The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes

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Abstract **We report for the first time the complete structure and sequence of the trichothecene biosynthesis gene cluster (i.e. *Tri5*-cluster) from *Fusarium graminearum* F15, a strain that produces 3-acetyldeoxynivalenol (3-ADON). A putative tyrosinase and polysaccharide deacetylase gene flank the *Tri5*-cluster: the number of pathway genes between them is less than half the total number of steps necessary for 3-ADON biosynthesis. In comparison with partial *Tri5*-cluster sequences of strains with 15-acetyldeoxynivalenol and 4-acetylnivalenol chemotypes, the *Tri5*-cluster from strain F15 contains three genes that are apparently unnecessary for the biosynthesis of 3-ADON (i.e. *Tri8* and *Tri3*, which are expressed, and pseudo-*Tri13*, which is not expressed). In addition, the *Tri7* gene was missing from the cluster. Recombinant TRI3 protein showed limited trichothecene C-15 acetylase activity. In contrast, recombinant TRI8 protein displayed no C-3 deacetylase activity, suggesting that the loss or alteration of function contribute directly to the chemotype difference.**

Key words: Biosynthesis gene cluster evolution; Comparative genomics; Fungal secondary metabolism; Plant pathogen; Trichothecene mycotoxin; Virulence factor

1. Introduction

Trichothecene mycotoxins are a large group of toxic secondary metabolites produced by fungi including several *Fusarium* species [1]. Their structural diversity arises from the position, number, and type of functional groups attached to the 12,13-epoxy-trichothec-9-ene skeleton responsible for inhibition of protein synthesis (see Fig. 1). Among trichothecene producers, *Fusarium graminearum* (teleomorph: *Gibberella zeae*) is a fungus of major concern to agriculture and food industries because this cereal pathogen poses serious threats to human and animal health by contaminating maize, wheat, and barley with the trichothecenes deoxynivalenol (DON), nivalenol (NIV), and their derivatives; e.g. 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), 4-acetylnivalenol (4-ANIV).

To understand the biological mechanisms of trichothecene biosynthesis, several pathway and regulatory genes have been isolated from a T-2 toxin producer *Fusarium sporotrichioides* NRRL 3299. Hohn and co-workers pioneered the research by first isolating *Tri5*, coding for the key enzyme trichodiene synthase [2]. Subsequently two biosynthesis pathway genes, *Tri3* and *Tri4*, were found to cluster around this sesquiterpene cyclase gene [3]. This finding provided a theoretical basis for a search for additional biosynthesis genes from the gene cluster (i.e. hereafter referred to as the *Tri5*-cluster).

Subsequent research revealed additional genes in the *Tri5*-cluster (i.e. *Tri8*-to-*Tri12*) from *F. sporotrichioides* NRRL 3299 [4–11] and *F. graminearum* GZ 3639 [6,8]. Recently, Lee and co-workers reported partial structural features of the *F. graminearum Tri5*-cluster (*FgTri5*-cluster) using two strains isolated in Korea, H-11 (15-ADON chemotype) and 88-1 (4-ANIV chemotype) [12]. Based on comparative and functional approaches, they demonstrated that *Tri13* codes for the P450 monooxygenase responsible for C-4 hydroxylation of the trichothecene ring [13]. However, the complete structure of the *Tri5*-cluster (i.e. size of the cluster in kb, number of genes in the cluster) has not yet been demonstrated. In the present study, we report for the first time the complete structure and sequence of the *FgTri5*-cluster in *F. graminearum* F15, a strain that produces 3-ADON [14].

2. Materials and methods

2.1. Strains, media, and chemicals

F. graminearum F15 and T-2 toxin producer *F. sporotrichioides* IFO 9955 [15] were used in this study. Fungal strains were maintained on potato dextrose agar (Difco). *F. graminearum* F15 was cultured in yeast glucose medium (0.5% w/v yeast extract and 2.0% w/v glucose) for DNA analysis and in potato dextrose broth (0.2% w/v potato

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Abbreviations: 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; CD, conserved domain; DON, deoxynivalenol; 4-ANIV, 4-acetylnivalenol; NIV, nivalenol; RT-PCR, reverse transcription-PCR

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starch and 1% w/v glucose) for trichothecene analysis and RNA extraction. Hygromycin B, trichothecenes, and acetyl CoA (trilithium salt) were purchased from Wako Chemical Co. (Osaka, Japan).

2.2. Plasmid construction and fungal transformation

The *FgTri6* expression vector pBF-*Tri6* was constructed for constitutive expression of *FgTri6* [16] in *F. graminearum* F15. *FgTri6* was amplified by PCR with primers T6-E (5′-GAATTCGAGATGATTTACATGGAGGAC-3′) and T6-P (5′-CTGCAGGGTCTCGGTTACTATCTGGAG-3′), digested with *Eco*RI and *Pst*I, and ligated to the *Eco*RI–*Pst*I vector fragment of pBF101 [17]. In the resulting vector, the promoter and terminator sequences of *Aspergillus nidulans trpC* (i.e. a gene that is constitutively expressed) [18] are placed upstream and downstream of *FgTri6*, respectively, to ensure constitutive expression of this regulatory gene.

F. graminearum F15 was co-transformed with pBF-*Tri6* and a hygromycin B resistance vector pCSN43 [18] by the PEG-$CaCl_2$ method. Protoplasts were prepared in 1.2 M $MgSO_4$ solution containing 3% w/v Glucanex (Novozymes Japan Ltd., Chiba, Japan) and 2% w/v driselase (Sigma), and purified as described previously [17]. Transformants were selected on potato dextrose agar containing 100 μg/ml of hygromycin B and used to screen for constitutive *FgTri6*-expressors by reverse transcription (RT)-PCR.

2.3. Cloning, sequencing, and analysis of the Tri5-cluster

Previously we isolated a cosmid clone pCosTr032 that contained *FgTri5* and *FgTri6*, but it lacked the complete coding region of *FgTri4* [14]. To clone the complete gene cluster, cosmids containing the upstream *Tri* genes were screened by PCR using the *FgTri4*-specific primers U4 and D4 [14]. Subsequently, we isolated another clone pCos-51-C-8 from the library distributed in 96-well plates.

The sequences of the *Tri5*-cluster and flanking regions were determined by primer walking. Amplified PCR fragments were purified by using QIAquick PCR Purification kit (Qiagen) and used as templates for the sequencing reactions.

Sequence similarity searches were performed by using the BLAST [19] program at NCBI (as of January 3, 2003).

2.4. DNA and RNA analyses

For Southern blot analysis of Tr-6-34, the full-length *FgTri6* was labeled with the PCR DIG Probe Synthesis kit (Roche) with 24 bp primers that were designed as follows: the U primers (sense strand) start with the first base (i.e. A) of start codons and the D primers (anti-sense strand) start with the last complementary base of stop codons. Genomic DNA digested with restriction enzymes was separated on an agarose gel and transferred to a Nytran N membrane (Schleicher and Schell, Dassel, Germany). Hybridization and washing techniques were conducted as described in the manufacturer's protocol. Probe-target hybrids were detected by using the DIG Nucleic Acid Detection kit (Roche).

RNA was isolated from mycelia ground in liquid nitrogen using the SNAP Total RNA Isolation kit (Invitrogen) and treated with RNase-free DNase I (Roche). For RT-PCR of the genes identified on the cosmids, the first-strand cDNA was synthesized using the Superscript First-strand Synthesis System for RT-PCR (Invitrogen). All primers used for the amplifications were designed as described above in preparation of the *FgTri6* probe.

2.5. Trichothecene analysis

Samples were extracted with ethyl acetate, concentrated under vacuum, and developed on a TLC plate (Merck F_{254} silica TLC) using ethyl acetate/toluene (3:1) as a solvent. Trichothecenes were visualized as described previously [20].

2.6. Recombinant enzyme assays

The coding region of *FgTri3* and *FgTri8* were amplified by PCR from the cDNA of *F. graminearum* F15 using LA-Taq (Takara, Kusatsu, Japan). The following primers were used: UFgTri3 (5′-ATGAGCGCTTCACCCTCCGCATTG-3′) and DFgTri3 (5′-AGCAAAGTTACAGTTTGAATGCCAGC-3′) for *FgTri3*, and UFgTri8 (5′-ATGGTTCTCGATCGTTTGTTGTTTC-3′) and DFgTri8 (5′-CTTCGGCTCACATTTCTTATCACAAC-3′) for *FgTri8*. The amplified fragments were cloned in pCR/T7-TOPO (Invitrogen), transformed to *Escherichia coli* DH5α, and sequenced. Inserts without PCR mutations (causing the coding amino acid sequence replacement) were used for the recombinant protein expression in *E. coli* BL21 (DE3) or Rosetta (DE3) (Novagen); *E. coli* cells were cultured at 22°C and harvested after a 14-h induction in the presence of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. Crude enzyme preparation and enzyme assay conditions were essentially the same as described for recombinant FgTRI101 protein [14] except that acetyl CoA was omitted from the reaction mixture for the deacetylation assay. In both the acetylation and deacetylation assays, pH of the reaction mixture was adjusted to 6.5, 7.5, and 9.5 with 10 mM Tris–HCl buffer.

2.7. Accession numbers

The nucleotide sequences of the complete *FgTri5*-cluster of *F. graminearum* F15 and *FsTri13* of *F. sporotrichioides* IFO 9955 have been deposited in DDBJ/EMBL/GenBank under accession numbers AB060689 (combined with AB088351 and AB088352) and AB088350, respectively).

3. Results and discussion

3.1. Constitutive expression of FgTri6 in F. graminearum leads to accumulation of trichothecenes in liquid shake culture

F. graminearum produces limited quantities of trichothecenes in liquid shake culture, making it unsuitable for expression analyses of the *Tri5*-cluster genes. Expression of the *Tri5*-cluster genes is positively regulated by *Tri6*, which codes for a Cys_2His_2 zinc-finger transcription factor [10,21]. Transcription of this regulatory gene was barely detectable in liquid shake cultures of *F. graminearum* F15 [16], while a significant amount of the *FsTri6* mRNA is produced in *F. sporotrichioides*. It has previously been demonstrated that targeted integration of heterologous *FsTri6* between the *FgTri5–FgTri6* regions greatly increases the expression level of 15-ADON in *F. graminearum* GZ3639 [22]. We thus attempted to generate a toxin overproducer by introducing an *FgTri6* expression vector pBF-*Tri6* (Fig. 2A) into strain F15.

Among 34 hygromycin B resistant transformants analyzed, one was identified that possessed several copies of pBF-*Tri6* integrated in the genome as assessed by Southern blot analysis; the banding pattern suggested the presence of at least two copies in tandem and one copy at an unlinked locus (Fig. 2B).

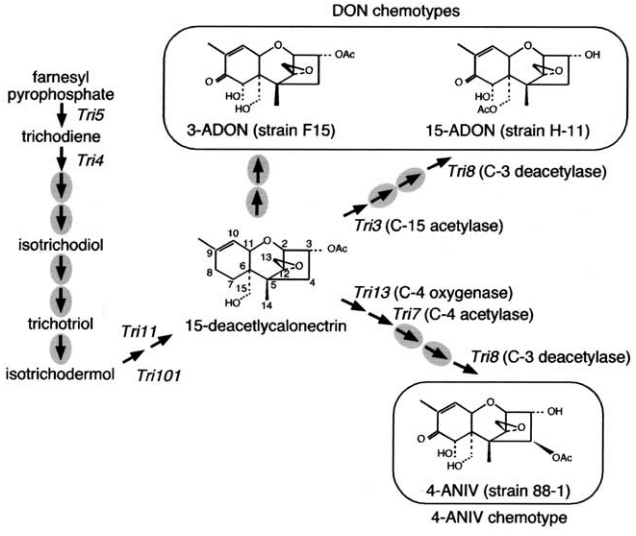

Fig. 1. Summary of trichothecene biosynthesis pathway of *F. graminearum*. Each arrow represents a single step in biosynthesis and the responsible pathway gene (if already identified) is indicated. Arrows in shaded ellipse indicate that the responsible genes have not yet been isolated.

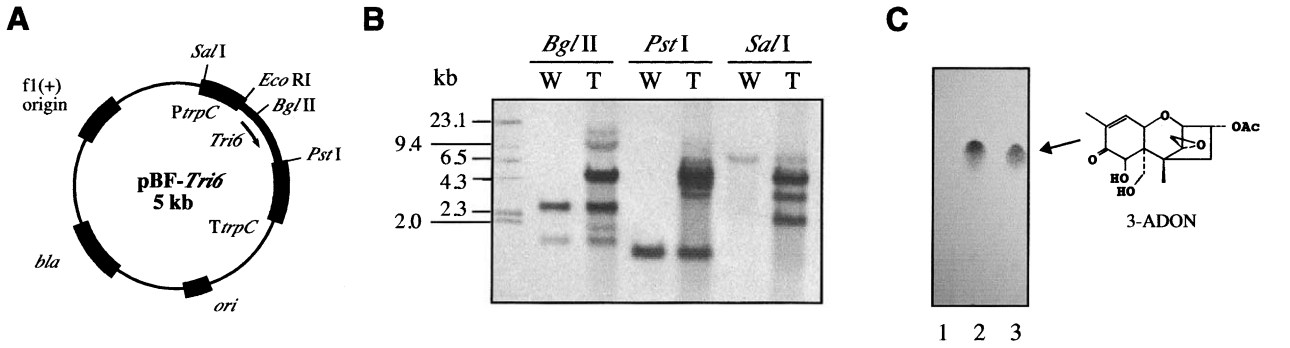

Fig. 2. Production of 3-ADON in liquid shake culture by Tr-6-34, a *Tri6*-expressor of *F. graminearum* F15. A: Structure of the vector used for transformation. *FgTri6* is flanked by the *trpC* promoter (P*trpC*) and terminator (T*trpC*) of *A. nidulans*. B: Southern blot analysis of Tr-6-34, a transformant that constitutively transcribes *FgTri6*. W, wild-type; T, Tr-6-34. *Bgl*II, *Pst*I, and *Sal*I cut only once in pBF-*Tri6*. Digoxigenin-labeled lambda *Hin*dIII marker (23.1, 9.4, 6.5, 4.3, 2.3, and 2.0 kb fragments) is shown on the left of the blot. C: Thin-layer chromatography of culture extracts (equivalent to 4 ml of culture) 2 days after inoculation of wild-type (lane 1) and Tr-6-34 (lane 2) strains. 3-ADON standard (30 μg) was loaded in lane 3.

The integration site of the vectors proved to be outside of the *Tri5*-cluster (entire cluster region defined in the next section) as assessed by PCR analyses (data not shown). This transformant (Tr-6-34), but not the wild-type strain, constitutively transcribed *FgTri6* in potato dextrose broth and accumulated a sufficient level of 3-ADON after 2 days of incubation (Fig. 2C). The result indicates that increased production of DON (including its acetylated derivatives) in *F. graminearum* can also be achieved by ectopic expression of *FgTri6*. The low production level of trichothecenes by *F. graminearum*, but not by *F. sporotrichioides*, is thus likely to be attributed to the difference of a signaling pathway leading to transcriptional activation of *FgTri6*, and not to the nature of the FgTRI6 transcription factor. Tr-6-34 was subsequently used for expression analyses of the *FgTri5*-cluster genes.

3.2. Analysis of the Tri5-cluster genes of F. graminearum F15

The *Tri5*-cluster of *Fusarium* species was reported to extend over an approximately 30 kb region, with *Tri8* and *Tri13* demarcating the ends of the characterized region [6,12,13,23]. However, the sequence and structure of cluster genes beyond this region have not yet been described. Thus we isolated the complete region of the *Tri5*-cluster with cosmids from strain F15 and analyzed individual *Tri* genes by RT-PCR.

Sequencing of the two cosmids pCosTr032 and pCos-51-

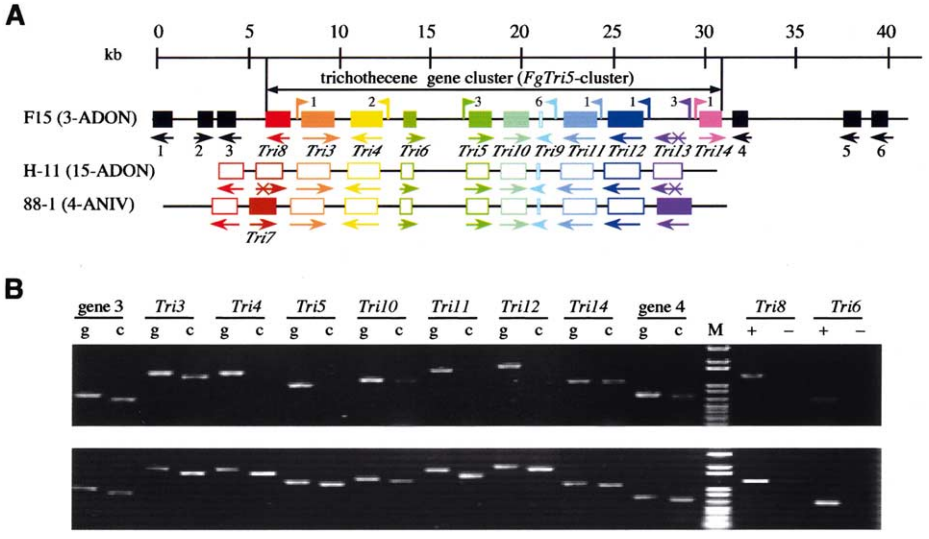

Fig. 3. Characterization of the complete *FgTri5*-cluster of *F. graminearum* F15. A: Comparison of the *FgTri5*-cluster from the producer *F. graminearum* strains F15 (upper), H-11 (middle), and 88-1 (lower). Thin black bars indicate regions with nucleotide sequence information (F15, AB060689; H-11, AF336366; 88-1, AF336365). The same biosynthesis gene is indicated by the same color. Arrows and the X symbol indicate direction and inactivation of genes, respectively. Closed and open boxes indicate the cluster genes for which experimental evidence of transcription has or has not been demonstrated, respectively. A box is not shown for strain F15 pseudo-*FgTri13* for which the transcript was not detected by RT-PCR. Color flags upstream of the *Tri* genes indicate the TRI6-binding consensus sequence [21]: the number of consensus sequences is shown next to the flags. Products of the non-cluster genes predicted by the BLASTX search are as follow: gene 1, β-1,3-glucosidase precursor; gene 2, putative esterase; gene 3, putative tyrosinase, gene 4, putative polysaccharide deacetylase; gene 5, 3-hydroxyacyl CoA dehydrogenase; gene 6, NADH-cytochrome *b5* reductase. B: RT-PCR analysis of the *FgTri5*-cluster genes of strain F15. Total RNA was isolated from wild-type strain (upper panel) or Tr-6-34 (lower panel). For those genes with introns, genomic DNA (g) and cDNA (c) were used as templates for PCR. For the biosynthesis genes without introns, RT-minus negative control reactions (−) are shown next to the RT-PCR samples (+) to demonstrate that genomic DNA contamination in the RNA samples was not detected. M: DNA size markers (1 kb Plus DNA Ladder; Invitrogen).

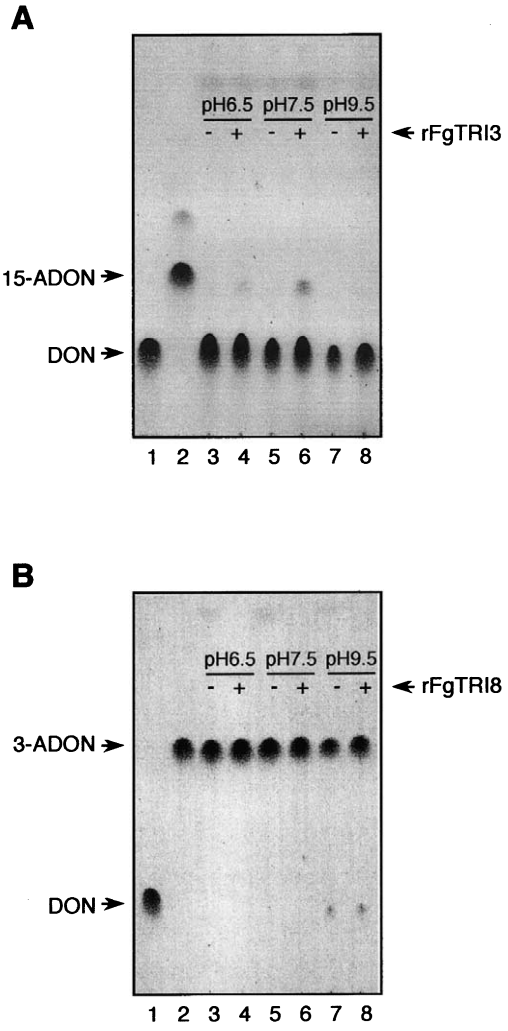

Fig. 4. In vitro enzyme assay of trichothecene biosynthesis gene products. A: 15-*O*-acetyltransferase assay of recombinant FgTRI3 using DON as a substrate. Lane 1: DON standard (30 μg); lane 2: 15-ADON standard (30 μg); lanes 3, 5, and 7: DON incubated with acetyl CoA and crude cell extracts of control *E. coli* BL21 (DE3); lanes 4, 6, and 8: DON incubated with acetyl CoA and crude cell extracts of *E. coli* BL21 (DE3) carrying a *FgTri3* expression plasmid. B: 3-*O*-deacetylation assay of recombinant FgTRI8 using 3-ADON as a substrate. Lane 1: DON standard (30 μg); lane 2: 3-ADON standard (30 μg); lanes 3, 5, and 7: 3-ADON incubated with crude cell extracts of control *E. coli* Rosetta (DE3); lanes 4, 6, and 8: 3-ADON incubated with crude cell extracts of *E. coli* Rosetta (DE3) carrying a *FgTri8* expression plasmid. In both A and B, enzyme assays were carried out at pH 6.5 (lanes 3 and 4), pH 7.5 (lanes 5 and 6), and pH 9.5 (lanes 7 and 8). Spontaneous deacetylation of 3-ADON was observed at pH 9.5 (B; lanes 7 and 8).

C-8, followed by BLAST similarity searches [19] at NCBI, revealed 11 (pseudo-)genes involved in trichothecene biosynthesis or regulation and six non-trichothecene genes over a 41 224 bp region (Fig. 3A). Minimum consensus sequences of TRI6 binding (YNAGGCC) [21] were found in the promoter region of trichothecene genes except for *FgTri6* [6,10,16], *FgTri10* [6,11] and *FgTri8* [8]. The absence of a TRI6 binding site upstream of *FgTri8* in strain F15 is unique among trichothecene producer strains that have been examined. The arrangement and orientation of the *FgTri5*-cluster genes was identical to previously characterized strains (15-ADON and 4-ANIV chemotypes) except that *Tri7* [6,13] was absent in strain F15. Comparative analyses of nucleotide sequences from the *Tri3-Tri8* region of 39 trichothecene producer strains characterized by O'Donnell et al. [24] and Ward et al. [25] indicate that the *Tri7* gene and TRI6 binding sites upstream of *Tri8* are absent from all 3-ADON strains, but present in 15-ADON and NIV strains (Ward; unpublished data). Previously, Lee et al. [12] developed a PCR assay based on sequence differences in *Tri7* to differentiate strains with DON and NIV chemotypes. However, the absence of *Tri7* in 3-ADON strains indicates a limitation on the utility of this PCR-based method for chemotype identification.

A putative tyrosinase gene (gene 3; highest BLASTP score of 86.3 bits with $E=5\times e^{-16}$ to P07524, tyrosinase from *Streptomyces antibioticus*; conserved domain (CD) search score of 65.4 bits with $E=7\times e^{-12}$ to pfam00264, tyrosinase) and a polysaccharide deacetylase gene (gene 4; highest BLASTP score of 127 bits with $E=e^{-28}$ to BAB79692, chitin binding protein from *Magnaporthe grisea*; CD search score of 119 bits with $E=2\times e^{-28}$ to pfam01522, polysaccharide deacetylase) flanked the *FgTri5* cluster, and were expressed in the wild-type strain at different growth stages independent of toxin biosynthesis (data not shown). A putative β-1,3-glucosidase precursor gene (gene 1; highest BLASTP score of 171 bits with $E=e^{-41}$ to BAA19145, glucan β-1,3-glucosidase from *Schizosaccharomyces pombe*; CD search score of 148 bits with $E=8\times e^{-37}$ to COG5309, exo-β-1,3-glucanase), an esterase gene (gene 2; highest BLASTP score of 90.5 bits with $E=2\times e^{-17}$ to NP_635537, conserved hypothetical protein from *Xanthomonas campestris*; CD search score of 46.8 bits with $E=2\times e^{-6}$ to COG2755, lysophospholipase L1 and related esterases), a 3-hydroxyacyl CoA dehydrogenase gene (gene 5; highest BLASTP score of 131 bits with $E=e^{-29}$ to NP_739385, putative 3-hydroxyacyl-CoA dehydrogenase from *Corynebacterium efficiens*; CD search score of 145 bits with $E=6\times e^{-36}$ to COG1250, 3-hydroxyacyl-CoA dehydrogenase), and a NADH-cytochrome *b5* reductase gene (gene 6; highest BLASTP score of 242 bits with $E=5\times e^{-63}$ to BAA85586, NADH-cytochrome *b5* reductase from *Mortierella alpina*; CD search score of 142 bits with $E=3\times e^{-35}$ to COG0543, 2-polyprenylphenol hydroxylase and related flavodoxin oxidoreductases) were also identified in the region surrounding the *FgTri5* cluster (Fig. 3A). Constitutive expression of these four genes was also confirmed by RT-PCR and their exon regions were determined experimentally (accession numbers AB060689 combined with AB088351 and AB088352).

RT-PCR of *FgTri5*-cluster genes (except *FgTri9* whose coding region is composed of only 132 bp) revealed differences among *Tri* genes in terms of their pattern of expression. *FgTri3*, *FgTri14*, and *FgTri8* transcripts were detected in the wild-type strain, while expression of the other *Tri* genes appeared to be extremely limited or non-existent (Fig. 3B); among these, translated products of *FgTri3* (coding for 15-*O*-acetyltransferase) [6,9] and *FgTri8* (coding for C-3 deacetylase) [8] are apparently unnecessary for biosynthesis of 3-ADON (see Fig. 1). In contrast, all of the *Tri* genes described in the lower panel of Fig. 3B were transcribed in the *FgTri6*-expressor strain Tr-6-34. Expression of *FgTri13*, whose gene product is not necessary for biosynthesis of 3-ADON, was not observed. In addition, the predicted ORF of *FgTri13* had deletions and insertions that cause the reading frame to be interrupted by stop codons. These observations indicate that *FgTri13* of strain F15 occurs as a pseudo-gene in the

3-ADON producer. *FsTri13* from *F. sporotrichioides* IFO 9955 (accession number AB088350) was able to complement the C-4 oxygenation function in strain F15 (data not shown).

3.3. Functional analyses of recombinant FgTRI3 and FgTRI8

Expression of two apparently unnecessary pathway gene (*Tri3* and *Tri8*) in strain F15 raises a question as to the function of the gene products in 3-ADON biosynthesis. Recent molecular evolutionary analyses indicated that the majority of the amino acid sites in *FgTri3* from 3-ADON producers were under strong purifying selection [25], suggesting that this gene is probably functional. To examine whether *FgTri3* and *FgTri8* respectively, code for a functional C-15 acetylase and C-3 deacetylase, we prepared recombinant FgTRI3 and FgTRI8 proteins and evaluated their activities in vitro. Recombinant FgTRI3 protein exhibited limited 15-*O*-acetyltransferase activity using DON as a substrate (Fig. 4A). This indicates that *FgTri3* codes for a functional enzyme. The 3-ADON chemotype of strains such as F15 could be attributed to a greatly impaired catalytic efficiency of TRI3, which is consistent with the production of minor amounts of 15-ADON by these strains [25]. Alternatively, a competing C-15 deacetylase (as yet unidentified), and/or a hypothetical inhibitor protein of TRI3 could be responsible for the profile of acetylated derivatives of DON produced by 3-ADON strains. Comprehensive understanding of complete genomes and TRI3 molecular enzymology will greatly facilitate tests of these alternative hypotheses. In contrast to *FsTri8* of T-2 toxin (i.e. C-3 deacetylated trichothecene) producing *F. sporotrichioides* NRRL 3299 [8], we were not able to detect the C-3 deacetylase activity from recombinant FgTRI8 protein of *F. graminearum* F15 (Fig. 4B). The same result was obtained by in vivo deacetylation assays using *Saccharomyces cerevisiae* carrying *FgTri8* (data not shown). Thus the simplest interpretation is that *FgTri8* of strain F15 is a transcribed pseudogene. These results suggest that loss or alteration of function for the *Tri8* gene product contributes directly to chemotype differences.

Considering that the impact of trichothecenes on the gene expression patterns of a model plant *Arabidopsis thaliana* differs depending on the functional groups attached to the 12,13-epoxy-trichothec-9-ene skeleton [26], chemotype differences might reflect the evolutionary consequences of complex interactions of *Fusarium* species with host plants.

3.4. Conclusions and perspectives

In this paper, we demonstrated for the first time that the *Tri5*-cluster of *Fusarium* species contains a limited number of genes for trichothecene biosynthesis (see Fig. 3A). *F. graminearum* with additional copies of *FgTri6* connected to a constitutive promoter facilitated identification of the cluster gene coding regions. Among the biosynthesis genes identified in the *FgTri5*-cluster, functions have not been assigned to *Tri9* and *Tri14*. It is likely that only three pathway genes in the *FgTri5*-cluster, *FgTri4*, *FgTri5*, and *FgTri11*, are involved in the biosynthesis of 3-ADON. Other genes in the *FgTri5*-cluster include the transcriptional regulators (*FgTri6* [6,16] and *FgTri10* [6,11]), a transporter gene (*FgTri12* [6,27]), and pseudo-genes or genes that are apparently unnecessary in the biosynthesis of 3-ADON (*FgTri13* [13], *FgTri8* [8], and *FgTri3* [6]). *FgTri9* codes for a polypeptide of 43 amino acid residues, which appears to be too small to code for a functional pathway enzyme. Thus, even if *Tri14* is assumed to be involved in the biosynthesis of 3-ADON, the number of pathway genes in the *FgTri5*-cluster is less than half of the 11 steps necessary for the synthesis of 3-ADON (see Fig. 1).

At present two biosynthetic genes have been shown to reside outside the *Tri5*-cluster: *Tri1* involved in hydroxylation of C-8 in T-2 toxin biosynthesis, but this gene has not been isolated [1], and *Tri101* which is responsible for self-protection of the producer. *Tri101* was cloned by cDNA expression cloning [14] and was shown to reside between a UTP-ammonia ligase and a phosphate permease gene [15]. It remains to be elucidated whether the majority of other biosynthesis genes are also clustered at one locus as a result of reciprocal translocation [28], or conversely, dispersed separately over the genome. Based on the complete *F. graminearum* genome sequence that will be available in the near future [29], strains with constitutive expression of *Tri6* might be useful for DNA microarray and/or comparative proteome analyses to identify and characterize the rest of the genes required for trichothecene biosynthesis.

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