Development of a versatile copper-responsive gene expression system in the plant-pathogenic fungus *Fusarium graminearum*

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**Abstract**

*Fusarium graminearum* is an important plant-pathogenic fungus that causes Fusarium head blight on wheat and barley, and ear rot on maize worldwide. This fungus has been widely used as a model organism to study various biological processes of plant-pathogenic fungi because of its amenability to genetic manipulation and well-established outcross system. Gene deletion and overexpression/constitutive expression of target genes are tools widely used to investigate the molecular mechanism underlying fungal development, virulence, and secondary metabolite production. However, for fine-tuning gene expression and studying essential genes, a conditional gene expression system is necessary that enables repression or induction of gene expression by modifying external conditions. Until now, only a few conditional expression systems have been developed in plant-pathogenic fungi. This study proposes a new and versatile conditional gene expression system in *F. graminearum* using the promoter of a copper-responsive gene, designated *F. graminearum* copper-responsive 1 (*FCR1*). Transcript levels of *FCR1* were found to be greatly affected by copper availability conditions. Moreover, the promoter (P<sub>FCR1</sub>), 1 kb upstream of the *FCR1* open reading frame, was sufficient to confer copper-dependent gene expression. Replacement of a green fluorescent protein gene and *FgENA5* promoter with a P<sub>FCR1</sub> promoter clearly showed that P<sub>FCR1</sub> could be used for fine-tuning gene expression in this fungus. We also demonstrated the applicability of this conditional gene expression system to an essential gene study by replacing the promoter of *FgIRE1*, an essential gene of *F. graminearum*. This enabled the generation of *FgIRE1* suppression mutants, which allowed functional characterization of the gene. This study reported the first conditional gene expression system in *F. graminearum* using both repression and induction. This system would be a convenient way to precisely control gene expression and will be used to determine the biological functions of various genes, including essential ones.
INTRODUCTION

Investigating the molecular mechanisms underlying various biological phenomena first requires examining the molecular functions of the related genes. The development of basic molecular techniques, such as gene knockout and overexpression, has accelerated molecular genetic research; however, few methods are available to study essential genes or fine-tune gene expression in nonmodel organisms. A conditional gene expression system can overcome this limitation by allowing repression or induction of gene expression simply by modifying the culture conditions. This technique is a convenient molecular tool and furthermore has potential for industrial applications (Mach & Zelling, 2003).

Fusarium graminearum is an important plant-pathogenic fungus that causes Fusarium head blight on wheat and barley, as well as ear rot on maize (Leslie & Summerell, 2006). F. graminearum infections result in severe yield losses worldwide (Goswami & Kistler, 2004), and contaminate grains with harmful mycotoxins such as trichothecenes and zearalenone (Desjardins, 2006). Genetic manipulations via highly efficient homologous recombination have led to this fungus being used as a model for large-scale, in-depth molecular genetic research of plant-pathogenic fungi (Jiang et al., 2020; Teng et al., 2018). The system has limitations in that it cannot be used in studies of zearalenone biosynthesis. Moreover, only one conditional gene expression system is currently available in F. graminearum. This system is based on the P_\text{ZEAR} promoter, which is activated by zearalenone or the oestrogenic compound β-estradiol (Lee et al., 2010, 2011a), and has been successfully applied to analyse the function of essential genes in this fungus (Bui et al., 2016; Lee et al., 2011b; Liu et al., 2019; Nguyen et al., 2020; Tang et al., 2018). The P_\text{ZEAR} system has limitations in that it cannot be used in studies of zearalenone biosynthesis. Moreover, only one-directional control (induction) is available when supplementing with zearalenone or β-estradiol. Therefore, there is the need to develop more versatile conditional gene expression systems in F. graminearum.

Several conditional gene expression systems have been developed in fungi. A doxycycline-dependent Tet-on/Tet-off system was established in Aspergillus fumigatus, Aspergillus niger, and Candida albicans (Meyer et al., 2011; Park & Morschhäuser, 2005; Vogt et al., 2005). In Saccharomyces cerevisiae and Cryptococcus neoformans, the GAL7 promoter has been widely used to activate or repress gene expression using galactose and glucose, respectively (Johnston, 1987; Wickes & Edman, 1995). The thiA promoter is repressible by thiamine in Aspergillus oryzae (Shoji et al., 2005). In Aspergillus nidulans and A. fumigatus, the alcA promoter is induced by glycerol, ethanol, or threonine, and is repressed during growth on glucose (Felenbok et al., 2001; Romero et al., 2003; Waring et al., 1989).

In this study, we focused on the copper-regulated promoters that have been used in several fungal species. Copper is an essential trace element that acts as a cofactor in many enzymes, but it can be detrimental to cells if accumulated in excess (Kim et al., 2008). Living organisms protect themselves from copper toxicity by maintaining cellular copper homeostasis. In F. graminearum, the P_{CTR4} transporter FgCrpA and transcription factor FgAceA play a role in copper tolerance (Liu et al., 2020). Previous studies of Schizosaccharomyces pombe and C. neoformans revealed that the promoter of the high-affinity copper transporter could drive strong copper-dependent regulation of target genes (Bellemare et al., 2001; Ory et al., 2004). Similarly, conditional gene expression systems using copper-regulated promoters have been proposed in several model fungal species (Gebhart et al., 2006; Lamb et al., 2013; Willyerd et al., 2009).

This study aimed to develop a new conditional gene expression system in F. graminearum based on a native copper-responsive promoter. We successfully identified F. graminearum copper-responsive 1 (FCR1) and demonstrated that the FCR1 promoter (P_{FCR1}) could drive gene expression in a copper-dependent manner. We further assessed the general applicability of this promoter for studying essential genes and fine-tuning gene expression. This study provides an easy and convenient method to generate conditional mutants. This should help determine the biological functions of the genes underlying fungal development, virulence, and mycotoxin production, which had previously proven difficult to study.

RESULTS AND DISCUSSION

2.1 Identification of a copper-responsive gene in F. graminearum

S. pombe ctr4+ (GenBank accession no. NP_587968), C. neoformans CTR4 (XP_775793), and Neurospora crassa tcu-1 (XP_964373) are representative copper-responsive genes that encode high-affinity copper transporters (Bellemare et al., 2001; Labbé et al., 1999; Lamb et al., 2013; Ory et al., 2004). The expression of these genes is regulated by copper availability, and they have previously been used for conditional gene expression systems. Based on amino acid sequence identity with S. pombe Ctr4, C. neoformans Ctr4, and N. crassa TCU-1, we identified three candidates for copper-responsive genes in F. graminearum: FGSG_00773, FGSG_06061,
and FGSG_07059. These genes have not been functionally characterized in *F. graminearum*.

To examine the responses of candidate genes to copper conditions, we investigated their relative transcript levels at 1, 4, 8, and 12 hr after the addition of 200 μM CuSO₄ and 300 μM of the copper chelator bathocuproinedisulfonic acid (BCS) in complete medium (CM) via quantitative reverse transcription PCR (RT-qPCR) assays (Figure 1). All three genes showed reduced transcript levels under CuSO₄-supplemented conditions (copper-sufficient conditions) and increased transcript levels when BCS was added (copper-deficient conditions). The highest levels of repression and induction were observed 12 hr after the addition of CuSO₄ and BCS. Under copper-deficient conditions, FGSG_00773 showed a higher induction effect (by about 8-fold) than those of FGSG_06061 and FGSG_07059 (about 5- and 4-fold, respectively). With sufficient copper, the transcript levels of FGSG_06061 decreased rapidly after 1 hr, while the transcript levels of FGSG_07059 and FGSG_00773 started to decrease after 4 hr. At 12 hr after inoculation, FGSG_00773 showed a maximum inhibitory effect (0.03-fold) while the transcript levels of FGSG_06061 and FGSG_07059 were 0.07- and 0.2-fold, respectively. These results identified gene FGSG_00773 as the most responsive to copper availability conditions. We designated this gene *F. graminearum* copper-responsive 1 (FCR1) and selected it for further characterization.

To start mapping the promoter region of FCR1, we compared the sequences upstream of the open reading frame (ORF) of FCR1 and its putative orthologs in *S. pombe* and *C. neoformans*. The upstream regions of copper transporter genes usually contain a cis-acting copper-signalling element (CuSE), which is similar to the metal regulatory element (MRE) in copper metalloregulatory transcription factors in *S. cerevisiae* and *Candida glabrata* (Beaudoin & Labbé, 2001; Koch & Thiele, 1996). The CuSE is represented as the consensus sequence 5′-D(T/A)DDHGCTGD-3′ (D = A, G or T; H = A, C or T). GCTG and a T/A located four nucleotides upstream of the first G are the core sequences of CuSE. While the CTR4 promoter regions of *S. pombe* and *C. neoformans* contained a CuSE, we could not find any CuSE within 2 kb upstream of the FCR1 ORF. Instead, we identified the sequence 5′-ATATCGCTGC-3′, which shows substantial similarity to a CuSE, at position −874. In this sequence, 9/10 nucleotides of the CuSE are present, including the core GCTC and T/A sequences. We predict that FCR1 might be regulated in a copper-dependent manner through a different pathway or mechanism than previously identified copper-responsive genes.

### 2.2 The optimal concentration of CuSO₄ and BCS for copper-responsive expression of FCR1

The relative transcript levels of FCR1 were analysed via RT-qPCR under various copper concentrations to confirm that FCR1 expression depends on the copper concentration. The results showed that all tested concentrations of CuSO₄ (10, 25, 50, 100, 150, 200 μM) and BCS (25, 50, 100, 150, 200, 300 μM) effectively repressed and induced the expression of FCR1, respectively (Figure 2a). We further investigated whether CuSO₄ and BCS could induce phenotypic alterations in this fungus to distinguish between the effects of the reagents themselves and those of altered gene expression. The *F. graminearum* wildtype strain Z-3639 did not show significant

![FIGURE 1](image_url) Identification of copper-responsive genes in *Fusarium graminearum*. Relative transcript levels of putative copper-responsive genes in copper-enriched and copper-deprived conditions. Fold-change values were analysed by quantitative reverse transcription PCR. Total RNA was extracted from the wildtype strain grown for 1, 4, 8, and 12 hr in complete medium (CM) and CM supplemented with 200 μM CuSO₄ or 300 μM bathocuproinedisulfonic acid (BCS). The relative transcript level of each gene in CM at each hour was arbitrarily set to 1 and omitted from the graph. Then, relative transcript abundances of each gene in the presence of CuSO₄ and BCS at each hour were analysed based on that value. Bars represent the mean ± SD of two biological and three technical replications, and significant differences (*p < 0.01, **p < 0.005, ***p < 0.001) are indicated.
defects in vegetative growth (Figure S2), conidiation, or sexual reproduction under treatment conditions of 10 μM CuSO₄ or 25 μM BCS (data not shown). Given that minimum experimental concentrations of CuSO₄ (10 μM) and BCS (25 μM) worked well in controlling FCR1 expression without inducing phenotypic alterations in F. graminearum, we used them for further experiments.

To examine the expression pattern of FCR1 over time under established working concentrations, we analysed the transcript levels of FCR1 at various time points (Figure 2b). The repressive effect of adding 10 μM CuSO₄ started 12 hr after inoculation and was maintained until at least 48 hr. The inducing effect of adding 25 μM BCS reached a maximum 8 hr after inoculation and gradually decreased thereafter. Similarly, a previous study of F. graminearum showed that the transcript levels of the zearalenone-inducible gene (ZEAR) decreased over time, even in the presence of a high concentration of extracellular zearalenone (Lee et al., 2010).

2.3 | P.FCRI can drive heterologous gene expression in a copper-dependent manner

To confirm that the promoter of FCR1 can regulate the expression of other genes in a copper-dependent manner, we used the green fluorescent protein (GFP) gene and FgENA5 gene. The −1.009 to −1 bp upstream region of FCR1 (P.FCRI) was fused to the GFP gene, and the P.FCRI-GFP cassette was randomly integrated into the genome of the F. graminearum Z-3639 strain. The GFP fluorescence pattern of the P.FCRI-GFP strain (Figure 3) was consistent with the expression pattern of FCR1 analysed by RT-qPCR (Figure 2b). At 8 hr after inoculation, GFP fluorescence began to increase in the presence of the indicated time points after the addition of 10 μM CuSO₄ and 25 μM BCS. Fold-change values were analysed by RT-qPCR. Bars represent the mean ± SD of two biological and three technical replications, and significant differences (*p < 0.01, **p < 0.005, ***p < 0.001) are indicated.

FIGURE 2 Relative transcript levels of FCR1 under various copper concentrations and time points. (a) Copper concentration-dependent relative transcript levels of FCR1. Total RNA was extracted from the wildtype strain 12 hr after inoculation in complete medium (CM) and CM supplemented with CuSO₄ (10–200 μM) or bathocuproinedisulfonic acid (BCS) (25–300 μM), and transcript levels were quantified by quantitative reverse transcription PCR (RT-qPCR). (b) Relative transcript levels of FCR1 at various time points. Total RNA was isolated from the wildtype strain cultured for the indicated time points after the addition of 10 μM CuSO₄ and 25 μM BCS. Fold-change values were analysed by RT-qPCR. Bars represent the mean ± SD of two biological and three technical replications, and significant differences (*p < 0.01, **p < 0.005, ***p < 0.001) are indicated.
KIM et al. showed almost as much lithium tolerance as the FgENA5 overexpression mutant. Although 10 μM CuSO₄ and 25 μM BCS were successful for copper-dependent gene regulation, higher concentrations of CuSO₄ (50 μM) and BCS (50 μM) led to a higher level of repression and induction. These results indicate that the conditional gene expression system using P_FCR1 can be applied to functional genetic
studies of target genes, particularly via concentration-dependent fine-tuning of gene expression.

2.4 | Functional analysis of the *F. graminearum* essential gene *FgIRE1* using *PFCR1*

The functional analysis of essential genes is difficult as it is not possible to apply the key tool of gene deletion. In fact, inability to obtain deletion mutants is an indication that the target gene might be essential. Ire1 is known to function as an endoplasmic reticulum (ER) stress sensor kinase in many fungal species. There is evidence that *IRE1* might be an essential gene in several fungi. For instance, no *ireA* (*IRE1* ortholog) deletion mutants were obtained in either *A. oryzae* (Tanaka et al., 2015) or *A. niger* (Carvalho et al., 2010; Mulder & Nikolaev, 2009). Consistent with this, the ortholog of *IRE1* (locus ID: FGSG_00775, *FgIRE1*) is known to be essential in *F. graminearum* (Wang et al., 2011). In this study, repeated trials to obtain a deletion mutant of *FgIRE1* were also unsuccessful. We concluded that *FgIRE1* is essential for basic growth and other physiological functions of *F. graminearum*, and generated conditional suppression mutants of *FgIRE1* by replacing its native promoter with *PFCR1* (Figure S3b). *PFCR1*-*FgIRE1* strains showed reduced vegetative growth on CM under copper-supplemented conditions, confirming that *FgIRE1* is an essential gene directly involved in survival (Figure 5).

*IRE1* disruption mutants have shown high sensitivity to ER stress in many fungal species (Cheon et al., 2011; Fan et al., 2015; Krishnan & Askew, 2014; Miyazaki et al., 2013). We expected *PFCR1*-*FgIRE1* strains to be more sensitive to ER stress under copper-enriched conditions and less sensitive under copper-depleted conditions. When copper was added under ER stress, induced by tunicamycin (TM), *PFCR1*-*FgIRE1* strains showed severe growth inhibition as the TM concentration increased (Figure 5a). When 5 μg/ml TM was applied, the *PFCR1*-*FgIRE1* strains hardly grew under copper-enriched conditions. In the presence of BCS, *PFCR1*-*FgIRE1* strains recovered tolerance to ER stress to a level similar to the wild type.

**FIGURE 5** Endoplasmic reticulum (ER) stress and heat stress sensitivity of *PFCR1*-*FgIRE1*. (a) ER stress sensitivity. The strains were inoculated on complete medium (CM) and CM supplemented with ER stress agents (0.02 μg/ml tunicamycin [TM], 0.05 μg/ml TM). Mycelial growth was observed under 10 μM CuSO$_4$ and 25 μM bathocuproinedisulfonic acid (BCS) treatment conditions. The pictures were taken 5 days after inoculation. (b) Heat stress sensitivity. Strains were incubated at 25 °C and 30 °C on CM and CM supplemented with 10 μM CuSO$_4$ and 25 μM BCS. The pictures were taken 3 days after inoculation.
Previous studies have demonstrated that heat stress and the heat shock response are closely related to the ER stress response (Liu & Chang, 2008; Liu et al., 2012). The C. neoformans ire1 deletion mutant showed extreme thermosensitivity compared to the wild type (Cheon et al., 2011; Jung et al., 2016). To analyse the heat sensitivity mutant showed extreme thermosensitivity compared to the wild type. When BCS was added, PFCR1 mutants in a fast and convenient way. We expect precise control of copper-enriched conditions, although the wild type did not show any growth defect in response to heat stress. When BCS was added, PFCR1-FgIRE1 strains recovered heat tolerance similar to the wild type. These results demonstrate that FgIRE1 was successfully suppressed by adding copper, and reduced expression of FgIRE1 led to higher sensitivity to ER and heat stress. We expect that an in-depth study of FgIRE1 and other essential genes will be possible using PFCR1, which enables both suppression and overexpression of target genes depending on the copper concentration. In conclusion, we found that the transcription of FCR1 is regulated in response to external copper concentrations, and that copper-dependent regulation of the target gene is possible by replacing the native promoter with PFCR1. This study is the first to develop a conditional gene expression system that enables both repression and induction in F. graminearum. The PFCR1 system is generally applicable for studying essential genes and would be a valuable tool to generate suppression or overexpression mutants in a fast and convenient way. We expect precise control of gene expression to facilitate extensive functional genetic studies of F. graminearum.

3 | EXPERIMENTAL PROCEDURES

3.1 | Strains and culture conditions

The F. graminearum wildtype strain Z-3639 (Bowden & Leslie, 1999) and all mutants derived from the wild type (Table S1) were stored as mycelial suspensions in 20% glycerol solution at −80 °C. Production of HK146 and HK147 (FgENA5 deletion mutant and FgENA5 overexpressing strain) is described elsewhere (Son et al., 2015). Culture medium was prepared as described in the Fusarium laboratory manual (Leslie & Summerell, 2006). The growth temperature was set at 25 °C unless otherwise indicated.

3.2 | Genetic manipulations and PCR primers

For genomic DNA isolation, each strain was cultured in 5 ml of CM for 3 days in a rotary shaker at 200 rpm, and genomic DNA was extracted according to the Fusarium laboratory manual (Leslie & Summerell, 2006). Total RNA was extracted from mycelia ground in liquid nitrogen using the Easy-Spin Total RNA Extraction Kit (Intron Biotech). Restriction endonuclease digestion and agarose gel electrophoresis were performed following standard protocols (Sambrook & Russell, 2001). Southern blot hybridization was performed with the NorthSouth Biotin Random Prime Labeling Kit and the NorthSouth Chemiluminescent Hybridization and Detection Kit (Thermo Scientific). The PCR primers used in this study (Table S2) were synthesized by an oligonucleotide synthesis facility (Bionics).

3.3 | Characterization of FCR1

To identify putative copper-responsive genes in F. graminearum, BLASTp was performed to compare the amino acid sequences of S. pombe Ctr4 (GenBank accession no. NP_587968), C. neoformans Ctr4 (XP_775793), and N. crassa TCU-1 (XP_964373) to the F. graminearum genome database (https://fungidb.org/). Genes that showed a significant match (E value <1e−10) were considered putative copper-responsive genes. The phylogenetic tree (Figure S1) was constructed using ClustalW and the MEGA X program, with 1,000 bootstrap replicates performed by the neighbour-joining method (Kumar et al., 2018).

3.4 | Genetic manipulations and fungal transformations

The double-joint (DJ) PCR method (Yu et al., 2004) was employed to construct the fusion PCR products required for targeted gene deletion and promoter replacement. Fungal transformation was performed as previously described via homologous recombination (Son et al., 2011).

To generate the PFCR1-GFP strain, hygromycin resistance gene cassette (HYG) and GFP were amplified from the pIGPAPA plasmid (Horwitz et al., 1999) using the primers HYG-F/HYG-R and GFP-F-Pfcr1/GFP-R. The −1,009 to −1 bp region upstream of the FCR1 translational site was amplified from the wildtype strain using the primers Pfcr1-F-HYG/Pfcr1-R1. Three fragments were fused by the DJ PCR method, and the resulting PCR product was used as a template to produce the final construct, HYG-PFCR1-GFP, with the primers HYG-F1 and GFP-R1. Subsequently, the HYG-PFCR1-GFP construct was cloned into the pGEM-T Easy vector following the manufacturer’s instructions for the pGEM-T and pGEM-T Easy Vector Systems Kit (Promega), using Escherichia coli DH10B. Plasmid DNA was extracted with the DNA-spin Plasmid DNA Purification Kit (Intron Biotech) and used to transform the F. graminearum wildtype protoplasts. For the promoter replacement of FgENA5 with PFCR1, HYG was amplified from the pIGPAPA plasmid (Horwitz et al., 1999) using the primers HYG-F/HYG-R, and PFCR1 was amplified from the wildtype strain with the primers Pfcr1-F-HYG/Pfcr1-R. The two fragments were fused by the DJ PCR method (Yu et al., 2004), and the HYG-PFCR1 construct was amplified with the primers HYG-F1 and Pfcr1-R1. The 5’ and 3’ flanking regions of FgENA5 were amplified from the wildtype strain using the primers ENA5-5F/ENA5-5R-Pfcr1 and ENA5-3F-Pfcr1/ENA5-3R-Pfcr1, respectively. After fusion PCR of the resulting three fragments, the final PCR construct was obtained with nested primers. The final PCR products were used to
transform fungal wildtype protoplasts. The $P_{FCR1}$- $FgIRE1$ strains were generated using the same strategy.

To generate $FgIRE1$ deletion mutants, the 5′ and 3′ flanking regions of $FgIRE1$ and a geneticin resistance cassette (GEN) were amplified from the wildtype strain and pII99, respectively. The three amplicons were fused by DJ PCR, and the third round of PCR was performed using nested primers. The resulting amplicons were transformed into the wildtype strain.

3.5 | Microscopic observation

Microscopic observation was performed with a DM6 B microscope (Leica Microsystems) equipped with a Leica DMC6200 camera using the fluorescent filter L5 (part no. 11504166). Conidial suspensions of the $P_{FCR1}$-GFP strain were inoculated in CM at $2 \times 10^5$ conidia/ml, and mycelia were harvested 24 hr after incubation on a rotary shaker (200 rpm). The mycelia were observed under UV light 8 and 12 hr after reinoculation in CM and CM supplemented with CuSO$_4$ (10 or 50 µM) or BCS (25 or 50 µM).

3.6 | RT-qPCR

Total RNA was extracted with an EasySpin Total RNA Extraction Kit (Intron Biotech). First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). RT-qPCR was performed with SYBR Green Supermix (Bio-Rad) and a 7500 real-time PCR system (Applied Biosystems) with the corresponding primers (Table S2). The endogenous housekeeping gene ubiquitin C-terminal hydrolase (UBH) was used as a reference gene for normalization. The PCR was repeated three times with two biological replicates. The relative transcript levels of target genes were calculated as previously described (Livak & Schmittgen, 2001).

To compare copper-dependent expression of FGSG_00773, FGSG_06061, and FGSG_07059, conidial suspensions of the wild type were inoculated in CM at $5 \times 10^4$ conidia/ml. Mycelia were harvested 36 hr after incubation on a rotary shaker (200 rpm), and recultured in CM and CM supplemented with 200 µM CuSO$_4$ or 300 µM BCS. Total RNA was extracted after 1, 4, 8, and 12 hr. Copper concentration-dependent relative transcript levels of FCR1 were examined using wildtype mycelia prepared by the same strategy. Total RNA was extracted 12 hr after reinoculation in CM and CM supplemented with CuSO$_4$ (10–200 µM) or BCS (25–300 µM). For analysis of the expression pattern of FCR1 over time, wildtype mycelia were prepared using the same approach and recultured for 1, 2, 4, 8, 12, 24, and 48 hr after the addition of 10 µM CuSO$_4$ or 25 µM BCS. Total RNA was isolated, and the relative transcript levels were analysed by RT-qPCR.

ACKNOWLEDGEMENTS

This work was supported by the Creative-Pioneering Researchers Program of Seoul National University, the Strategic Initiative for Microbiomes in Agriculture and Food funded by the Ministry of Agriculture, Food and Rural Affairs (918012-4), National Research Foundation of Korea (2021R1C1C10044200), and the New Breeding Technologies Development Program (PJ014836042020), Rural Development Administration, Republic of Korea. The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Kim, S., Park, J., Kim, D., Choi, S., Moon, H., Young Shin, J. et al (2021) Development of a versatile copper-responsive gene expression system in the plant-pathogenic fungus Fusarium graminearum. Molecular Plant Pathology, 22, 1427–1435. https://doi.org/10.1111/mpp.13118