Trichothecene Nonproducer Gibberella Species Have Both Functional and Nonfunctional 3-*O*-Acetyltransferase Genes

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ABSTRACT

The trichothecene 3-*O*-acetyltransferase gene (*FgTri101*) required for trichothecene production by ***F**usarium **g**raminearum* is located between the phosphate permease gene (*pho5*) and the UTP-ammonia ligase gene (*ura7*). We have cloned and sequenced the *pho5*-to-*ura7* regions from three trichothecene nonproducing Fusarium (*i.e.*, *F. oxysporum*, *F. moniliforme*, and Fusarium species IFO 7772) that belong to the teleomorph genus Gibberella. BLASTX analysis of these sequences revealed portions of predicted polypeptides with high similarities to the TRI101 polypeptide. While *FspTri101* (**F**usarium **sp**ecies *Tri101*) coded for a functional 3-*O*-acetyltransferase, *FoTri101* (***F**. **o**xysporum Tri101*) and *FmTri101* (***F**. **m**oniliforme Tri101*) were pseudogenes. Nevertheless, *F. oxysporum* and *F. moniliforme* were able to acetylate C-3 of trichothecenes, indicating that these nonproducers possess another as yet unidentified 3-*O*-acetyltransferase gene. By means of cDNA expression cloning using fission yeast, we isolated the responsible *FoTri201* gene from *F. oxysporum*; on the basis of this sequence, *FmTri201* has been cloned from *F. moniliforme* by PCR techniques. Both *Tri201* showed only a limited level of nucleotide sequence similarity to *FgTri101* and *FspTri101*. The existence of *Tri101* in a trichothecene nonproducer suggests that this gene existed in the fungal genome before the divergence of producers from nonproducers in the evolution of Fusarium species.

TRICHOTHECENES are a large group of toxic sesquiterpene epoxides that inhibit protein biosynthesis in eukaryotic systems (Desjardins *et al.* 1993). Their structural diversity arises from the position, number, and type of the functional groups attached to the 12,13-epoxy-trichothec-9-ene skeleton. The mycotoxin is produced by certain Fusarium species such as *Fusarium graminearum* (type B trichothecene producer) and *F. sporotrichioides* (type A trichothecene producer), but not by most other species (*e.g.*, *F. oxysporum*, *F. moniliforme*, *F. decemcellulare*, and *F. solani*). Recently, about half of the biosynthesis genes have been identified in a gene cluster (*i.e.*, hereafter termed the *Tri5*-cluster since it contains *Tri5*, which is responsible for the first key step in the biosynthesis pathway) of *F. graminearum* and *F. sporotrichioides* (Brown *et al.* 2001; Lee *et al.* 2001). However, the rest of the pathway genes were missing from the *Tri5*-cluster (Kimura *et al.* 2001); so far, with the exception of only *Tri101* (Kimura *et al.* 1998a), genes outside of the *Tri5*-cluster have not yet been isolated from producers.

Tri101 encodes trichothecene 3-*O*-acetyltransferase that is involved in the protection of *F. graminearum* against its own toxin. It appears to be the only functional 3-*O*-acetyltransferase gene of the producer (Kimura *et al.* 1998a,c) and is under the transcriptional control of *Tri6*, a regulatory gene within the *Tri5*-cluster (McCormick *et al.* 1999). The resistance gene product acts mainly on the first step after the second cyclization (*i.e.*, conversion of isotrichodermol to isotrichodermin) in *F. sporotrichioides* (McCormick *et al.* 1999). Although the extent to which *Tri101* contributes to self-protection appears to differ between the type A and type B trichothecene producers, the resistance gene is similarly located between the phosphate permease gene (*pho5*) and the UTP-ammonia ligase gene (*ura7*) in their respective genomes. These housekeeping genes are also close together in the genome of the nonproducer *F. oxysporum*, suggesting that *pho5* and *ura7* may be linked in other Fusarium species (Kimura *et al.* 1998b). Since *F. oxysporum*

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appeared not to have apparent homologs of *Tri101* as assessed by standard hybridization conditions in genomic Southern analysis, the resistance gene has previously been assumed to have been acquired horizontally as a defense option in the evolution of Fusarium species (Kimura *et al.* 1998b).

Horizontal transfer of fungal nuclear genes has so far been suggested from (1) the occurrence of the gene in a particular strain or species and not in a large number of their close relatives and (2) the inconsistency of the phylogeny of the gene in question from the traditionally accepted phylogeny of the organism (Rosewich and Kistler 2000). However, instances of horizontal gene transfer are mostly considered "anecdotal" in fungi (Walton 2000). Clear evidence for horizontal transfer is expected as more genomic sequences become available for analysis.

To find clear evidence of horizontal gene transfer without whole genome sequences, we sought to verify previous Southern analysis for the lack of *Tri101* in several Fusarium species by cloning and sequencing their *pho5*-to-*ura7* regions. Sequence and functional analysis of these regions led to an unexpected finding with interesting implications regarding the evolutionary history of *Tri101* in that the nonproducers contained related pseudogenes in this region. Further analysis revealed that trichothecene nonproducer Fusarium strains possessed an additional functional 3-*O*-acetyltransferase gene different from *Tri101* (designated as *Tri201*).

MATERIALS AND METHODS

Fungal strains: *F. graminearum* F15 is a 3-acetyldeoxynivalenol (3-ADON) producer strain used in our previous studies (Kimura *et al.* 1998a,c). Other strains were purchased from the Institute for Fermentation (IFO; Osaka, Japan): *F. oxysporum* IFO 31983, *F. moniliforme* (*Gibberella fujikuroi*) IFO 31251, *F. decemcellulare* IFO 30918, *F. solani* IFO 31094, Fusarium species (sp.) IFO 7772 (strain IFO 7772 was incorrectly deposited as "*Gibberella zeae*"), *F. sporotrichioides* IFO 9955, and *Trichothecium roseum* IFO 5772. In the following paragraphs, a gene name is preceded by the abbreviated name of the fungus (*e.g.*, *Fopho5* for ***F. o****xysporum pho5*).

Cloning of a portion of *pho5* and *ura7* from the trichothecene nonproducing Fusarium species: To obtain the nucleotide sequences of *pho5* and *ura7* from the nonproducers, we first amplified a portion of these genes by PCR. Primers that successfully worked for the amplifications were (1) stPho51 (5′-ACTACTTT CATCGTCCCTGGTGAA-3′) and stPhod1 (5′-GATACCAAGAAG CATGAAAAGAGC-3′) for *Fopho5*, (2) stUra72 (5′-GGCGAGTGC TTTGTTCTCGATGAC-3′) and stUrad2 (5′-CTGAGTGGGCTTT GTCTTCTCCTC-3′) for *Foura7*, (3) stPho53 (5′-CTTCTGTGTCT CCATGTTGACCATCATGCTCGG-3′) and stPhod5 (5′-GGGCC AAAGTTGAAAAAGAATTGGGCAAGAACG-3′) for *Fmpho5*, (4) stUra74 (5′-TGCTGGTCTTCTTCTCAAGACTCTTGGTCTTCG-3′) and stUrad2 for *Fmura7*, (5) stPho53 and stPhod3 (5′-CTC CGAAGCCAACAAGTGTTCGCCACATCTTGT-3′) for *Fsppho5*, and (6) stUra73 (5′-CCGATCTCGATCTTGGAAACTACG-3′) and stUrad2 for *Fspura7*. The PCR products were cloned in pGEM-TEasy (Promega, Madison, WI) and sequenced.

Amplification of the syntenic regions by long PCR: On the basis of the partial sequence of *phos5* and *ura7*, the syntenic region of each nonproducer was amplified by long PCR from the genomic DNA using the LA-PCR kit (Takara Biomedicals, Kusatsu, Japan). Primers that worked well for the amplifications were (1) UP-P3 (5′-CGCTGCCTCAGGAAAGATTGGT TCCATCATTGG-3′) and rUP-U2 (5′-GCATCCGCATAAAA CAACAATCTCAAACAAAAC-3′) for *F. oxysporum*, (2) GfUP-P3 (5′-TTCCTGGCTACTGGGTCTCGGTTGCTACTATTG-3′) and rGfUP-U3 (5′-GCCAAGCAATCGGTAATCGGTAATCGG TAGGAG-3′) for *F. moniliforme*, and (3) GzUP-P1 (5′-AGCT CCCACACGCTCTGACAATGCTATCAAGC-3′) and GzUP-U2 (5′-CTGAGCATCAGCGTTTCCTACGTCCCTATTATC-3′) for Fusarium sp. IFO 7772. The amplified fragments (>8 kb) were cloned in pGEM-TEasy. Sequence templates were generated using the GPS1 genome priming system (New England Biolabs, Beverly, MA). Primers were synthesized on the basis of the above provisional sequences and used for both the PCR amplification and direct DNA sequencing.

Sequence comparison of the syntenic regions: Sequence identities were calculated by using the CLUSTALW program in the LASERGENE software (DNAstar, Madison, WI). The syntenic regions that contain all four (pseudo)genes oriented in the same direction (*i.e.*, *F. graminearum vs.* Fusarium sp. IFO 7772 and *F. oxysporum vs. F. moniliforme*) were analyzed by Harr plot analyses (Harr *et al.* 1982). The plots were made using the Harrplot 2.0 program (GENETYX-MAC 10.1, Software Development, Tokyo) with a window of 100 and a stringency of 75.

Construction of a phylogenetic tree: The phylogenetic relationships of the Fusarium species used in this study were confirmed by constructing a neighbor-joining tree (CLUSTALW at DDBJ) based on the 28S rDNA sequences. Fifteen of the reference 28S rDNA sequences were retrieved from GenBank (accession numbers indicated), 7 sequences were generated from strains obtained from the IFO, and the final sequence was generated from *F. graminearum* F15. Primers F65 and R635 (see Mule *et al.* 1997) were used for the PCR amplification. The sequences are deposited with GenBank under accession nos. AB084297–AB084304. Bootstrap tests were performed with 1000 replications for the consensus tree. The values are shown only at the nodes of major clades for simplicity.

Cloning of *FoTri201* from *F. oxysporum*: The cDNA library of *F. oxysporum* was constructed using the *Schizosaccharomyces pombe* expression vector pcDSP21 (Kimura *et al.* 1994); mRNA was isolated from spores germinated in the presence of 100 μg/ml T-2 toxin, and the *Sal*I-*Not*I directional library was constructed using a Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA). *S. pombe* cells carrying the cDNA expression library were selected with T-2 toxin as described previously (Kimura *et al.* 1998a). The cDNA in the shuttle vector was recovered from a resistant colony and its nucleotide sequence was determined. Using the LA PCR *in vitro* cloning kit (Takara Biomedicals), the genomic regions upstream and downstream of *FoTri201* were amplified by vectorette PCR from *Mun*I and *Xho*I cassette libraries, respectively. The following primers were used for the amplifications: primer Fo201-longD1 (5′-CATTTTTGATGGTGGCG GTGATGGCAGGATGTG-3′) and nested primer Fo201-longD2 (5′-TCGGTGATTGGGAAGCAGAAGCTGACTTGGGTGTAG GATC-3′) for cloning of the upstream region and primer Fo201-longU1 (5′-CCGATGGAGAAATTACTGCTGCGTTAT CGCTG-3′) and nested primer Fo201-longU2 (5′-TATCGCT GAGGGATGAGGATACGGAGACATTG-3′) for cloning of the downstream region.

Cloning of *FmTri201* from *F. moniliforme*: On the basis of the sequence of *FoTri201*, we designed primers Fo201-cloneU1 (5′-GACCTAGACATAGAGCTGGACATCATCG-3′) and Fo201-cloneD2 (5′-GCCAATGAATTGCCCATACTTTTTCCAC-3′) and used them to amplify a portion of *FmTri201* from *F. monili-*

forme. The PCR product was cloned in pGEM-TEasy and sequenced. The regions upstream and downstream of this fragment were amplified by vectorette PCR from *Eco*T22I and *Fba*I cassette libraries, respectively. Primer Gf201-longD1 (5′-TGAATACGCCAGTGCCATCATCTTTAACCTGG-3′) and nested primer Gf201-longD2 (5′-ACCCAAGGGAAGCTCTGA GATAGACGCTGTAG-3′) were used for amplification of the upstream region, and primer Gf201-longU1 (5′-CTTGGATT GGGGAATCCTGAGAGTGTGAGGAG-3′) and nested primer Gf201-longU2 (5′-TCATGCCCAAGAGACCAGACGGAGAAA TAAC-C-3′) were used for amplification of the downstream region.

***In vitro* acetyltransferase assays:** The coding regions of *Tri101* and *Tri201* were amplified by PCR using the following primers (mismatched bases are underlined): Nco-Fm101 (5′-TACACCATGGTCGATCTAGACGTCGAAATCAACC-3′) and Bam-Fm101 (5′-GAATGGATCCTGTGATCAATGCTGATCGAC GTCTG-3′) for *FmTri101*; Nco-Fsp101 (5′-TTCTACACCATG GCCAACATAGATACTCCTCATC-3′) and Bam-Fsp101 (5′-ATGGGATCCAAGCTCATCAATGCGCAGGCCCAAG-3′) for *FspTri101*; and Nde-Fo201 (5′-GTTACACATATGGTCGACC TAGACATAG-3′) and Bam-Fo201 (5′-CATTGGATCCCCCGC GAGCAACCGGAAAC-3′) for *FoTri201*. The *Nco*I-*Bam*HI or *Nde*I-*Bam*HI fragments of the PCR products (without nonsynonymous substitutions in the coding region) were cloned in pET-19b or pET-12a (Novagen), respectively, and propagated in *Escherichia coli* DE3 (BL21). Recombinant enzyme was prepared from the bacteria and assayed for 3-*O*-acetyltransferase activity as described (Kimura *et al.* 1998b); the sample was developed on a TLC plate (Merck silica TLC) using ethylacetate/toluene (3:1) as the solvent.

Northern blot analysis and reverse transcriptase-PCR: Total RNA was isolated from mycelia as described previously (Kimura *et al.* 1998b). Ten micrograms of RNA samples were denatured, separated on agarose gels containing formaldehyde, and transferred to nylon membranes (Schleicher & Schuell, Dassel, Germany). Probes were prepared by labeling the DNA fragments (entire length of *FspTri101* or *FoTri201* cloned in pGEM-TEasy) with digoxigenin using the RNA labeling kit (Roche Applied Science). Hybridization, washing, and detection were accomplished according to the manufacturer's protocol. For reverse transcriptase (RT)-PCR, the cDNA was synthesized from total RNA (treated with the RNase-free DNase I) using the Superscript first-strand synthesis system (Invitrogen, San Diego). The following primers were used for the amplification of the *Tri201* cDNA: FoTri201-U (5′-CACTAAACGTTACAAA CATGGTCGAC-3′) and FoTri201-D (5′-GCCAATGAATTGC CCATACTTTTTCCAC-3′).

RESULTS

Cloning of the *pho5*-to-*ura7* regions from trichothecene nonproducing Fusarium species: A portion of *pho5* and *ura7* could be amplified from all nonproducers examined by PCR based on the corresponding gene sequences of *F. graminearum* (Kimura *et al.* 1998b). Primers were designed on the basis of the sequence of each fungal strain and used for long PCR. By this strategy, we were able to amplify the *pho5*-to-*ura7* regions from *F. oxysporum*, *F. moniliforme*, and Fusarium sp. IFO 7772, but no PCR products were obtained from *F. decemcellulare* and *F. solani* (see also supplemental figures at http://www.genetics.org/supplemental/).

In sequence analyses of the regions between *pho5* and *ura7*, an unexpected result was obtained. The BLASTX search (similarity at the translated level) generated significantly high alignment scores of these regions against the FgTRI101 polypeptide (accession no. BAA24430; *i.e.*, *FoTri101*, 143 bits, $E = 4 \times e^{-33}$; *FmTri101*, 125 bits, $E = 1 \times e^{-27}$; and *FspTri101*, 612 bits, $E = 1 \times e^{-174}$). In addition, pseudo succinyl-CoA synthase genes (*scs*) were identified between *pho5* and *ura7* in *F. oxysporum* (293 bits, $E = 3 \times e^{-78}$, to CAB11045) and *F. moniliforme* (242 bits, $E = 7 \times e^{-71}$, to CAB11045); this gene was not present between *pho5* and *ura7* in *F. graminearum*, but was located nearby. We were able to amplify the region upstream of *ura7* from *F. graminearum* (1188 bp; GenBank accession no. AB076252) and Fusarium sp. IFO 7772 using primer rstSCS2 (5′-GACACTGGTTTGGGG CGATGATACCAGGACAAT-3′), which is based on the sequence of *Foscs*. In this manner, the regions from all four Fusarium species proved to be syntenic; they all contained *pho5*, *Tri101*, *ura7*, and *scs* (Figure 1A). All exon and intron regions were determined by RT-PCR of individual (pseudo)genes. The final sequences deposited with GenBank contain the regions flanked by the following primer annealing sites: between stPho53 and stUrad2 for the sequences of *F. oxysporum* (8856 bp; AB076253) and *F. moniliforme* (8435 bp; AB076254) and between stPho53 and rstSCS2 for the sequence of Fusarium sp. IFO 7772 (9362 bp; AB076255).

Characterization of the syntenic regions: Four genes (including pseudogenes) were in the syntenic region of these Fusarium strains; the directions and orders of these genes, however, were different as shown in Figure 1A. Such rearrangements involve inversion processes (marked by crossed lines between gene lines of Fusarium sp. IFO 7772 and *F. oxysporum*), which are often associated with inversely oriented repeat sequences situated close to the inversion end points. However, the inverted repeat motifs (marked by IR in Figure 1A) were evident only upstream and downstream of *FmTri101* (bp 1959–2021 and bp 3918–3856 of AB076254, with 9 bp of mismatches). RT-PCR of candidate housekeeping genes (*i.e.*, *pho5*, *ura7*, and *scs*) revealed that all of them are transcribed; subsequent sequence analyses indicated that the corresponding coding regions of *Fgscs*, *Foscs*, and *Fmscs* were interrupted by stop codons as determined from cDNA sequencing (Figure 1A).

The syntenic regions of Fusarium strains were compared by Harr plot analyses with pairs of *F. graminearum vs.* Fusarium sp. IFO 7772 (Figure 1B, left) and *F. oxysporum vs. F. moniliforme* (Figure 1B, right). The coding regions of *pho5* and *ura7* were significantly conserved among Fusarium strains (see also supplementary tables at http://www.genetics.org/supplemental/). No significant similarities were found between the *scs* sequences of *F. graminearum* and Fusarium sp. IFO 7772; small regions of similarities, however, were noticed downstream (labeled region S; Figure 1, A and B) and at the C terminus of

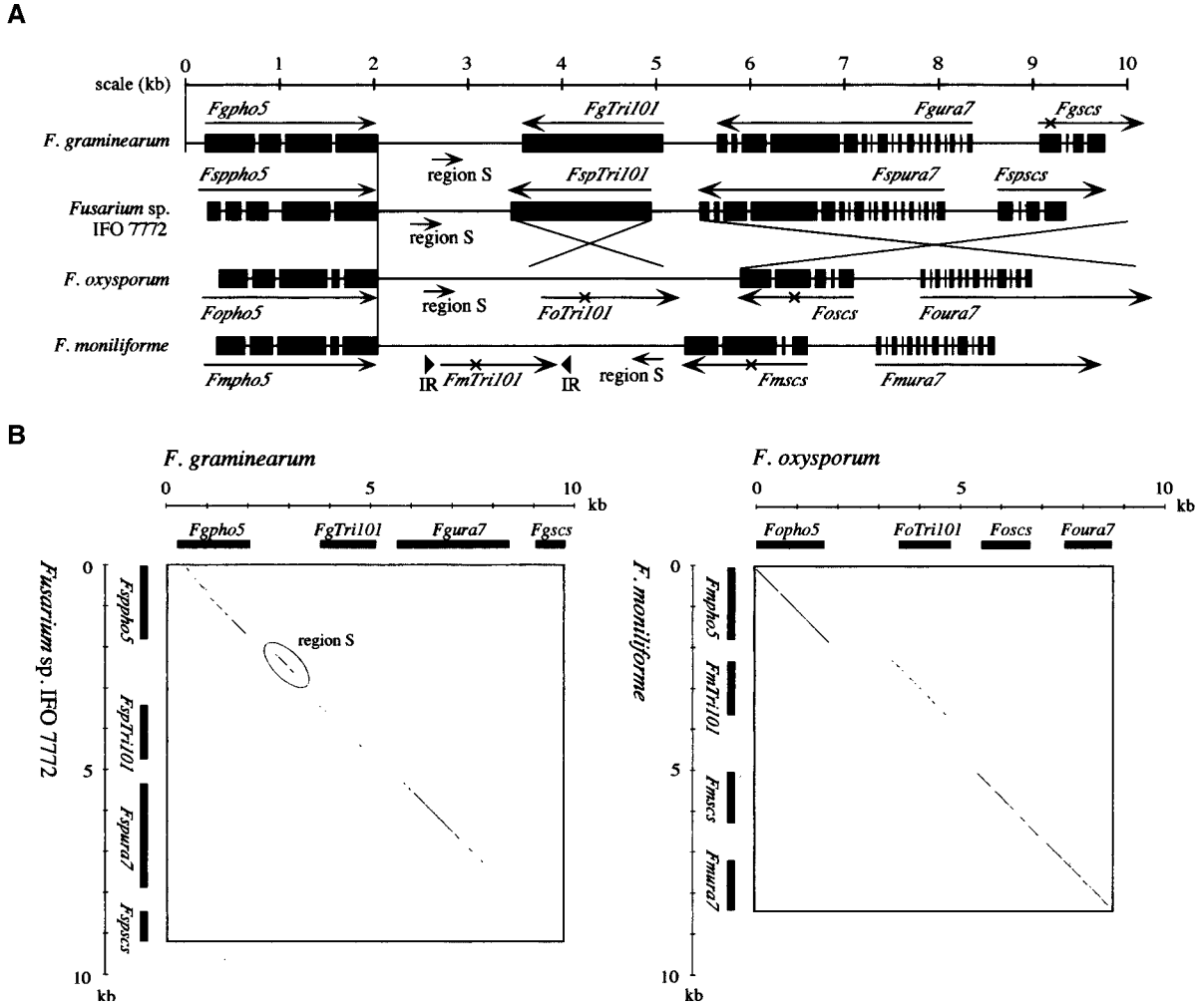

FIGURE 1.—Comparative analyses of trichothecene producer and nonproducer Fusarium species. (A) The *pho5*-to-*ura7* syntenic regions from four Fusarium species are illustrated. Exons are shown in solid boxes. Arrows (over the solid boxes) represent directions of the genes and pseudogenes. X indicates that the gene function is inactivated by mutations (*e.g.*, the presence of stop codons or substitution of active site residues of the encoded enzyme). A set of imperfect inverted repeats (IR) was found adjacent to *FmTri101* (total 63 bp with 9 bp of mismatches; AB076254), but not adjacent to *FgTri101*, *FoTri101*, and *FspTri101*. Crossed lines denote inversions of the indicated regions. Inverted repeat motifs (including imperfect ones) of significant length were found only upstream and downstream of *FmTri101* from *F. moniliforme*, but not around other (pseudo) genes examined in this study. (B) Harr plot analysis showing the nucleotide sequence similarity of the syntenic region; *F. graminearum vs.* Fusarium sp. (left) and *F. oxysporum vs. F. moniliforme* (right). The comparison was performed with a base window of 100 and stringency of 75. A local region of significant nucleotide sequence similarity (identities >80%) was found in the intergenic region of the four strains (marked by region S; see also Figure 1A in which the directions of alignments are indicated by arrows).

Tri101. In alignment of the syntenic regions of *F. oxysporum* and *F. moniliforme*, gaps of significant sizes appeared upstream and downstream of *Tri101*. Except for these gaps, these two strains showed relatively high similarities over the entire length of these regions (including the *scs-ura7* intergenic region).

Phylogenetic position of Fusarium sp. IFO 7772: Since the phylogenetic position of Fusarium sp. IFO 7772 was not certain, we examined the evolutionary relationships of the fungal strains used in the study using 28S rDNA sequence data. As shown in Figure 2A, Fusarium sp. IFO 7772 was separated from *F. decemcellulare* and *F. solani* and clustered together with other strains that belong to the teleomorph genus Gibberella. Strain IFO 7772 did not fall into a group that produces trichothecenes, which is in accordance with our failure to detect *Tri5* by Southern blot analysis (data not shown).

Characterization of *Tri101* from the trichothecene nonproducing strains: The putative coding region of *FoTri101* was interrupted by an ochre codon and an opal codon. This observation indicates that the gene occurs as a pseudogene in *F. oxysporum*. In contrast, *FmTri101* and *FspTri101* did not contain any stop codons; each revealed an open reading frame (ORF) that encodes a predicted

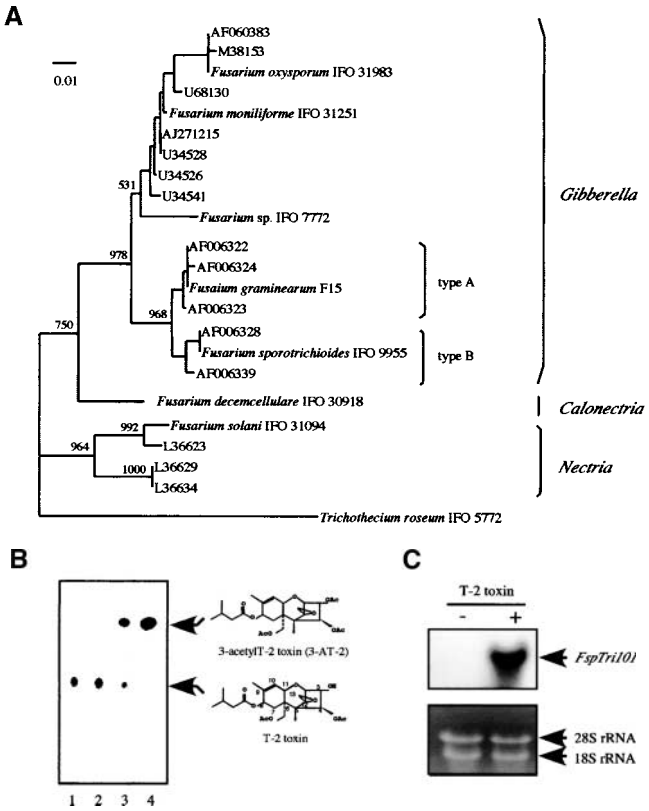

FIGURE 2.—The nonproducer strains that belong to the teleomorph genus Gibberella have either a functional or a nonfunctional *Tri101*. (A) Phylogenetic relationships of the Fusarium strains used in the study determined from 28S rDNA sequence comparisons. The neighbor-joining tree was constructed by using the reference sequences in GenBank (accession numbers are shown). The type of trichothecenes produced by the fungal strains is indicated as type A or type B; the teleomorph genus (*e.g.*, Gibberella) is shown on the right part of the tree. The scale bar indicates one base change per 100 nucleotide positions. Bootstrap values from 1000 replications are shown at the nodes of major clades. Fusarium sp. IFO 7772 was positioned as a nonproducer strain that belongs to the teleomorph genus Gibberella. (B) *In vitro* acetylation assay of trichothecene by recombinant TRI101. Lane 1, T-2 toxin standard; lanes 2 and 3, ethylacetate extract of T-2 toxin incubated with acetyl-CoA and the crude extract of bacteria expressing *FmTri101* (lane 2) or *FspTri101* (lane 3); lane 4,3-acetylT-2 toxin (3-AT-2) standard. (C) Northern blot analysis. Spores of Fusarium sp. IFO 7772 were germinated with or without 100 μg/ml of T-2 toxin on YG medium. Total RNA samples were run on a 0.8% formaldehyde-agarose gel (stained with ethidium bromide; bottom) and then hybridized with the *FspTri101* riboprobe after transfer to a nylon membrane (top).

protein of 458 and 453 amino acids, respectively. Among these sequences, the *FspTri101* ORF, but not the *FmTri101* ORF, maintained a perfect conservation of the "HXXXDG" motif, which is known to be the catalytic domain of an acetyltransferase family (ST-PIERRE *et al.* 1998). No TRI6-binding consensus sequences (HOHN *et al.* 1999) were upstream of the initiation codons.

In accordance with the structural features of the encoded proteins, only recombinant FspTRI101 expressed in *E. coli* showed trichothecene 3-*O*-acetyltransferase activity (Figure 2B). To determine if the resistance (pseudo)genes were transcribed in the three nonproducers, we isolated total RNA from the spores germinated in the presence or absence of 100 μg/ml T-2 toxin. As shown in Figure 2C, induction of *FspTri101* expression by T-2 toxin was demonstrated by Northern blot analysis. However, the transcripts of *FoTri101* and *FmTri101* were not detected even by the sensitive RT-PCR assay under either culture condition (data not shown).

Cloning and characterization of *FoTri201* from *F. oxysporum*: Although inactivating mutations are present in the coding regions of *FoTri101* and *FmTri101*, germinating spores of *F. oxysporum* and *F. moniliforme* showed resistance to trichothecenes and also possessed activities to transfer an acetyl group to the C-3 position of trichothecenes *in vivo* (data not shown). This implied that they possess another unidentified 3-*O*-acetyltransferase gene. We thus attempted to clone the responsible gene from *F. oxysporum* as described in MATERIALS AND METHODS.

S. pombe transformants carrying the cDNA expression library were transferred to YEA plates containing 50 μg/ml T-2 toxin. Among 5000 Ura^+ transformants, one T-2 resistant clone was obtained. Analysis of the nucleotide sequence of the cDNA insert revealed a putative ORF (designated as *FoTri201*) with significant similarity to FgTRI101 protein: this cDNA clone was subsequently determined to specify a truncated but active form (7 bp deleted from the first A of the initiation codon, which resulted in deletion of 8 amino acid residues from the N terminus; see below). The active site motif HXXXDG was conserved in FoTRI201 (Figure 3). On the basis of the cDNA sequence, the coding region of *FoTri201* was amplified by PCR from *F. oxysporum* genomic DNA. Sequence analysis of the product showed that the coding region was not interrupted by introns. The promoter and terminator regions of *FoTri201* were cloned by vectorette PCR (GenBank accession no. AB083516). The upstream region contained an original initiation codon 24 bp upstream from the first ATG of the cloned cDNA; the transcription of this upstream ATG codon was confirmed by RT-PCR analysis (data not shown). The complete *FoTri201* ORF encoded a predicted protein of 454 amino acids with 63.0% similarity to the FgTRI101 polypeptide (BLASTP score; 582 bits, $E = 1 \times e^{-165}$). As for the *Tri101*-related genes of the nonproducers, the TRI6-binding consensus sequence (HOHN *et al.* 1999) was not found in the promoter region of *FoTri201*.

In the Northern blot analysis, only a small amount of the *FoTri201* transcript was detected from spores germinated in the presence of T-2 toxin (Figure 4A); induction of the resistance gene by addition of trichothecene was not as evident as we have observed in *F. graminearum* (KIMURA *et al.* 1998c) and Fusarium sp. IFO 7772 (this

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FgTRI101      1 ----------MAFKIQLDTLGQLPGLLSIYTQISLLYPVSDPSQYPTIVSTFEQGLKRFSEAVPWVAGQVKAEGISEGNTGTSFIV-PFEDVPRVVVKDLRDDPSAPTIEGMRKAGYPMA  109
FspTRI101     1 ---MANI-DTPHLHMELDTLGQQPLLR-IYTQISLCFPVADPSKHSTIVATLQHGLSRLAQSFPWVAGQVKCDAKEGTS-GI-FSIVPFEKVPRLVVKDLRDDPSAPTMEGLRKAEFPMR  113
FoTRI201      1 MTALN-VTNMVDLDIELDIIGQQPFMVKIYTQVSFCFPITDPSTHPAITATIKNGLQRLSQSFPWVAGQVKDDV---T--GV-FKIKPFEATPRLVVKDLRDDPSAPTMEDLRKAEFPMS  113
                  . ..**..**.* .  ****.*...*. *** ...*..*   ** *.....******** .    .  * * . *** .**.**************.*..***..**

FgTRI101    110 MFDENIIAPRKTLPIGPGTGPDDPKPVILLQLNFIKGGLILTVNGQHGAMDMVGQDAVIRLLSKACRNDPFTEEEMTAMNLDRKTIVPYLENYTIGPEVDHQIVKPDVAGGDAVLTPVSA  229
FspTRI101   114 MFDENVIAPRKTLPIGPDYSPNDPEPVLLVQVNFIEGGLILTVNAQHACMDMMGQDAVVRLLSKACRKEEFTEEEITTINIDRKNAVPLIENYEVGPELDHQLVKSPPPTEQSPPPPAS-  232
FoTRI201    114 MFDENKIAPRKTLPIGPDYSPDDPLPVLIFQLNFIEGGLILTVNGQHGCMDMTGQDELIRLLSKACRDEAFTQEEISTMNLERKTIVPPLENYELGPELDHQIIKPPPTTETPPAPPKAS  233
                ***** ***.*******...*.** **.. *.***.********.**..*** ***...******** . **.**....*..**..** .***. ***.***..*.... ..  . .* .

FgTRI101    230 SWAFFKFSPKAMSELKDAATKTLDASTRFVSTDDALSAFIWKSASRVRLERIDGSAPTEFCRAVDARPAMGVSNNYPGLLQNMTYHNSTIGEIANESLGATASRLRSELDPASMRQRTRG  349
FspTRI101   233 -WVLFSFSPQALSELKDKATQSLDPSTKFISTDDAVSAFIWQSVTRVRLAHLDASAPTNFRRAVDVRAALDAPKEYPGMLQNMTYSISTLSQIANEPLGAVASRLRRELSPSSLRQRTRG  351
FoTRI201    234 -WAFFSFSPQALSDLKDKATQTLDAGTRFVSTDDALSAFIWQSVSRARRARLDDSTSTQFCRAVDVRTQLDVPKNYPGILQNMTYSVSKLSQIANEPLGIVASRLRSELGRDDLRRRTQA  352
                 *..*.***.*.*.***.**..**..***.*****.*****.*..*.*....* *..* *.****.* .......*** ******. *....****.**..*****.** . ..*.**..

FgTRI101    350 LATYLHNNPDKSNVSLTADADPSTSVMLSSWAKVGLWDYDFGFGLGKPETVRRPIFEPVESLMYFMPKKPDGEFCAALSLRDEDMDRLKADKEWTKYAQYVG  451
FspTRI101   352 LVTYLHNQPDKSVVSLTADANSSTDIMLSSWAKTGFWGYDFGLGLGKPESVRRPLFEPFESLAYLLPKRPDGEITAALSFRDEDIERLKVDEEWVKYAQYIG  453
FoTRI201    353 LVTYLHDQTNRASVSVTADANPSTDIMLSSWAKLKCWDYDFGLGLGKPESVRRPLFEPFESLMYLMPKRPDGEITAALSLRDEDTETLKQDEKWKKYGQFIG  454
                *.****..... **.****..**..******** . *.****.***.**.****.***.***.*...**.****..****.**** ..** *..* **.*..*
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FIGURE 3.—Amino acid sequence of TRI201 from the nonproducer *F. oxysporum*. The deduced amino acid sequence of *F. oxysporum Tri201* (FoTRI201; bottom) was compared to TRI101 from *F. graminearum* (FgTRI101; top) and Fusarium sp. IFO 7772 (FspTRI101; middle). The first ATG of the truncated *FoTri201* clone recovered in *S. pombe* corresponds to the ninth Met residues (see text). Asterisks and dots indicate identical and similar amino acids, respectively. Residues that comprise the catalytic triad (*i.e.*, the HXXXDM motif) are boxed.

study). A sensitive and semiquantitative RT-PCR assay also showed slightly elevated expression of *FoTri201* in the trichothecene-treated culture (Figure 4B). As expected from the conservation of the active site sequence motif, recombinant FoTRI201 produced in *E. coli* could specifically transfer an acetyl group to C-3 of T-2 toxin, nivalenol (NIV), and deoxynivalenol (DON) (Figure 4C).

Cloning of *FmTri201* from *F. moniliforme*: The coding region of *Tri201* was highly conserved between *F. oxysporum* and *F. moniliforme*: *FmTri201* from *F. moniliforme* could easily be amplified by PCR with primers based on the sequence of *FoTri201*. The *Eco*T22I-*Fba*I fragment (2216 bp; AB089164) containing the upstream and downstream regions of *FmTri201* was cloned by vectorette PCR as described in MATERIALS AND METHODS. The complete ORF of *FmTri201* coded for a polypeptide of 454 amino acid residues with high similarity to *FoTri201* (85.8 and 89.2% identities at the nucleotide and peptide sequence level, respectively); the active site motif was also conserved.

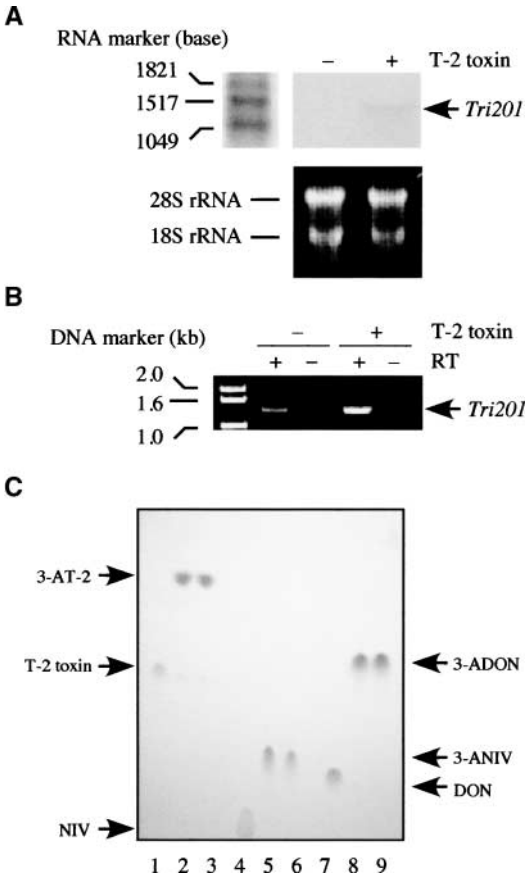

FIGURE 4.—Expression analyses of *Tri201*. (A) Northern blot analysis. The RNA probe of *FoTri201* was hybridized to the total RNA of the fungus in either the presence (+) or the absence (−) of T-2 toxin. (B) RT-PCR. Amplification of *FoTri201* was done using the cDNA samples (RT; +) from both the trichothecene-treated (T-2 toxin; +) and untreated (T-2 toxin; −) cultures. Control reactions (RT; −) did not yield the PCR products. (C) TLC of *in vitro* acetylation assay. Lanes 1–9, standard samples of T-2 toxin (lane 1), 3-AT-2 (lane 3), NIV (lane 4), 3-acetylnivalenol (3-ANIV; lane 6), DON (lane 7), and 3-ADON (lane 9); ethylacetate extract of T-2 toxin (lane 2), NIV (lane 5), or DON (lane 8) after 3 hr of incubation with acetyl-CoA and recombinant FoTRI201.

DISCUSSION

This study indicated that *Tri101* is located in the *pho5*-to-*ura7* syntenic region of Gibberella strains that do not produce trichothecenes. Our previous false conclusion regarding the horizontal acquisition of *Tri101* (KIMURA *et al.* 1998b), which was based on the suggestion that nonproducers lacked *Tri101*, could be attributed to the lack of significant nucleotide sequence similarities between the corresponding sequences of producers and nonproducers. Since long PCR (using several pairs of perfect match long primers) has not been successful for cloning the corresponding region from *F. decemcellulare* and *F. solani*, we have not isolated *Tri101* from these fungal strains. It remains to be elucidated whether *Tri101* was acquired after the major diversification of

Gibberella from Calonectria or earlier in the evolutionary history of Fusarium species (Figure 3C).

Of the three *Tri101* regions cloned from trichothecene nonproducing Fusarium, only *FspTri101* proved functional with expression responsive to the addition of T-2 toxin to the culture. This feature is similar to that of *F. graminearum* (Kimura *et al.* 1998c), in which the resistance gene is under the transcriptional control of *Tri6* (Hohn *et al.* 1999). However, no TRI6-binding consensus sequences were in the promoter region of *FspTri101.* One possible explanation is that the extant producer species (*e.g.*, *F. graminearum*) evolved with fortuitous mutations in the *Tri101*-promoter regions to allow a noncluster trichothecene gene to be efficiently coregulated with other pathway genes. Alternatively, it is possible that the ancestor of Fusarium sp. IFO 7772 was a trichothecene producer and fortuitous mutations wiped out existing TRI6-binding sites or that it elaborated an alternative system for transcriptional activation of *FspTri101.*

Surprisingly, *F. oxysporum* and *F. moniliforme* possessed trichothecene 3-*O*-acetyltransferase activities although *Tri101* was a nonfunctional pseudogene in these nonproducers. To search for the gene responsible for the acetylation, we isolated *FoTri201* from *F. oxysporum* by cDNA expression cloning. On the basis of this sequence, *FmTri201* was then cloned from *F. moniliforme* by PCR techniques. At the nucleotide sequence level, the similarity of *Tri201* to *FgTri101* was so low that it failed to be detected by standard Southern blot analysis. Perhaps the presence of functional (*i.e.*, *Tri201*) and nonfunctional (*i.e.*, *Tri101*) 3-*O*-acetyltransferase genes represents a classical example of gene duplication (Kistler *et al.* 1995) and the absence of significant nucleotide sequence similarity reflects a long evolutionary history subsequent to the duplication event. Analyses of the neighbor genes to *Tri201* may provide additional clues concerning function (other than conferring resistance to added trichothecenes) of this gene in *F. oxysporum* and *F. moniliforme.*

The aflatoxin biosynthesis genes exist in the aflatoxin nonproducer *Aspergillus oryzae*, which has traditionally been used as a *koji* mold for food and beverage fermentation in Asian countries (Klich *et al.* 1995; Kusumoto *et al.* 1996). In this context we should not work on an implicit assumption that the trichothecene biosynthesis gene cluster has been absent from the genome in the evolutionary history of *F. oxysporum* and *F. moniliforme.* It is also possible that the ancestor of the extant nonproducer had once acquired a cluster of functional biosynthesis genes and subsequently lost functions and similarities by the accumulation of random mutations (due to the lack of selective advantage to having this gene cluster for *F. oxysporum* and *F. moniliforme*). In contrast, there is a selective pressure to maintain the functions of the cluster genes in *F. graminearum* [*i.e.*, trichothecenes have a role as a pathogenicity factor for *F. graminearum* during its parasitism to the sensitive host plant (Proctor *et al.* 1995)].

Complete genome databases will contribute significantly in understanding the phytopathogenicity of fungi and the molecular biology of trichothecene biosynthesis (Pennisi 2001). In addition, as we have learned from the unexpected discovery of trichothecene biosynthesis genes (*i.e.*, pseudo-*Tri101* and *Tri201*), the genomics of related nonproducer species may also lead to understanding the evolutionary history of the trichothecene biosynthesis genes. In fact, recent progress of the *Magnaporthe grisea* genome sequence project has revealed a region that is syntenic to a portion of the *Neurospora crassa* genome (Hamer *et al.* 2001). In view of the conservation of gene organization across those distant genera, the existence of syntenic regions within the genome of a single genus is not surprising. Hence, comparative studies of the trichothecene producers and closely related nonproducers will be important to fully understand the evolution of the genes necessary for trichothecene biosynthesis.

We thank Dr. Kerry O'Donnell for confirmation of Fusarium sp. IFO 7772 as a trichothecene nonproducer strain.

Note added in proof: Region S (Figure 1) proved to correspond to a recently released EST (GenBank accession no. BU065253) of *F. graminearum* (nitrogen-starved mycelia).

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