Journal of Microbiology (2013) Vol. 51, No. 6, pp. 858–865
DOI 10.1007/s12275-013-3472-3

Evaluation of Bakanae Disease Progression Caused by *Fusarium fujikuroi* in *Oryza sativa* L.[§]

In Sun Hwang[1], Woo-Ri Kang[1], Duk-Ju Hwang[1], Shin-Chul Bae[1], Sung-Hwan Yun[2], and Il-Pyung Ahn[1*]

[1]*National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Republic of Korea*
[2]*Department of Medical Biotechnology, Soonchunhyang University, Asan 336-745, Republic of Korea*

(Received September 9, 2013 / Accepted October 7, 2013)

Bakanae disease caused by *Fusarium fujikuroi* is an important fungal disease in rice. Among the seven strains isolated from symptomatic rice grains in this study, one strain, FfB14, triggered severe root growth inhibition and decay in the crown and root of rice seedlings. The remaining six strains caused typical Bakanae symptoms such as etiolation and abnormal succulent rice growth. To reveal the relationship between mycelial growth in the infected tissues and Bakanae disease progression, we have established a reliable quantification method using real time PCR that employs a primer pair and dual-labeled probe specific to a unigene encoding *F. fujikuroi PNG1* (*FfPNG1*), which is located upstream of the fumonisin biosynthesis gene cluster. Plotting the crossing point (CP) values from the infected tissue DNAs on a standard curve revealed the active fungal growth of FfB14 in the root and crown of rice seedlings, while the growth rate of FfB20 in rice was more than 4 times lower than FfB14. Massive infective mycelial growth of FfB14 was evident in rice stems and crown; however, FfB20 did not exhibit vigorous growth. Our quantitative evaluation system is applicable for the identification of fungal virulence factors other than gibberellin.

Keywords: Bakanae disease, Taqman real time PCR

Introduction

Fusarium fujikuroi Niremberg is an anamorph of *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura and is a member of the *G. fujikuroi* species complex (GFSC) (O'Donnell *et al.*, 1998). GFSC is a polyphyletic group composed of species from sections *Liseola* and *Elegans* and more than nine mating populations, and *F. fujikuroi* is mating population C (MP-C) (Leslie and Summerell, 2006). This seed-borne pathogen is a causal agent of Bakanae or foolish seedling disease in rice (*Oryza sativa* L.) and is widely distributed in Asia, Africa, North America, and Italy (Ou, 1985; Prà *et al.*, 2010). In particular, outbreaks of this disease during the rice growth rate stage provoke severe yield losses up to 70% (Ou, 1985). Infected rice turns pale yellow and exhibits chlorosis, poor grain ripening such as empty panicles, and foot and stem rot. Despite its readily distinguishable symptoms, exact diagnosis and evaluation of this disease are frequently confusing because several other fungal species also belong to GFSC, and section *Liseola* also inhabits the infected plants to a degree (Carter *et al.*, 2008; Izzati and Salleh, 2010; Wulff *et al.*, 2010). Resistance breeding and biological control are most ideal, and some of them are now available (Watanabe *et al.*, 2007; Ma *et al.*, 2008; Kazempour and Anvary, 2009; Kato *et al.*, 2012). Bakanae disease management is mainly dependent on fungicide treatments such as prochloraz and carbendazim; however, these are not environmentally friendly, and there have been recent reports of chemical-resistant *F. fujikuroi* in Korea (Kim *et al.*, 2010; Yang *et al.*, 2012).

F. fujikuroi is known for its production of the plant hormone gibberellic acid (GA), which is involved in plant growth regulation. GAs accumulate within and around rice roots during host recognition, pre-penetration morphogenesis, and/or pathogen growth in the plants, and GAs are believed to be responsible for abnormal internode elongation of the stem because high concentrations of this hormone cause hypertrophy of the cells in the parts of rice found above ground. In extreme cases, infected plants topple over and die because they are no longer sturdy enough to support their own weight. Although several studies have been performed to elucidate the chronological interactions between *F. fujikuroi* and rice, no explanations for the roles of GA in disease progression and pathogen growth in the host are available.

Accurate quantification is indispensable in the evaluation of resistance germplasm, Bakanae disease forecasting, and management. Like other disease assessment, enumeration of this disease have been dependent on the observation of visible symptoms. Although this method is easiest and is still useful for some objectives such as Bakanae forecasting in the field, it does not produce quantitative values and is not applicable for chronological Bakanae estimation and in particular, early disease initiation. In addition, this evaluation does not reflect fungal growth in the host but rather measures alterations to plants. To develop sensitive techniques specific to the pathogen itself, several alternative approaches have been designed (Jarnagin and Harris, 1985; Harrison *et*

*For correspondence. E-mail: jinhyung@korea.kr; Tel.: +82-31-299-1724; Fax: +82-31-299-1722
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

al., 1990; Martin *et al.*, 1990). However, the above approaches have drawbacks with respect to sensitivity, time and labor efficiency, and reproducibility. Several polymerase chain reaction (PCR)-based approaches have been described; however, they were also qualitative because the number of amplicons does not mirror the amount of fungal DNA in the input plant DNA. Finally, crossing point (CP) value-based quantitative real time PCR was introduced to solve this problem (Lievens *et al.*, 2006; Selma *et al.*, 2008). The use of fluorophores made it possible to measure detectable amplification-initiation steps and CP values. As CP values are inversely related to the logarithm of the initial copy number of target genes, objective and reproducible measurements of fungal DNA can be calculated (Kulik, 2011; Pavon *et al.*, 2012; Su'udi *et al.*, 2012; Zhao *et al.*, 2012).

The aim of this study was to reveal the relationship between symptom progression and *F. fujikuroi* proliferation in rice. We have developed a reliable method to produce a digitalized estimation of *F. fujikuroi* among infected tissue DNA by employing a primer pair and dual-labeled probe that is specific to the unigene *FfPNG1*, which encodes transglutaminase deemed to confer resistance against proteolysis to a processed protein.

Materials and Methods

Fungal strains and pathogenicity assay

The *Fusarium fujikuroi* isolates and other fungal species used in this study (Table 1) were obtained from Dr. Sun-Hwan Yun at Soonchunhyang University and the National Academy of Agricultural Science in Korea. *Fusarium* species were grown on *Fusarium* complete medium containing 2% agar (Leslie and Summerell, 2006). *Magnaporthe oryzae* was grown on oatmeal agar (50 g oatmeal flakes and 20 g agar per 1 L distilled water), *Cochliobolus miyabeanus* was grown on sucrose proline agar (SPA) (Ahn *et al.*, 2005), and *Rhizoctonia solani* was grown on potato dextrose agar (PDA, Difco Lab, USA). All strains were grown at 22°C under continuous florescent light. Subsequently, each inoculum was transferred into complete liquid media and grown for 3 days at 25°C and 150 rpm in the dark. Prior to sampling, the mycelial mass was filtered through miracloth (Calbiochem, Germany) and was subsequently frozen in liquid nitrogen and stored at -80°C.

Virulence of *F. fujikuroi* on rice plants (*Oryza sativa* L. cv. 'Dongjin') was analyzed as described previously (Wiemann *et al.*, 2009). After surface sterilization of rice seeds by submerging in 1% (v/v) sodium hypochlorite for 30 min with gentle shaking, excess surface disinfectant was thoroughly washed out with a sufficient amount of sterilized distilled water. Rice seeds were germinated on half strength Murashige and Skoog agar for 7 days at 25°C under a 16 h photoperiod of fluorescent light. The *F. fujikuroi* strains tested were grown on oatmeal agar as described above. Ten agar blocks (6 mm in diameter) were placed in a 2 × 5 matrix on vermiculite soil contained in block-shaped pots (width × length × height = 16 × 7.5 × 7 cm^3). Additional vermiculite was evenly poured on the soil up to 2 cm. Ten healthy rice seedlings were planted on the surface of the vermiculite layer just above the sites of inoculum placement. The plants were returned to the greenhouse with natural light conditions. Whole seedlings were harvested every other day after inoculation, and their weight and height were measured. The seedlings were kept at -70°C for DNA or RNA preparation. Pictures of symptom progression were taken at 14 days post inoculation (dpi). A pathogenicity assay was performed at least three times, and similar results were obtained.

Table 1. Fungal strains used to evaluate primer pair specificity

Scientific name	Strain/ collection no.	PCR results[a]	Hosts isolated[b]
Fusarium fujikuroi	F7fB14	+	*O. sativa*
	FfB20	+	*O. sativa*
	FfB66	+	*O. sativa*
	FfB98	+	*O. sativa*
	FfB100	+	*O. sativa*
	FfB101	+	*O. sativa*
F. proliferatum	Fp45823	-	*O. sativa*
F. graminearum	Fg46434	-	*H. vulgare*
F. oxysporum	Fo40032	-	*O. sativa*
Magnaporthe oryzae	KI197	-	*O. sativa*
Cochliobolus miyabeanus	Cm85	-	*O. sativa*
Rhizoctonia solani	Rs40101	-	*S. tuberosum*

[a] +, strain was detected; -, strain was not detected.
[b] Scientific name of the original hosts harboring each strain

Standard molecular methods

Genomic DNA of the fungal mass or infected plant materials were prepared as described previously (Stewart and Via, 1993). DNA concentrations were estimated by comparing band brightness with the largest 23.1-kb fragment of *Hin*dIII-digested lambda DNA (Bioneer, Korea) using ImageJ software (NIH, 2011). The DNA concentrations of the fungal and plant samples were adjusted to 50 ng/ml, and DNA was subsequently prepared in serial dilutions as indicated for Taqman real time PCR.

For Southern blot analysis, genomic DNA was isolated from seven *F. fujikuroi* strains: FfB14, FfB20, Ff66, FfB90, FfB98, and FfB100. DNA (5 μg) was digested with *Xho*I or *Hin*CII and transferred to a nylon membrane (Hybond N+, Amersham). The blot was hybridized with a 320-bp fragment of *FfPNG1* amplified using the primer pair FfPNG1_232F (5′-CTG CGA CAT CTC CCC AAG ATC-3′) and FfPNG1_551R (5′-CTG TGT TAG TCA ATC CA TCC-3′) (Fig. 1). The primers were designed based on the nucleotide sequence of the scaffold 41_2 in the draft genome of FfB14 (GenBank accession No. KB205993.1) (Jeong *et al.*, 2013) and compared with the whole coding DNA sequence of *F. fujikuroi PNG1* (*FfPNG1*) and the same gene in *F. verticillioides* strain M-3125 (Supplementary data Fig. S1). The probe was labeled with [α-^{32}P] dCTP through random priming (Feinberg and Vogelstein, 1983) using a Rediprime II DNA Labeling system kit (GE Healthcare, USA). After washing with an SSPE series ranging from 2 × to 0.1× supplemented with 0.1% SDS, the membrane was exposed to BAS film (Fujifilm, Japan).

The specificity of a primer pair for real time PCR, FfPNG1_232F and FfPNG1_355R (5′-CAA CAG ACC GGG GTT CTC-3′), for *F. fujikuroi* was enumerated. Conventional

PCR was performed using genomic DNA (83.3 ng) from seven *F. fujikuroi* strains as template, including the three *Fusarium* species *F. proliferatum*, *F. graminearum*, and *F. oxysporum*, as well as three were other fungal species frequently found in rice paddy fields, specifically *Magnaporthe oryzae*, *Cochliobolus miyabeanus*, and *Rhizoctonia solani*, and finally rice (*Oryza sativa* L. cv. Dongjin). PCR conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. The primer specificity was determined by *F. fujikuroi*-specific 134-bp amplicon production. A picture was taken using a Gel Documentation System (Bio-Rad, USA).

For the molecular identification of the fungal strains, 520-bp ITS regions were amplified from the genomic DNA of seven *F. fujikuroi* strains using the primer pair ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al.*, 1990). After cloning them into the pGEM-T Easy vector, the nucleotide sequences of the cloned ITS regions were identified using M13F and M13R primers.

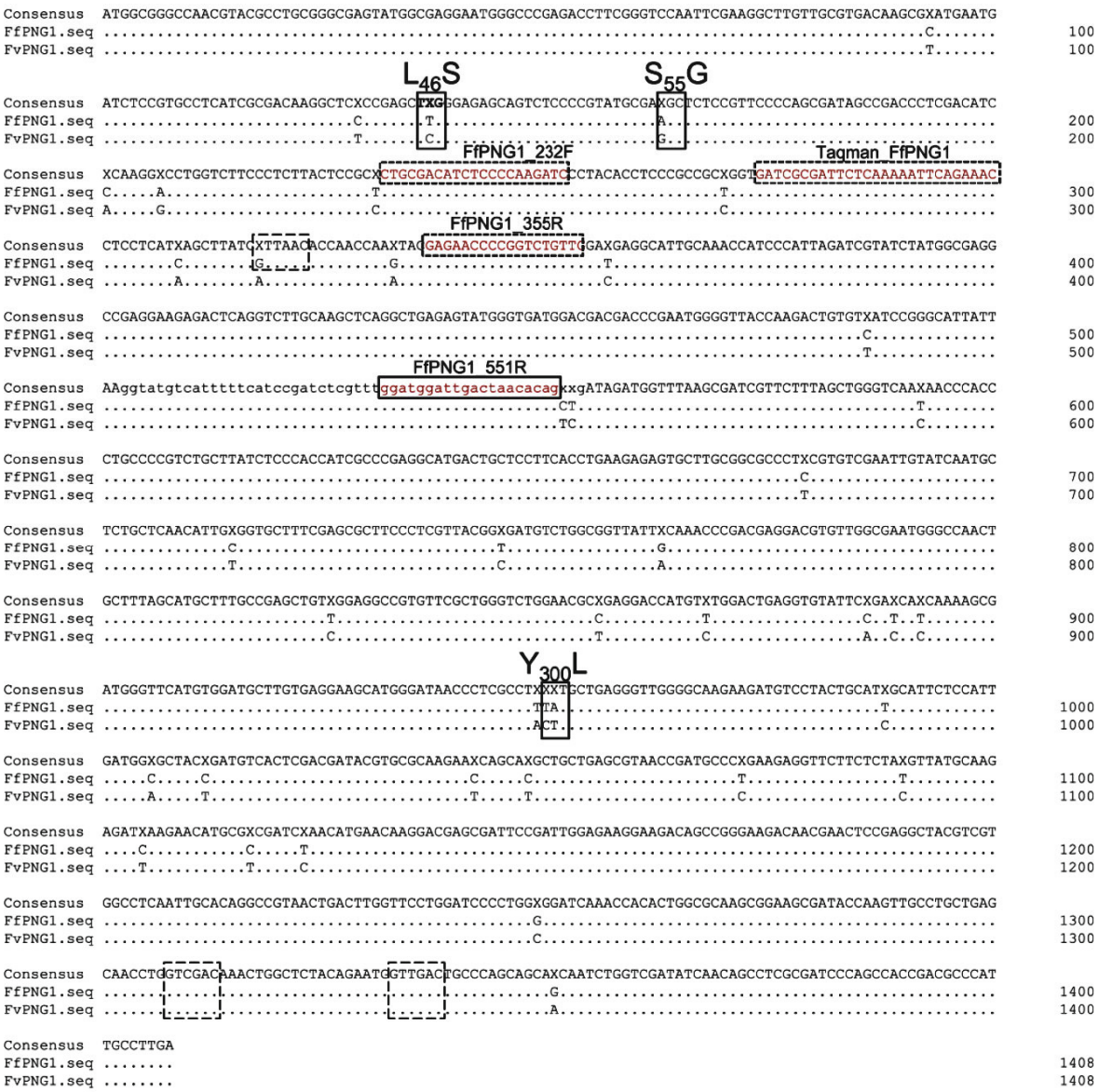

Fig. 1. Genomic DNA sequence of *FfPNG1* in *Fusarium fujikuroi* strain FfB14 and *FvPNG1* in *F. verticillioides* strain M-3125. Three different amino acids between FfPNG1 and FvPNG1 are indicated with solid boxes, and the items remain above. Two exon regions are indicated with capital letters, and the translated amino acid sequences are shown. Primer pairs (FfPNG1_232F and FfPNG1_355R) and Taqman-FfPNG1, the probe sequence for Taqman real time PCR, are designated with dotted boxes and an empty arrow. The primer pair consisting of FfPNG1_232F and FfPNG1_551R is indicated with solid boxes, and the probe for Southern blot analysis was amplified using this primer pair for PCR. The three *Hinc*II sites are indicated with dashed boxes, and one of them is located between FfPNG1_232F and FfPNG1_551R. This *Hinc*II site is specific to *F. fujikuroi* and is absent in *F. verticillioides*. However, there was no *Xho*I site within *FfPNG1*. Four *Hinc*II sites in the upstream region of *FfPNG1*, designated with a double-sided arrow, are absent.

Taqman real time PCR

Two fluorescent dyes, Texas Red and matching black hole quencher 2 (BHQ2), were added at the 5′ and 3′ ends for probe design. The probe for Taqman real time PCR was 5′-GAT CGC GAT TCT CAA AAA TTC AGA AAC-3′. A 10-fold dilution series (10^{10} to $10^{-0.1}$ gene copies) of pGEM-T Easy::*FfPNG1* (134-bp) plasmid was used as a template for preparation of a standard curve to indicate the relationship between crossing point (CP) values and *FfPNG1* copy number. Similarly, *F. fujikuroi* genomic DNA was adjusted in a 3-fold dilution series from 83.3 ng to 1 pg to construct the standard curve to indicate the relationship between CP values and the amount of fungal DNA. Real time PCR was performed in a Light Cycler 480 II (Roche, Germany) in triplicate for 40 cycles (15 sec at 95°C and 15 sec at 60°C), starting with initial incubation at 95°C for 4.5 min. The CP values obtained from this reaction were set automatically. For the evaluation of pathogen growth *in planta*, genomic DNA of the 20 rice seedlings infected with *F. fujikuroi* were combined when DNA was prepared. Quantification in ng of pathogen DNA among 250 ng of plant DNA was performed by plotting CP values on the standard curve.

Fungal ramification *in planta*

To observe fungal growth within plants, the crown and stem parts were recovered at 7 dpi. The tissues were stained with 2.5 mg/ml aniline blue staining solution resuspended in lactophenol (equal volumes of glycerol, lactic acid, and phenol). After slight boiling for 1 min, the samples were further stained in the same solution for a day. Excessive aniline blue was washed away several times with lactophenol, and then mycelial growth within the infected tissue was observed

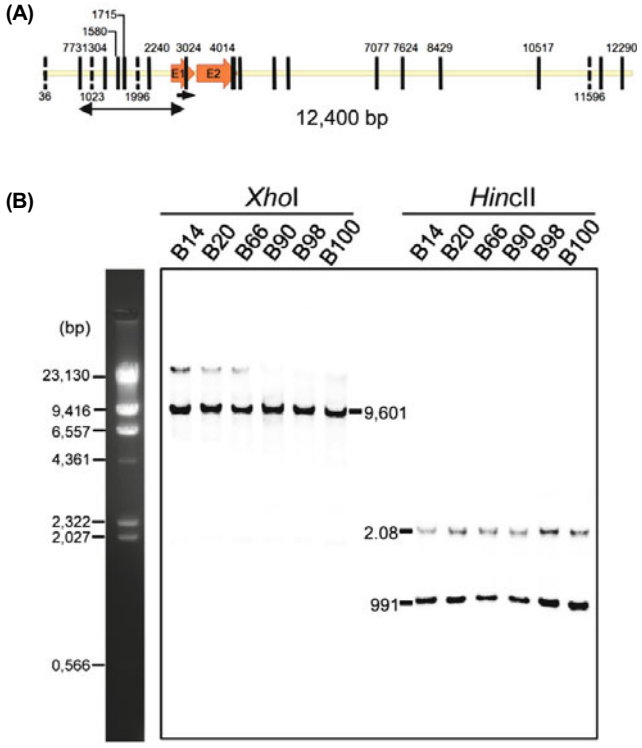

Fig. 2. Restriction map of the genomic sequence around *FfPNG1* and Southern blot analysis. (A) Restriction map of *FfPNG1* and its neighboring 12,400-nucleotide sequence. Solid line and dashed lines represent the *Xho*I and *Hinc*II sites, respectively. The numbers under the dashed line are the relative positions of the *Xho*I sites, and those above the solid lines are *Hinc*II sites. The two exons of *FfPNG1* are marked with empty arrows, E1 and E2. The probe region for Southern blot analysis is designated with a small arrow within the first exon. (B) Southern blot analysis using the cloned amplicon between FfPNG1_232F and FfPNG1_551R as the probe. This probe hybridized with the *FfPNG1*-containing 9,601 bp-long *Xho*I fragment and two *Hinc*II fragments approximately 2.08 kb and 991 bp in length.

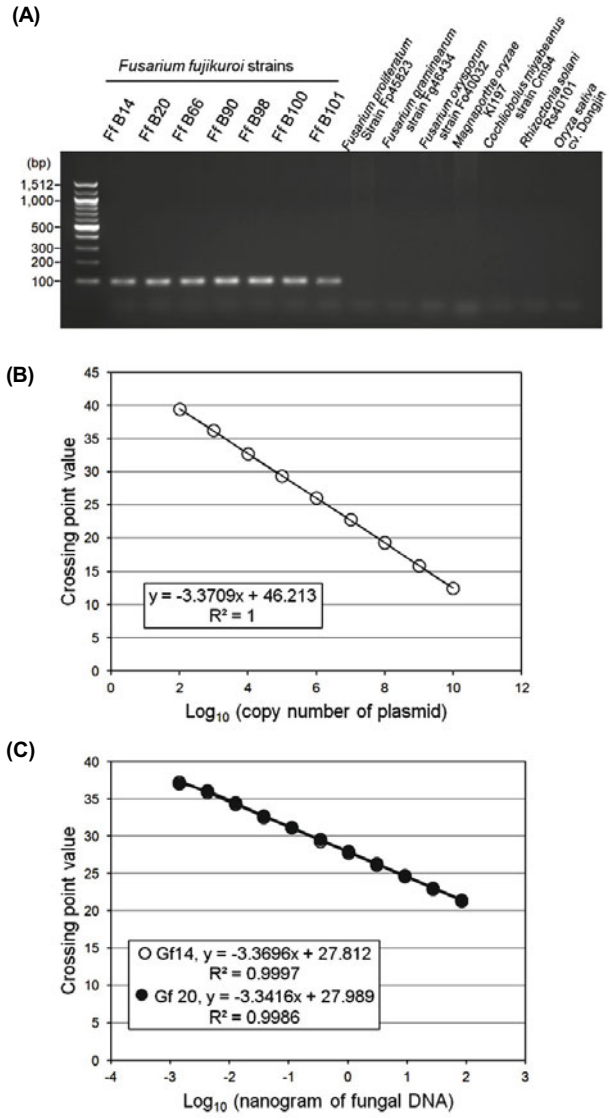

Fig. 3. Specificity of the primer pair for *FfPNG1*and the relationship between crossing point (CP) values, copy number of the *FfPNG1*/genome, and amount of fungal DNA. (A) The primer pair FfPNG1_232F and FfPNG1_355R was used for all PCR reactions. Each reaction contained 250 ng of DNA from the seven strains of *F. fujikuroi*, other fungal pathogens, and host DNA indicated. (B) Linear regression of the CP values and copy numbers of *FfPNG1* or fungal genome. A 10-fold dilution series of *FfPNG1* cloned into the pGEM-T easy vector was used as the template. (C) Linear regression of the CP values and amount of *F. fujikuroi* DNA from the FfB14 and FfB20 strains. A three-fold dilution series of *F. fujikuroi* DNA was used as the template.

under the light microscope (Peng *et al.*, 1986).

Results and Discussion

Mycological and molecular identification of *F. fujikuroi*

We analyzed morphological characteristics and molecular evidence and then comparatively enumerated these results with *F. fujikuroi* to rule out taxonomic confusion (O'Donnell *et al.*, 1998; Leslie and Summerell, 2006). As briefly described in the "Introduction", the *Gibberella fujikuroi* species complex (GFSC) is composed of several mating populations (MPs), and *F. fujikuroi* shares visible characteristics and habitats with other fungal species belonging to the same complex. The colony morphology on PDA was completely identical to descriptions of *F. fujikuroi* (data not shown). Approximately 5 to 6 days after inoculation, colony diameter reached 7 to 8 cm, and its color became whitish pink with active sporulation (data not shown). All seven strains produced micro- and macroconidia, and in particular, they produced microconidia in chains on conidiophores, which is one of the important mycological characteristics of *F. fujikuroi*. Subsequently, the nucleotide sequence of the internal transcribed spacer (ITS) region on ribosomal DNA (rDNA) was identified to support above classical identification. ITS region sequencing has been widely adopted in mycology, and furthermore, this DNA region-based barcode system was recently deemed useful for fungal numerical taxonomy (Schoch *et al.*, 2012). As shown in Supplementary data Fig. S1, the ITS sequence of our strains was absolutely identical to *F. fujikuroi* strain DC-1-77 from *Dracaena cambodiana* (GenBank accession No. KC215122). These results provide evidentiary support that all our strains were *F. fujikuroi*.

FfPNG1 as a target for quantitative real time PCR

In previous studies, the fumonisin biosynthesis gene cluster of *Fusarium verticillioides* was characterized, and its nucleotide sequence is now accessible (Proctor *et al.*, 2003; Kistler *et al.*, 2007). A draft genome of *F. fujikuroi* strain FfB14 was recently opened, and the sequence was deposited in GenBank (Jeong *et al.*, 2013). *FfPNG1*, which is located upstream region of this cluster, spans two exons separated by one short intron sequence (Fig. 1). Despite 38 nucleotide changes within the exon regions, amino acid sequences were relatively well conserved, and only three amino acid changes were observed. The open reading frame (ORF) consisted of 1,353 nucleotides encoding 450 amino acids.

A Southern blot was performed to confirm the copy number(s) of *FfPNG1* within the fungal genome. A 320-bp fragment of this gene, which was amplified using the primer pair FfPNG1_232F and FfPNG1_551R, was used as the probe (Figs. 1 and 2). *Xho*I and *Hin*cII digestion of the amplicon and its nucleotide sequencing indicated the absence of a *Xho*I site and the presence of a single *Hin*cII site (data not shown). In particular, this *Hin*cII site is specific to *FfPNG1* and absent in *FvPNG1*. As shown in Fig. 2B, the probe hybridized with single *Xho*I-digested fragments and double *Hin*cII-digested fragments of all strains analyzed. These data indicated that *FfPNG1* exists as a single copy in the fungal genome. Therefore, *FfPNG1* was a proper target for real time PCR aimed at quantification of fungal DNA because amplicon copy number reflects the number of fungal genomes within the tested DNA from infected tissue. The draft genome sequence around the *FfPNG1* indicated that two *Hin*cII fragments, 784- and 991-bp-long, should hybridize with our 320-bp probe. A 991-bp *Hin*cII fragment was evident in the tested strains; however, the remaining signals were shifted to 2.08 kb. To confirm these results, we amplified a 2,252-bp fragment via PCR and analyzed its nucleotide sequence. Sequencing results revealed the absence of four *Hin*cII sites located upstream of *FfPNG1* (data not shown).

Next, the specificity of our primer pair was evaluated based on the specific amplification pattern that is peculiar to *F. fujikuroi* species and distinguishable from other fungal species sharing the same host and habitat (rice and paddy field). Our primer pair was able to specifically amplify the target genomic region, a 124-bp genomic sequence from *FfPNG1*, from *F. fujikuroi* listed in Table 1, and the primers did not react with DNA from fungal species belonging to the same genus *Fusarium* and those included in Dothidiomycetes and Basidiomycota, which are taxonomically far from *F. fujikuroi* (Fig. 3A). They also did not produce any amplicon from rice DNA. These results guaranteed our system's specificity for *F. fujikuroi* and its ability to discern this pathogen in biological material and in an agricultural environment.

Linear regression construction through Taqman real time PCR

Crossing point (CP) values from real time PCR are the most reliable criteria in determining a target organism's existence and assessing the number of organisms present, as this value negatively reflects the copy number of template DNA with high accuracy. This value is the cycling number at which the fluorescence of a sample rises above background fluorescence. In addition to specific primer pair above, we also adopted a dual-labeled Taqman probe, Taqman_FfPNG1, to further improve the specificity of our reaction (Yin *et al.*, 2001; Matsenko *et al.*, 2008; Gunel *et al.*, 2011). Accumulated results have shown that employment of a Taqman probe in real time PCR is recommended for enhancement of the reliability of a standard curve depicting with the relationships between (CP) values, copy number, and amount (weight) of pathogen genomic DNA (Su'udi *et al.*, 2013a, 2013b). The relationship between the log values of the cloned *FfPNG1* copy number and their CP values was negative straight regression; its slope value was -3.3709, and the Y intercept was 46.213 (Fig. 3B). The regression showed absolute reliability. Subsequently, we estimated CP values of a known amount of fungal DNA from two strains, FfB14 and FfB20. The log values of the DNA and corresponding CP values also showed negative straight regressions; their slope values were -3.3696 and -3.3416 and Y intercepts were 27.812 and 27.989, respectively (Fig. 3C). They were also highly reliable (R^2>0.998). If all the experimental conditions, such as template DNA quantification, are ideal, then the above two standard curves should be identical regardless of strain differences. As expected, the two standard curves almost completely merged with a high degree of reliability. These results indicated

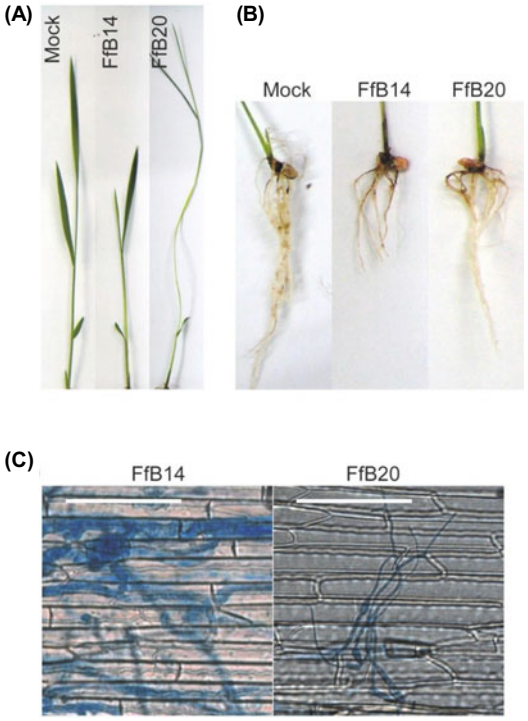

Fig. 4. Progression of Bakanae disease on rice (*Oryza sativa* cv. Dongjin) seedlings infected with FfB14 and FfB20. (A) After placing each fungal inoculum with FfB14 or FfB20 on the vermiculite, the surface was evenly covered with 2-cm-thick vermiculite again, and the seedlings were placed on the upper layer. Oatmeal agar blocks were placed as the mock treatment. FfB14 challenge resulted in poor growth; however, FfB20 infection triggered rice seedling etiolation and foolish growth. (B) Symptoms on the rice roots inoculated with FfB14 or FfB20 harvested at 7 days post inoculation (dpi). Compared to the mock-treated rice roots, FfB14 drastically inhibited root growth, and the roots and crowns were decayed. Although FfB20 also inhibited root growth, this inhibition was relatively minute. (C) Invasive mycelial growth of FfB14 and FfB20 in the rice stem harvested at 7 dpi. FfB14 vigorously infected rice stems, and active invasive mycelial growth was observed. FfB20 also grew in the rice stem; however, the growth was relatively lower. Bars=50 μm.

that our DNA evaluation is preferable and reliable for fungal quantification of *A. brassicicola* biomass. These results further confirmed that our system can be trusted to enumerate fungal biomass within infected rice tissues.

Pathogenicity assay and enumeration of pathogen growth

Etiolation and chlorosis of the aboveground parts of rice seedlings are the typical, representative symptoms of Bakanae disease due to gibberellin production of the pathogen *F. fujikuroi* and frequently result in the toppling of whole plants. However, our pathogenicity analysis in this research revealed that some strains preferred to induce crown and/or root rot and dark brown coloration of the infection sites, usually accompanied by poor root growth. This tendency was much more evident in the rice seedlings directly exposed to the fungal inoculum; all infected rice seedlings were dead within 3 days. To prevent direct contact between rice root and fungal inoculum, we placed fungal inoculum, in the form of a 6-mm diameter mycelial block, in soil and covered them with 2 cm-thick-vermiculite layer. Prior to covering the fungal inoculum, we marked the inoculum's position with a toothpick. The rice seedlings were placed on the vermiculite layer precisely above the location of each inoculum block. Despite preventing the direct contact between inoculum and plants, the growth of almost all rice seedlings challenged by FfB14 was noticeably retarded at 5 dpi compared to mock-treated controls, and more than 90% of them were dead by two weeks after inoculation (Figs. 4A and 5A). FfB14 did not trigger the promotion of growth of the aboveground parts of the seedling and furthermore induced clear stunting at 5 dpi and thereafter. In contrast, infection with strain FfB20 triggered typical hypertrophy and abnormal growth promotion. The rice seedlings did not exhibit observable differences compared to the mock (oatmeal agar block)-treated seedlings up to 2 dpi for all inoculated plants. Approximately 5 dpi, strain FfB20-inoculated rice seedlings showed peculiar faster growth, and the differences between inoculated and mock-treated plants increased and reached 61.3% at 13 dpi. Rather than provoking the accelerated rice growth, the FfB14 strain ferociously infected the rice seed-

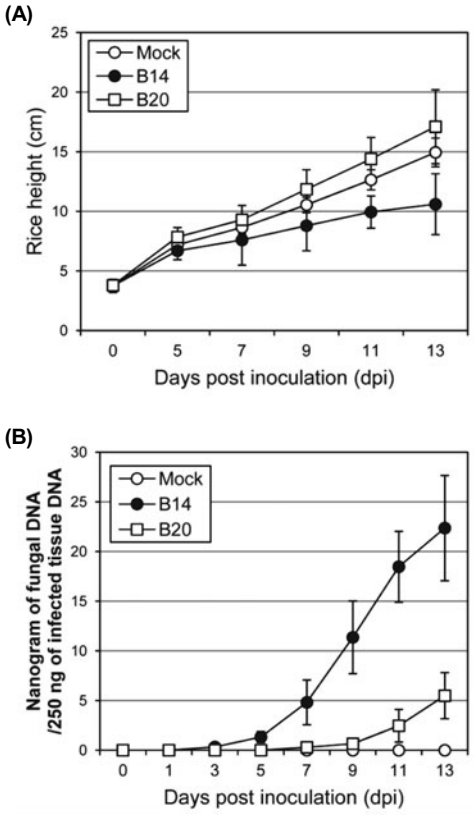

Fig. 5. Alterations in the growth of rice challenged by FfB14 and FfB20 and quantification of pathogen growth *in planta* with Taqman real time PCR. (A) FfB14 infection resulted in the inhibition of rice growth; however, FfB20 promoted seedling growth. After pathogen inoculation, 10 rice plants were harvested at the indicated times, and their growth was estimated. (B) Quantification of pathogen DNA among DNA from plants inoculated with F. fujikuroi at the indicated times. Roots of 5 rice seedlings challenged by FfB14, FfB20, or mock treated were harvested at the indicated time, and DNA was prepared. The ng of fungal DNA in 1 μg of infected root DNA were quantified by plotting CP values on the standard curve. The pathogenicity assay was performed at least 3 times, and representative results are shown.

lings and actively propagated within and around the rice stems and roots. The rice roots infected by FfB14 also exhibited poor growth and turned brown to dark brown within 5 dpi (Fig. 4B). Although FfB20 infection also restricted root growth, the inhibition was very low compared to the FfB14-infected rice. To observe the mycelial propagation of the pathogen, we harvested stems and roots at 7 dpi and analyzed them under the microscope after staining with aniline blue. Massive invasive mycelial growth was evident in the seedling stems and roots, and active conidiogenesis was observed on the surface of stems inoculated with FfB14; however, this fungal infection and sporulation were very rare in the seedlings challenged by FfB20. Although FfB20 infected the rice plants, the infectivity was much lower (Fig. 4C). To estimate the fungal growth in the rice seedlings infected by FfB14 and FfB20, we harvested rice seedlings at the indicated times after inoculation and evaluated the amount of fungal DNA within the infected rice seedlings through Taqman real time PCR (Fig. 5B). Seedling infection by FfB14 was detected at 3 dpi; however, FfB20 growth in the rice was initiated at 7 dpi. FfB14 growth was 16 times higher than that of FfB20 at 7 dpi, and the differences were amplified from 4.5 ng at 7 dpi to 16.9 ng at 13 dpi. Our real time PCR analysis revealed that fungal growth of FfB14 was more than 4 times higher than that of strain FfB20 at 13 dpi. Although the other strains did not exhibit discrete differences, their inoculation usually culminated in etiolation and abnormal growth of the internode. Compared to other strains, strain B90 did not provoke typical the foolish seedling appearance, but this strain did induce seedling withering and finally death.

In summary, we have developed a reliable method to evaluate *F. fujikuroi* mycelial growth by demonstrating that CP values inversely reflect the amount of fungal DNA present. Our method is dependent on a specific primer pair and an additional dual-labeled probe, which guaranteed accurate and objective enumeration. Based on the results presented here and additional observations not described here, we can create a brief protocol for to establish this dual-labeled probe and primer pair; confirmation of target gene copy numbers and nucleotide sequences are indispensible. After evaluation of the specificity of the primer pair, sequencing of the resulting amplicons and Southern analyses employing the amplicons as probes are recommended. Exact quantification of fungal template DNA and infected tissue DNA is a critical step for the construction of a reliable standard curve and estimation of fungal DNA among tissue DNA. Several trials based on absorbance at 260 nm did not give us constitutive values, but measurement of band brightness was reproducible. Although the aim of this investigation was to establish a quantification system, our data here strongly emphasize the value of symptom development results and exact characterization of tested fungal strains.

Acknowledgements

This research was supported by grants from the Research Program for Agricultural Science and Technology Development (Project No. PJ008840) and the Next-Generation BioGreen 21 Program (Plant Molecular Breeding Center No. PJ008021072011), Rural Development Administration, Korea.

References

Ahn, I.P., Kim, S., Kang, S., Suh, S.C., and Lee, Y.H. 2005. Rice defense mechanisms against *Cochliobolus miyabeanus* and *Magnaporthe grisea* are distinct. *Phytopathology* **95**, 1248–1255.

Carter, L.L.A., Leslie, J.F., and Webster, R.K. 2008. Population structure of *Fusarium fujikuroi* from California rice and water grass. *Phytopathology* **98**, 992–998.

Feinberg, A.P. and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6–13.

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.

Harrison, J.G., Barker, H., Lowe, R., and Rees, E.A. 1990. Estimation of amounts of *Phytophthora infestans* mycelium in leaf tissue by enzyme-linked immunosorbent assay. *Plant Pathol.* **39**, 274–277.

Izzati, N.A. and Salleh, B. 2010. Variability of *Fusarium* species associated with Bakanae disease of rice based on their virulence, vegetative and biological compatibilities. *Sydowia* **62**, 89–104.

Jarnagin, J.L. and Harris, S.K. 1985. A modified plastic culture flask for microscopic observation of fungi. *Mycopathologia* **90**, 55–58.

Jeong, H., Lee, S., Choi, G.J., Lee, T., and Yun, S.H. 2013. Draft genome sequence of *Fusarium fujikuroi* B14, the causal agent of the bakanae disease of rice. *Genome Announc.* **1**, e00035-00013.

Kato, A., Miyake, T., Nishigata, K., Tateishi, H., Teraoka, T., and Arie, T. 2012. Use of fluorescent proteins to visualize interactions between the Bakanae disease pathogen *Gibberella fujikuroi* and the biocontrol agent *Talaromyces* sp. KNB-422. *J. Gen. Plant Pathol.* **78**, 54–61.

Kazempour, M.N. and Anvary, M. 2009. Isolation of *Fusarium fujikuroi* antagonistic bacteria and cloning of its phenazine carboxylic acid genes. *African J. Biotechnol.* **8**, 6506–6510.

Kim, S., Park, M., Kim, Y., Lee, S., Choi, B., Lee, S., and Kim, I. 2010. Degradation of prochloraz by rice bakanae disease pathogen *Fusarium fujikuroi* with differing sensitivity: a Possible explanation for resistance mechanism. *J. Korean Soc. Appl. Biol. Chem.* **53**, 433–439.

Kistler, C., Xu, J.R., Trail, F., Kang, S., Shim, W.B., and Woloshuk, C. 2007. *Fusarium* comparative project. http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html

Kulik, T. 2011. Development of TaqMan assays for 3ADON, 15ADON and NIV *Fusarium* genotypes based on *Tri12* gene. *Cer. Res. Comm.* **39**, 200–214.

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

Lievens, B., Brouwer, M., Vanachter, A.C., Cammue, B.P., and Thomma, B.P. 2006. Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. *Plant Sci.* **171**, 155–165.

Ma, L.Y., Ji, Z.J., Bao, J.S., Zhu, X.D., Li, X.M., Zhuang, J.Y., Yang, C.D., and Xia, Y. 2008. Responses of rice genotypes carrying different dwarf genes to *Fusarium moniliforme* and gibberellic acid. *Plant Prod. Sci.* **11**, 134–138.

Martin, F., Delaruelle, C., and Hilbert, J.L. 1990. An improved ergosterol assay to estimate fungal biomass in ectomycorrhizas. *Mycol. Res.* **94**, 1059–1064.

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. ImageJ.
O'Donnell, K., Cigelnik, E., and Nirenberg, H.I. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**, 465–493.
Ou, S.H. 1985. Rice Diseases. Commonwealth Mycological Institute, Kew, England.
Pavon, M.A., Gonzalez, I., Martin, R., and Lacarra, T.G. 2012. ITS-based detection and quantification of *Alternaria* spp. in raw and processed vegetables by real-time quantitative PCR. *Food Microbiol.* **32**, 165–171.
Peng, Y.L., Shishiyama, J., and Yamamoto, M. 1986. A whole-leaf staining and clearing procedure for analyzing cytological aspects of interaction between rice plant and rice blast fungus. *Ann. Phytopathol. Soc. Jpn.* **52**, 801–808.
Prà, M.D., Tonti, S., Pancaldi, D., Nipoti, P., and Alberti, I. 2010. First report of *Fusarium andiyazi* associated with rice Bakanae in Italy. *Plant Disease* **94**, 1070–1070.
Proctor, R.H., Brown, D.W., Plattner, R.D., and Desjardins, A.E. 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genet. Biol.* **38**, 237–249.
Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., and Consortium, F.B. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci. USA* **17**, 6241–6246.
Selma, M.V., Martínez-Culebras, P.V., and Aznar, R. 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. *Int. J. Food Microbiol.* **122**, 126–134.
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.
Su'udi, M., Park, J.M., Kang, W.R., Park, S.R., Hwang, D.J., and Ahn, I.P. 2012. Quantification of rice brown leaf spot through Taqman real-time PCR specific to the unigene encoding *Cochliobolus miyabeanus* SCYTALONE DEHYDRATASE1 involved in fungal melanin biosynthesis. *J. Microbiol.* **50**, 947–954.
Su'udi, M., Park, J.M., Kang, W.R., Hwang, D.J., Kim, S., and Ahn, I.P. 2013a. Quantification of rice sheath blight progression caused by *Rhizoctonia solani*. *J. Microbiol.* **51**, 380–388.
Su'udi, M., Park, J.M., Park, S.R., Hwang, D.J., Bae, S.C., Kim, S., and Ahn, I.P. 2013b. Quantification of *Alternaria brassicicola* infection in the *Arabidopsis thaliana* and *Brassica rapa* subsp. *pekinensis*. *Microbiology (Reading, England)* **159**, 1946–1955.
Watanabe, S., Kumakura, K., Izawa, N., Nagayama, K., Mitachi, T., Kanamori, M., Teraoka, T., and Arie, T. 2007. Mode of action of *Trichoderma asperellum* SKT-1, a biocontrol agent against *Gibberella fujikuroi*. *J. Pesticide Sci.* **32**, 222–228.
White, T.J., Bruns, T., Lee, S., and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds.), PCR Protocols: A Guide to Methods and Applications, pp. 315–322. Academic Press, Inc., New York, USA.
Wiemann, P., Willmann, A., Straeten, M., Kleigrewe, K., Beyer, M., Humpf, H.U., and Tudzynski, B. 2009. Biosynthesis of the red pigment bikaverin in *Fusarium fujikuroi*: genes, their function and regulation. *Mol. Microbiol.* **72**, 931–946.
Wulff, E.G., Sørensen, J.L., Lübeck, M., Nielsen, K.F., Thrane, U., and Torp, J. 2010. *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. *Environ. Microbiol.* **12**, 649–657.
Yang, Y., Kim, Y., Lee, S., Lee, S., An, G., and Kim, I. 2012. Involvement of an efflux transporter in prochloraz resistance of *Fusarium fujikuroi* CF245 causing rice bakanae disease. *J. Korean Soc. Appl. Biol. Chem.* **55**, 571–574.
Yin, J.L., Shackel, N.A., Zekry, A., McGuinness, P.H., Richards, C., Van Der Putten, K., McCaughan, G.W., Eris, J.M., and Bishop, G.A. 2001. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. *Immunol. Cell Biol.* **79**, 213–221.
Zhao, S., Clarke, B.B., Shen, Q.R., Zhang, L.S., and Zhang, N. 2012. Development and application of a TaqMan real-time PCR assay for rapid detection of *Magnaporthe poae*. *Mycologia* **104**, 1250–1259.