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Quantification of Rice Sheath Blight Progression Caused by *Rhizoctonia solani*[§]

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Rhizoctonia solani **has a wide host range, including almost all cultivated crops and its subgroup anastomosis group (AG)-1 IA causes sheath blight in rice. An accurate measurement of pathogen's biomass is a convincing tool for enumeration of this disease. Mycological characteristics and molecular diagnosis simultaneously supported that all six strains in this study were *R. solani* AG-1 IA. Heterokaryons between strains Rs40104, Rs40105, and Rs45811 were stable and viable, whereas Rs40103 and Rs40106 did not form viable fused cells, except for the combination of Rs40106 and Rs40104. A primer pair was highly specific to *RsAROM* gene of *R. solani* strains and the amplified fragment exists as double copies within fungal genome. The relationship between crossing point (CP) values and the amount of fungal DNA was reliable (R^2>0.99). Based on these results, we determined *R. solani*'s proliferation within infected stems through real time PCR using a primer pair and a Taqman probe specific to the *RsAROM* gene. The amount of fungal DNA within the 250 ng of tissue DNA from rice cv. Dongjin infected with Rs40104, Rs40105, and Rs45811 were 7.436, 5.830, and 5.085 ng, respectively. In contrast, the fungal DNAs within the stems inoculated with Rs40103 and Rs40106 were 0.091 and 0.842 ng. The sheath blight symptom progression approximately coincided with the amount of fungal DNA within the symptoms. In summary, our quantitative evaluation method provided reliable and objective results reflecting the amount of fungal biomass within the infected tissues and would be useful for evaluation of resistance germplasm or fungicides and estimation of inoculum potential.**

***Keywords*:** rice sheath blight, Taqman real-time PCR

Introduction

Rhizoctonia solani Kühn is the anamorph of the basidiomycete fungus *Thanatephorus cucumeris* (A. B. Frank) Donk and has a wide host range including almost all cultivated crops and worldwide distribution (Ou, 1985). This fungal species is composed of 13 anastomosis groups (AGs) according to the affinity of hyphal fusion between the isolates or strains (Parmeter, 1970; Sneh *et al.*, 1991; Carling *et al.*, 2002). If the paired isolates form heterokaryons at the mycelial interface, both strains fall into the same AG. AGs differ in their host range and pathogenicity and are further subdivided into somatic or vegetative compatibility groups (SCG or VCG). This classification is dependent on the viability and stability of the heterokaryon between the two strains (Carling, 1996). Sheath blight is one of the most devastating diseases in rice (*Oryza sativa* L.) cultivation and is caused by *R. solani* which belongs to the AG-1 IA. Because there is no available major genes conferring dominant resistance to most of the commercially grown rice cultivars, sheath blight control is particularly dependent on fungicide applications. Although quantitative traits loci (QTLs) of rice contributing to the partial resistance against *R. solani*'s infection have been reported (Loan *et al.*, 2004; Pinson *et al.*, 2005; Liu *et al.*, 2009, 2012; Jia *et al.*, 2012), their introduction into commercial cultivars through traditional breeding is not successful and especially hampered by the lack of resistance-evaluation method of rice capable of providing objective and quantitative values (Fu *et al.*, 2011).

Accurate disease assessment is a critical step in sheath blight forecasting and resistance evaluation. Its importance is emphasized in evaluation of partial, quantitative resistance. Lesion progression from the soil surface and the percentage of symptomatic seedlings or tillers among the tested plants has been widely applied in the evaluation of sheath blight severity and incidence (International Rice Research Institute, 1988). Although these methods are convenient and still useful in some cases, these methods are not able to represent the fungal growth within the symptom, which is one of the most reliable indicators of disease severity. In addition, visual rating is not applicable in chronological estimation or the early disease initiation period. A more serious problem is the target of this evaluation. In fact, visible symptom is a response of hosts induced by pathogen infection and following fungal ramification and pathogen-associated molecular patterns (PAMPs) including repertoire of diverse enzymes and secondary metabolites. The latter frequently triggers symptoms such as halo beyond and around the pathogen-growing area and results in overestimation of disease severity. To solve this problem, a polymerase chain reaction

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(PCR)-based strategy was developed for fungal evaluation within and around host. This technique is theoretically applicable for all fungal species and is relatively simple and sensitive, but it is still qualitative, not quantitative, because of the inconsistent relationship between the amplicon amount and the amount of template DNA. The introduction and application of fluorescence dyes such as SYBR Green has enabled input DNA's evaluation in a real-time manner (Sayler and Yang, 2007; Okubara *et al.*, 2008; Selma *et al.*, 2008). The crossing point (CP) value is believed to be the most reliable standard because it correctly reflects the amount of DNA within the sample. CP value is the cycling number at which the fluorescence of a sample rises above the background fluorescence. The additional introduction of a target region-specific oligo probe, a Taqman probe bearing a fluorescent reporter dye at the 5′-end and a quencher molecule at the 3′-end, has enhanced the specificity and accuracy of real time PCR (Lees *et al.*, 2002; Budge *et al.*, 2009).

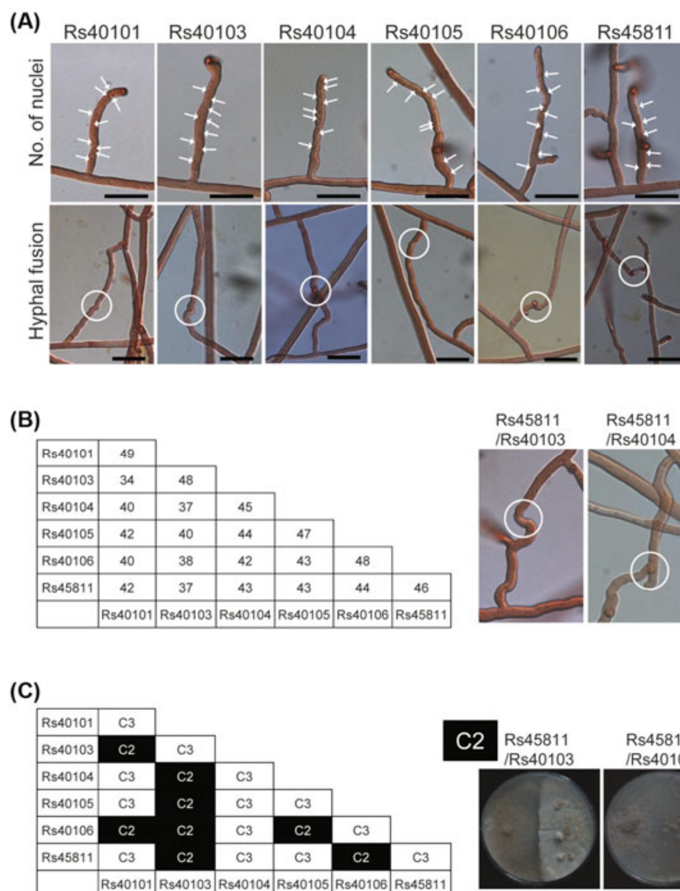
Internal transcribed spacer (ITS) sequences have been used to find species-specific primer sets in many fungal species (Salazar *et al.*, 2000; Martin and Rygiewicz, 2005; Koetschan *et al.*, 2010). However, above conservation is a double-edged sword with regard to specificity of the reaction. In addition, these regions frequently exist within a fungal genome as the uncertain multiple copies variable according to the strain (Nilsson *et al.*, 2008; Bellemain *et al.*, 2010). In this case, it is difficult to determine a consistent relationship between CP values and the number of fungal genomes within the samples.

Table 1. Fungal strains used in the specificity testing of the Taqman real time PCR assay

Scientific name	Strain/collection no.	PCR results[a]	Hosts isolated[b]
Rhizoctonia solani	KACC 40101	+	*Oryza sativa*
Rhizoctonia solani	KACC 40103	+	*Arachis hypogaea*
Rhizoctonia solani	KACC 40104	+	*Cyperus exaltatus*
Rhizoctonia solani	KACC 40105	+	*Cyperus exaltatus*
Rhizoctonia solani	KACC 40106	+	*Oryza sativa*
Rhizoctonia solani	KACC 45811	+	*Oryza sativa*
Magnaporthe oryzae	KI197	-	*Oryza sativa*
Cochliobolus miyabeanus	Cm94	-	*Oryza sativa*
Fusarium moniliforme	KACC 41032	-	*Oryza sativa*
Fusarium graminearum	KACC 46434	-	*Hordeum vulgare*
Alternaria brassicicola	KACC 44877	-	*Brassica rapa*
Botrytis cinerea	KACC 40574	-	*Cucumis sativus*

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

In this work, we provide a quantification method for *R. solani* within infected rice sheaths through Taqman real-time PCR employing a primer pair and a probe specific to the fungal *AROM* gene (*RsAROM*), which is involved in the shikimate biosynthetic pathway. This pathway mediates the transition of quinate, a major component of decaying plant debris, into protocatecuate. In addition, the relationship between heterokaryon formation and virulence degree of the tested strains is presented.



(B)

Rs40101	49					
Rs40103	34	48				
Rs40104	40	37	45			
Rs40105	42	40	44	47		
Rs40106	40	38	42	43	48	
Rs45811	42	37	43	43	44	46
	Rs40101	Rs40103	Rs40104	Rs40105	Rs40106	Rs45811

(C)

Rs40101	C3					
Rs40103	C2	C3				
Rs40104	C3	C2	C3			
Rs40105	C3	C2	C3	C3		
Rs40106	C2	C2	C3	C2	C3	
Rs45811	C3	C2	C3	C3	C2	C3
	Rs40101	Rs40103	Rs40104	Rs40105	Rs40106	Rs45811

Fig. 1. Hyphal fusion and heterokaryon formation of *R. solani* strains. After paired growth of the tested strains on a slide glass and subsequent safranin O staining, both characteristics were analysed under a light microscope. (A) Mycelial branching, number of nuclei, and hyphal fusions in *R. solani*. Nuclei and self hyphal fusions are indicated with white arrows and white circles, respectively. (B) Hyphal fusions between the *R. solani* strains. The numbers in the table indicate the frequencies of observed hyphal fusions among 50 mycelial contact points between the strains. Typical hyphal fusions between strains Rs45811/Rs40103 (left) and Rs45811/Rs40104 (right) are marked with white circles (right panel). (C) Heterokaryon's viability from the pair-wise cultivation of strains on oatmeal agar plate. Perfect fusion and killing reactions are indicated with C3 and C2 in the table, respectively. The right panel shows the representative C2 reaction between strains Rs45811 and Rs40103 (left) and C3 reaction between strains Rs45811 and Rs40104 (right).

Materials and Methods

Biological materials

The fungal isolates used in this study (Table 1) were obtained from Korean Agricultural Culture Collection (KACC), Rural Development Administration (Korea). Fungal inocula were prepared as follows: *R. solani*, *Magnaporthe oryzae*, and *Alternaria brassicicola* were grown on oatmeal agar (50 g oatmeal and 25 g agar per 1 L of distilled water), *Cochliobolus miyabeanus* was grown on sucrose proline agar (SPA) (Ahn *et al*., 2005), and *Fusarium* and *Botrytis cinerea* were grown on potato dextrose agar (PDA, Difco Lab., USA). All strains were grown at 22°C under a continuous florescent light. Each inoculum was transferred into complete liquid medium and grown for 3 days at 25°C, and 150 rpm in the dark. Prior to sampling, the mycelial mass was filtered with Miracloth (Calbiochem, Germany), frozen in liquid nitrogen, and stored at -80°C.

Mycological and molecular characterization of *R. solani*

The presence of multiple nuclei within a cell, hyphal fusion between the thalli, and heterokaryon viability are the most important keys in the fungal identification and determination of anastomosis groups. Actively growing colony edges from the same strain or two tested strains grown for 2 days on oatmeal agar were transferred on a 2% water agar-coated sterile slide glass in three replicates. The samples were kept in a humid chamber overnight and stained with safranin O as described previously (Yamamoto and Uchida, 1982). The number of nuclei within a cell and inter-thallus hyphal fusion was examined under a bright field microscope (Zeiss Axioplan 2). More than 20 cell nuclei and the number of hyphal fusions among a 50 mycelial contact points were estimated. For the macroscopic observation of stable and viable heterokaryon formation, two actively growing mycelial blocks from the tested strains were paired 4 cm apart on oatmeal agar and incubated at 22°C under a continuous fluorescent light. Stable heterokaryon formation resulting in merged growth (C3) or empty lane formation due to fused heterokaryon suicide (C2) at the interface of the two paired colonies was determined as described previously after 5 to 8 days of incubation (Toda and Hyakumachi, 2006; Qu *et al*., 2008). Pair-wise incubations of the tested strains were performed with all possible combinations and all the experiments were repeated three times.

For the molecular identification of the fungal strain, the 609- or 611-bp internal transcribed spacer (ITS) regions were

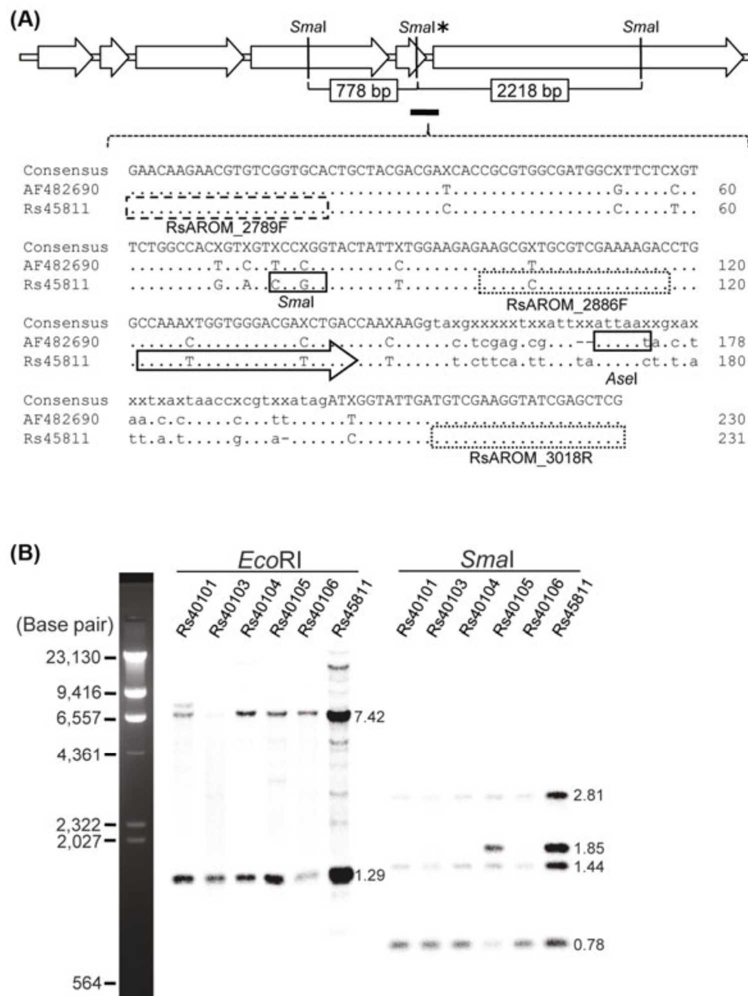


Fig. 2. Comparison nucleotide sequences of the *RsAROM* gene in Rhs 1AP strain infecting potato and that in Korean Rs45811 strain virulent on rice plant. (A) Restriction map of the *RsAROM* gene in Rhs 1AP is presented in the upper diagram. Empty arrows indicate the 6 exon regions. A *Sma*I site specific to Korean Rs45811 strain is indicated with an asterisk. The small black bar under the diagram indicates the 231 bp-long amplified fragment used for Southern blot analysis. In the lower panel, the exon regions are indicated with capital letters. The primer pair (RsAROM_2886F and RsAROM_3018R) and Taqman probe are designated with dotted boxes and an empty arrow. The probe for Southern blot analysis was the amplicon ranging between RsAROM_2789F (dashed box) and RsAROM_3018R primers. The Korean Rs45811-specific *Sma*I site and Rhs 1AP-specific *Ase*I site are indicated with a solid box. (B) Southern blot analysis of *RsAROM*. After the digestion of genomic DNA with *Eco*RI and *Sma*I, the membrane was hybridized with a 231-bp amplicon as the probe. There were no *Eco*RI sites and a single *Sma*I site within the probe region.

amplified from the genomic DNA of six *R. solani* strains using the primer pair ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) (White *et al.*, 1990).

Standard DNA works

DNA from fungal mass or infected plant materials was prepared using the hexadecyl trimethyl ammonium bromide (CTAB) method (Stewart and Via, 1993). After dissolving the precipitated DNA with 10 mM Tris (pH 8.0), the concentration was quantified based on the absorbance at 260 nm and further confirmed through gel electrophoresis and subsequent comparison of the relative brightness of the known amount of the largest 23.1-kb lambda DNA fragment digested with *Hin*dIII using the Java-based software ImageJ (NIH, 2011). Two PCR primer pairs were employed for amplification of *RsAROM* gene; the first primer pair was RsAROM_2886F (5′-AAG CGC TGC GTC GAA AAG AC-3′) and RsAROM_3018R (5′-CGA GCT CGA TAC CTT CGA CA-3′) (Fig. 2A) and was used for Taqman real time PCR. Therefore, specificity of this primer set for *RsAROM* was evaluated through PCR containing genomic DNAs (83.3 ng) from *R. solani*, other fungal plant pathogens, and rice. The amplicon size was 133-bp. The second primer set was RsAROM_2789F (5′-GAA CAA GAA CGT GTC GGT GC-3′) and RsAROM_3018R (Fig. 2A). Ampicon's size of this PCR was 231-bp harboring one *Sma*I restriction site and this fragment was the probe in Southern blot analyses. The PCR conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, 62°C for 15 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. PCR products were analyzed by 2% (w/v) agarose gel electrophoresis. Common PCR in this research were done with high fidelity *Pfu* polymerase (Nanohelix, Korea) and then ligated into pGEM-T Easy vector (Promega, USA) after A-tailing as indicated by manufacturer. After transformation of the ligation mixture into *Escherichia coli* strain DH5α, cloning was confirmed through *Eco*RI digestion and nucleotide sequencing using M13F and M13R primers. Amplification, cloning, and sequencing of ITS regions were done just like above. Espe-

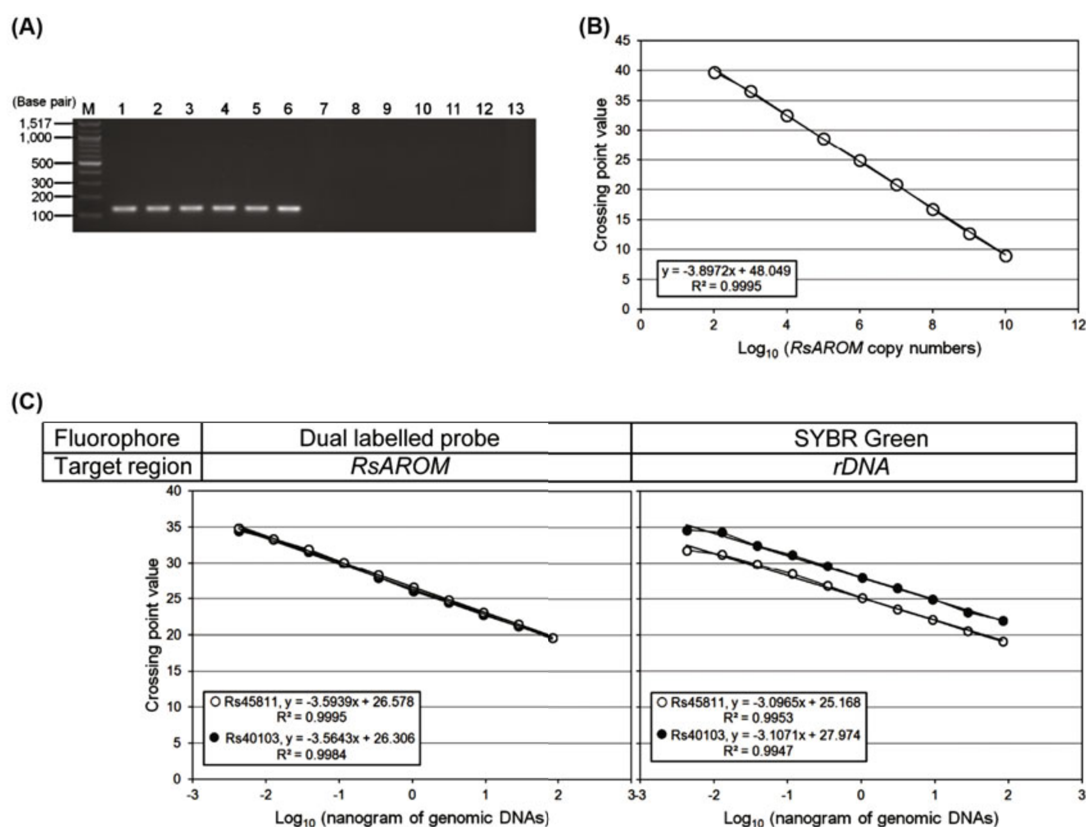


Fig. 3. Specificity of primer pair for *RsAROM* and relationships among crossing point (CP) values, copy numbers of *RsAROM*, and the mount of genomic DNA. (A) In all reactions, RsAROM_2886F and RsAROM_3018R primers were employed to generate a 133-bp amplicon, and 83.3 ng genomic DNA from each fungal strain and rice was used as the template. Lanes: M, 100-bp DNA ladder; 1, *R. solani* strain Rs40101; 2, *R. solani* strain Rs 40103; 3, *R. solani* strain Rs 40104; 4, *R. solani* strain Rs 40105; 5, *R. solani* strain Rs 40106; 6, *R. solani* strain Rs 45811; 7, *Magnaporthe oryzae* race KI197; 8, *Cochliobolus miyabeanus* strain Cm94; 9, *Fusarium fujikuroi* KACC 41032; 10, *Fusarium graminearum* KACC 46434; 11, *Alternaria brassicicola* KACC 44877; 12, *Botrytis cinerea* KACC 40574, and 13, *Oryza sativa* cv. Dongjin. (B) Standard curve obtained by plotting CP values versus log values of *RsAROM* copy numbers. A 10-fold dilution series of the *RsAROM* clone was used as the template. (C) Standard curve obtained by plotting CP values versus log values of genomic DNA extracted from *R. solani* strain Rs45811 (closed circle) and Rs40103 (open circle). In the left panel, CP values were obtained based on the Cy5 fluorescence cleaved from the 5′ end of Taqman probe specific to *RsAROM*. Probe and primer set for this real time PCR were shown in Fig. 2A. In the right panel, CP values were obtained based on the SYBR Green fluorescence attached to the minor groove of amplified rDNA region. Primer pair for this real time PCR was shown in Supplementary data Fig. S1.

cially, 133-bp amplicon-containing clone, *pGEM-T Easy::RsAROM*, was used as the template in analysis of relationship between crossing point (CP) values and copy numbers of 231 bp-long amplicon (Fig. 3B).

Genomic DNA was prepared from six *R. solani* strains (Rs 40101, Rs40103, Rs40104, Rs40105, Rs40106, and Rs45811). DNA (5 μg) was digested with *Eco*RI or *Sma*I, incubated in 37°C for 4 h and loaded in a 0.7% agarose gel. The gel was blotted onto a nylon membrane and further processed with a standard DNA blot procedure (Southern, 1975). A 231-bp fragment of *RsAROM* was used as the radio-labeled probe. The probe was labeled with [α-^{32P}] dCTP through random priming (Feinberg and Vogelstein, 1983) using the Rediprime II DNA Labeling system kit (GE Healthcare, USA). After hybridisation, the membranes were washed with 2 × SSPE, 0.5 × SSPE, 0.1 × SSPE supplemented with 0.1% SDS, exposed to BAS film (Fujifilm, Japan) and developed using a Personal Molecular Imager (PMI) system (Bio-Rad, USA).

Pathogenicity assay

Rice seeds (cultivars Dongjin and Nakdong) were surface-sterilized by submerging in a 100 μg/ml solution of thiophanate-methylthiram for overnight and five seeds were sown in a commercial soil mixture (Bunong, Korea)-containing pots (12 cm diameter × 10 cm height). The rice plants were grown in a greenhouse at 28°C (day time)/24°C (night time), 75% humidity, and under the natural light conditions. After growing the tested strains on a 9-cm diameter oatmeal agar plate for a week as described above, the colonies and light-brown sclerotia were chopped into 3×3 mm^2 mycelial blocks and placed evenly on the soil surface of the pots containing six-week-old rice plants. Inoculated plants were returned to the greenhouse and alterations including symptom development were estimated daily. Sheath blight-specific lesion formation and its lesion length from the soil surface were measured at the indicated times. Disease symptoms were acquired at 21 days post inoculation (dpi). All experiments were independently repeated more than three times and each treatment was composed of 10 replicates.

Real time PCRs

The fluorescent dye cyanine 5 (Cy5) and the matching black hole quencher 2 (BHQ2) were added to the 5′ and 3′ ends of the dual labeled probe (5′-CCA AAT TGG TGG GAC GAT CTG AC-3′). A 10-fold dilution series (10^{10} to $10^{-0.1}$ gene copies) of *pGEM-T Easy::RsAROM* plasmid was used as the template to determine the relationship between copy numbers of 231 bp-long amplicon in *RsAROM* and CP values. Similarly, *R. solani* genomic DNA was diluted three-fold ranging from 83.3 ng to 1 pg to construct a standard curve of the relationship between the amount of genomic DNAs and CP values. Real time PCR was performed using a Light Cycler 480 II (Roche, Germany) with triplicates for 40 cycles (15 sec at 95°C and 15 sec at 60°C), beginning with an initial incubation at 95°C for 4.5 min. The CP values obtained from this reaction were set automatically. SYBR Green-mediated real time PCR was also performed as described previously (Sayler and Yang, 2007) employing the primer pair Rs1F (5′-GCC TTT TCT ACC TTA ATT TGG CAG-3′) and Rs2R (5′-GTG TGT AAA TTA AGT AGA CAG CAA ATG-3′) (Supplementary data Fig. S1) and the same DNAs described above. To evaluate pathogen proliferation *in planta*, 25-cm-long segments from five infected rice stems were ground at the same time and the DNA was prepared. DNA preparation, quantification, and Taqman real time PCR were performed as described above. The weight (ng) of the pathogen DNA within 250 ng of the infected tissue DNA was determined by plotting CP values on a standard curve.

Results

Mycological and molecular identification of *R. solani* AG-1 IA

To determine the fungal species, branching morphologies of mycelia and the number of nuclei within a cell were analyzed. All strains grew rapidly on oatmeal agar and formed dark brown, irregular sclerotia within a week. Under a bright field microscope, almost all mycelia showed right-angled branching and safranin O-staining revealed that there were more than five nuclei within each cell (Fig. 1A). ITS region is widely applicable in the species identification and phylogenetic analyses, and we also have cloned these regions from all tested *R. solani* strains and their sequences were aligned with ITS sequences of *R. solani* isolated from rice belonging to AG-1 IA (accession number AY185115) (Sayler and Yang, 2007), AG-2-1, AG-3, AG-5, and AG-12 (Supplementary data Fig. S1). All ITS sequences from our strains showed more than 99% similarity with AY185115. In contrast, the similarities were decreased to 88%, 82% (AG-3 and AG-5), and 79% with strains in AG-2-1, AG-3 and AG-5, and AG-12, respectively. These results and the mycological characterizations clearly confirmed that our strains were all AG-1 IA. In spite of the high similarities with AY185115, some polymorphisms were evident in the sequences from strains Rs40101 and Rs45811. Compared with AY185115, the 96th nucleotide guanine was absent, and three additional nucleotides, ATC, were inserted between the 177th and 178th nucleotides. These results indicated that all strains in this experiment were *R. solani*. All strains showed self hyphal fusion. To investigate the tested strain's anastomosis group, all strains were grown on slide glasses in a pair-wise fashion and the hyphal fusions were analyzed in all possible combinations of the strains. Rs45811 was the representative strain in AG-1 IA, and all strains in this research showed successful hyphal fusion with Rs45811 (Fig. 1B).

Next, we investigated the viabilities of heterokaryons between the strains. Pair-wise cultivations between Rs40104, Rs40105, and Rs45811 showed merged growth (C3 reaction) (Fig. 1C). This result indicated that the heterokaryons between the above strains were stable and viable. In contrast, pair-wise growth between Rs40103 and other strains resulted in empty lane formation at the interface regions of the colonies. Therefore, these heterokaryons were lethal (C2 reaction). Except for in combination with Rs40104, Rs40106 didn't form stable heterokaryons and killing reactions (C2) were evident (Fig. 1C). Taken together, the competence of Rs40103 and Rs40106 in generating heterokaryon progeny is restricted compared to other strains.

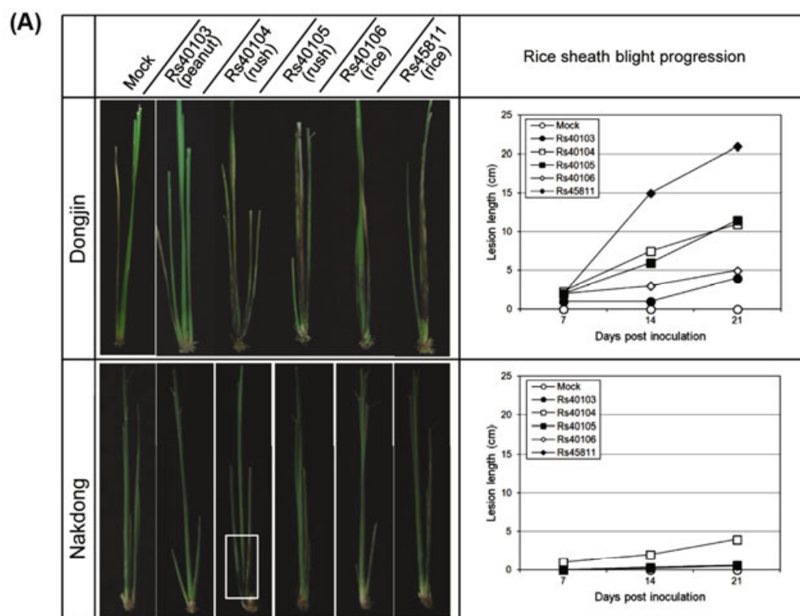
RsAROM-specific rice sheath blight evaluation system

In this investigation, we established a method to measure *R. solani* proliferation within rice with real time PCR employing a primer pair and a dual labeled probe specific to the fungal *AROM* gene. In the genome database (Center for Integrated Fungal Research, 2008) for *R. solani* strain Rhs 1AP, which is virulent to potato and is a member of the AG-3, *RsAROM* was located in RsContig4004. The entire sequence of *RsAROM* was 5,323 bp and consisted of six exons (GenBank accession no. AF482690, Fig. 2A).

We tested two primer pairs composed of three primers to select a specific and appropriate region in the genomic sequence of *RsAROM* for PCR amplification (Fig. 2A). Comparative analysis of the 231-bp amplicon sequence, between RsAROM_2789F and RsAROM_3018R, with the corresponding region of RsContig4004 revealed polymorphism and resulted in the absence of the *Ase*I site and presence of a *Sma*I site in Korean *R. solani* strains (Fig. 2A). The amplicon from this primer combination was used as the probe in Southern blot analysis.

Southern blot analysis was performed to confirm the copy numbers of 231 bp-long amplicon in *RsAROM* within the genome (Fig. 2B). Based on the above sequence information, genomic DNAs were digested with *Eco*RI and *Sma*I. There was only one *Sma*I site in the probe region and no *Eco*RI site was found. Interestingly, the probes hybridized with two fragments 7.42 kb and 1.29 kb, among the genomic DNA fragments digested with *Eco*RI. *Sma*I-digestion led to three fragments, 2.81 kb, 1.44 kb, and 0.82 kb, hybridised with the same probe. Additional 1.85 kb-long-*Sma*I fragments appeared in the Rs40105 and Rs45811.

Specificity is a prerequisite for the quantification method, and we tested the specificity of the primer set, RsAROM_2886F and RsAROM_3018R, with PCR using genomic DNA from six *R. solani* strains, six other fungal species, and rice (Table 1) as templates. The result indicated that the primer pair was specific and only amplified *RsAROM* from *R. solani*. In addition, no product was amplified in PCR using rice DNA as the template, one of preferred hosts for *R. solani* AG-1 IA (Fig. 3A).



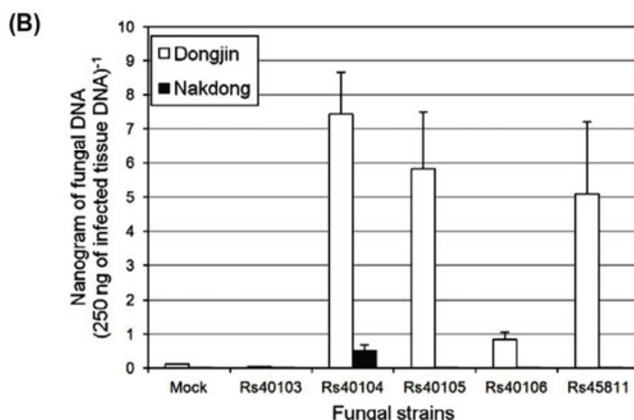


Fig. 4. Rice sheath blight progressions on the two rice cv. Dongjin and Nakdong and quantification of pathogen growth *in planta* by Taqman real time PCR. (A) Rice cv. Dongjin and Nakdong were infected or treated with each *R. solani* strains and mock (chopped oatmeal agar), and grown in the greenhouse for three weeks until symptoms developed. The rice stems were harvested and the picture of the representative sample of each treatment was taken at 21 days post inoculation (dpi). Each treatment was composed with 10 rice plants. Right panel shows lesion progressions on cv. Dongjin and cv. Nakdong measured at 7, 14, and 21 dpi. (B) Quantification of pathogen's DNAs (ng) within plant DNA (250 ng) infected with six *R. solani* strains. After harvesting 25 cm-long rice stems at 21 dpi, DNA was prepared. The amount of fungal DNA within 250 ng of infected sheath DNAs was quantified by plotting the CP values on the standard curve as described in 'Materials and Methods'. Mean and standard deviation of ten rice plants was presented.

Relationship between the amount/copy numbers of genomic DNA and CP values

Two standard curves were constructed to determine the relationship between CP values and copy numbers of 231 bp-long amplicon in *RsAROM* and between CP values and the amount of fungal DNA. The slope generated from the standard curve was -3.8972 with high linear correlations (R^2>0.999) between log values of copy numbers and CP values (Fig. 3B). The genomic DNA from two strains of *R. solani* (Rs45811 and Rs40103) and their serial diluents were used as templates. The reliabilities between the CP value and the amount of genomic DNAs of Rs45811 and Rs40103 were also high (R^2>0.99), and the regression slopes were -3.5939 and -3.5643, respectively (Fig. 3C).

In addition, standard curves describing the relationships between CP values dependent on the fluorescence intensity of SYBR Green and two serial diluents of genomic DNA from Rs45811 and Rs40103 were also constructed (Supplementary data Fig. S1). The genomic DNAs and their serial diluents were the same as in the above experiments. Approximately similar reliable relationships were observed (R^2> 0.99) with slopes of -3.0965 and -3.1071, respectively (Fig. 3C). In contrast with the almost same intercept values of Y axes between the two linear regressions in the *RsAROM*-targeting method above, the difference in intercept values of the Y axes between the two linear regressions in the ribosomal DNA-specific method was 2.806.

Pathogenicity assays of *R. solani*

At three days post inoculation (dpi), the soil surface of the pot inoculated with *R. solani* strain Rs45811 was covered with web-shaped fungal mycelia. However, the sheath parts of the rice (*O. sativa* cv. Dongjin) plants didn't show any visible symptoms and remained healthy until 5 dpi, similar to the rice treated with mock (chopped oatmeal agar). The lower surface of the sheath was slightly covered with thin mycelia at 6 dpi, and small circular, green-greyish lesions were visible on the sheath at 7 dpi (Fig. 4). At approximately 10 dpi, the symptoms progressed to circular, oblong, ellipsoid lesions and the colour changed to dark brown, and symptoms actively progressed. The lesion expansion reached 15 cm at 14 dpi, and symptom progression was also evident in the inner sheath. The lesion reached 22 cm at 21 dpi, and the entire sheath part was covered with dark brown, grayish-centered ellipsoid lesions, and almost all the plant was withered and dead. This is the typical progressions of sheath blight in rice. In spite of the active symptom development in the above ground part, root did not show any visible alterations (data not shown).

In conventional lesion length measurement-based sheath blight evaluation, Rs45811 is the strongest virulent strain on the rice cv. Dongjin. This strain was originally isolated from rice. Rs40104 and Rs40105 were also highly virulent on the rice cv. Dongjin. Both strains were isolated from the monocot Poales weed, rush (*Cyperus exaltatus* Retz.). Although the virulence of Rs40106, which was isolated from rice, was weaker than those of Rs40104 and Rs40105, this strain evidently triggered typical rice sheath blight symptoms. In contrast, Rs40103 did not cause any symptoms on the rice sheath. This strain was isolated from the stem of the peanut, *Arachis hypogaea*.

In contrast to the rapid symptom development observed on the rice cv. Dongjin, the rice cv. Nakdong was generally resistant against *R. solani* infection (Fig. 4A). Rs40104 was the only strain that triggered typical sheath blight symptoms. However, lesion length progression in the cv. Dongjin by this strain was also 3.75 times higher than that in the cv. Nakdong.

Quantification of *R. solani* within infected rice tissues

Rice stems infected with five *R. solani* strains were recovered at 21 dpi and the amount of fungal DNA within the infected tissue DNA was evaluated. The amount of fungal DNA within tissue DNA was calculated by plotting the CP values on the standard curve. Southern blot analysis revealed that 231 bp-long amplicon in *RsAROM* exists as double copies within a fungal genome. That is, each genome contains two copies of above fragment. Therefore, the copy numbers of the fungal genome within the sample is one-half of the number of 231 bp-long amplicon in *RsAROM*. The amounts and copy numbers of fungal DNAs within 250 ng of tissue DNAs from cv. Dongjin infected with Rs40104, Rs40105, and Rs45811 were 7.435, 5.829, and 5.086 ng and 352,418, 276,598 and 241,451 genomes respectively (Fig. 4B). Similarly, the amounts and copy number of fungal DNAs were 0.841 and 0.514 ng and 40,300 and 24,678.5 genomes within stem DNAs inoculated with Rs40106 and Rs40104. In contrast, fungal DNA was not detectable in the DNA of cv. Dongjin challenged with Rs40106 or treated with mock.

Discussion

Reliable and reproducible quantification of rice sheath blight is indispensible for successful disease management and correct evaluation of germplasms or inbred lines of rice conferring partial resistance. In this research, we have settled down real time PCR method able to quantify rice sheath blight based on the amount of *R. solani* DNA within an infected tissue DNA. This system was composed of a primer pair and dual-labeled oligo probe specific to *RsAROM*, a *R. solani* gene existing double copies in the fungal genome. Relationships between CP values and amount of genomic DNAs from two strains were highly reliable (Fig. 3C, left pannel). Further, these two linear regressions showed almost same slopes and Y axis intercepts. These results indicate that these two relationships should be applicable for enumeration of *R. solani* strains harboring two copies of 231 bp-long amplicon in *RsAROM* and sheath blight forecasting in the rice fields. Previous research employed SYBR Green-dependent real time PCR and a primer pair specific to ribosomal DNA (rDNA) in the evaluation of *R. solani* AG-1 IA (Sayler and Yang, 2007). We attempted an almost identical strategy (Fig. 3C, right pannel). Although relationship between CP values and the amount of DNA was reliable, the Y axis intercept values of the two linear regressions from the genomic DNAs of Rs45811 and Rs40103 were significantly different by about 2.806. This value means that the copy numbers of rDNA within the genome of Rs40103 were

$2^{2.806}$ (6.99) times larger than that within the genome of Rs45811 because analyzed amounts of two strain's DNAs were identical in each data point. Several descriptions also support the uncertain copy numbers of rDNAs within the fungal genome (Nilsson *et al*., 2008; Bellemain *et al*., 2010). The application of an rDNA-specific standard curve should be limited strains sharing identical rDNA copy number. In addition, this system would not be able to quantify the inoculum potentials in paddy fields harboring diverse strains of *R. solani* AG-1 IA with various copy numbers of rDNA. Confirmation of the target gene's copy number is a critical requisite for estimation of fungal DNA through real time PCR.

Maximum lesion length within a stem has been the criterion for the visual rating of rice sheath blight. However, this method is not applicable for the exact estimation of disease severity due to irregular symptom progression around the rice sheath and stems. For example, typical sheath blight symptoms were frequently observed on the middle part of the rice stem despite of no symptom formation below them (Fig. 4A, Rs40106-infected Dongjin sheath). Active lesion formation limited to the longitudinal half of the stem was also observed. These uneven symptom developments indicated that visual evaluation is not able to reflect actual sheath blight progressions. Rs40104, Rs40105, and Rs45811 showed strong virulence on the rice cv. Dongjin and this was coincided with a larger amount of these strains' fungal DNAs within the infected tissue DNAs (Fig. 4). Rs40106 and Rs40104 were also virulent on cv. Dongjin and cv. Nakdong, however, symptom development was something restricted in these interactions. These strains' ramifications in cv. Dongjin were also much lower than those by above three strains. These results indicate that the fungal DNA amount was positively correlated with lesion progression. Therefore, our method should be applicable for the estimation of *R. solani* virulence or evaluation of a rice cultivar's resistance against sheath blight.

Although Rs45811 inoculation provoked the most superior sheath blight progressions, the mycelia growth of Rs40104 was higher than that of Rs45811in the susceptible cv. Dongjin (Fig. 4B). Therefore, there was no absolute positive relationship between lesion progression and pathogen propagation, which implies the possible involvement of other virulence factors contributing to Rs45811's virulence on the susceptible host cv. Dongjin. *R. solani* is a representative rice necrotrophic pathogen and in many cases, toxic secondary metabolites or enzymes are necessary for this pathogen's full virulence (Morrissey and Osbourn, 1999; Howlett, 2006; Collemare and Lebrun, 2011).

Interestingly, comparative analysis of our Southern blot data for *RsAROM* in rice-infecting *R. solani* AG-1 IA and nucleotide sequence data (http://rsolani.org/download.html) around the *RsAROM* in potato pathogen Rhs 1AP belong to AG-3 revealed huge differences. An 18-kb fragment from the *Eco*RI digestion of the genomic DNA and two 778 bp and 2,218 bp fragments from the *Sma*I digestion were expected to be hybridized with the tested probe due to the presence of a *Sma*I site specific to Korean *R. solani* belonging to AG-1 IA (Fig. 2A). However, two 7.42 kb and 1.29 kb *Eco*RI fragments and three 2.81 kb, 1.44 kb, and 0.78 kb *Sma*I fragments hybridized with the probe. The genomic DNA sequence of *RsAROM*'s open reading frame (ORF) in Rhs 1AP is 5.1 kb. The longer 7.42 kb fragment is able to contain full length of RsAROM, 1.29 kb fragment, however, the shorter 1.29 kb fragment can't harbor full length of this ORF. Although we have searched for nucleotide sequences exhibiting similarities with 231 bp-long amplicon in this research in the Rhs 1AP's genome database, no region showed high degree of similarities able to hybridize with our amplicon. These results indicate the presence of 231 bp-long fragments in *RsAROM* as double copies in the genome of *R. solani* and they are located in two *Eco*RI fragments. One of the *RsAROM* harboring 231-bp-long amplicon might contain one or more *Eco*RI sites within its ORF. In addition, above differences indicates significant structural and sequence variations between Rhs 1AP and our *R. solani* strains.

Rush (*C. exaltatus*) is frequently cultivated in the paddy field because of its market value in Korea. Five strains isolated from rice and rush were virulent on rice cv. Dongjin, whereas Rs40103 didn't infect rice in spite of its identity with respect to hyphal anastomosis. Rs40104 and Rs40105 isolated from rush were severely virulent on rice plants. Their virulence on rice plants was even stronger than that of Rs40106 from rice. Therefore, rush could be a host plants of *R. solani* AG-1 IA and further, this plant could be a potent carrying plants of *R. solani* virulent on rice because rush is usually cultivated in the rice paddy field and in turn, rice is planted in the next year. If rush debris carrying *R. solani* remained in rice fields, it could be a primary inoculum. The roles of several weeds as carriers of *R. solani* AG-1 IA have been investigated through a transfer study using soybean as the susceptible crop (Black *et al*., 1996). In the extreme case, the pathogen's virulent sclerotia or propagules were recovered from all the weeds and soybean plants placed adjacent to the weeds infected with the same pathogens. These results suggest the importance of weed control for successful sheath blight management in rice ecosystems.

R. solani AG-1 IA is a pathogen of diverse host species such as rice, corn, sorghum, bean, soybean, crimson clover, turfgrass, and camphor seedlings (Sneh *et al*., 1991). Major populations of *R. solani* infecting peanut plants are members of AG-4 (Woodard and Jones, 1983). Among 130 isolates from peanut fields, 129 isolates were determined to be in AG-4. The presence of avirulent *R. solani* AG-1 IA on rice from peanut stems further suggests that the rotation of rice with peanut is preferable for the management of sheath blight in the rice paddy fields.

In sum, we have established a reliable and constitutive evaluation method to quantify fungal colonization with Taqman real time PCR employing a primer pair and a dual labeled probe specific to *R. solani*'s *RsAROM*. This method would be applicable in the evaluation of resistance germplasm or fungicides and estimation of inoculum potential in the rice field.

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