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NOTE

Evaluation of Fusarium Head Blight in Barley Infected by *Fusarium graminearum*

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Fusarium head blight, which is primarily caused by *Fusarium graminearum*, is a devastating disease in the barley field. A real-time PCR protocol was developed to evaluate the growth of this pathogen in the host plant tissues. All four strains harbored the gene encoding ATP-BINDING CASSETTE TRANSPORTER (*FgABC*; FGSG_00541) as a single copy within their genomes. Our Southern blot result was identical with the genomic data for *F. graminearum* strain PH-1. Based on the crossing point (CP) values obtained in our TaqMan real-time PCR analysis, two standard curves describing the relationship among the CP value, *FgABC* copy number, and amount of fungal DNA were constructed. Chronological enumeration of fungal growth was coincided with the symptom development.

Keywords: *Gibberella zeae*, TaqMan real-time PCR

Fusarium graminearum (teleomorph: *Gibberella zeae*) causes a devastating Fusarium head blight (FHB) in small grain cereals, such as wheat, barley, and corn. This fungus is also famous for its mycotoxin production in the infected tissues and grains. In addition to yield losses and poor grain quality, the contamination of grains by mycotoxin causes serious health problems in humans and animals.

The visible symptoms of FHB include an overall and comprehensive host response that is induced by the pathogenic infection. This fungal infection involves penetration, *in planta* ramification and pathogen-associated molecular patterns (PAMPs), which require a series of diverse enzymes and secondary metabolites that are specific or non-specific to the host plants. Diffusion of the secondary metabolites provokes additional damage to the host, such as a halo beyond and around the growth area of the pathogen, and causes confusion or overestimation of the disease severity. FHB severity is assessed according to visible symptom development on the host plants (http://ohioline.osu.edu/ac-fact/0049.html; Hallen-Adams *et al.*, 2011). The severity and incidence of this disease are described based on the ratio of symptomatic spikelets on each head and the proportion of infected heads among the tested plants. Although this method is widely used in the screening of resistant germplasms and fungicides and is easy to perform, the results are subjective and not quantitative. Furthermore, symptom-based characterization is not applicable in the early phase of the interaction between barley and *F. graminearum*.

To evaluate pathogen growth in a quantitative and objective manner, a crossing point (CP) value-based evaluation technique was developed. A growing body of evidence has indicated the exact quantitative relationship between the CP value of each sample and the amount of target DNA within the sample. Briefly, the CP value is the PCR cycling number at which the fluorescence of a sample rises above the background fluorescence. Recent approaches have confirmed the existence of primer pair-dependent specificity by supplementing a dual-labeled oligonucleotide probe, a TaqMan probe, in the PCR reaction. Because of its specificity, this technique has been applied in diverse research studies on *F. graminearum*, such as the analysis of species discrimination based on the species-specific fungal genes involved in mycotoxin production (Bluhm *et al.*, 2004), the measurement of fungal growth within infected plant leaves and grains (Reischer *et al.*, 2004; Sarlin *et al.*, 2006; Hogg *et al.*, 2007), the identification of the fungal mycotoxin chemotype (Fredlund *et al.*, 2010), the monitoring of crown rot populations in wheat fields (Hogg *et al.*, 2010), and the simultaneous evaluation of multiple fungal species (Vegi and Wolf-Hall, 2013). The primer pairs and/or TaqMan probes used in these studies were designed based on the ribosomal DNA (rDNA) and the genes encoding MAT, which determines mating type and sexual recombination, TUBULIN, and enzymes involved in mycotoxin production, such as TRI5. However, rDNA and *TUBULIN* genes have a drawback of their unfixed number and/or multicopy presence within a genome. It will be not easy to reveal the confirmative relationship between copy numbers of rDNA/*TUBULIN* genes and fungal genome numbers. *MAT* gene-specific detection system will be specific to one of either mating type populations. Comparative analysis of the trichothecene cluster from Korean and American *F. graminearum* strains revealed the significant sequence

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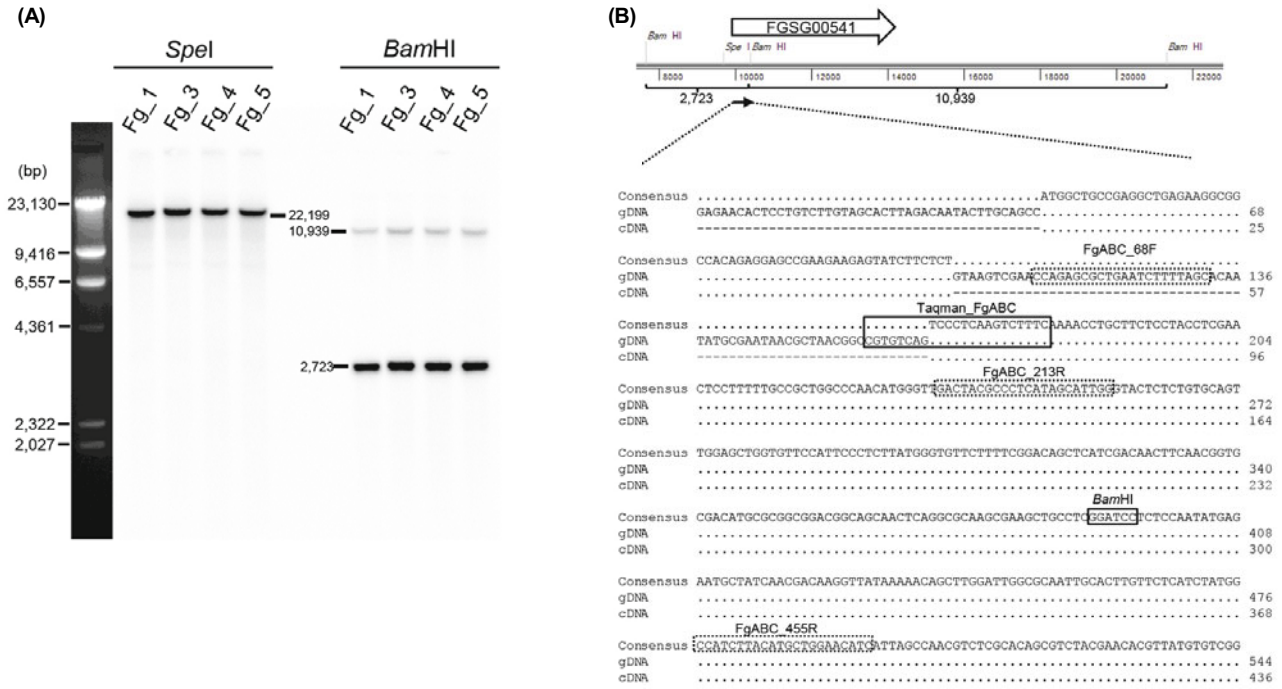

Fig. 1. Nucleotide sequence of the *F. graminearum* ATP-BINDING CASETTE TRANSPORTER (*FgABC*; FGSG_00541) gene and its presence as a single copy in the fungal genome. (A) The 22-kb region of the *F. graminearum* strain PH-1 genome that harbors *FgABC* is presented in the upper panel. The numbers below the map correspond to the sizes (bp) of the *Bam*HI fragments containing the sequence of our probe for the Southern blot analysis (indicated with a black arrow). In the lower panel, the genomic and complementary DNA sequences of *FgABC* and the positions of the primers and TaqMan probe, Taqman_FgABC, are presented. The primers and TaqMan probe are indicated with dotted boxes and a solid box, respectively. The single *Bam*HI site is also indicated with a solid box. (B) Southern blot analysis was performed using the cloned amplicon between FgABC68F and FgABC_455R as the probe. The *FgABC*-containing *Spe*I fragment of *F. graminearum* strain PH-1 was 22,199 bp, and the *Bam*HI fragments harboring the 5′ and 3′ regions of the probe used in the Southern blot analysis were 10,939 bp and 2,723 bp, respectively.

differences approximately 12%. Our objective was to develop quantitative method specific to all virulent *F. graminearum* and reflecting fungal genome numbers.

To determine the severity of FHB based on quantitative and objective results, we developed an easy and reliable method that is able to quantify fungal growth *in planta* using real-time PCR with a primer pair and TaqMan probe specific to *FgABC* (FGSG_00541), which encodes *F. graminearum* ATP-BINDING CASETTE transporter (Becher *et al.*, 2011). The sequence of the *FgABC* gene was retrieved from the genome database of *F. graminearum* strain PH-1 (NRRL 31084) (JGI, 2010), and its locus name is FGSG_00541. This strain was isolated from symptomatic wheat spikelets. This gene is composed of two exons that are separated by a short, 65-bp intron (Fig. 1A). The open reading frame consists of 4,047 nucleotides, encoding 1,348 amino acids. Nucleotide sequencing also indicated that the *FgABC* genes in our strains are the same as that in strain PH-1 (data not shown).

All 4 fungal strains Fg_1, Fg_3, Fg_4, and Fg_5 were obtained from the National Academy of Agricultural Science, Rural Development Administration, Korea. Fungal mycelia were grown in complete medium (Leslie and Summerell, 2006) for 3 days at 25°C and 150 rpm in the dark, and the culture filtrate was removed by filtering through Miracloth (Calbiochem, USA). DNA from the pulverized fungal mass or infected plant materials was prepared using the hexadecyl trimethyl ammonium bromide (CTAB) method (Stewart and Via, 1993). The DNA concentration was measured based on the absorbance at 260 nm and by comparing the brightness of its band on an agarose gel to that of a known amount of the 23.1-kb fragment of lambda DNA digested with *Hin*dIII using the Java-based software ImageJ (NIH, 2011).

Prior to all, the copy number of *FgABC* within the *F. graminearum* genome was determined. Genomic DNA was prepared from four *F. graminearum* strains (Fg_1, Fg_3, Fg_4, and Fg_5). The DNA (5 μg) was digested with *Spe*I or *Bam*HI and the gel was blotted onto a nylon membrane. Conventional PCR was performed with high fidelity *Pfu* polymerase (Nanohelix, Korea), and the two resulting amplicons were subsequently cloned into the pGEM-T Easy vector (Promega, USA). A 388-bp amplicon (FgABC_68F and FgABC_455R) harboring a *Bam*HI site was used as the probe in this experiment. No *Spe*I site was observed in the nucleotide sequence of *FgABC*. As shown in Fig. 2B, a single *Spe*I fragment containing full-length *FgABC* was observed at ~22 kb and two *Bam*HI fragments corresponding to the 5′ and 3′ regions of *FgABC* were present at 11 kb and 2.7 kb, respectively. Genomic sequencing revealed that the size of the *Spe*I fragment is 22,199 bp and that the sizes of the two *Bam*HI fragments are 10,939 and 2,723 bp. These results indicate the conservation of *FgABC* as a single copy gene in the Korean *F. graminearum* strains and the PH-1 strain.

Next, a sensitive and specific primer pair to *FgABC* was developed to assess the growth of *F. graminearum* within

the infected tissue. To examine the specificity of our primer pair (FgABC_68F, 5′-CCAGAGCGCTGAATCTTTTAGC-3′ and FgABC_213R, 5′-CCAATGCTATGAGGGCGTAGTC-3′) for *FgABC* in real-time PCR, we performed conventional PCR with these primers and genomic DNA from our *F. graminearum* strains, other fungal species, and the host plants (Fig. 2A). The PCR program and cloning protocol were performed as described above, and the resulting construct was named *pGEM-T Easy*::*FgABC*. This experiment confirmed the specificity of this primer pair for *FgABC* because a 146-bp amplicon was evident in the reaction containing the genomic DNA of *F. graminearum* and no amplification was observed in other reactions containing the genomic DNA of the other fungi or host plants. Therefore, this primer pair was applied in the subsequent real-time PCR analysis, together with a dual-labeled oligonucleotide probe, Taqman _ FgABC (Fig. 1A).

With respect to sensitivity, real-time PCR using a TaqMan probe promises a more accurate assessment than real-time PCR using SYBR green (Matsenko *et al.*, 2008; Gunel *et al.*, 2011). We prepared two standard curves to describe the relationship between the CP value, copy number, and amount of fungal genomic DNA (Figs. 2B and C). The fluorescent dyes 6-carboxyfluorescein (FAM) and Black Hole quencher 1 (BHQ1) were added to the 5′ and 3′ ends to generate a dual-labeled probe, FgABC-Taqman.

A standard curve of the CP value vs. the copy number of *FgABC* was generated using *pGEM-T Easy*::*FgABC* plasmid DNA as the template in real-time PCR with the TaqMan probe. The template was serially diluted 10-fold, from 10^{10} to $10^{-0.1}$. The slope obtained from this standard curve was -3.4725, with a high linear correlation (R^2=1) (Fig. 2B). Because *FgABC* is a unigene, the copy number of *FgABC* within the infected tissue samples should be identical to the genome copy number. A standard curve of the CP value vs. the amount of fungal DNA was generated using a similar strategy. Known concentrations of fungal genomic DNA were serially diluted 3-fold, from 83.3 ng to 1 pg. The linear correlation between the DNA concentration and CP value was high (R^2>0.997), with regression slopes of -3.5755 and -3.5609 for Fg_1 and Fg_3, respectively (Fig. 2C). The linear regressions of Fg_1 and Fg_3 were almost identical. This result indicates the accuracy of our technique used to quantify the genomic DNA based on the relative brightness of its band on an agarose gel. By comparing the relative brightness of the genomic DNA band with the band for the 23.1-kb fragment of lambda DNA digested with *Hin*dIII, we were able to calculate the fungal or tissue DNA concentration. In addition, because the standard curves for the DNAs from different strains were almost identical, the standard curves are applicable for *F. graminearum*, which harbors one copy of the *FgABC* gene within its genome. Hence, we estimated the genome copy number and fungal DNA concentration by plotting the CP values from the infected samples on the aforementioned linear regressions.

We next investigated the interaction between barley (*Hordeum vulgare* cv. Keunal) and *F. graminearum* strain Fg_1. Barley seeds were husked and surface-sterilized by submerging them in a 100 μg/ml solution of thiophanate-methyl thiram overnight. Three seeds were sown per pot (25 cm in diameter, 30 cm in height) in a commercial soil mixture (Bunong, Korea) and allowed to germinate for one week in a dark, humid chamber. The barley plants were grown at 25°C (day)/18°C (night) in a greenhouse under natural light until they reached the heading stage. Macroconidia of *F. graminearum* strain Fg_1 were grown for 5 to 7 days in ethyl methyl cellulose liquid medium (Yu *et al.*, 2008) at 25°C and 150 rpm in the dark. After removal of the mycelia

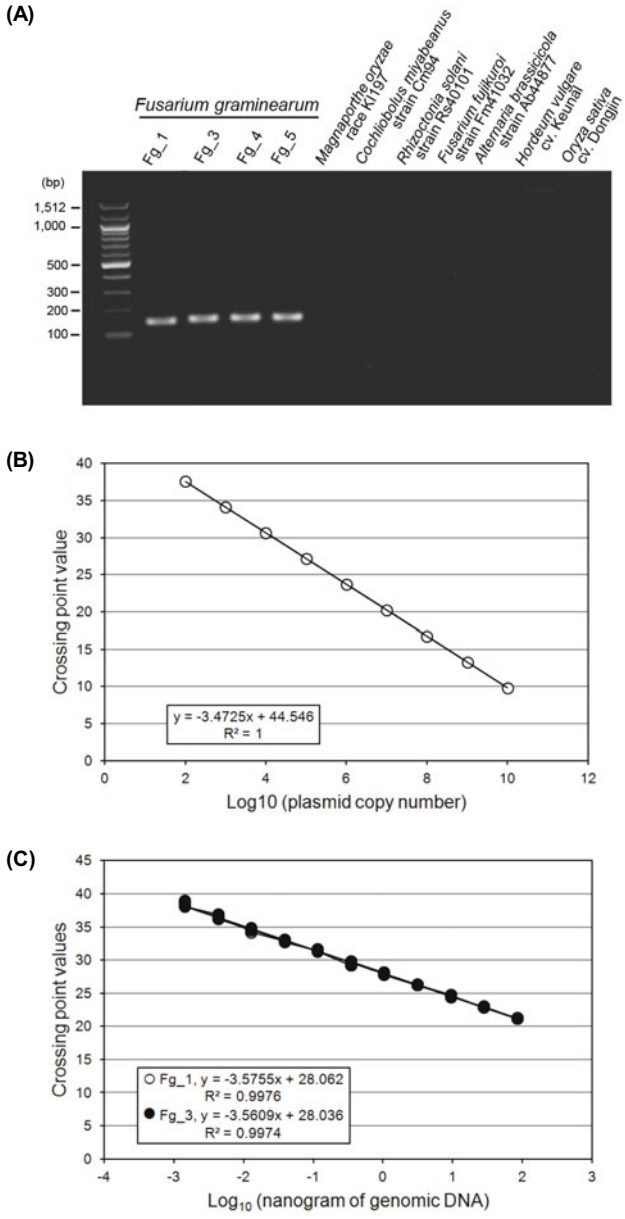

Fig. 2. Specificity of the primer pair for *FgABC* and the relationship between the crossing point (CP) value, the *FgABC*/genome copy number, and the amount of fungal DNA. (A) The primer pair FgABC_68F and FgABC_213R was used for all PCR reactions. Each reaction contained 250 ng of DNA from each of four strains of *F. graminearum*, other fungal pathogens, or the host as indicated. (B) Linear regression of the CP values and copy numbers of *FgABC* or the fungal genome. A 10-fold dilution series of *FgABC* cloned into the pGEM-T Easy vector was used as the template. (C) Linear regression of the CP values and *F. graminearum* DNA concentrations. Two 3-fold-dilution series of *F. graminearum* DNA from Fg_1 and Fg_3 were used as templates, respectively.

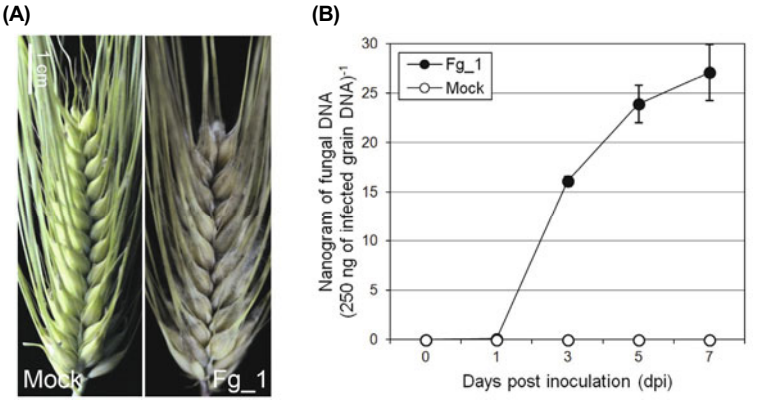

Fig. 3. Quantification of Fusarium head blight (FHB) in barley heads (*Hordeum vulgare* cv. Keunal) inoculated with *F. graminearum* strain Fg_1. (A) FHB progression on the barley heads. Images were captured 7 days post inoculation (dpi). The mock (250 μg/ml Tween 20)-treated barley heads remained healthy at 10 dpi. However, the Fg_1-infected barley heads were almost completely covered with pathogen mycelia at 7 dpi and changed color to light brown at 10 dpi. Among the three replicates, a representative head is presented. (B) Chronological quantification of the pathogenic DNA within the infected heads. Each data point indicates the Mean±SD of three barley heads.

by filtering with Miracloth, the macroconidia were precipitated by centrifugation at 1,000 rpm and 25°C for 10 min. Subsequently, the macroconidia were washed twice with sterilized distilled water, and their concentration was adjusted to 1×10^6 ml^{-1} with 250 μg/ml Tween 20. Barley heads at the early anthesis stage were sprayed with this macroconidial suspension until the spikelets were sufficiently filled with the inoculum. The infected plants were then placed in a dark chamber with absolute relative humidity. After 72 h, the plants were returned to the greenhouse, and their disease progression was monitored daily. After inoculation, for three replicates, the infected barley heads were allowed to recover for the indicated times and used for DNA extraction. Active FHB progression was evident for the barley heads infected with strain Fg_1. At 3 dpi, mycelial growth on the heads was observed, and almost all of the heads were covered with aerial mycelia as time progressed (Fig. 3A). The color of almost all of the infected heads changed to light brown at 14 dpi. The fungal growth on the barley heads was quantified by plotting the CP value of the DNA from the infected tissue (250 ng) on the standard curve. Consistent with the evaluation of visible FHB symptoms, distinguishable pathogen growth occurred at 3 dpi, and the amount of fungal DNA continuously increased up to 16.1 ng.

The primer pair used in this study is highly specific for *FgABC*. Chronological evaluation of fungal growth based on the linear regression was coincided with the symptom development. This unigene-based quantification method for fungal growth might be a powerful alternative for the evaluation of resistant germplasms, the screening of effective fungicides, and, possibly, the quantification of primary inoculums in the field.

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References

Becher, R., Weihmann, F., Deising, H., and Wirsel, S. 2011. Development of a novel multiplex DNA microarray for *Fusarium graminearum* and analysis of azole fungicide responses. *BMC Genomics* **12**, 52.

Bluhm, B.H., Cousin, M.A., and Woloshuk, C.P. 2004. Multiplex real-time PCR detection of fumonisin-producing and trichothecene-producing groups of *Fusarium* species. *J. Food Prot.* **67**, 536–543.

Fredlund, E., Gidlund, A., Pettersson, H., Olsen, M., and Borjesson, T. 2010. Real-time PCR detection of *Fusarium* species in Swedish oats and correlation to T-2 and HT-2 toxin content. *World Mycotoxin J.* **3**, 77–88.

Gunel, T., Kalelioglu, I., Surmeli, Y., Turken, B., Ermis, H., and Aydinli, K. 2011. Comparison of real-time polymerase chain reaction assay methods for detection of *RHD* gene in amniotic fluid. *J. Nat. Sci. Biol. Med.* **2**, 193–197.

Hallen-Adams, H.E., Wenner, N., Kuldau, G.A., and Trail, F. 2011. Deoxynivalenol biosynthesis-related gene expression during wheat kernel colonization by *Fusarium graminearum*. *Phytopathology* **101**, 1091–1096.

Hogg, A.C., Johnston, R.H., and Dyer, A.T. 2007. Applying real-time quantitative PCR to fusarium crown rot of wheat. *Plant Disease* **91**, 1021–1028.

Hogg, A.C., Johnston, R.H., Johnston, J.A., Klouser, L., Kephart, K.D., and Dyer, A.T. 2010. Monitoring Fusarium crown rot populations in spring wheat residues using quantitative real-time polymerase chain reaction. *Phytopathology* **100**, 49–57.

JGI. 2010. http://genome.jgi.doe.gov/Fusgr1/Fusgr1.download.html.

Leslie, J.F. and Summerell, B.A. 2006. The Fusarium Laboratory Manual, Blackwell Professional Publishing, Ames, Iowa, USA.

Matsenko, N.U., Rijikova, V.S., and Kovalenko, S.P. 2008. Comparison of SYBR Green I and TaqMan real-time PCR formats for the analysis of *her2* gene dose in human breast tumors. *Bull. Exp. Biol. Med.* **145**, 240–244.

NIH. 2011. http://rsbweb.nih.gov/ij/download.html. ImageJ.

Reischer, G.H., Lemmens, M., Farnleitner, A., Adler, A., and Mach, R.L. 2004. Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan probe. *J. Microbiol. Methods* **59**, 141–146.

Sarlin, T., Yli-Mattila, T., Jestoi, M., Rizzo, A., Paavanen-Huhtala, S., and Haikara, A. 2006. Real-time PCR for quantification of toxigenic *Fusarium* species in barley and malt. *Eur. J. Plant Pathol.* **114**, 371–380.

Stewart, C.N. and Via, L.E. 1993. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. *BioTechniques* **14**, 748–749.

Vegi, A. and Wolf-Hall, C.E. 2013. Multiplex real-time PCR method for detection and quantification of mycotoxigenic fungi belonging to three different genera. *J. Food Sci.* **78**, M70–M76.

Yu, H.-Y., Seo, J.-A., Kim, J.-E., Han, K.-H., Shim, W.-B., Yun, S.-H., and Lee, Y.-W. 2008. Functional analyses of heterotrimeric G protein Gα and Gβ subunits in *Gibberella zeae*. *Microbiology* **154**, 392–401.