesized cDNA was placed between the *SalI* and *NotI* cloning site in a correct orientation relative to the transcripational elements on pcDSP21. After digestion of pTR19-1-4 with *PstI* and *BamHI*, the cDNA insert was cloned into the corresponding sites of pUC18. The cloned insert in pUC18 was then recovered after digestion with *HindIII* and *SmaI*. It was replaced with the BSD fragment of pUCSVBSD (40) following *PstI* digestion, blunt-ending, and *HindIII* digestion of the vector.
resistance gene.

T-2 Toxin Resistance Is Conferred by O-Acetylation at the C-3 Position—S. pombe cells transformed by this resistant plasmid were incubated with T-2 toxin to confirm the specific O-acetylation of the trichothecene ring at the C-3 position. A bright blue spot having \( R_B = 0.83 \), a value of 3-AT-2 toxin, was detected. However, HT-2 toxin (\( R_B = 0.20 \)) was not detected in extracts of the fisson yeast transformant. The transformed product showed the following signals on \(^1H\) NMR analysis: 5.92 (d, \( J = 2.9 \) Hz, H-4), 5.75 (br d, \( J = 5.9 \) Hz, H-10), 5.29 (br d, \( J = 5.9 \) Hz, H-8), 5.18 (d, \( J = 2.9 \) Hz, H-3), 4.35 (d, \( J = 12.2 \) Hz, H-15a), 4.22 (d, \( J = 5.9 \) Hz, H-11), 4.10 (d, \( J = 12.2 \) Hz, H-15b), 3.86 (d, \( J = 4.9 \) Hz, H-2), 3.07 (d, \( J = 3.9 \) Hz, H-13a), 2.82 (d, \( J = 3.9 \) Hz, H-13b), 2.37 (dd, \( J = 5.9 \) Hz and 15.1 Hz, H-7a), 2.16, 2.10, 2.07 (each s, AcO-3, 4, and 15), 2.17–2.05 (m, H-2' and 3'), 0.96 (d, \( J = 6.3 \) Hz, H-5'), and 0.96 (d, \( J = 6.3 \) Hz, H-5'). The \(^1H\) NMR result was consistent with the structure of 3-AT-2 toxin. This implies that the resistance phenotype should be attributed to the 3-O-acetyl conjugation of T-2 toxin, not to the deacetylation at the C-4 position, which also reduced the toxicity to less than one-tenth (Table I). Therefore, the cloned cDNA insert proved to encode Tri101.

Sequencing of the cdNA for Tri101—The primary structure of the 1.6-kb cdNA insert in pUC18 (i.e. pUCTR19-1-4 in Fig. 3) was determined from both directions by sequencing a series of deletion clones. The cdNA contained a single potential open reading frame that encoded a polypeptide of 451 amino acid residues (Fig. 4). Around the putative initiation codon, a consensus sequence for filamentous fungi (22) was observed; consistencies at \(-5 (C \text{ or } T), -3 (C \text{ or } A), \text{ and } -1 (C \text{ or } A) \) were noticed in addition to the highly conserved C, A, and C at positions \(-4, -3, \text{ and } +5 \), respectively. No sequence similarities were found when the nucleotide and amino acid sequences were compared with DDBJ/EMBL/Genbank\(^TM\) and SWISS-PROT data base sequences, including those of Tri3 (23), the trichothecene 15-O-acetyltransferase gene.

Use of Tri101 as a Selectable Marker in S. pombe—There are no selectable marker genes of eukaryotic origin, except blasticidin S (BS) resistance BSD (15), which confer resistance to drugs by means of inactivation. To compare the frequency of transformation with URA3 and BSD, pBRURA3 and pBRARS were constructed from pAU9 (16) by eliminating the EcoRI fragment of ARS or the Poul1-HindIII fragment of URA3, respectively. S. pombe cells were cotransformed by either pBRURA3, pUCSVBSD (Fig. 3), or pUCSVTri101 (Fig. 3) with the transducing vector pBRARS and plated on agar media that are sensitive to both the 3-O-acetyl conjugation of T-2 toxin, not to the deacetylation at the C-4 position, which also reduced the toxicity to less than one-tenth (Table I). Therefore, the cloned cDNA insert proved to encode Tri101. Sequencing of the cdNA for Tri101—The primary structure of the 1.6-kb cdNA insert in pUC18 (i.e. pUCTR19-1-4 in Fig. 3) was determined from both directions by sequencing a series of deletion clones. The cdNA contained a single potential open reading frame that encoded a polypeptide of 451 amino acid residues (Fig. 4). Around the putative initiation codon, a consensus sequence for filamentous fungi (22) was observed; consistencies at \(-5 (C \text{ or } T), -3 (C \text{ or } A), \text{ and } -1 (C \text{ or } A) \) were noticed in addition to the highly conserved C, A, and C at positions \(-4, -3, \text{ and } +5 \), respectively. No sequence similarities were found when the nucleotide and amino acid sequences were compared with DDBJ/EMBL/Genbank\(^TM\) and SWISS-PROT data base sequences, including those of Tri3 (23), the trichothecene 15-O-acetyltransferase gene.

**Is Tri101 a Self-defense Gene for the t-type Trichothecene Producer?—**Although T-2 toxin was inactive on spor germination of F. graminearum strain F15, trichothecin (Fig. 1), a type trichothecene similarly toxic as T-2 toxin to S. pombe (Table I), exerted a potent inhibitory action on the fungal germination at 100 \( \mu \text{g/ml} \) in YPD medium. This inhibitory action would be attributed to the lack of the 3-hydroxyl group to be acetylated in d-type trichothecenes. Other t-type trichothecenes did not inhibit spore germination of the fungus due to the presence of the 3-O-acetyltransferase activity. The result indicates that resistance in t-type-producing strains does not involve modification or replacement of the target ribosomes, as opposed to the d-type resistance mechanisms of the Saccharomyces cerevisiae mutant (24) and the trichothecin-producing strain of Trichothecium roseum (25).

Due to the commercial unavailability of the intermediates, we could not check whether S. pombe cells expressing Tri101 have an ability to convert isotrichodermol to isotrichodermol. However, it seems very likely that Tri101 is the only gene responsible for the reaction, considering that all of the 25 T-2 toxin-resistant S. pombe transformants carried Tri101 and that no homologous genes were contained in the genome of the t-type-producing fungus. Furthermore, recombinant TRI101
from *E. coli* was specific to the C-3 hydroxyl group of the trichothecene ring irrespective of the substituents at other positions. These results suggest that all the compounds with 3-hydroxy-12,13-epoxytrichothec-9-ene structure served as substrates for the enzyme. Thus, the primary defensive strategy of *F. graminearum* would be modification of t-type trichothecenes by TRI101 through O-acetylation at the C-3 position.

**FIG. 4.** Nucleotide sequence and deduced amino acid sequence of the 1.6-kb cDNA insert for Tri101. The nucleotide sequence is numbered from the translation initiation site; starting at bp 1 and ending at bp 1356. The predicted protein is 49.4 kDa in size. The restriction enzymes that recognize the sequences are indicated above the underlined nucleotide sequences.

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N-Acetylation and O-phosphorylation are representatives of substrates for the enzyme. Thus, the primary defensive strategy of *F. graminearum* would be modification of t-type trichothecenes by TRI101 through O-acetylation at the C-3 position.
self-defense mechanisms against various kinds of antibiotics. Inactivation of a drug by O-acetylation is known to occur in the case of chloramphenicol, but it was not found in organisms that produce chloramphenicol. To our knowledge, Tri101 is the first antibiotic-inactivating O-acetyltransferase gene derived from a producing organism. The existence of an apparent 3-O-acetyl exchange mechanism in the biosynthetic pathway (11) may possibly be explained by the relative instability of the 3-O-acetyl group attached to the trichothecene ring and the importance of the substituent that has to be re-acetylated for the well being of the producer.

Utility of Tri101—The idea that the tricyclic intermediates of t-type trichothecenes are modified by Tri101 would have important implications for understanding the mechanism of trichothecene production. If the gene is essential for the self-defense mechanism, the Tri101 mutant generated through a genetic transformation technique would be lethal when the toxic intermediates accumulated within the cell. An alternative way to rescue the lethal Tri101 mutation is to introduce a second mutation on genes that catalyze a trichothecene biosynthetic step before the formation of isotrichodermol. Such oxygenation genes can be inactivated by integration of a selectable marker using the restriction enzyme-mediated integration technique (26, 27). This implies that use of a Tri101 mutant provides an opportunity to clone other biosynthetic genes before the second cyclization reaction by simply selecting for the ability of the restriction enzyme-mediated integration transformants.

**TABLE II**

| Plasmidsa | Selective media b | Efficiencyc |
|-----------|------------------|-------------|
| pBR/URA3  | MMA              | 2.8 × 10^4  |
| pUCSVBSD  | YEA (BS 20 μg/ml)| 1.2 × 10^4  |
| pUCSTri1  | YEA (T-2 toxin 20 μg/ml) | 5.7 × 10^2 |

a Each plasmid was cotransformed with pBR322.
b Media formulation is described in Ref. 16. MMA, modified minimal medium.
c Efficiency is expressed as a number of transformants/μg of vector DNA.

**FIG. 5.** In vitro specific 3-O-acetylation of trichothecene ring by recombinant TRI101. A, overexpression of recombinant Tri101 in E. coli. Total proteins were analyzed by 15% SDS-polyacrylamide gel electrophoresis after heat treatment in the denaturation buffer. The gel was stained with Coomassie Brilliant Blue. The positions of the markers are shown in kilodaltons. Lane 1, total proteins from uninduced cells; lane 2, total proteins from induced cells. B, TLC of trichothecenes acetylated by recombinant TR101. The crude extract of E. coli expressing the gene was used for the acetyltransferase assay. Lane 1, DON standard; lane 2, ethyl acetate extract of DON incubated with the crude extract and acetyl-CoA; lane 3, 3-acetyldeoxynivalenol standard; lane 4, DAS standard; lane 5, ethyl acetate extract of DAS incubated with the crude extract and acetyl-CoA; lane 6, 3-ADAS standard; lane 7, T-2 toxin standard; lane 8, ethyl acetate extract of T-2 toxin incubated with the crude extract and acetyl-CoA; lane 9, 3-AT-2 toxin standard.

**FIG. 6.** Analysis of large genomic regions on both sides of Tri101. A, the restriction map of cosmids clones containing Tri101. T3 and T7 indicate the direction of insert DNA in SuperCos1 cosmid vector. Two cosmids clones, pCosTr135 and pCosTr137, were shown to cover 35 kb on both sides of Tri101. C, ClaI, E, EcoRI; K, kpnI. B, agarose gel electrophoresis of the trichothecene biosynthetic genes amplified from the cosmids clones. The templates used for PCR were as follows: lanes 1–4, pCosTr135 (0.5 ng); lanes 5–8, pCosTr137 (0.5 ng); lanes 9–12, pCosTr032 (0.5 ng); lane 13, genomic DNA (5 ng); lane 14, Perfect DNA marker (Novagen). Primers D4 and U4 were used in lanes 1, 5, 9, and 13; primers D5 and U5 were used in lanes 2, 6, and 10; primers D6 and U6 were used in lanes 3, 7, and 11; and primers D101 and U101 were used in lanes 4, 8, and 12.
nants to grow under conditions that permit trichothecene biosynthesis.

Another possible application of the gene comes out of the observations of several researchers (28–30) that trichothecenes play a role as a virulence factor in a number of plant diseases. The availability of the Tri5 gene made it possible to evaluate the role of trichothecene at the molecular level (18, 31), and recently the Tri5 disruptant of Gibberella zeae has been shown to be less virulent on wheat than the trichothecene-producing parental strains (32, 33). Therefore, a transgenic plant expressing Tri101 might be valuable for control of wheat head scab and reduce the use of agricultural chemicals.

**Location of Tri101 in the Genome of the Producer Fungus—**

The first successful cloning of a trichothecene biosynthetic gene yielded Tri5 (formerly Tox5), encoding trichodiene synthase (34). Tri5 was cloned by screening a λgt11 library of F. sporotrichioides with antiserum raised against the purified enzymes (35). Recently, other trichothecene biosynthetic genes, Tri3 (23) and Tri4 (17), and a regulatory gene, Tri6 (19), have been identified in cosmid clones that cover a region of 60 kb around Tri5. Thus, it was of interest whether Tri101 was also clustered in the same region in the genome of the producer fungus. PCR analysis indicated that Tri101 was not located near these biosynthetic genes, but this does not necessarily rule out the possibility of its existence in the trichothecene biosynthetic gene cluster. Rather, the Tri101-containing cosmid clones might be useful in isolating other genes, not linked to Tri5 in the biosynthetic gene cluster. In fact, transformations with the Tri5-containing cosmid clones did not result in restoration of T-2 toxin production in a strain carrying mutations at the Tri1 locus (36), suggesting that the gene cluster is too large to be covered by several cosmid clones. Further investigation may shed light on the structure of the trichothecene biosynthetic gene cluster and the mechanism of trichothecene production.

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**Note Added in Proof—**Tri101 shows a high similarity to open reading frame YLL063c of S. cerevisiae, which appeared in the GenBank™ after submission of this manuscript (accession number Z73168). The BLASTX 2.0.3 program at NCBI (Nov. 14, 1997) generated a statistically significant alignment of its translated sequence to this yeast hypothetical protein at an E value of e⁻¹⁰³ (similarities = 63%, identities = 44%). Open reading frame YLL063c is located on chromosome XII and codes for a probable membrane protein of unknown function. The yeast homolog would not be a functional trichothecene 3-O-acetyltransferase, but its existence raises a question as to the evolutionary origin of this biosynthetic gene.

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