The Journal of Microbiology (2012) Vol. 50, No. 6, pp. 947–954 DOI 10.1007/s12275-012-2538-y
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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

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(Received October 5, 2012 / Accepted October 25, 2012)

Rice brown leaf spot is a major disease in the rice paddy field. The causal agent *Cochliobolus miyabeanus* is an ascomycete fungus and a representative necrotrophic pathogen in the investigation of rice-microbe interactions. The aims of this research were to identify a quantitative evaluation method to determine the amount of *C. miyabeanus* proliferation *in planta* and determine the method's sensitivity. Real-time polymerase chain reaction (PCR) was employed in combination with the primer pair and Taqman probe specific to *CmSCD1*, a *C. miyabeanus* unigene encoding *SCYTALONE DEHYDRATASE,* which is involved in fungal melanin biosynthesis. Comparative analysis of the nucleotide sequences of *CmSCD1* from Korean strains with those from the Japanese and Taiwanese strains revealed some sequence differences. Based on the crossing point (CP) values from Taqman real-time PCR containing a series of increasing concentrations of cloned amplicon or fungal genomic DNA, linear regressions with a high level of reliability (R^2>0.997) were constructed. This system was able to estimate fungal genomic DNA at the picogram level. The reliability of this equation was further confirmed using DNA samples from both resistant and susceptible cultivars infected with *C. miyabeanus*. In summary, our quantitative system is a powerful alternative in brown leaf spot forecasting and in the consistent evaluation of disease progression.

Keywords: rice brown leaf spot, Taqman real-time PCR

Introduction

Rice brown leaf spot, which is caused by ascomycete, necrotrophic fungal pathogen *Cochliobolus miyabeanus* (Ito & Kuribayashi), is one of the most devastating diseases in rice cultivation worldwide. The epidemic caused by this disease is more serious during the grain-ripening season and in paddy fields lacking nitrogen fertilizer, such as manure. Recent agricultural regulation restricting the application of nitrogen fertilizer and climate change with accompanying temperature increases had synergized crop losses and poor grain quality due to this disease. Furthermore, this pathogen is seed transmitted, and an outbreak of brown leaf spot during the harvesting season frequently results in the amplification of the primary inoculum (Savary *et al.*, 2000; Maliha *et al.*, 2010).

Breeding and introducing the resistant cultivar should be an ideal solution in rice brown leaf spot management; however, no resistant germplasm is available for japonica rice. In addition, some rice mutants or cultivars that are resistant to rice blast, caused by *Magnaporthe oryzae*, showed hyper susceptibility to this disease (Jung *et al.*, 2005, 2006). Agrochemicals, including ferimzone, tricyclazole, and isoprothiolane, have been recommended as protectants or curative agents for rice brown spot.

Like other fungal disease management strategies, accurate disease forecasting and the timely application of fungicides are indispensable for maintaining brown leaf spot under the economic threshold level. Lack of proper disease forecasting frequently results in unplanned fungicide application and culminates in time-consuming, economically wasteful, and environmentally unfriendly disease management. Until now, rice brown spot forecasting was dependent on the 0 to 9 scale-based naked eye observation designed by the International Rice Research Institute (1988). Although this method has been widely used for brown leaf spot forecasting in the field, the estimation might be inconsistent and requires a high level of experience. One of the serious problems of disease forecasting by depending on naked eye observation is its latency, as it takes 3 to 4 days from artificial inoculation to symptom development specific to *C. miyabeanus* visible to the naked eye. In the field, the interval from infection by primary inoculum to observable pathogen species-specific disease development is much longer than the above period. During this latent period, reproduction of the fungal inoculum and its spread make disease management more difficult. The efficiency of disease control by curative agents is highly affected by the treatment time; the treatment is more effective the sooner is its applied after pathogen infection. A quantitative and reliable detection method for *C. miyabeanus* is indispensable for successful rice brown spot management and its consistent evaluation.

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Several methods are available for the quantitative evaluation of fungal pathogens *in planta,* and most of them employ crossing value-based fungal DNA quantification through real-time polymerase chain reaction (PCR) to overcome the amplicon saturation problem of normal PCR. During the last decade, SYBR Green or Taqman probe-dependent amplification quantification has been developed for *Fusarium oxysporum* and *F. culmorum*, the causative agents of host wilt and kernel infection (Jimenez-Fernandez *et al*., 2011; Kim and Yun, 2011; Hudcovicova *et al*., 2012), soil-borne *Verticillium dahliae*, which also causes host wilt (Bilodeau *et al*., 2012), *Rhizoctonia solani*, which infects bean seedlings (Friberg *et al*., 2009), and the rice blast pathogen *Magnaporthe oryzae* (Qi and Yang, 2002). These convincing methods have provided specific, quantitative measurement on the crossing point value of the indwelling pathogens within the infected host tissues and agricultural environments, including soils and water. Several approaches have employed primer pairs based on nucleotide sequences of genes present as multiple copies or as a member of families within the genome, such as ribosomal DNA (rDNA) (Borneman and Hartin, 2000; Martin and Rygiewicz, 2005). If the copy number of the target gene within the genome is not fixed or if the target region sequence is not specific, the reliability of the evaluation methods may be hampered. Despite the assay's improvements in other diseases, no method for the quantitative assessment of *C. miyabeanus* has been developed.

In this approach, we provide a Taqman probe and a primer pair-based method for the quantitative evaluation of *C. miyabeanus*. The Taqman probe and primer pair are specific to the *C. miyabeanus scytalone dehydratase1* (*CmSCD1*) gene, which is indispensable for fungal melanin biosynthesis and exists as a single copy within the fungal genome (http://genome.jgi.doe.gov/Cocmi1/Cocmi1.home.html) (Kihara *et al*., 2004). These methods should be a powerful alternative for the consistent evaluation of rice brown leaf spot.

Materials and Methods

Fungal strains and growth conditions

All fungal strains (Fig. 2A) were obtained from the National Institute of Agricultural Science and Technology in the Rural Development Administration (Korea). To prepare the fungal inoculum, *Cochliobolus miyabeanus* was grown on sucrose proline agar (SPA) (Ahn *et al*., 2005). *M. oryzae* and Alternaria brassicicola were grown on oatmeal agar (50 g oatmeal flakes and 20 g agar per 1 L distilled water), and *Botrytis cinerea*, *Rhizoctonia solani*, and *Fusarium* were grown on potato dextrose agar (PDA, USA). All strains were grown at 22°C under continuous florescent light. Subsequently, each inoculum was transferred into complete liquid media (Turgeon *et al*., 1985) and grown for 3 days at 25°C at 150 rpm in the dark. The mycelial mass was then filtered with miracloth (Calbiochem, Germany), frozen in liquid nitrogen and stored at -80°C.

Pathogenicity assay

The rice (*Oryza sativa*) cultivars (cv.) Dongjin and Tetep were grown in a greenhouse as described previously until six to seven leaves were fully expanded, and *C. miyabeanus* strain Cm94 was then inoculated (Ahn *et al*., 2005). Briefly, conidia of *C. miyabeanus* strain Cm94 were collected from cultures grown in SPA, and the concentration was adjusted into 1×10^5 conidia/ml using distilled water. Rice plants were sprayed with conidial suspension supplemented with Tween 20 to a final concentration of 250 μg/ml until all the leaves were covered with fine droplets. The inoculated plants were kept at 25°C in the dark at almost absolute relative humidity for 20 h and then transferred to a greenhouse under natural light conditions (70–75% relative humidity, 26°C). Pictures were taken at 4 days post inoculation (dpi). Each treatment was composed of 70 plants, and six or seven leaves from 10 plants were harvested for DNA preparation. Experiments were repeated three times, and almost identical trends were observed.

DNA preparation and PCR amplification

Genomic DNA from fungi and leaves was prepared using the hexadecyl trimethyl ammonium bromide (CTAB) method (Stewart and Via, 1993). In addition, the grain DNA was prepared from hundreds of symptomless rice grains collected from the panicles of 10 plants. Each sample was harvested from two paddy fields in Suwon and Gunwi, Korea, in September 2011. Rice brown leaf spot was heavily manifested in the Gunwi field, but the Suwon field was free from the disease. Briefly, approximately 50 mg of sample was placed into a 2 ml Eppendorf tube containing 0.8 ml CTAB buffer [100 mM Tris-HCl, 10 mM EDTA, 0.7 M NaCl, 1% (w/v) CTAB, 1% (v/v) β-mercaptoethanol] and pulverized using a TissueLyser (QIAGEN, Germany) at 30 Hz for 2 min. The samples were treated with chloroform and incubated at 65°C for 30 min. After centrifugation, the supernatant was recovered and mixed with the same volume of isopropanol, and the DNA was then precipitated. The DNA concentration was determined based on the absorbance at 260 nm using a spectrophotometer and adjusted to 50 ng/μl (Agilent Technologies, USA). In addition, the quantification was further confirmed through the comparison of band brightness of the sample DNA with that of the largest 23.1 kb fragment of lambda DNA digested with *Hin*dIII (Invitrogen, USA) using the Java image processing software ImageJ after gel electrophoresis and post staining with ethidium bromide (http://rsbweb.nih.gov/ij/download.html). In total, 50 ng of genomic DNA isolated from several fungal races and species was used as the template in polymerase chain reaction (PCR) using primers CmSCD1_44F (5′-CATGTGTGCAGTAAA GTGACTC-3′), located in the intron region, and CmSCD1_302R (5′-GTCTTGAGGAGGGGGTT-3′), located in the second exon region, for Taqman real-time PCR. The amplicon was 258 bp. The CmSCD1_-33F (5′-ACCAAGTAATTGA ACCAAGGTC-3′) and CmSCD1_742R (5′-GCATTTCAT CATGATTCGCATT-3′) primer pair was used to amplify a 775-bp region of genomic DNA harboring the full-length *CmSCD1* gene using Speed *Pfu* polymerase (Nanohelix, Korea) to compare the nucleotide sequence of Korean *CmSCD1* with those from Japan and Taiwan. In addition, 335-bp amplicons were amplified using the CmSCD1_-33F and CmSCD1_302R primers and digested with *Bme* 1580I (New England Biolabs)

to confirm the limited presence of the additional nucleotide insertion of CACGG in the intron region. The recognition site of this enzyme is 5′-G(K; T or G)GC(M; A or C)C-3′, and the cutting site is between the fifth M and last C. The PCR program was denaturation at 95°C for 5 min, 30 cycles at 95°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 7 min. The PCR products were analyzed in a 2% agarose gel and visualized on a UV transilluminator.

Synthesis of Japanese *CmSCD1* and amplicon digestion with *Bme* 1580I

To conform the Korean *C. miyabeanus* strain-specific GCA/CGG presence within the 335-bp amplicons (derived from the primer pair CmSCD1_-33F and CmSCD1_302R), we synthesized the Japanese 330-bp nucleotide sequence region and cloned it into the pGEM-T vector (T-D9-F6/69_330) (Bioneer, Korea). After PCR, the amplicons from the Korean strains and those from T-D9-F6/69_330 were digested with *Bme* 1580I and electrophoresed in a 2% agarose gel.

Plasmid construction

Amplicons (775 and 258 bp, respectively) from each PCR assay using the above two primer pairs and genomic DNA from *C. miyabeanus* strains Cm1, Cm36, Cm50, Cm85, and Cm94 were purified using a PCR purification kit (Solgent, Korea) and A tailed using normal *Taq* polymerase. The A-tailed amplicons were ligated into the pGEM®-T Easy vector

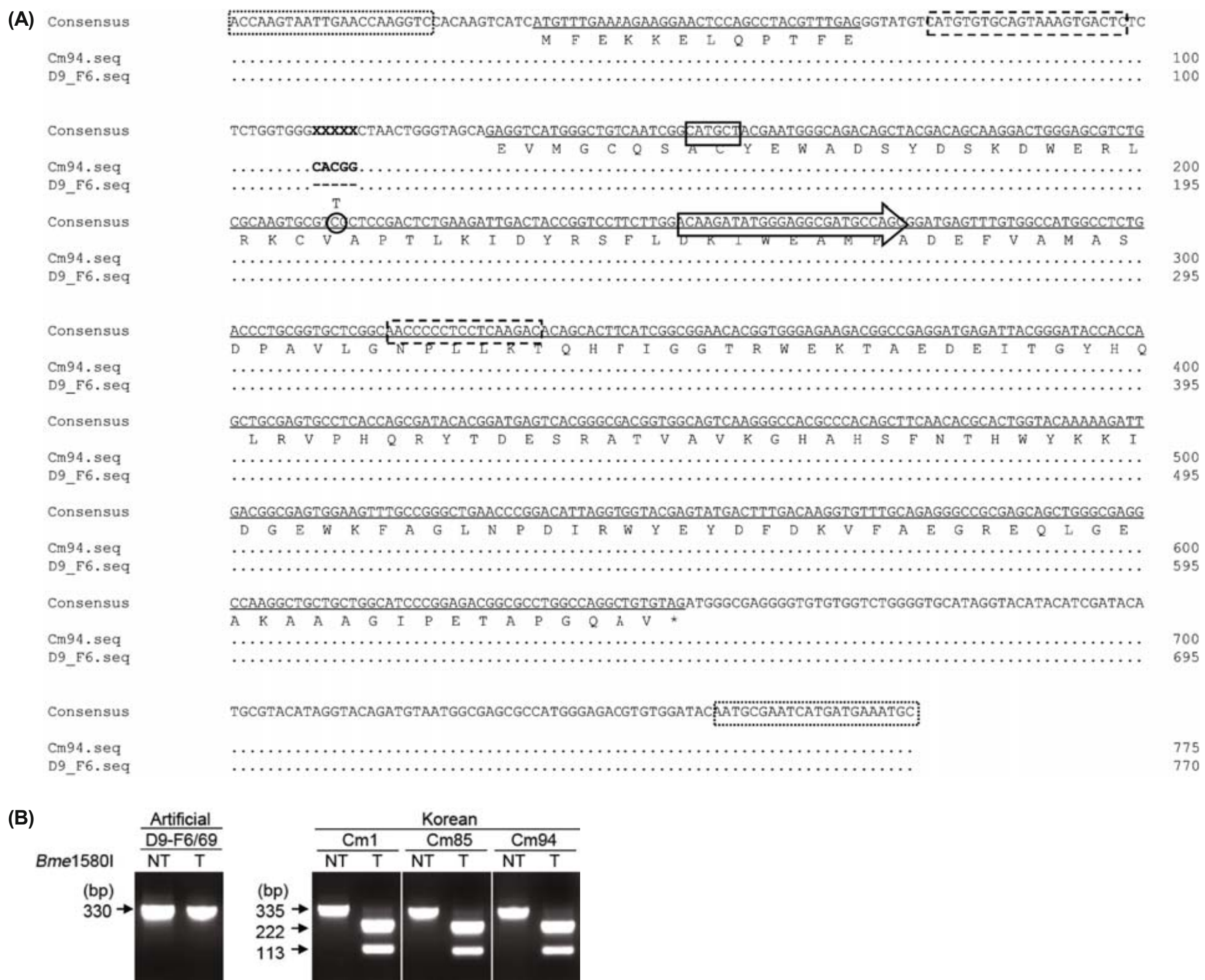

Fig. 1. Comparison of the genomic DNA sequence of *SCYTALONE DEHYDRATASE1*. The nucleotide sequences of *CmSCD1* from Korean *C.s miyabeanus* strains, Japanese *C. miyabeanus* strain D9-F6/69 (GenBank accession no. AB100172) (Kihara *et al.*, 2004) and Taiwanese strain ATCC 44560 are presented. (A) Two exon regions are underlined, and the translated amino acid sequences are shown. Except for the 5-nucleotide (CACGG) insertion in the intron region (bold), both nucleotide sequences are identical. Primer pairs (CmSCD1_44F and CmSCD1_302R) and probe sequence for Taqman real-time PCR are designated with dashed boxes and an empty arrow. The primer pair CmSCD_-33F and CmSCD1_742R is indicated with dotted boxes. The circle indicates the different nucleotide in *CmSCD1* from Cm94 (cytosine) and ATCC44560 from Taiwan (thymine). The single *Sph*I restriction site is indicated with solid box. (B) Amplicon digestion patterns using *Bme* 1580I. Artificial *CmSCD1* was synthesized based on the D9-F6/69 sequence and lacks CACGG. The restriction pattern of amplicons using the primer pair CmSCD_-33F and CmSCD1_742R directly indicates the limited distribution of CACGG in the 5 tested Korean *C. miyabeanus* genomes. NT designates the amplicon not treated with *Bme* 1580I, and T indicates the amplicon treated with *Bme* 1580I.

(Promega, USA) at 4°C overnight. The ligation products were introduced into *E. coli* DH5 alpha competent cells through heat shock transformation. Several colonies containing insert were grown in LB broth containing ampicillin at 37°C overnight, and the plasmid was isolated using a plasmid miniprep kit (Solgent). Clones containing the target insert were confirmed through *Eco*RI digestion and DNA sequencing and named T-CmSCD1_775 and T-CmSCD1_258. The molecular weight of T-CmSCD1_258 was calculated based on the number of bases of the pGEM-T vector and 258-bp inserts (Daniell *et al.*, 2012).

Southern blot analysis

Fungal genomic DNA (5 mg) was digested with restriction enzymes (*Eco*RI or *Sph*I) and separated by 0.7% agarose gel electrophoresis overnight. The gel was treated with depurination solution (0.2 N HCl), denaturation solution (1.5 M NaCl, 0.5 M NaOH), and neutralization solution (1.5 M NaCl, 1 M Tris-HCl, pH 7.5). Subsequently, the gel was blotted onto a nylon membrane and hybridized with a *CmSCD1* probe labeled with [a-^{32}P] dCTP through random priming (Feinberg and Vogelstein, 1983) using the Rediprime II DNA Labeling system kit (GE Healthcare, USA). The DNA probe was prepared from the T-CmSCD1_258 plasmid via *Eco*RI digestion and gel extraction (Solgent). After hybridization, the membranes were washed with 2× SSPE and 0.1% SDS, exposed to BAS film (Fujifilm, Japan) and developed using the Personal Molecular Imager (PMI) system (BioRad, USA).

Taqman real-time PCR

The probe (5′-ACAAGATATGGAGGCGATGCCAG-3′) harbored a reporter fluorescent dye 6-carboxyfluorescein (FAM) at the 5′ end and a black hole quencher 1 (BHQ1) at the 3′ end. Based on the molecular weight of T-CmSCD1_258, the copy number was adjusted in a 10-fold dilution series ranging from 1×10^{10} to 1×10^{1} copies. The dilution series was used as the template to evaluate the relation between the copy number of *CmSCD1* and CP values. In addition, fungal genomic DNA extracted from *C. miyabeanus* (Cm1, Cm85, and Cm94) was used as the template and adjusted in a 3-fold dilution series ranging from 83.3 to 1 pg to evaluate the CP values. Real-time PCR was performed using the Light Cycler 480 II (Roche, Germany) in triplicate for 40 cycles (15 sec at 95°C and 15 sec at 60°C) after an initial incubation at 95°C for 4.5 min. For the evaluation of the fungal number *in planta* through Taqman real-time PCR, 250 ng of DNA from the infected tissue was used as the template under the same conditions. The CP values obtained from this reaction were set automatically by the system.

Results and Discussion

Nucleotide sequence analyses of *CmSCD1*

Using the primer pairs CmSCD1_-33F and CmSCD1_742R and CmSCD1_44F and CmSCD1_302R, 755- and 258-bp amplicons were amplified from the genomic DNA of *C. miyabeanus* strains Cm1, Cm36, Cm50, Cm85, and Cm94 and cloned into the pGEM-T easy vector (Fig. 1A). Sequencing results indicated the common additional insertion of CACGG in the Korean strains in the intron region of *CmSCD1*, but these 5 nucleotides were absent in the Japanese strain D9-F6/69 (Kihara *et al.*, 2004). Together with the upstream G, this sequence was recognized by the restriction enzyme *Bme* 1580I (Fig. 1A, the different intron region is designated in bold). Digestion of the 330-bp amplicon from the synthesized D9-F6/69 DNA and the 335-bp amplicons from the Cm1, Cm85, and Cm94 DNA with *Bme* 1580I also supported the Korean strain-specific presence of the CACGG insertion in the intron region (Fig. 1B). Although the genomic DNA of Japanese D9-F6/69 is most ideal for these analyses, the international distribution of *C. miyabeanus* is impossible due to the Biological Weapon Convention (BWC), and synthe-

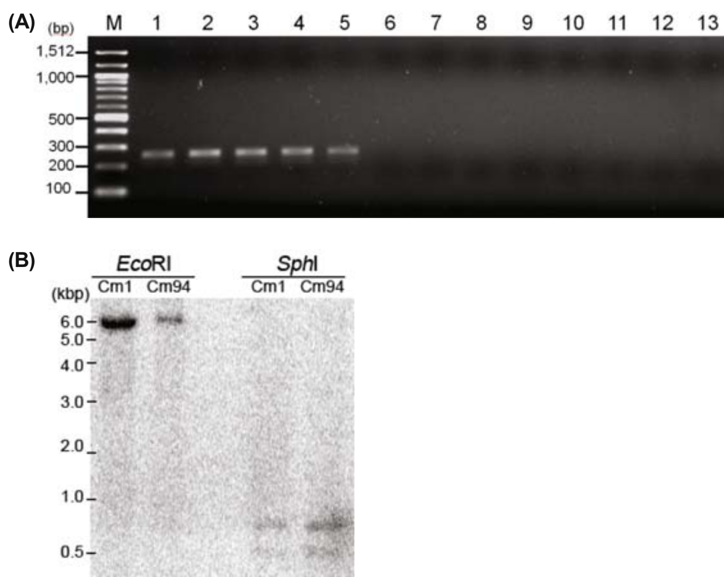

Fig. 2. Specific PCR amplification of the unigene *CmSCD1*. (A) In all reactions, the CmSCD1_44F and CmSCD1_302R primer pair was employed. Each reaction contained genomic DNA (250 ng) from the following fungal mycelia or rice leaf tissue as the template, except M. M, 100-bp DNA ladder; 1, *C. miyabeanus* Cm1; 2, *C. miyabeanus* Cm36; 3, *C. miyabeanus* Cm50; 4, *C. miyabeanus* Cm85; 5, *C. miyabeanus* Cm94; 6, *Magnaporthe oryzae* KI197; 7, *M. oryzae* KJ401; 8, *Botrytis cinerea* KACC40574; 9, *Fusarium moniliforme* KACC41032; 10, *Fusarium graminearum* KACC46434; 11, *Rhizoctonia solani* KACC40101; 12, *Alternaria brassicicola* KACC42464; and 13, *Oryza sativa* cv. Dongjin. (B) Southern blot analysis of *CmSCD1*. After digestion of Cm1 and Cm94 genomic DNA with *Eco*RI and *Sph*I, the membrane was hybridized with the 258-bp amplicon as the probe. There are no *Eco*RI sites and a single *Sph*I site within the probe region of *CmSCD1*.

sized the 330-bp nucleotide as the alternative control. Our results suggest that the CACGG insertion-based *Bme* 1580I restriction polymorphism could be a molecular marker to discriminate Korean and Japanese *C. miyabeanus* with cleaved amplified polymorphic sequences (CAPS) markers. CAPS markers have been widely used to discriminate the genotype of crossed lines harboring point mutation(s) (Xie *et al*., 1998; Kim *et al*., 2009). This result implies the possibility of this sequence-dependent restriction polymorphism as a molecular marker for the discrimination of Korean and Japanese *C. miyabeanus* populations; however, obtaining sequence information from a statistically sufficient number of strains should be a prerequisite.

In the agricultural field, fungal nucleotide variation occasionally provokes a serious problem for disease managment. During the last two decades, strobilurin (Q_oI) has been widely applied to control several pathogens infecting aerial part of the hosts. Analyses of Q_oI–resistant *Magnaporthe grisea*, which is virulent in perennial ryegrass turf, revealed that nucleotide variation(s) occurring in the fungal mitochondrial *cytochrome b* gene should confer a blast pathogen with chemical resistance (Kim *et al*., 2003).

Recently, the genome of Taiwanese *C. miyabeanus* (ATCC 44560) was made publically available (http://genome.jgi.doe.gov/Cocmi1/Cocmi1.home.html), and the *CmSCD1* sequence of this strain was compared with the sequence from the Korean strain. With the exception of one nucleotide difference in the second exon, the entire nucleotide sequence was identical, and this difference did not affect the amino acid sequence or protein structure of CmSCD1 because CTC and CTG commonly encode the same amino acid, valine.

Specificity of the primer set

The primer pair CmSCD1_44F and CmSCD1_302R was designed based on the nucleotide sequence of *CmSCD1*. Previously, the specificity of this primer pair was evaluated based on the presence or absence of amplification of a 258-bp fragment. The location of the primer set and probe are illustrated in Fig. 1A. Polymerase chain reactions employing this primer pair successfully amplified the target sequence from all strains of *C. miyabeanus* (Fig. 2A). In contrast, no PCR product was amplified from the genomes of other fungal species or the rice (*Oryza sativa*) cultivar Dongjin. These results indicate that this primer pair is highly specific for *CmSCD1* within the *C. miyabeanus* genome. In addition, a Southern blot was performed to confirm the copy number of *CmSCD1* using the 258-bp amplicon as the hybridization probe, and the result verified the presence of *CmSCD1* in the genome of *C. miyabeanus* as a single copy (Fig. 2B). Because of the absence of an *Eco*RI site and the presence of a single *Sph*I site within the probe region (Fig. 1A), single and signals in response to *Eco*RI and *Sph*I simultaneously suggest that *CmSCD1* is a unigene. Therefore, *CmSCD1* is a good candidate for the construction of a primer pair and probe in the quantitative analyses of fungal DNA.

Linear regression of crossing point value for *CmSCD1*

Taqman real-time PCR using the T-CmSCD1_258 clone as a template was performed to generate a standard curve to estimate the copy number of *CmSCD1*. Figure 3A illustrates the mean crossing point (CP) value obtained from PCR versus $\log_{10}$ copy number. The copy number of *CmSCD1* was adjusted in a 10-fold dilution series that ranged from 1×10^{10} to 1×10^{1} copies, and the reliability of the linear regression was high (R^2>0.999). The limit of this regression was between 10^2 and 10^1. The regression slope and intercept of the Y axis were -3.4112 and 41.295, respectively. The remaining three regressions address the relationship between the amount of genomic DNA from *C. miyabeanus* strains and CP values. The genomic DNA was adjusted in a 3-fold dilution series ranging from 83.3 ng to 1 pg. The R^2 values of Cm1, Cm85, and Cm94 regressions were more than 0.997; therefore, the above regressions were all convincing. The slope values and intercepts of the Y axes were -3.3845, -3.4868, and -3.4450 and 27.732, 27.375, and 27.831, respectively (Fig. 3B). The linear regression CP values from the three independent serial dilutions of genomic DNA from Cm1, Cm85, and Cm94 were almost completely identical. These results imply that the relationship between the CP values and the concentration of fungal DNA presented in this research is reliable. In addition, the comparison of the linear regression and CP values strongly indicates that this system is able to detect *C. miyabeanus* genomic DNA at the picogram level. Until now,

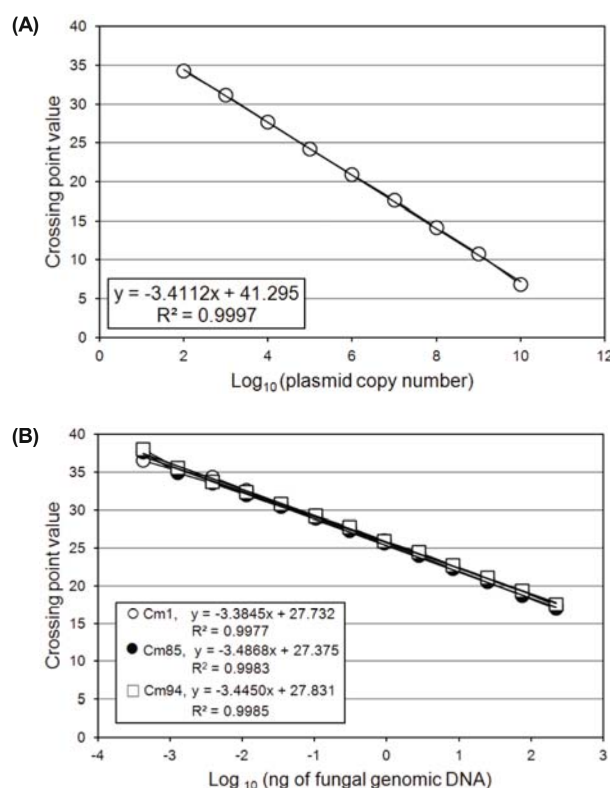

Fig. 3. Relation between crossing point (CP) values, copy numbers, and amount of genomic DNA. (A) Linear regression of the CP values and copy numbers. A ten-fold dilution series of the *CmSCD1* clone was used as the template. (B) Linear regressions of the CP values and the amount of fungal genomic DNA from the *C. miyabeanus* strains Cm1, Cm85, and Cm94. Each experiment was performed in three replicates, and almost identical results were obtained from the three independent experiments.

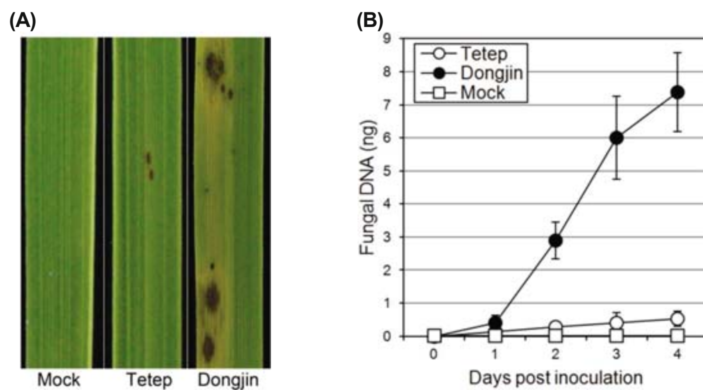

Fig. 4. Quantification of brown leaf spot progression in the rice cultivars Tetep and Dongjin. Tetep is incompatible with *C. miyabeanus* strain Cm94, while Dongjin is compatible. Dongjin was also mock treated (250 μg/ml Tween 20). (A) Six-week-old rice plants were inoculated with Cm94, and pictures were taken one week after infection. (B) Chronological quantification of fungal DNA within infected plant DNA. Each data point indicates the mean and standard deviation from 10 DNA samples from the infected leaves, and all DNA was analyzed with three replicates. Each treatment was composed of 50 plants, and fully expanded 6th or 7th leaves were harvested for DNA preparation.

several investigations have addressed criteria to determine whether chemical application is necessary, and they indicate that fungal mass corresponding to genomic DNA at the picogram level was able to act as a primary inoculum (Qi and Yang, 2002; Bilodeau *et al.*, 2012). These results support that our evaluation system could be applied to disease forecasting, which is indispensable for successful rice brown leaf spot management. Although we tried to use SYBR green-based real time PCR to determine the relationships among CP values, copy numbers, and amount of genomic DNA several times, the R^2 values of all regressions obtained were less than 0.99 (data not shown).

Chronological evaluation of rice brown leaf spot progression

To evaluate rice brown leaf spot progression in compatible and incompatible interactions, we have employed *C. miyabeanus* strain Cm94 using the incompatible, resistant cultivar Tetep and the compatible, susceptible cv. Dongjin. In addition, we estimated the amount of *C. miyabeanus* DNA in the DNA of whole rice grains harvested from the brown leaf spot-infected field and symptomless rice grains from the disease-free field.

On the Dongjin leaves inoculated with Cm94, visible host cell death was observed approximately 16 h post inoculation (hpi). At 36–40 hpi, microscopic lesions began to form and became enlarged as time progressed. At 72–94 hpi, the average lesion area was approximately 0.6×3.4 mm (width by length), and chlorosis began to appear around the margin of lesions (Fig. 4A). Although rapid cell death was also observed within 16 hpi on the Tetep cultivar inoculated with the same strain, neither significant lesion enlargement nor chlorosis ensued thereafter. The inoculated 7th leaves were harvested from the 10 plants daily, and genomic DNA was prepared from each sample. Compared to the progression pattern in rice blast, one of the most representative biotrophic rice diseases caused by *Magnaporthe oryzae*, the first visible symptom appearance was faster, approximately 16 to 36 h, as described previously. Minute cell death preceded lesion enlargement in the compatible interaction (Ahn *et al.*, 2005). Water-soaked tissue alteration, which is abundant in the beginning period of blast infection, was also absent in brown leaf spot. However, yellowing and browning around the lesion margins were serious approximately 73 hpi and thereafter. All of these disease progressions are typical characteristics of necrotrophic diseases.

For application of the above method, genomic DNA extracted from the leaves infected with Cm94 was used as a template in real-time PCR (Fig. 4B). Quantification was performed by interpolating the CP values from the Taqman real-time PCR into the trendline equation standard (Fig. 3B). In contrast to rice blast, there were observable differences between compatible and incompatible interactions in Dongjin and Tetep, respectively. Fungal growth at 1 dpi reached 395 and 133 pg among the 250 ng of Dongjin and Tetep DNA inoculated with Cm94, respectively. Fungal DNA reached 2,892 pg at 2 dpi and sharply increased to 5,996 and 7,377 pg at 3 and 4 dpi. In the incompatible interaction, fungal DNA was 279 pg and slightly increased to 411 and 525 pg at the same time point (Fig. 4B). In rice blast, there was no difference with respect to fungal numbers in the compatible and incompatible interactions at 1 dpi (submitted for publication). Furthermore, disease progression from 12 h post inoculation (hpi) to 60 hpi was slower in rice blast than in rice brown leaf spot. A recent hypothesis and subsequent investigations suggest that the plant's defense system is composed of an early defense layer of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and later effector-triggered immunity (ETI) (Jones and Dangl, 2006). In the compatible interaction between *M. oryzae* and the host, the decreased 16 hpi to 48 hpi fungal quantification supports the PTI-dependent activation of host defense. In addition, the necrotrophic pathogen's faster ramification *in planta* compared with the hemibiotrophic *M. oryzae* suggests the rapid nullification of PTI by the host-infecting interactions produced by *C. miyabeanus*. Similar suggestions regarding distinctive biotrophic and necrotrophic pathogen progression have been described (Mengiste, 2012).

Due to its highest reliability, the linear regression from Cm94 was employed in the estimation of the primary inoculum within the grain. Quantitative PCR using grain DNA from Gunwi revealed that the amount of *C. miyabeanus* DNA in the 250 ng of total DNA was 2.1728 pg, whereas no fungal DNA was detected in the grain DNA from Suwon. Therefore, seed surface sterilization is indispensable for the eradication of the primary inoculum and successful rice brown leaf spot management, although no visible symptom is observed. Seed contamination by and the transmission of *C. miyabeanus* has already been reported, but the quantitative

evaluation of the primary inoculum (Ba and Sangchote, 2006) and the quantitative analysis in this research elucidate the potential of our methods for rice seed health management. Until now, the primary method of seed health management and the identification of concealed pathogens has been several days for the incubation and identification through microscopic analyses. Our methods might be a powerful alternative, saving both time and labor.

Conclusions

Comparative analyses of the *CmSCD1* nucleotide sequence in Korean *Cochliobolus miyabeanus* strains with those from Japan and Taiwan revealed some differences. Based on these differences, we tested a *Bme* 1580I-dependent amplicon restriction polymorphism that can easily discriminate Korean *C. miyabeanus* strains from Japanese ones. This method is similar to the cleaved amplified polymorphic sequence (CAPS) markers frequently used for genotype determination in *Arabidopsis* and crops. More *CmSCD1* sequence results will be necessary for further confirmation.

Our primer pair is highly specific for *CmSCD1*. In addition, the subsequent real-time PCR employing a fluorescence dye-labeled probe was able to quantify the propagation of *C. miyabeanus* in infected leaf tissues. Our unigene-based quantitative analysis method should be a powerful alternative, saving both time and labor with efficient disease forecasting, because the relation between disease control efficiency and the interval from infection to chemical application is inversely proportional. To improve the pathogen detection and quantification system, the following three factors should be carefully considered: 1) rather than the genes existing in the genome as variable or multiple copies, the genes should exist as a single copy or fixed copies; 2) the primer pair and probe should be highly specific for the target gene; and 3) the amplicon must be specific and distinguishable from those generated from the different species or hosts.

In sum, our scheme is a reliable protocol for the quantification of a fungal pathogen in its host *via* Taqman real-time PCR and includes target gene selection, the confirmation of target gene copy number, and standard curve-based fungal mass estimation. The presented quantification method should be a powerful tool for rice brown leaf spot quantification and an exact forecasting prerequisite for efficient disease management.

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, PJ00802107), Rural Development Administration, Korea.

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