A set of heterologous promoters useful for investigating gene functions in *Fusarium graminearum*

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Abstract
The activities of four constitutive promoters from *Aspergillus nidulans* were compared in *Fusarium graminearum* by using β-glucuronidase (GUS) as a reporter gene. The promoter-GUS constructs were integrated into the Tri14 locus at the terminus of the trichothecene gene cluster and crude cell extracts were used for the reporter assay. The translation elongation factor 1-alpha (TEF1α) promoter yielded by far the strongest induction of GUS with little or no effect seen with the glyceraldehyde 3-phosphate dehydrogenase (GPD), polyubiquitin (UBI), and β-tubulin (TUB) gene promoters. The promoters of TUB and TEF1α, with or without an original trichothecene regulator gene (Tri6) opal codon, were connected to a transcriptional fusion of Tri6 and enhanced green fluorescent protein (EGFP) gene, and targeted to a locus downstream of the trichothecene 3-O-acetyltransferase gene (Tri101). Northern blot analysis revealed expression levels of these fusion genes to be proportional to the activities of the promoters as demonstrated by the GUS assay. In addition, analysis of trichothecene levels demonstrated drastically decreased activity of a translational fusion of TRI with EGFP (TRI::EGFP) as a trichothecene transcription factor. These results indicate that the set of promoters reported in this study could be used to investigate biological functions of master genes by modulating their expression levels in *F. graminearum*.

*Fusarium graminearum* is a mycotoxin-producing fungus for which genetic manipulation techniques were established more than a decade ago. Despite considerable achievements in our understanding, at a molecular level, of the growth, differentiation, and metabolism of this important cereal pathogen, tools for engineering its gene expression systems are still in great demand. Promoters with differing strengths are useful for basic research because the functions of key genes associated with the biological phenomena under study can be examined by modulating their expression levels. The use of heterologous promoters as a component of transformation vectors has an advantage over the use of promoters of *F. graminearum* in that the heterologous promoters are not integrated into the genome by a single crossover homologous recombi-
nation event through the promoter sequences. Therefore, using heterologous promoters is beneficial as a component of transformation vectors when targeting vectors for comparative functional analysis of a series of related genes. This is because the heterologous promoters can also be used for modulating the transcription level of master genes, such as the mycotoxin regulator genes, that affect the specific phenotypes under study.

In the current study, promoters of glyceraldehyde 3-phosphate dehydrogenase (GPD; AN8041), polyubiquitin (UBI; AN 2000), and β-tubulin (TUB; AN 1182) genes were cloned by polymerase chain reaction (PCR) from the genomic DNA of Aspergillus nidulans using KOD plus (TOYOBO Life Science, Osaka) with the following primers: gpdAPro-U 3 and gpdAPro-D 2 (amplon size 2.2 kb); AN20polyUbiPro-D (amplon size 1.3 kb); and AN1182BtubPro-U3 and AN1182B-tubPro-D (amplon size 1.0 kb), for GPD, UBI, and TUB, respectively (see Aspergillus Genome Database AspGD [http://www.aspergillusgenome.org/] for accession numbers and Supplementary Table 1 for primer sequences). The amplified promoter fragments were cloned into pGR-Srf-hph-MFTri14, a vector that contains a SrfI cloning site upstream of β-glucuronidase (GUS), and Tri14 flanking regions near the end of the trichothecene gene cluster, to yield pP$_{GPD}$GR-hph-MFTri14, pP$_{UBI}$GR-hph-MFTri14, and pP$_{TUB}$GR-hph-MFTri14, for GPD, UBI, and TUB, respectively.

Because pP$_{GPD}$GR-hph-MFTri14 contains an internal NheI site in the GPD promoter fragment in addition to the unique NheI site in pGR-Srf-hph-MFTri14, the restriction site was eliminated as follows: (1) pP$_{GPD}$GR-hph-MFTri14 (12.5 kb) was digested with NheI to yield a large vector fragment (11.2 kb); (2) a small region corresponding to the discarded DNA fragment (1.3 kb containing upstream regions of a GPD promoter and Tri14) was amplified by PCR using pP$_{GPD}$GR-hph-MFTri14 as a template with the primers DLTri14-U/Nhe and gpdAPro-walkSpeD (amplon size 1.3 kb) (NheI and SpeI sites underlined, Supplementary Table 1); and (3) the amplified fragment, digested with NheI and SpeI (1.3 kb), was ligated into the large NheI fragment of the vector (11.2 kb) to yield pP$_{GPD\Delta Nhe}$GR-hph-MFTri14.

The promoter evaluation vectors that were constructed to be integrated at the Tri14 locus (pP$_{GPD\Delta Nhe\_GR-hph-MFTri14}$, pP$_{UBI\_GR-hph-MFTri14}$, and pP$_{TUB\_GR-hph-MFTri14}$) were linearized with NheI and transformed into F. graminearum MAFF 111233 (renamed Fusarium asiaticum$^9$) according to the method described previously$^8$. Strains with double crossover homologous recombination at the Tri14 locus were screened for by long PCR and further verified by Southern blot analysis (data not shown). Using the wild-type cells, and the transformants carrying the promoter-GUS fusions at the Tri14 locus (P$_{TEF1α}$::GUS$^9$, P$_{GPD\Delta Nhe\_::GUS}$ [this study], P$_{UBI\_::GUS}$ [this study], P$_{TUB\_::GUS}$ [this study], P$_{Bio10\_::GUS}$, and P$_{Bio4\_::GUS}$), the GUS activities were measured as described previously$^9$.

Fig. 1 shows the GUS activity of the crude protein extracts from each strain cultured for 24 h in a trichothecene non-inducing YG medium (2% glucose and 0.5% yeast extract). The assay was carried out using 4-methylumbelliferyl β-d-glucuronide as a substrate of GUS as described previously$^9$. Compared to the promoter of the TEF1α gene, promoters of GPD, UBI, and TUB induced over 90% less GUS activity. There were no marked differences between the activities of these three promoters (Fig. 1; inset). The Tri4 and Tri101 promoters induced marginal to no change in GUS activity, which was comparable to the background activity level seen in wild-type cells.

To verify that these heterologous promoter fragments can be used to examine gene functions by controlling their expression levels, we focused on a trichothecene regulator gene (Tri6) encoding a Cys$_2$His$_2$ zinc finger transcription factor$^{11}$. A region downstream of trichothecene 3-O-acetyltransferase gene (Tri101)
was chosen as a locus where these promoters could be integrated for the comparative analyses (Fig. 2A). The promoters of TUB and TEF1a, with or without an original opal codon at the end of the Tri6 coding region, were connected to Tri6 that was fused to an enhanced green fluorescent protein (EGFP) gene. The resulting constructs (pP_{TUB} Tri6::EGFP@DwTri101 [type 1], pP_{TUB} Tri6stopEGFP@DwTri101 [type 2], pP_{TEF1a} Tri6::EGFP@DwTri101 [type 3], and pP_{TEF1a} Tri6stopEGFP@DwTri101 [type 4]; Supplementary Fig. 1) were transformed to a Tri6 disruption (null) mutant of F. graminearum JCM in 9873 that was generated by targeted gene disruption of Tri6 in the gene cluster (Supplementary Fig. 2). The transformants carrying each vector downstream of the Tri101 coding region were selected with high efficiencies (46% of homologous integration on average). PCR amplifications of the upstream and downstream regions (amplicons U and D, respectively), which are indicative of successful double crossover homologous recombination, are shown for the four strains representing each type of transformant (strains 1-#1, 2-#1, 3-#1, and 4-#1) (Supplementary Fig. 3).

The four transformed strains were grown in 100 ml of a trichothecene-inducing YS_60 medium (6% sucrose and 0.1% yeast extract) in a 300-ml Erlenmeyer flask. Total RNA was blotted to a Nytran® membrane (Schleicher & Schell GmbH; Dassel, Germany) and hybridized with a digoxigenin (DIG)-labeled Tri6 probe prepared by using a PCR DIG Probe Synthesis kit (Roche Diagnostics GmbH; Mannheim, Germany) with the primers CjTri 6_S and CjTri 6_AS (see Supplementary Table 1). As shown in Fig. 2B, these constitutive promoters of A. nidulans also proved to be active in F. graminearum grown in different media. The mRNA levels of the Tri6 fusion genes transcribed from the TUB promoter (i.e., P_{TUB}::Tri6::EGFP and P_{TUB}::Tri6stopEGFP) were approximately an order of magnitude lower than those from the TEF1a promoter (i.e., P_{TEF1a}::Tri6::EGFP and P_{TEF1a}::Tri6stopEGFP), as estimated from the intensity of the bands on the northern blot (compare lanes 2 and 4, and lanes 3 and 5). This result is consistent with the GUS activity induced by the TUB and TEF1a promoters in YG medium (Fig. 1). However, smear signals were detected in the region slightly below the Tri6 band for both Tri6::EGFP and Tri6stopEGFP mRNAs; the intensities of the smear were much greater in the transformants obtained with type 3 and type 4 constructs carrying the strong TEF1a promoter (lanes 4 and 5). The smear corresponds to partially degraded or shorter incomplete mRNAs.
Fig. 2. Expression of the Tri6 fusion genes using promoters of differing strengths. A. Diagram showing targeted integration of the Tri6 fusion vectors downstream of Tri101. The Tri6 fusion genes of EGFP, with or without an original opal codon of Tri6 (Tri6stopEGFP or Tri6::EGFP), were connected to the TUB or TEF1α promoter, and the resulting constructs (types 1–4) were used to create a Tri6 disruption mutant of F. graminearum JCM 9873 (see Supplementary Fig. 2). After the legitimate recombination between homologous sequences, the Tri6 fusion genes connected to the TUB or TEF1α promoter were placed downstream of Tri101 in the same orientation. Arrowheads indicate primers used for screening of the recombinants. B. Expression levels of the Tri6 fusion genes in relation to the amount of trichothecene produced by each transgenic strain. Conidia of the wild-type (WT) and each transformant were inoculated into YG medium (with final conidia concentration of \(1 \times 10^7\) ml) and pre-cultured for 18 h. One milliliter of pre-cultured mycelia was inoculated into 1 ml of YS medium and cultured for 48 or 79 h before the RNA and trichothecene metabolites were analyzed. Trichothecenes were extracted and analyzed as described previously\(^{10}\). Upper panel: northern blot analysis of the transformants probed with the Tri6 probe; middle panel: ethidium bromide staining of rRNA samples to show equal loading of RNA samples used for the blot; lower panel: thin layer chromatography (TLC) analysis of the 15-ADON metabolite extracted from 1 ml of the culture medium. Trichothecenes were developed on a TLC plate (Merck Fsilica TLC) using ethyl acetate/toluene (3:1), and visualized with a chromogenic reagent as previously described\(^{16}\). C. TLC of 15-ADON produced by the transgenic strains. Fungal strains (WT and each transformant) were cultured in 1 ml of YS_60 medium in a 24-well culture plate at 25 °C with gyration shaking (135 rpm). After 55 h of incubation, 15-ADON was extracted from 600 µl of the medium and analyzed as described above.
transcribed from the strong and constitutive promoters, presumably due to the extensive accumulation or instability of the mRNA within the fungal cells under the culture conditions.

The presence of an original opal codon of Tri6 in the Tri6 transcriptional fusion gene resulted in somewhat decreased accumulation of the fusion transcripts (Fig. 2B; compare lanes 2 and 3, and lanes 4 and 5), suggesting that nonsense-mediated mRNA decay (NMD), or mRNA surveillance, may function to degrade mRNAs containing premature termination codons. Nonetheless, the Tri6stopEGFP mRNA transcribed from the TEF1α promoter was more abundant than the Tri6 mRNA in the wild-type strain in YS_60 medium, and caused an earlier accumulation of trichothecene 15-acetyldeoxynivalenol (15-ADON) (Fig. 2B; compare lanes 1 and 5). These results suggest that the amount of TRI within the fungal cells is a major factor determining when trichothecene biosynthesis is initiated under these culture conditions. The experiment also revealed that the transcriptional activity of the TRI6::EGFP fusion protein is absent, or much weaker, compared to the TRI6 protein (Fig. 2B; compare lanes 4 and 5). Under conditions using a 24-well culture plate containing 1 ml of YS_60 medium, which is known to stimulate trichothecene production much more than using an Erlenmeyer flask (our unpublished observation), 15-ADON was detected in the transgenic fungal culture medium (Fig. 2C). Although EGFP fluorescence could not be detected in the P_{TEF1α}::Tri6::EGFP transformant under the epifluorescence microscope (implying improper folding of the EGFP moiety), the fusion gene was able to function weakly as a positive transcription factor. Hence, the C-terminal fusion of TRI with EGFP retain some ability to form a transcription complex with other activator proteins to transcribe the Tri biosynthetic pathway genes.

In conclusion, we isolated and characterized promoters that induced different transcription levels of foreign genes in F. graminearum. Gene cassettes containing these promoters could be integrated at designed loci with reasonable efficiencies. These promoters afford the means to establish experimental designs of our Tri6 functional studies that are now underway.

Acknowledgements

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References


**Supplementary materials**

Supplementary materials may be found in the online version of this article:

Supplementary Table 1. Primers used in this study.

Supplementary Fig. 1. Structures of vectors used to demonstrate the usefulness of the heterologous promoters in the functional analysis of the master regulatory gene.

Supplementary Fig. 2. Generation of a *Tri6* disruption (null) mutant of *F. graminearum* JCM 9873.

Supplementary Fig. 3. PCR amplification of the regions with a double crossover homologous recombination.
### Supplementary Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
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<tr>
<td>gpdAPro-U3</td>
<td>5’-CTGAAATACGTCGAGCCTGCTCCGCTTG-3’</td>
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<tr>
<td>gpdAPro-D2</td>
<td>5’-CATGGTGATGTCTGCTCAAGCGGGGTAG-3’</td>
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<tr>
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<td>5’-CGCAGTATCAACAGCCCCAACCTG-3’</td>
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<td>5’-GATGTCGACAACGTAGCTTGGATCG-3’</td>
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<td>5’-TGTGAAGGGTGACGGGTATAG-3’</td>
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<td>DLTri14-U/Nhe</td>
<td>5’-AAGCTAGCGTATCGTCTCCCAATCGGTC-3’</td>
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<td>gpdAPro-walkSpeD</td>
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<td>5’-CTCGAAATGATTTACATGGAGGC-3’</td>
</tr>
<tr>
<td>CjTri6_AS</td>
<td>5’-CACACCGATCCCTCGTCAACA-3’</td>
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Supplementary Fig. 1. Structures of vectors used to demonstrate the usefulness of the heterologous promoters in the functional analysis of the master regulatory gene. pdJTri6-tk_neo, a Tri6 disruption vector; pP_TUB_Tri6::EGFP@DwTri101, pP_TUB_Tri6::EGFP@DwTri101, pP_TEF1a_Tri6::EGFP@DwTri101, and pP_TEF1a_Tri6::EGFP@DwTri101, Tri6 fusion vectors for targeted integration into the region downstream of Tri101.
Supplementary Fig. 2. Generation of a Tri6 disruption (null) mutant of F. graminearum JCM 9897. A. Diagram showing targeted integration of the Tri6 disruption vector pdJTri6-tk_neo (Supplementary Fig. 1) at the Tri6 locus. B. Southern blot analysis of the Tri6 disruption mutant. The DNA of the WT and Tri6 disruption mutant (∆Tri6 tk) were digested with SmaI or SpeI. A DIG-labeled probe (indicated as filled box) was prepared by PCR with primers neo-specific_F (5’- GCGATACCGTAAAGCACGAG -3’) and neo-specific_R (5’- TGGAGAGGCTTATTGGCTATG -3’), and used for the analysis. M, DNA Molecular Marker II (Roche Diagnostics GmbH; Mannheim, Germany).
Supplementary Fig. 3. PCR amplification of the regions with a double crossover homologous recombination. Targeted integration of the vector was demonstrated by successful amplification of amplicon U with the primers TT101D_12 (5′-TCTCAGCGCAGCATTCTAT-3′) and TT101D_13 (5′-GACATCGACACCAACGATCT-3′) (5′ portion of Tri101 plus trpC terminator), and amplicon D with primers TT101D_14 (5′-AAGTACTCGCCGATAGTGGA-3′) and TT101D_15 (5′-GCGTTTACGGAGGTGTCACT-3′) (3′ portion of hph plus Tri101 terminator). Strains 1-#1, 2-#1, 3-#1, and 4-#1 are representative transformants obtained with type 1, type 2, type 3, and type 4 constructs, respectively. The left lane was loaded with 1 Kb Plus DNA ladders (Life Technologies; Gaithersburg, MD).