Concordant evolution of trichothecene 3-O-acetyltransferase and an rDNA species phylogeny of trichothecene-producing and non-producing fusaria and other ascomycetous fungi

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The cereal pathogen Fusarium graminearum species complex (e.g. Fusarium asiaticum, previously referred to as F. graminearum lineage 6) produces the mycotoxin trichothecene in infected grains. The fungus has a gene for self-defence, Tri101, which is responsible for 3-O-acetylation of the trichothecene skeleton in the biosynthetic pathway. Recently, trichothecene non-producers Fusarium oxysporum and Fusarium fujikuroi (teleomorph Gibberella fujikuroi) were shown to have both functional (Tri201) and non-functional (pseudo-Tri101) trichothecene 3-O-acetyltransferase genes in their genome. To gain insight into the evolution of the trichothecene genes in Gibberella species, the authors examined whether or not other (pseudo-)biosynthesis-related genes are found near Tri201. However, sequence analysis of a 12 kb region containing Tri201 did not result in identification of additional trichothecene (pseudo-)genes in F. oxysporum. In a further attempt to find other trichothecene (pseudo-)genes from the non-producer, the authors examined whether or not the non-trichothecene genes flanking the ends of the core trichothecene gene cluster (i.e. the Tri5 cluster) comprise a region of synteny in Gibberella species. However, it was not possible to isolate trichothecene (pseudo-)genes from F. oxysporum (in addition to the previously identified pseudo-Tri101), because synteny was not observed for this region in F. asiaticum and F. oxysporum. In contrast to this unsuccessful identification of additional trichothecene (pseudo-)genes in the non-producer, a functional trichothecene 3-O-acetyltransferase gene could be identified in fusaria other than Gibberella: Fusarium decemcellulare and Fusarium solani; and in an ascomycete from a different fungal genus, Magnaporthe grisea. Together with the recent functional identification of Saccharomyces cerevisiae ScAYT1, these results are

Abbreviations: 3-ADON, 3-acetyldeoxynivalenol; DON, deoxynivalenol; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are AB181459–AB181481, AB193099 and AB193100.

A list of hypothetical genes identified on Fusarium oxysporum cosmids is shown in Supplementary Table S1, an analysis of the region containing genes A–O in F. oxysporum and Fusarium fujikuroi in Supplementary Fig. S1 and an analysis of the trichothecene 3-O-acetyltransferase of Magnaporthe grisea in Supplementary Figure S2 with the online version of this paper at http://mic.sgmjournals.org.
INTRODUCTION

Trichothecenes are a large group of protein synthesis inhibitors in eukaryotes. These secondary metabolites are also known as mycotoxins and cause contamination of agricultural and food products. Fungi in several different genera, including Fusarium, Trichothecium and Myrothecium, produce various types of trichothecenes (Desjardins et al., 1993). The biosynthesis of trichothecene has perhaps been most extensively studied in Fusarium species, such as the T-2 toxin (a highly toxic trichothecene) producer Fusarium sporotrichioides (Beremand, 1987; Brown et al., 2001; Keller & Hohn, 1997), and in wheat head blight fungi such as Fusarium asiaticum (previously referred to as Fusarium graminearum) lineage 6 (O’Donnell et al., 2004; Kimura et al., 2003b; Lee et al., 2001, 2002; Wuchiyama et al., 2000) and F. graminearum lineage 7 (Brown et al., 2001, 2002). Following the isolation of Tri5 (Hohn & Beremand, 1989), some but not all trichothecene genes were found to cluster around this first gene in the biosynthetic pathway (Hohn et al., 1993). Recently, at least two new genes in the pathway, Tri1 (McCormick et al., 2004; Meek et al., 2003) and Tri16 (Peplow et al., 2003), were identified at another single locus in the genome of F. sporotrichioides and F. graminearum (Brown et al., 2003). However, most of the other trichothecene genes (either dispersed or clustered) remain to be identified (Kimura et al., 2003b).

The resistance genes of antibiotic producers are often linked to the biosynthesis gene clusters in the genome of the producers (Cundliffe, 1989). We previously found that 3-O-acetylation of the trichothecene skeleton is a self-protection mechanism for F. asiaticum. Based on this finding, the gene responsible, Tri101, was cloned by expression cloning using fission yeasts (Kimura et al., 1998a). However, this gene, involved in resistance and biosynthesis (McCormick et al., 1999), was located between a UTP-ammonia ligase gene (ura7) and a phosphate permease gene (pho5); that is, it was not clustered with other trichothecene genes (Kimura et al., 1998b). Furthermore, the trichothecene non-producers Fusarium oxysporum and Fusarium fujikuroi [incorrectly referred to as Fusarium moniliforme in our previous study (Kimura et al., 2003a); teleomorph Gibberella fujikuroi] were unexpectedly found to carry both functional and non-functional trichothecene 3-O-acetyltransferase genes (Tri201 and Tri101, respectively). These trichothecene non-producers possess a pseudo-Tri101 between pho5 and ura7, which comprises a region of microsynteny in Gibberella species. These findings raise the possibility that the ancestor of F. oxysporum was a trichothecene producer and that Tri201 is an original copy of a duplicated 3-O-acetyltransferase gene involved in biosynthesis (Kimura et al., 2003a). In fact, non-aflatoxigenic Aspergillus species are known to have a dysfunctional aflatoxin gene cluster in their genome (Klich et al., 1995; Kusumoto et al., 1998) and it is a question of whether or not a similar case holds true for the trichothecene gene cluster in Gibberella species.

In non-aflatoxigenic Aspergillus species that are phylogenetically close to aflatoxin-producing species (i.e. where both belong to Aspergillus section Flavi), the genes for aflatoxin biosynthesis have been identified by Southern blot analysis (Klich et al., 1995). In contrast to these Aspergillus species, the divergence of trichothecene producer and non-producer Gibberella species occurred long ago, and functional 3-O-acetyltransferase genes have not been detected on a Southern blot (Kimura et al., 1998b). There are no extant strains with a near-isogenic background which may be used for a comparative analysis. Chromosome numbers and gene orders could differ between the producer and non-producer strains, and it is difficult to trace the evolutionary events that have happened to the ancestral Fusarium species (Kimura et al., 2001). However, if the non-biosynthesis genes surrounding the trichothecene genes are found in syntenic regions of the F. graminearum species complex and of the F. oxysporum genomes (e.g. the pho5–ura7 region containing Tri101), it might be possible to gain insight into the evolution of the gene cluster in the absence of the entire genome sequence.

We therefore constructed a cosmid library of F. oxysporum to (1) identify genes on both sides of Tri201 and (2) isolate orthologues of non-trichothecene genes demarcating the core trichothecene gene cluster (i.e. the Tri5 cluster) (Kimura et al., 2003b). These non-trichothecene genes were used for a comparative analysis of F. asiaticum and F. oxysporum. We also describe the identification and characterization of functional trichothecene 3-O-acetyltransferase genes from diverse fungal genera. These results are discussed in relation to the evolutionary history of the trichothecene genes in Gibberella species.

METHODS

Strains and growth conditions. F. asiaticum F15, F. oxysporum IFO 31983, F. fujikuroi IFO 31251, Fusarium acuminatum IFO 7772 [referred to as Fusarium species IFO 7772 in our previous study (Kimura et al., 2003a)], Fusarium decemcellulare (teleomorph; Albonectria rigidisscula) IFO 30918, Fusarium solani IFO 31094 and Magnaporthe grisea (anamorph; Pyricularia oryzae) P2 have been described previously (Banno et al., 2003; Kimura et al., 1998a, 2003a). Fusarium nygamai MAFF 239069, Fusarium niskaidoi MAFF 237507 and Fusarium avenaceum TNP-G-3 are in Dr Aoki’s fungal collection at the Ministry of Agriculture, Fishery, and Forestry of Japan (MAFF) Genebank. Aspergillus nidulans (teleomorph, Emericella nidulans) IFO 33017 was purchased from NITE Biological Resource Center (NBRRC), Kisarazu, Japan. Saccharomyces cerevisiae INVSc1 was
obtained from Invitrogen and used throughout this study. Fungal strains were maintained on Difco potato glucose agar (Becton Dickinson) plates at 20 °C. Mycelia were grown on YG medium (0.5% yeast extract, 2% glucose) and used for extraction of DNA and RNA.

**Construction of a *F. oxysporum* cosmid library and PCR screening of positive clones.** A cosmid library of *F. oxysporum* was constructed using the SuperCos1 cosmid vector (Stratagene), following the manufacturer’s instructions. Individual cosmid clones were distributed in 96-well plates (52 plates containing 4992 clones) and aliquots (48 cosmid clones each) were used as templates for the screening of positive clones by PCR. The following primers were used for the isolation of *F. oxysporum* genes from the cosmid library (see Table 1 for primer sequences): primers Tri201-F and Tri201-D3 for *Tri201*, primers tyrosinase-F and tyrosinase-R for a putative tyrosinase gene (gene B, orthologue of gene 3), primers acetylclon1-F and acetylclon1-R for a putative poly saccharide deacetylase gene (gene S, orthologue of gene 4), primers 1.3clone-F and 1.3clone-R for a putative β-1,3-glucosidase gene (gene Q, orthologue of gene 1), and primers NADH-F and NADH-R for a putative NADH-cytochrome b5 reductase gene (gene J, orthologue of gene 6).

**Sequence analysis of cosmids containing non-trichothecene genes.** The internal sequence of pCosF017-12-G was partially determined and mapped using PCR techniques. Briefly, the insert DNA was divided into three regions, which are demarcated by gene B (orthologue of gene 3) and/or gene J (orthologue of gene 6), and these regions were amplified by long PCR with the following primer pairs: Fotyrosinase-U32 and SuperT7, Fotyrosinase-D32 and FonNADH-D32, and FonNADH-U32 and SuperT3. The amplified PCR products were partially digested with Sau3AI and cloned in pUC18. Randomly selected clones were sequenced by a single pass analysis and putative gene-coding regions were predicted using the BLASTX analysis. To determine the partial nucleotide sequences of these DNA fragments (fragment I–fragment XV), primers were synthesized on the basis of their provisional (i.e., with possible PCR errors) sequences and used for direct DNA sequencing. The *F. oxysporum* genes identified on pCosF017-12-G (gene A–gene O) were mapped to the cosmids by sequentially determining their relative positions by PCR.

**Cloning of trichothecene 3-O-acetyltransferase genes (TAT) from *F. decemcellulare* and *F. solani*.** Twenty-one primers were designed based on the nucleotide sequences of known trichothecene 3-O-acetyltransferase genes (i.e. *Tri101*, *Tri201* and *FdTAT* for cloning of *FsoTAT*) and used for RT-PCR of TAT. To concentrate the TAT mRNA species, RNA was prepared from mycelia treated with 100 µg T-2 toxin ml⁻¹. Portions of *FdTAT* and *FsoTAT* were amplified after extensive RT-PCR trials with possible pairs of primers (see Table 1 for successful primer pairs). Based on the internal sequences of the RT-PCR products, we designed primers for vectorette PCR (TaKaRa LA PCR in *vitro* cloning kit; TAKARA BIO) to clone the upstream and downstream regions of TAT. Primers and cassette libraries listed in Table 1 were used for genomic walking by vectorette PCR.

**In vitro acetyltransferase assays.** The candidate 3-O-acetyltransferase genes, with the exception of *FdTAT*, were amplified by PCR using KOD-plus DNA polymerase (TOYOBO) and the amplified products (without non-synonymous substitutions) were cloned into an expression vector, pET-12a (Novagen), between the NdeI and BamHI sites. For construction of an *FdTAT* expression vector, the PCR product (without non-synonymous substitutions) amplified by LA-Taq (TAKARA BIO) was directly cloned in pCRT7/NT-TOPO (Invitrogen). Primers listed in Table 1 were used for PCR.

The expression vectors were transformed to *Escherichia coli* Rosetta (DE3) (Novagen) and grown on CircleGrow medium (Bio 101) containing 100 µg ampicillin ml⁻¹ and 34 µg chloramphenicol ml⁻¹. The bacterial cultures were incubated at 20 °C overnight with 1 mM IPTG to induce expression of the acetyltransferase gene. Crude recombinant enzymes were prepared from the bacteria and the trichothecene 3-O-acetyltransferase activities were assayed as described previously (Kimura et al., 1998a). Ethylacetate-extracted reaction mixtures were developed on TLC plates (Kieselgel; Merck) with ethylacetate/toluene (3 : 1 v/v) as the solvent. Trichothecenes were visualized with the chromogenic reagent 4-(p-nitrobenzyl)pyridine following the standard detection method (Takita et al., 1979).

**Northern analyses of the 3-O-acetyltransferase genes.** Fungal spores (*F. solani*) or young mycelial plugs (*F. decemcellulare* and *M. grisea*) were grown in YG medium containing T-2 toxin (20 or 100 µg ml⁻¹) for 3 days at 28 °C. Yeast cells were inoculated on YPD medium (1% yeast extract, 2% peptone and 2% glucose) with 20 µg T-2 toxin ml⁻¹ and cultured for 2 days at 28 °C. Fungal cells were disrupted by a bead-beater (Micro Smash MS100R; Tomy) and total RNA was extracted using TRIzol (Invitrogen). Twenty micrograms of RNA was used for Northern blot analysis, as described previously (Kimura et al., 1998b). Digoxigenin-labelled RNA probes (DIG RNA Labelling kit SP6/T7; Roche Diagnostics) were prepared by in *vitro* transcription of the entire coding region of the TAT and ScAYT1 genes (PCR products used for construction of *E. coli* expression vectors in the previous section) cloned in pGEM-T Easy (Promega).

**Sequence alignment and construction of phylogenetic trees.** Multiple alignment of the deduced trichothecene 3-O-acetyltransferase sequences was done using the CLUSTALW program (Thompson et al., 1994) in the GENETYX-MAC (version 12.0) software package (Software Development, Tokyo). For construction of unrooted neighbour-joining (NJ) trees, we used sequence analysis tools available at DDBJ (http://spiral.gen.nig.ac.jp/homology/welcome-e.shtml). Alignment was made with the program CLUSTALW (DDB version) with the BLOSUM scoring matrix (GAOPEN, 15 for DNA and 10 for protein; GAPEXT, 6.66 for DNA and 0.2 for protein; GAPDIST, 8; MAXDIV, 40; ENDGAPS, off; NOPGAPS, off; NOHGAPS, off) and then automatically converted to tree files using the tree-making program TREE (DDB version). The trees were calculated with 1000 bootstrap replications (KIMURA, on; TOSSGAPS, on; SEED, 111), and the results (downloaded as MME type files) were visualized using the Macintosh program TREEVIEW (Page, 1996). The following 288/265 rDNA sequences were used for construction of species phylogeny: *AB084297* for *F. asiaticum*; *AB084298* for *F. sporotrichoides*; *AB084299* for *F. oxysporum*; *AB084300* for *F. fusarium*; *AB084301* for *F. acuminatum*; *AB084302* for *F. decemcellulare*; *AB084303* for *F. solani*; *AF362554* for *F. grisea*; *AY130346* for *S. cerevisiae*. The phylogenetic analysis of the acetyltransferases was also done using a maximum-likelihood method with the program PUZZLE (Strimmer & von Haeseler, 1996) at the Institute Pasteur (http://www.pasteur.fr/english.html).

**RESULTS**

**Tri201 is absent from a microsynteny region of the *F. graminearum* species complex and some trichothecene non-producer Gibberella species.** We isolated two cosmid clones containing *Tri201* from a *F. oxysporum* genome library distributed in 96-well plates. The nucleotide sequence of the region around *Tri201* (12 396 bp; AB181461) was determined by sequencing these cosmid clones. By using RNA isolated from mycelia grown on YG medium, the transcription of candidate genes was examined by RT-PCR. A BLASTX search revealed a
Table 1. Primers used for cloning and characterization of the 3-O-acetyltransferase genes

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Primer sequences</th>
<th>Comments</th>
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<tr>
<td>Tri201-F</td>
<td>5′-CACCTACCATACAACTAACTCATGCGAC-3′</td>
<td>Primer for isolation of cosmids containing <em>FoTri201</em></td>
</tr>
<tr>
<td>Tri201-D3</td>
<td>5′-AGCTCGGATGATCTGCAATATGCTCA-3′</td>
<td>Primer for isolation of cosmids containing <em>FoTri201</em></td>
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<td>Gibbe-201checkU</td>
<td>5′-GATCCACAAACGCCGAGAAGGCGGACG-3′</td>
<td>Primer for amplification of the microsynteny region of <em>Gibberella</em> species</td>
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<td>tyrosinase-F</td>
<td>5′-CAAGATCCCGGTTGCCGAAAAAGGCGG-3′</td>
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<td>tyrosinase-R</td>
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<td>acetylclone-R</td>
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</tr>
<tr>
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<td>Primer for isolation of cosmids containing orthologue of gene 6</td>
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<td>Tri201-DwU1</td>
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<td>decem-L-U-CAS</td>
<td>5′-ACGCTGCTGGAAGGAGGAGCAGAAGG-3′</td>
<td>Primer for vectorette PCR of FdTAT downstream region (FbaI cassette)</td>
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<td>decem-L-U-nest-CAS</td>
<td>5′-CGAACACTGACTACAACTACACACAAGCAGAAGG-3′</td>
<td>Nested primer for vectorette PCR of FdTAT downstream region (FbaI cassette)</td>
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<tr>
<td>decem-L-D-CAS</td>
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<td>Primer for vectorette PCR of FdTAT upstream region (XbaI cassette)</td>
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<tr>
<td>solani-L-U-CAS</td>
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<td>Primer for vectorette PCR of FdTAT downstream region (HindIII cassette)</td>
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<td>solani-L-D-nest-CAS</td>
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transcribed hypothetical gene similar to *Drosophila melanogaster* CG15021 (encoding a protein similar to RE17165p; accession no. AAL48746) and a putative \(\beta\)-D-galactosidase gene (non-transcribed under the above experimental conditions) around Tri201, but no trichothecene (pseudo-)genes were found nearby (Fig. 1). The hypothetical gene, Tri201, and a putative \(\beta\)-D-galactosidase gene were also arranged in the same manner in *F. fujikuroi* with respect to gene order and orientation. In *F. asiaticum* F15 and *F. acuminatum* IFO 7772 (which have a functional Tri101), however, these two non-trichothecene genes were adjacent to each other, and there was no (pseudo-)Tri201 between these genes.

We further investigated the arrangement of genes at this locus using other trichothecene non-producing *Gibberella* species. For this purpose, the regions between the hypothetical gene and a putative \(\beta\)-D-galactosidase gene were amplified by long PCR with LA-Taq using primers Gibbe-201checkU and Gibbe-201checkD3 (shown as arrowheads in Fig. 1). Sequence analysis of the amplified products revealed that *F. nygamai* (AB193099) and *F. nisikadoi* (AB193100) carry Tri201 between these two genes. In addition to *F. acuminatum*, *F. avenaceum* did not have this (pseudo-)gene. These results suggest that some trichothecene-non-producing *Gibberella* species lack Tri201 in the microsynteny region.

### Table 1. cont.

<table>
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<td>Reverse primer for cloning of MgTAT into pET-12a</td>
</tr>
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**Fig. 1.** Comparison of the microsynteny regions of *Gibberella* species, which contain a transcribed hypothetical gene (similar to *D. melanogaster* CG15021) and a putative \(\beta\)-D-galactosidase gene. Experimentally determined exons are represented as filled boxes (black for Tri201 and grey for other genes); predicted exons of non-transcribed (i.e. vegetatively growing mycelia) genes are shown as open boxes. While the trichothecene non-producers *F. oxysporum* and *F. fujikuroi* had a functional Tri201 between a hypothetical gene and a putative \(\beta\)-D-galactosidase gene (accession nos AB181461 and AB181462, respectively), Tri201 was missing from this region in *F. asiaticum* and *F. acuminatum* (AB181459 and AB181460, respectively); the latter two species carry a functional Tri101 in the pho5-ura7 syntenic region. Filled and empty arrowheads denote primers Gibbe-201checkU and Gibbe-201checkD3, respectively. Three additional *Fusarium* strains, *F. nygamai*, *F. nisikadoi* and *F. avenaceum*, were used for the PCR analysis. The PCR products amplified by these primers are shown below the map.
Non-trichothecene genes flanking the Tri5 cluster do not comprise a region of synteny in Gibberella species

The 25 kb Tri5 cluster is surrounded by putative tyrosinase (gene 3) and polysaccharide deacetylase (gene 4) genes in F. asiaticum F15 (Kimura et al., 2003b). On both sides of these non-trichothecene genes, there are a putative β-1,3-glucosidase gene (gene 1), an esterase gene (gene 2), a 3-hydroxyacyl-CoA dehydrogenase gene (gene 5), and a NADH-cytochrome b5 reductase gene (gene 6). Among these genes, we focused on the cluster-flanking genes, gene 3 and gene 4, and the most distal genes, gene 1 and gene 6 (see Fig. 2). Primers were synthesized on the basis of the sequences of these non-trichothecene genes (see Table 1) and used to amplify portions of their F. oxysporum orthologues. By sib selection, the non-trichothecene genes were identified in a pool of cosmids distributed in 96-well plates, and a single cosmid was subsequently isolated. Although orthologues of gene 3 and gene 6 were located on a single cosmid, pCosFo17-12-G (gene B and gene J, respectively), gene 1 and gene 4 were identified on different cosmids, pCosFo15-3-H (gene Q) and pCosFo1-12-D (gene S), respectively (Fig. 2). These three cosmids did not overlap each other, as assessed by PCR with primers on the basis of the insert T3 and T7 end-sequences (data not shown). Shotgun sequencing of pCosFo17-12-G, combined with PCR mapping and BLASTX analysis, resulted in the identification of 15 putative non-trichothecene genes (genes A to O, with identification of gene L as an orthologue of gene 5; see also Supplementary Table S1 with the online version of this paper at http://mic.sgmjournals.org for BLASTX results of these F. oxysporum genes) in the neighbourhood of gene B and gene J, but no candidate trichothecene (pseudo-)genes were found nearby. As shown in Fig. 2, most orthologues of these genes were identified on different chromosomes (see also Supplementary Table S1; genes A, C, D, E, F, G, K, M, N and O are on different contigs) of F. graminearum PH-1 (NRRL 31084) (Trail & Common, 2000), for which the complete genome sequence is available (Broad Institute; http://www.broad.mit.edu/annotation/fungi/fusarium/). Orthologues of the five non-trichothecene genes on pCosFo15-3-H and pCosFo1-12-D (i.e. genes P to T in Fig. 2) were not mapped to a single contig of F. graminearum PH-1 (Supplementary Table S1). In F. oxysporum and F. fujikuroi, most genes identified on pCosFo17-12-G comprised a region of synteny, as assessed by PCR mapping (Supplementary Fig. S1 with the online version of this paper at http://mic.sgmjournals.org). These results indicate that non-trichothecene genes demarcating the Tri5 cluster in the F. graminearum species complex are not organized in a region of synteny in trichothecene-producing (e.g. F. asiaticum, F. graminearum) and non-producing (e.g. F. oxysporum, F. fujikuroi) Gibberella species.

Trichothecene 3-O-acetyltransferase genes in Fusarium species that belong to teleomorph genera other than Gibberella

Although our previous analysis focusing on plo5 and ura7 did not result in the identification of trichothecene 3-O-acetyltransferase genes (TAT) from Fusarium strains

![Fig. 2. Comparative analysis of F. asiaticum and F. oxysporum focusing on the non-trichothecene genes at either end of the Tri5 cluster. Solid and dotted lines represent sequenced and non-sequenced (but mapped) regions, respectively. F. oxysporum genomic DNA regions are enclosed by dotted ovals. The scale bar (5 kb) applies to both F. asiaticum and F. oxysporum genomic DNA regions. Although orthologues of F. asiaticum non-trichothecene genes (gene 3, gene 5 and gene 6) were found on a single cosmid pCosFo17-12-G (gene B, gene L and gene J, respectively), further sequence analyses (i.e. BLASTX analysis and PCR mapping of randomly sequenced regions) of this cosmid did not support the presence of (pseudo-)trichothecene genes or microsyntenic regions in F. oxysporum. Chromosome numbers (e.g. Fg: chr-1) of F. graminearum PH-1 orthologues are shown in parentheses.](image-url)
belonging to teleomorph genera other than Gibberella (Kimura et al., 2003a), we found that F. decemcellulare and F. solani were able to acetylate C-3 of trichothecenes (data not shown). Portions of the 3-O-acetyltransferase genes (referred to as FdTAT and FsoTAT from F. decemcellulare and F. solani, respectively) were amplified by RT-PCR using RNA isolated from T-2 toxin-treated mycelia. Concentration of the TAT mRNA species by treatment with T-2 toxin was necessary to achieve a successful PCR with primers (designed on the basis of the sequences of other Fusarium strains) that do not perfectly match the TAT genes. Based on the internal sequences of the amplified cDNA fragment, upstream and downstream coding regions of TAT were obtained from genomic DNA by vectorette PCR with the primers listed in Table 1. We further obtained the regions upstream and downstream of TAT by conducting extensive rounds of PCR walking using vectorette PCR (primers not shown). The combined nucleotide sequences containing FdTAT and FsoTAT are available from GenBank under the accession numbers AB181463 (5692 bp) and AB181464 (5392 bp), respectively. At position -3 relative to the coding regions of FdTAT and FsoTAT (1374 bp and 1380 bp, respectively), we found ‘A’, which is highly conserved in translational initiation sites of fungal genes (Ballance, 1986). Also, the coding regions of the genes were not interrupted by introns. These features are similar to those of other trichothecene 3-O-acetyltransferase genes of Gibberella. At the amino acid sequence level, FdTAT and FsoTAT were found to be 61-9% identical to FgTRI101 (BAA24430), while FdTAT and FsoTAT were 73-5% identical to each other. They contained the sequence motifs HXXMDMXG and DFGXGLGXP (see Fig. 3, bold), which are well conserved among trichothecene 3-O-acetyltransferase genes of Gibberella species.

**Characterization of the trichothecene 3-O-acetyltransferase genes from ascomycetous fungi**

The recent completion of genome sequencing projects for several fungi (Broad Institute/MIT; as of June, 2004) has revealed hypothetical genes homologous to Tri101 from M. grisea (hypothetical genes; locus ID MG097777.4 encoding a hypothetical protein XP_364932, designated MgTAT in this study, and locus ID MG08440.4 encoding a hypothetical protein XP_362997), A. nidulans (hypothetical gene; locus ID AN3384.2 encoding a hypothetical protein EAA63352) and S. cerevisiae (gene name, ScAYT1), but not from Neurospora crassa. In addition, there is a trichothecene 3-O-acetyltransferase pseudogene in the genome of Schizosaccharomyces pombe (SPCC338.19; see annotation of AL023781). None of these ascomycetous fungi produce trichothecenes. Among these genes, ScAYT1 is known as a functional acetyltransferase gene (Alexander et al., 2002).

The function of these hypothetical genes was examined with *in vitro* acetyltransferase assays using trichothecenes as a substrate. FdTAT, FsoTAT, MgTAT, ScAYT1 and the hypothetical genes MG08440.4 and An3384.2 were expressed in *E. coli* using the T7 expression system. Almost equal amounts of recombinant protein in soluble fraction were used for the assay (Fig. 4a). As shown in Fig. 4(b), deoxynivalenol (DON) was specifically acetylated at C-3 of the trichothecene skeleton by the crude recombinant enzyme fractions, except those of MG08440.4 and An3384.2 (data not shown). While TAT from fusaria showed equally strong 3-O-acetyltransferase activities with DON, MgTAT was less active (see TLC at 1 min incubation). ScAYT1 showed much less DON acetylase activity than MgTAT, but an expected product, 3-acetyldeoxynivalenol (3-ADON), became detectable after 3 h incubation. In addition to DON, T-2 toxin also served as a substrate for these recombinant proteins (data not shown). These results demonstrate that several ascomycetous fungi distinctly related to the trichothecene producers (i.e. *M. grisea* and *S. cerevisiae*) have genes that show trichothecene 3-O-acetyltransferase activity. In support of the results, the deduced amino acid sequences of these functional homologues showed the conserved sequence motifs HXXMDXXG and DFXXGXXGXP (Fig. 3, bold and boxed), and were 43-9% (MgTAT) and 43-5% (ScAYT1) identical to that of FasTRI101 from *F. asiaticum* F15 (Kimura et al., 1998a).

Expression of the gene in these fungi was also investigated by Northern blot analysis. Fungal cells were treated with T-2 toxin (100 μg ml⁻¹ for *F. decemcellulare* and *F. solani* and 20 μg ml⁻¹ for *M. grisea* and *S. cerevisiae*, respectively) and total RNA was isolated before and after the toxin treatment to compare the transcript level of the resistance gene. Acetyltransferase gene expression was significantly induced by the toxin treatment in *F. solani* and to a lesser extent in *F. decemcellulare*, but was unchanged in *M. grisea* and *S. cerevisiae* (Fig. 4c). While *S. cerevisiae* constitutively expressed the ScAYT1 gene irrespective of the culture
when overexpressed in E. coli inclusion body in [1]. (a) Recombinant proteins from ascomycetous fungi. (a) Recombinant proteins including FasTRI101, most of which was recovered as an inclusion body in E. coli HMS174 (DE3) in our previous study (Kimura et al., 1998a) were recovered in soluble fractions when overexpressed in E. coli Rosetta (DE3) grown at 20°C. Total proteins were separated by 12.5% SDS-PAGE and the gel was stained with Coomassie brilliant blue. Soluble enzyme fractions (equal amounts included in the reaction mixture) used in the acetyltransferase assay were loaded on each lane. M, protein marker; C, crude cell extract of control E. coli; Fas, crude cell extract of E. coli overexpressing FasTRI101; Fd, crude cell extract of E. coli overexpressing FdTAT; Fso, crude cell extract of E. coli overexpressing FsoTAT; Mg, crude cell extract of E. coli overexpressing MgTAT; Sc, crude cell extract of E. coli overexpressing ScAYT1. (b) TLC of in vitro acetylation assay using DON as a substrate. Forty micrograms each of DON (lane 1) and 3-ADON (lane 2) were loaded as standard samples. DON was incubated with acetyl-CoA and soluble E. coli cell extract of control (lane 3), recombinant FasTRI101 (lane 4) (Kimura et al., 1998a), recombinant FdTAT (lane 5), recombinant FsoTAT (lane 6), recombinant MgTAT (lane 7), and recombinant ScAYT1 (lane 8) for 3 h (right panel) or 1 min (left panel). (c) Northern blot analysis of the acetyltransferase genes. The complementary strand RNA probes were hybridized to the total RNA of the fungi grown in the presence (+) or absence (−) of T-2 toxin.

conditions and specifically acetylated C-3 of T-2 toxin (20 μg ml−1) in the medium, expression of MgTAT was not detected in M. grisea. However, DON (20 μg ml−1) was acetylated at C-3 by the M. grisea culture and the transcript of MgTAT was detected in the toxin-treated mycelia by RT-PCR (Supplementary Fig. S2 with the online version of this paper at http://mic.sgmjournals.org), which was used to detect small amounts of RNA below the detection limit of Northern blot analysis (Kimura et al., 1998c).

Phylogenetic relationships

Although there is no evidence of orthologous relationships between these trichothecene 3-O-acetyltransferase genes, they all have similar functions in that the gene products confer trichothecene resistance to the fungal strains in which they exist. We therefore constructed an unrooted phylogenetic tree based on the amino acid sequences of these trichothecene 3-O-acetyltransferases to establish the evolutionary links among this enzyme family. As shown in Fig. 5, the NJ tree was basically concordant with the species phylogeny (see 28S/26S rDNA tree of the fungi in Fig. 5), except for the phylogenetic positions of TAT from F. decemcellulare and F. solani. An essentially identical tree was obtained by the maximum-likelihood method with the program PUZZLE (data not shown).

DISCUSSION

The discovery of Tri201 in trichothecene-non-producer Gibberella species raised the possibility that they were once trichothecene producers and that extant non-producers (e.g. F. oxysporum) arose subsequently by accumulating mutations in the trichothecene genes (Kimura et al., 2003a). In this study, we examined restricted genomic regions of F. oxysporum (i.e. the Tri201 locus and hypothetical Tri5 cluster locus) on the basis of the assumption that trichothecene (pseudo-)genes, if they exist, may be located in microsynteny regions of F. oxysporum and F. asiaticum, as are the (pseudo-)Tri101 genes (Kimura et al., 2003a). However, we were not able to obtain evidence either for or against the presence of putative trichothecene (pseudo-) genes in F. oxysporum (see Figs 1 and 2), other than the previously identified Tri201.

Horizontal gene transfer is one possible explanation for the acquisition of a secondary metabolism gene cluster (Walton, 2000). However, only a few cases are supported by experimental data, such as the T-toxin biosynthesis gene cluster of Cochliobolus heterostrophus (Yang et al., 1996), the HC-toxin biosynthesis gene cluster of Cochliobolus carbonum (Ahn & Walton, 1996) and the AK-toxin biosynthesis gene cluster of Alternaria alternata (Tanaka et al., 1999). In these cases, the toxin biosynthesis genes are present only in some isolates of a species and are completely absent from others in the near-isogenic background. Unlike these cases, the evolutionary history of the trichothecene genes (e.g. whether or not the biosynthesis genes were acquired by horizontal gene transfer...
predating the evolutionary radiation of the trichothecene-producing *Fusarium* species) currently remains unclear due to limited sequence information on phylogenetically close trichothecene-non-producer *Gibberella* species. The entire genome sequence of *F. oxysporum* and other related species may be necessary for comparative intraspecific sequence analysis of trichothecene-producer and non-producer fusaria. It should be noted, however, that Ward and co-workers specifically ruled out horizontal gene transfer in the case of the type B trichothecene-producing *Fusarium* species; the toxin gene cluster evolution was discordant with the species phylogeny inferred from genes outside the trichothecene core cluster, and a novel form of balancing selection was suggested to explain this discordance (Ward et al., 2002).

The occurrence of *Tri201* only in the trichothecene-non-producer *Gibberella* species cannot be explained by recent horizontal gene acquisition, since the phylogeny of these trichothecene 3-O-acetyltransferases is concordant with the species phylogeny. This indicates that the acetyltransferase gene experienced duplication (i.e. *Tri201* generated) and inactivation (i.e. *Tri101* inactivated) in the evolutionary history of *F. oxysporum* and *F. fujikuroi*. In addition to the teleomorph genus *Gibberella*, we also extended our investigations of trichothecene 3-O-acetyltransferase genes to *F. decemcellulare* and *F. solani*, which belong to the teleomorph genera *Albonectria* and *Neocosmospora*, respectively. The unexpected discovery of a functional TAT in these phylogenetically distant *Fusarium* species raised an alternative possibility: that the trichothecene 3-O-acetyltransferase gene is just an antibiotic resistance gene and has a different evolutionary history from other trichothecene biosynthesis-related genes. In fact, O’Donnell and co-workers previously reported that the evolution of *Tri101* tracks with the species phylogeny within the *F. graminearum* species complex (O’Donnell et al., 2000) and, in this context, our present results extend the applicability of their findings to other fusaria.

As more genomes are being sequenced and made publicly available, homology-based searches have revealed the existence of unexpected genes. For example, the occurrence of phytochelatin (formerly believed to be a plant-specific peptide) in animals was not documented until a homologue of the phytochelatin synthase gene was found in the genome of *Caenorhabditis elegans* (Clemens et al., 2001; Vatamaniuk et al., 2001). Recently, sequencing efforts have also been made for several filamentous ascomycetes (*A. nidulans*, *M. grisea*, *F. graminearum*, *S. pombe* and *N. crassa*) in addition to *S. cerevisiae* (Mannhaupt et al., 2003). The genome information has revealed the presence of putative trichothecene 3-O-acetyltransferase gene homologues in most of these fungi. Functional identification of the trichothecene 3-O-acetyltransferase genes of *M. grisea* (i.e. MgTAT) and *S. cerevisiae* (i.e. ScAYT1) appears to be in support of the alternative idea that this resistance gene is widely distributed among various fungal species and has a different evolutionary history from other trichothecene genes of the *F. graminearum* species complex. If this is the case, the ancestors of *N. crassa* and *S. pombe* may once have possessed the 3-O-acetyltransferase gene, which was subsequently lost or inactivated due to a lack of selective constraints.

After we finished the analysis of these functional trichothecene 3-O-acetyltransferase genes, more fungal genomes were sequenced and released into the public domain in GenBank. A tblastn search of these acetyltransferase genes further revealed highly homologous sequences in...

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**Fig. 5.** Evolutionary relatedness of ascomycetous fungi used in this study (left) and their trichothecene 3-O-acetyltransferase protein family (right). Unrooted phylogenetic trees were constructed using the NJ method and downloaded from the DDBJ Web server. The scale bars indicate the branch length corresponding to the mean number of differences (0.1) per residue along each branch. Bootstrap values supporting the branches connecting the subgroups are indicated. A 3-O-acetyltransferase tree with the same topology was constructed using the maximum-likelihood method accomplished with PUZZLE. An enhanced view of the *Fusarium* species branch is shown in the species phylogeny (28S/26S rDNA tree) of the fungi used in this study. Fas, *F. asiaticum*; Fs, *F. sporotrichioides*; Fo, *F. oxysporum*; Ft, *F. fujikuroi*; Fac, *F. acuminatum*; Fd, *F. decemcellulare*; Fso, *F. solani*; Mg, *M. grisea*; Sc, *S. cerevisiae*. The phylogenies of the fungi used in this study. Fas, *F. asiaticum*; Fs, *F. sporotrichioides*; Fo, *F. oxysporum*; Ft, *F. fujikuroi*; Fac, *F. acuminatum*; Fd, *F. decemcellulare*; Fso, *F. solani*; Mg, *M. grisea*; Sc, *S. cerevisiae*.
the genome of Coccidioides posadasii (minimum E value of e^{-134}; Query=FdTAT; score=476) and Coprinopsis cinerea (minimum E value of e^{-121}; Query=FdTAT; score=435). Although the functions of these genes are not known, the conserved sequence motifs HXXMDXXG and DFXXGXGXP were conserved in these homologues, suggesting that they may also code for functional trichothecene 3-O-acetyltransferases.

It is rather a surprise that several fungi [at least seven distinct species functionally identified in this study and previous studies (Alexander et al., 2002; Kimura et al., 2003a)] that do not produce trichothecenes carried functional trichothecene 3-O-acetyltransferase genes without accumulating mutations during their evolution. Unlike prokaryotic antibiotic resistance genes, which are often found on plasmids or transposons, there are no structural features that greatly accelerate horizontal transmission of the trichothecene resistance genes. In such cases, the persistence of this non-essential gene depends on the selective advantage that it confers to the organism in which it exists (Walton, 2000). For example, certain phytopathogenic fungi possess inactivating genes for the plant antibiotics phytoalexins (Covert et al., 1996; Weltring et al., 1988) and 'phytoanticipins' (Bowyer et al., 1995; Glenn et al., 2002; Sandrock et al., 1995), which is reasonably explained by the functional advantages for the fungi. However, unlike these cases, the presence of 3-O-acetyltransferase genes in diverse groups of trichothecene-non-producer fungi is unusual in that these fungi do not necessarily inhabit an environment frequently exposed to this group of antibiotics.

To our knowledge, there are only two other examples of fungal antibiotic resistance genes (by means of inactivation) that are not subject to selective constraints: MPR1 of S. cerevisiae strain Σ1278b (Kimura et al., 2002) and BSD of Aspergillus terreus (Kimura et al., 1994). MPR1 and BSD code for acetyltransferase and deaminase, respectively, and they confer resistance to the toxic proline analogue L-azetidine-2-carboxylic acid and the aminoacyltransferase antibiotic blasticidin S, respectively. Both genes have highly conserved homologues in other fungi (e.g. the MPR1 homologue in N. crassa and the BSD homologue in C. posadasii), suggesting that these resistance genes are widely distributed across genera in fungi. Although the eukaryotic antibiotic resistance genes apparently have no physiological functions, they may actually contribute to 'marginal fitness', as demonstrated for seemingly non-essential genes in yeasts (Thatcher et al., 1998).

ACKNOWLEDGEMENTS

This research was supported in part by a grant for ‘Integrated Research on the Safety and Physiological Function of Food’ to M. K. from the Ministry of Agriculture, Fishery, and Forestry of Japan (MAFF). T. T. was financially supported from RIKEN by a Junior Research Associate (JRA) programme for graduate students.

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Evolutionary history of trichothecene genes


