Fusarium graminearumのTri4遺伝子のプロモーターをトリコテセン生合成遺伝子クラスターの末端に配置すると機能しない

誌名	Mycotoxins
ISSN	
著者名	東海,武史 中嶋,佑一 市川,雛代 前田,一行 西内,巧 小林,哲夫 木村,真
発行元	日本マイコトキシン学会
巻/号	63巻1号
巻号補足	
掲載ページ	p. 17-25
発行年月	2013年1月

Research Paper www.jstage.jst.go.jp/browse/myco

A promoter of *Fusarium graminearum Tri4* does not function when placed at the end of the trichothecene gene cluster

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Key words : chromosomal location; *Fusarium graminearum*; gene cluster; GUS reporter; transcriptional activation

(Received January 14, 2013; Accepted January 29)

Abstract

The central region of the trichothecene gene cluster contains two pathway and two regulatory genes (four *Tri* genes) that are necessary for forming the trichothecene skeleton. One of these genes, *Tri4*, encodes a multifunctional cytochrome P 450 monooxygenase, and the expression of this gene is highly induced under trichothecene-inducing conditions. Apart from this gene cluster, *Tri101* occurs as a single non-cluster gene, and the product of this gene contributes to the function of self-protection in *Fusarium graminearum*. The promoter activity of these *Tri* genes was compared with that of *TEF1α* (a highly expressed translation elongation factor 1-alpha) gene under toxin-inducing conditions by using the β-glucuronidase (GUS) gene as a reporter. The *Tri101* promoter- and *TEF1α* promoter-reporter fusions integrated at the end of the gene cluster showed activities that were similar to those of the original loci with respect to the timing and the level of GUS protein accumulation. However, as opposed to the normal pattern of *Tri4* transcription from the original locus, no GUS activity was detected when the *Tri4* promoter was placed at the end of the gene cluster. Thus, we can conclude that the position of the promoter in the gene cluster is important for native transcriptional regulation of *Tri4* in trichothecene biosynthesis.

Introduction

Some plant pathogenic *Fusarium* species, such as the cereal pathogen *F. graminearum*, produce sesquiterpenoid mycotoxin trichothecenes that affect and thereby threatens the safety of cereal grains and related food products[1)]. Four genes located at the center of the trichothecene gene cluster, *Tri4*, *Tri6*, *Tri5*, and *Tri10*, are necessary for the formation of the basic trichothecene skeleton in the biosynthesis of *Fusarium* trichothecenes (Fig. 1). *Tri5* encodes trichodiene synthase, which is necessary for the first cyclization step[2)], and *Tri4* is

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a highly expressed multifunctional cytochrome P 450 monooxygenase gene required in the four subsequent oxygenation steps[3, 4]. The first trichohecene skeleton of *Fusarium* species, isotrichodermol, is biosynthesized as a result of the non-enzymatic second cyclization after the oxygenation steps. In addition to these trichothecene genes in the gene cluster, *Tri101* occurs as a single non-clustered gene in the genome of *F. graminearum*[5]. *Tri101* expression is responsible for 3-*O*-acetylation of isotrichodermol, which is implicated in the self-protection of the fungus from the toxicity of trichothecenes.

Tri4 shares its promoter region with *Tri6* (a Cys_2His_2 zinc finger transcription factor gene)[6], whose activation is dependent on a functionally unidentifed regulatory protein encoded by *Tri10* (Fig. 1). Expression of these *Tri* genes is regulated in response to various environmental stimuli, including carbon source, nitrogen source, pH, water activity, and oxidative stress[7]. When *F. graminearum* is grown under trichothecene-inducing conditions, *Tri4* is induced and is expressed at a high level prior to the oneset of toxin production; expression of *Tri101* then follows in accordance with the timing of toxin accumulation[1]. We were thus interested in whether these promoters could be used as versatile tools for regulated expression of heterologous genes with respect to the expression patterns of the native *Tri4* and *Tri101* genes.

Promoter-reporter fusions are often used for comparing promoter activities, and must be integrated at the same locus to avoid chromosomal effects on transgene expression. In addition, the integration should not affect fungal growth or metabolism. With these criteria met, the activities of *Tri4* and *Tri101* promoters were evaluated using a promoter of the highly expressed *TEF1α* (translation elongation factor 1-alpha) gene[8] as a reference for comparison.

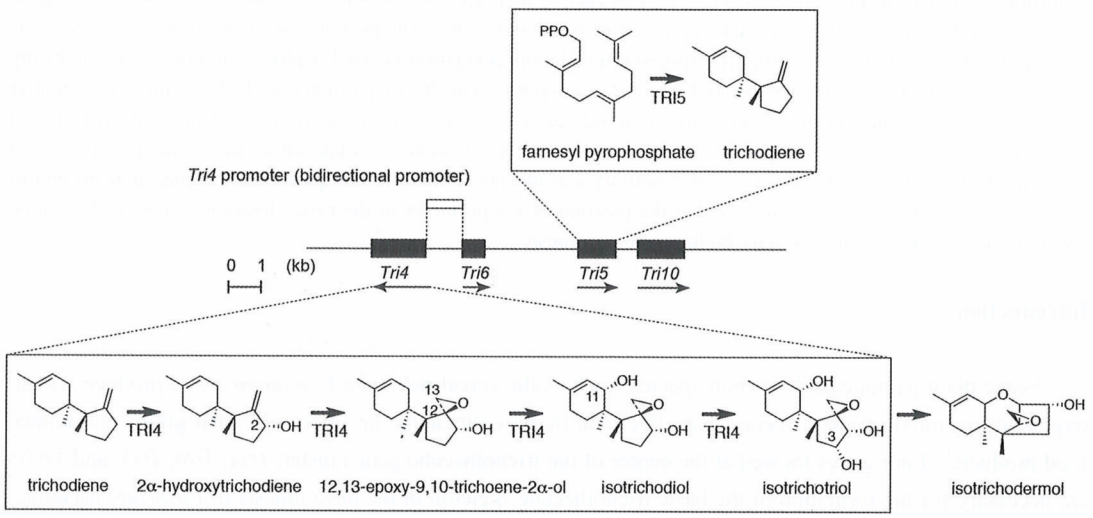

Fig. 1. The central region of the trichothecene gene cluster, comprising *Tri4*, *Tri6*, *Tri5*, and *Tri10*. The entire intergenic region (1.1 kb) between the start codons of *Tri4* and *Tri6* were used as a shared bidirectional promoter of *Tri4* for the experiments. Enzymatic reactions catalyzed by TRI4 and TRI5 and described in the boxes. Note that these two pathway genes are sufficient to build the trichothecene skeleton because the second cyclization (isotrichotriol to isotrichodermol) proceeds spontaneously[1].

Materials and Methods

Fungal strains and culture conditions *F. graminearum* MAFF 111233, a strain that produces nivalenol (NIV)-type trichothecenes, was used as a host for the expression of a β-glucuronidase (GUS) gene (*uidA*)[9]. For induction of trichothecene production, fungal strains were cultured in 100 ml of modified rice flour (MRF) medium[10] (containing 4 % rice (boiled for 30 min and glass filtered), 3 % sucrose, and 0.1 % yeast extract) on a gyratory shaker (160 rpm) at 22 °C. Strain MAFF 111233 mainly produces 4,15-diacetylnivalenol (4,15-diANIV) in MRF medium.

Amplification of promoters used for the assay *Tri4* and *Tri101* promoters were amplified from the genomic DNA of *F. graminearum* by performing PCR using KOD Neo (ToYoBo Life Science) with primers MFTri 4 Pro-U2 (5′-AAAAAGATGGCTTGATGGGAGGGG-3′) and MFTri 4 Pro-D (5′-CTTTTCAAAGTTCAAGTCTTTGATAG-3′) (with an amplicon size of 1.1 kb), and primers MFTri101Pro-U (5′-AATGGATGAGTCAATCGATACGCA-3′) and MFTri 101 Pro-D (5′-TTTGGTGGTGTTTGTAAATCACTT- 3′) (amplicon size 0.7 kb), respectively. The promoter of *TEF1α* (AN4218.3) was amplified by perfoming PCR of the genomic DNA of *Aspergillus nidulans* NBRC 33017 with primers AN4218TEFPro-U3 (5′- TAGCGACTTAGAGTGCACGCTCAAAAGA-3′) and AN4218TEFPro-D (5′-GGTGAAGGTTGTGTTATGTTTTGT-3′) (amplicon size 1.1 kb).

Construction of promoter-GUS fusion vectors for integration into the* Tri14 *locus pGR−Srf−hph (Fig. 2A), a derivative of pHF312 (Tokai et al. unpublished; constructed from pBF312[11] by replacing *bsd*, a blasticidin S deaminase gene, with *hph*, a hygromycin phosphotransferase gene), is a promoter-cloning GUS vector that contains *hph* for selection of fungal transformants and a *Srf*I site for insertion of promoters amplified by PCR. For targeted integration of this vector into the *Tri14*[12] locus, we constructed pGR−Srf−hph-MFTri14 by replacing the short *Not*I-*Spe*I linker fragment of pGR−Srf−hph with a *Not*I-*Spe*I digested inverse PCR (IPCR) product containing each side of the *Tri14* region. This IPCR product was generated in the following manner: (1) the *Tri14* region was amplified by perfoming PCR with *Nhe*I site-containing primers DLTri14-U/Nhe (5′-AAGCTAGCGTATCAGTCTCCAATCGGTC- 3′) and DLTri 14 -D/Nhe (5′-CGGCTAGCGTTGACGAACATTGCTGACC-3′), (2) the amplified product was digested using *Nhe*I and then was self-ligated, and (3) this self-ligated DNA was used as a template for performing IPCR with primers INVTri 14 -D/Not (5′-ACAGCGGCCGCGTATAGGTTGCTTCAAG- 3′) and INVTri 14 -U/Spe (5′-GAACTAGTGTTGTGATCAAGCAGACTCC- 3′) containing *Not*I and *Spe*I sites, respectively. The promoter fragments amplified by performing PCR were cloned into the *Srf*I-cloning site of pGR−Srf−hph-MFTri 14 (yielding vectors pP_{Tri4}-GR-hph-MFTri14, pP_{Tri101}-GR-hph-MFTri14, and $P_{TEF1\alpha}$-GR-hph-MFTri14), linearized with *Nhe*I, and were used to transform *F. graminearum* according to the method described previously[4].

Screening of transformants Transformants with homologous recombination were screened by performing long PCR (LA-PCR) using LA-Taq (Takara Bio Inc.). Primers FgTri 14 -LAU (5′-TAGCTGGAATGTCGCCAAAAAGACTCTG-3′) and FgTri 14 -LAD (5′-CCTTACTTTAGCTTCAACGACCGAACAC-3′) were used for amplifying the *Tri14* region (Fig. 2B).

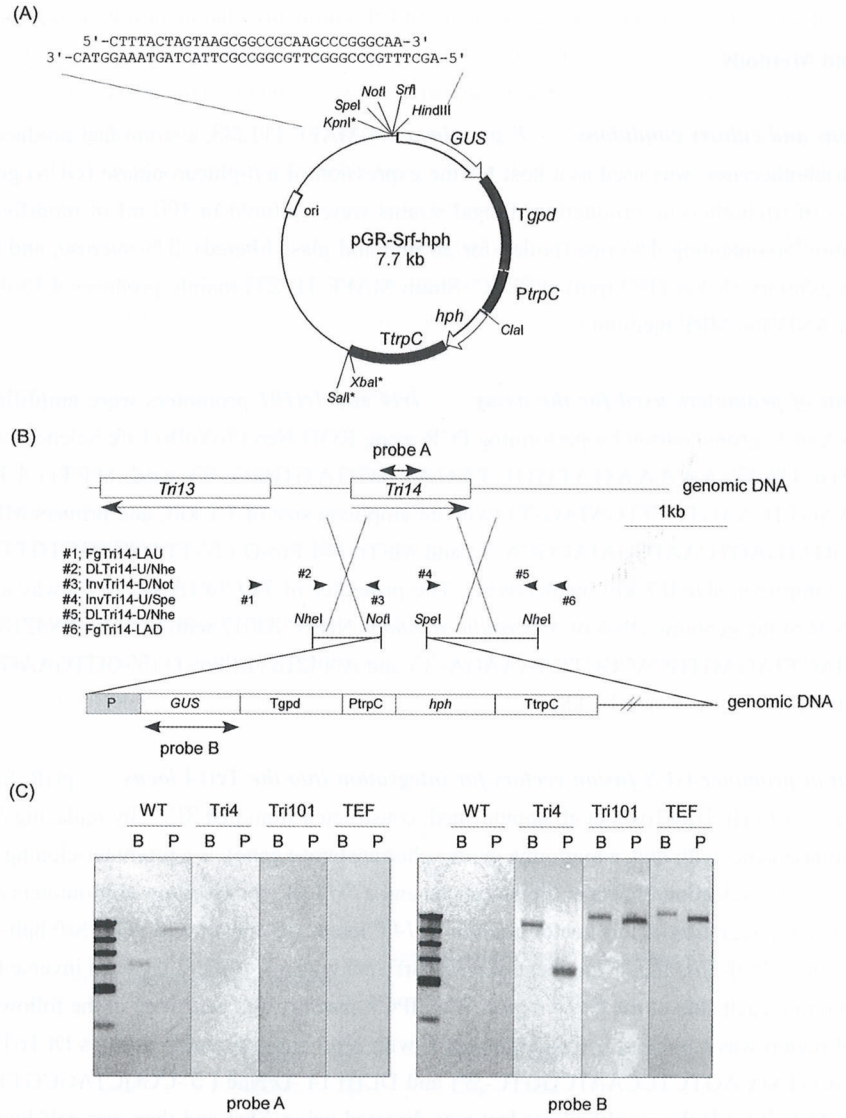

Fig. 2. Generation of *F. graminearum* transformants for evaluating promoter activities. A. Structure of pGR-Srf-hph used for constructing pGR-Srf-hph-MFTri 14 , a promoter-reporter fusion vector designed for targeted integration at the *Tri14* locus. A *Kpn*I-*Hin*dIII fragment of the *A. nidulans trpC* promoter in pGR 101[5] was replaced with a synthetic linker containing *Kpn*I, *Spe*I, *Not*I, *Srf*I, and *Hin*dIII sites (shown in the figure). After *Sac*I digestion and blunting, the larger vector fragment was ligated to a blunted *Sph*I fragment of pHF312 containing the *Cryphonectria parasitica gpd* terminator (T*gpd*), the *A. nidulans trpC* promoter, *hph*, and the *A. nidulans trpC* terminator. B. Diagram showing targeted integration of the promoter-GUS fusion vectors to the *Tri14* locus. Promoters of *F. graminearum Tri4*, *Tri101*, and *A. nidulans TEF1α* were fused to GUS in pGR-Srf-hph-MFTri 14 , and the resulting vectors (pP_{Tri4}_GR-hph-MFTri 14 , pP_{Tri101}_GR-hph-MFTri14, and p$P_{TEF1\alpha}$_GR-hph-MFTri14) were targeted to the *Tri14* locus. Arrow heads indicate annealing sites of primers used for screening of the transformants (#1 and #6) and for constructing pGR-Srf-hph-MFTri14 (#2 - #5). Probes used for Southern blot analyses are indicated by double arrows. C. Southern blot analyses of wild-type (WT) and transformants. The result of a representative transformant is shown. DNA was digested using *Bam*HI (B) or *Pst*I (P), and was hybridized with probe A or probe B. "Tri4", "Tri101", and "TEF" refer to the transformants carrying P_{Tri4}::GUS, P_{Tri101}::GUS, and $P_{TEF1\alpha}$::GUS at the *Tri14* locus, respectively. λ*Hin*dIII maker (23, 9.4, 6.5, 4.4, 2.3, 2.0, 0.56 kb) was loaded to the left of the WT lanes.

Nucleic acid hybridizations DNA probes for Southern and northern blot analyses were generated by labeling fragments with digoxigenin using the PCR DIG probe synthesis kit (Roche Applied Science). For Southern blot analyses, probe A was obtained by performing PCR of genomic DNA with primers Fgtri-14 probe-U (5′-TTGAATTCCCAGGTCTCAGTCACG-3′) and Fgtri 14 probe-D (5′-CCAATGATGTTGTC-GGGAACAATC-3′); probe B was amplified from pGR-Srf-hph by using primers GUS-ATG (5′-ATGTTAC-GTCCTGTAGAAACCCCA-3′) and GUS-TGA (5′-TTCATTGTTTGCCTCCCTGCTGCG-3′). For northern blot analyses, a *Tri4* probe was amplified from cDNA with primers GzTri4/TAA and GzTri4/ATG/SacI[4]; a *Tri101* probe was amplified with primers U101 and D101[5]. Standard hybridization techniques recommended by the manufacturer were used for Southern and northern blot analyses.

In vitro *GUS assay* One gram of mycelia was ground in a mortar in liquid nitrogen and suspended in 2 ml of GUS extraction buffer (50 mM phosphate buffer, pH 7.0, containing 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% *N*-lauroylsarcosine sodium salt, 0.1% Triton X-100). The mycelial suspension was centrifuged at 4 °C for 15 min at 20,400 × *g*. The supernatant was filtered through a Millex syringe filter unit (0.45 μm, Millipore) and used to evaluate the promoter activity by performing GUS assay. The GUS assay was performed using a β-glucuronidase (GUS) Reporter Gene Activity Detection kit (MGT Inc.), as per the manufacturer's instructions. Briefly, 10 μl of crude enzyme was mixed with 90 μl of GUS Assay Buffer supplied in the kit (final 0.09 mM 4-methylumbelliferyl β-D-glucuronide), incubated for 30 min at 37 °C, and then the reaction was terminated by adding 200 μl of Carbonate Stop buffer (included in the kit). The concentration of 7-hydroxy-4-methylcoumarin (4-MU) formed was determined by measuring the fluorescence of 4-MU with a Wallac ARVO SX 1420 MultiLabel Counter (Perkin Elmer) using excitation and emission wavelengths of 365 and 469 nm, respectively. The GUS activity was calculated in pmol of 4-MU formed per min per μg of protein.

Chemical and biochemical analyses Trichothecenes were extracted using ethyl acetate, developed on a TLC plate (Merck F_{254} silica TLC) using ethyl acetate/toluene (3 : 1) as a solvent, and visualized with a chromogenic reagent, as per a previously described method[13]. Protein concentrations were determined using a Pierce BCA ™ Protein assay kit (Thermo Scientific).

Results and Discussion

A β-glucuronidase (GUS) gene was introduced downstream of each promoter and was used as a reporter to evaluate the promoter activity. A portion of the coding region of *Tri14*[12], which is located at the end of the trichothecene gene cluster, was chosen as the integration site for the promoter-GUS fusions; this gene is not necessary for the trichothecene biosynthesis steps, and the deletion mutant normally produces trichothecenes when the fungus is grown under toxin-inducing conditions. Promoter fragments amplified by PCR were cloned into the *Srf*I site of pGR-Srf-hph-MFTri 14, and the sequence was verified by sequencing. After digestion with *Nhe*I, the vectors were transformed to *F. graminearum*.

The genomic DNA of the transformants were screened for the occurrence of the promoter-GUS fusions at the *Tri14* locus (Fig. 2 B). Strains with double crossing-over homologous recombination gave LA-PCR products that were approximately 10 kb in length; primers FgTri14-LAU and FgTri14-LAD (see **Materials**

and Methods), whose annealing sites are separated by 3 kb in the wild-type genome (Fig. 2B) had been used. Figure 2 C shows representative Southern blot data of such transformants carrying P_{Tri4}::GUS, P_{Tri101}::GUS, and $P_{TEF1\alpha}$::GUS, further confirming the integration of the promoter-GUS fusions at the *Tri14* locus. When hybridized with probe A (a deleted region of *Tri14* containing neither the *Bam*HI nor the *Pst*I site), single bands detected on the DNA blots of the wild-type (a 5 kb *Bam*HI fragment and a *Pst*I fragment larger than 10 kb) were lost from those of the transformants carrying P_{Tri4}::GUS, P_{Tri101}::GUS, and $P_{TEF1\alpha}$::GUS at the *Tri14* locus (Fig. 2 C; left panel). In contrast, single bands were detected on the blots of these transformants with probe B (a coding region of GUS containing neither the *Bam*HI nor the *Pst*I site), and these bands were absent in the case of the wild-type (Fig. 2C; right panel).

The GUS activity of these transformants were measured at various time points after transfer to the trichothecene-inducing MRF medium. The wet weight of these transformants showed less than 20 % of variability (compared to that of the $P_{TEF1\alpha}$::GUS transformant) at each time point (data not shown). The timing and level of trichothecene accumulation was not significantly altered by the disruption of *Tri14* (Fig. 3 A). Constitutively, high levels of GUS activitiy was observed in the mycelia of the $P_{TEF1\alpha}$::GUS transformant; the activity was highest at 24 h, and then it gradually decreased at 48, 72, and 96 h (Fig. 3 B). This pattern of expression is reasonable because the promoters of housekeeping genes are transcribed during active growth, and their expression declines after the transition of the organism to the stationay phase. In contrast, the P_{Tri101}::GUS transformant showed a GUS expression pattern consistent with the acumulation of trichothecenes under trichothecene-inducing culture conditions. However, integration of the P_{Tri4}::GUS fusion vector (pP_{Tri4}_GR-hph-MFTri14) into the *Tri14* locus resulted in undetectable levels of GUS activity at all time points. This lack of detectable GUS activity cannot be attributed to an unexpected mutation that may have been accidentally acquired during transformation because other pP_{Tri4}_GR-hph-MFTri 14 transformants carrying the promoter-reporter fusion at the *Tri14* locus (and also those carrying it at ectopic sites) also failed to produce the GUS protein (data not shown). Data obtained from the northern blot analyses in the same time course experiment confirmed a normal transcript accumulation pattern of *Tri4* like that in the native promotors[14], which was also observed for *Tri101* (Fig. 3C). These results suggest that the *Tri4* promoter does not support sufficient transcription levels of heterologous genes when integrated at non-native sites.

The 1.1 kb intergenic region of divergently transcribed *Tri4* and *Tri6* constitutes a shared bidirectional promoter. In strain MAFF 111233 used in this study, the *Tri4* promoter region contains two closely positioned putative TRI6-binding sites, $T4A_{Fg}$ (TCAGGCCT) and $T4B_{Fg}$ (CCAGGCCT), between bp −377 and −361 with respect to the ATG start codon of *Tri4* (underlined nuclotides indicate matches to consensus sequences[15], TNAGGCCT, and/or the minimum sequence required for TRI 6 binding YNAGGCC[15]). These putative TRI 6 -binding sites correspond to $T\,4\,A_{Fs}$ (TCAGGCCC) and $T\,4\,B_{Fs}$ (CCAGGCCT), two experimentally dertermined TRI 6 -binding sites in the *Tri4* promoter of *Fusarium sporotrichioides*, with regard to their positions from the ATG start codon[15]. Therefore, the affinity of the TRI6 protein to the *F. graminearum Tri4* promoter is comparable and cannot be the cause of undetectable levels of GUS expression from the *Tri14* locus.

Tri4 is situated 17.5 kb upstream of *Tri14* that demarcates the end of the gene cluster. The inability of the *Tri4* promoter to cause heterologous gene expression from a non-native locus may suggest the necessity of additional elements located in the coding region of *Tri4* for transcriptional activation of its own gene. Alternatively, the physical linkage of *Tri* genes or chromatin remodeling at the central region of the gene cluster may

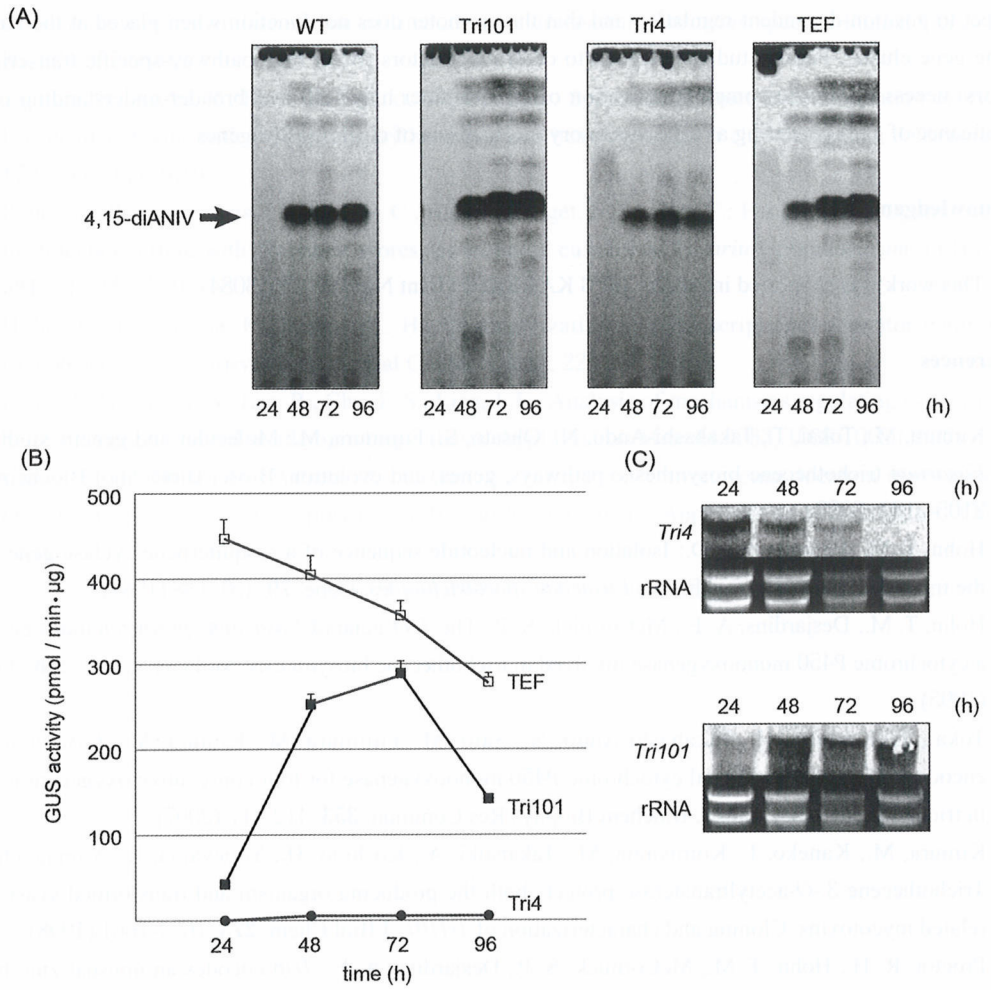

Fig. 3. The *Tri4* promoter does not work when integrated at the *Tri14* locus. In A and B, "Tri4", "Tri101", and "TEF" refer to the same transformants as described in Fig. 2 C. A. TLC analysis of trichothecenes extracted from the culture of the wild-type (WT) and the transformants at each time point of the GUS assay. Strain MAFF 111233 produces 4,15-diANIV in liquid culture. Ethyl acetate extract from 5 ml of the culture was loaded in each lane. In consideration of the fact that the cultures are derived from independent strains, the data indicate that their trichothecene levels do not significantly differ each other. B. GUS activities of the transformants at 24, 48, 72, and 96 h after transfer to MRF medium. C. Northern blot analyses of *Tri* genes. Expressions of *Tri4* and *Tri101* in the transformants were monitored at each time point of the GUS assay.

have a regulatory role in the epigenetic activation of *Tri4* and some other *Tri* genes. In either case, the distance between the native *Tri4* locus and the P_{Tri4}::GUS reporter fusion is too long to exert regional control over the transcriptional activity of the *Tri14* locus. Similar position-dependent regulation of clustered secondary metabolite (SM) gene expression has also been demonstrated for the *ver-1*[16] and *nor-1*[17] genes in aflatoxin biosynthesis; GUS expression from their promoters that were engineered to reside outside of the aflatoxin gene cluster was marginal to absent. These results led to the conclusion that positional effects are important in the expression of aflatoxin biosynthetic genes[18].

In conclusion, we found that a gene located in the centeral region of the trichothecene gene cluster is

subject to position-dependent regulation and that the promoter does not function when placed at the the end of the gene cluster. Future studies designed to determine factors (other than pathway-specific transcription factors) necessary for the complete activation of the promoter may lead to a broader understanding of the significance of gene clustering and the regulatory mechanisms of clustered SM genes.

Acknowledgement

This work was supported in part by JSPS KAKENHI Grant Number 23658084.

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Fusarium graminearum の *Tri4* 遺伝子のプロモーターをトリコテセン生合成遺伝子クラスターの末端に配置すると機能しない

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トリコテセン生合成遺伝子クラスターの中心の領域には，2つの経路遺伝子と2つの調節遺伝子の合計4つの生合成遺伝子（*Tri* 遺伝子）が存在し，トリコテセン基本骨格を形成する．このうちの1つ *Tri4* 遺伝子は多機能シトクロム P450 をコードし，トリコテセン産生条件下で高レベルに発現が誘導される．遺伝子クラスター外の全く関係のないところには *Tri101* が単一遺伝子として存在し，*Fusarium graminearum* においてはトリコテセンの毒性からの自己耐性に寄与している．これらのプロモーターの活性を，毒素産生誘導条件下で GUS 遺伝子をレポーターとして用い，*TEF1α* 遺伝子（高レベルで発現する翻訳伸張因子をコードする）のプロモーターの活性と比較した．クラスターの末端に組み込んだ *Tri101* プロモーターと GUS 遺伝子との融合および *TEF1α* プロモーターと GUS 遺伝子との融合によって示されるレポーター活性は，もともとの位置にそれらのプロモーターがある場合に示す活性と一致した．しかしもともとの位置からの正常な *Tri4* の転写とは対照的に，遺伝子クラスターの末端に配置した *Tri4* プロモーターからは GUS 活性が検出されなかった．従ってクラスター内におけるプロモーターの位置が *Tri4* の本来の発現制御にとって重要であると考えられる．

キーワード：遺伝子クラスター；染色体上の位置；転写活性化；*Fusarium graminearum*; GUS レポーター

オクラおよびゴマの種子から単離されたフザリウム種，生産するマイコトキシンおよびその毒性

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2系統のオクラと17サンプルのゴマの種子のフザリウム汚染について調査を行った．2系統のオクラからは6菌株，10サンプルのゴマからは31菌株，合計37種のフザリウム菌株が得られた．得られた菌株は *F. oxysporum*，*F. semiticum*，*F. verticillioides* の3つの種に属した．オクラからの全て