Detection and Quantification of *Rhizoctonia solani* AG-1 IA, the Rice Sheath Blight Pathogen, in Rice Using Real-Time PCR

Ronald J. Saylor and Yinong Yang, Department of Plant Pathology, University of Arkansas, Fayetteville 72701

ABSTRACT

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Rhizoctonia solani Kühn is the causal organism of sheath blight, a major rice disease worldwide that severely impairs yield and quality. It is difficult to identify the pathogen in the early phase of the infection and to accurately quantify the fungal development based on visual inspection. Therefore, a rapid and reliable method is advantageous for the detection and quantification of the pathogen causing this important rice disease. In this study, a real-time, quantitative polymerase chain reaction (QPCR) assay was developed to detect and quantify *R. solani* AG-1 IA DNA from infected rice plants. A specific primer pair was designed based on the internal transcribed spacer region of the fungal ribosomal DNA. The specific detection of *R. solani* DNA was successful with quantities as low as 1 pg. The QPCR assay could be used for detecting the rice sheath blight pathogen, quantifying fungal aggressiveness, and evaluating the resistance level of rice cultivars.

Additional keywords: early detection, *R. oryzae*, *R. oryzae sativa*, *R. solani* AG-1 IB

Sheath blight of rice (*Oryza sativa* L.) is one of the most economically important disease of rice and occurs wherever rice is grown (37), including the southern United States. This disease is caused by the soil-borne pathogen *Rhizoctonia solani* Kühn, with the teleomorph *Thanatephorus cucumeris* (A.B. Frank) Donk, belonging to anastomosis group 1 intraspecific group IA (AG-1 IA). Symptoms typically appear in the late tillering or early internode elongation growth stages and consist of circular, oblong, or ellipsoid green-gray, water-soaked spots about 1 cm long near the water line (28).

No major gene for resistance has been found for sheath blight; however, partial resistance was observed during a survey of 6,000 rice cultivars (16), and genes contributing to partial resistance have been identified (22). One disadvantage to polygenic or partial resistance, like that found to sheath blight, is the difficulty in selection of resistance genes (9). Although several efforts have been made to identify quantitative trait loci (QTLs) for resistance to sheath blight (18,19,23), success has been limited. Cultural methods such as crop rotation, fertilizer management, plant

spacing, and silica amendment have been shown to provide modest disease control (6,26,27), but are insufficient for most of the susceptible semidwarf cultivars grown commercially. As a result, disease control is achieved primarily through fungicide application at the onset of disease. Rapid and accurate diagnosis of the disease is important if fungicide applications are to be efficacious as the timing of foliar fungicide applications is critical for achieving effective disease control. However, diagnosis of this disease by visual symptoms is difficult at the early stages of lesion development when sheath blight may be confused with sheath rot (*Sclerotium oryzae*) or lesions caused by the rice stem borer (*Scirpophaga* sp.). In addition, the use of fungicides may be limited in the future due to environmental concerns, expense, and the potential development of fungicide-resistant pathogen populations. Early detection of this disease may allow growers to apply fungicides in the early phase of the disease to prevent the spread of the disease to a wider area requiring much greater application of fungicides.

Real-time, quantitative polymerase chain reaction (QPCR) has been used for rapid identification and quantification of numerous plant-pathogenic fungi (4,5,8, 11–15,24,34), viruses (3,7,10), and bacteria (2,21,29,30,36) from infected host tissue. Currently, rice breeders assess the severity of sheath blight visually in their efforts to select resistant cultivars and to identify QTLs for resistance (18,19,22). Visual rating of sheath blight infection is subject to the bias of the individual investigator and this is particularly a problem when comparing studies performed by

different investigators, such as in QTL mapping. The assessment of partial resistance to sheath blight is especially difficult due to the effects of environmental variability on disease pressure. Quantification of *R. solani* DNA in infected rice tissue by real-time PCR may be useful to researchers by providing an unbiased method for measuring pathogen growth in infected plants. In addition to the ability to quantify pathogen DNA, real-time PCR is faster than conventional PCR for pathogen detection because it does not require the analysis of amplicons on an agarose gel.

In this study, we report the development of a real-time PCR assay for the detection and quantification of *R. solani* AG-1 IA DNA from infected rice tissue. The internal transcribed spacer (ITS) regions from different groups of *R. solani* were compared and used for designing a pair of PCR primers that is specific for the AG-1 IA isolates, but not other AG groups of *R. solani*. We have demonstrated that the real-time PCR method is very specific and highly sensitive for detecting and quantifying the sheath blight pathogen in rice leaves.

MATERIALS AND METHODS

Fungal isolates and plant materials.

The fungal species, host, geographic origin, isolate code, anastomosis group, and GenBank accession numbers of their 18-28S ITS used in this study are listed in Table 1.

The cultivars of rice (*Oryza sativa* subsp. *japonica*) used in this study were Nipponbare and Jasmine 85. Nipponbare is susceptible to sheath blight, whereas Jasmine 85 is partially resistant.

Pathogen culture and inoculation.

Rice plants were grown in a computer-controlled greenhouse at temperatures of approximately 28°C during the day and about 24°C at night to the early tillering stage. Pathogen inoculations were conducted with a detached leaf method for convenience (Y. Jia, *personal communication*). The second-youngest leaf of 2-month-old plants were cut into 16-cm-long segments and the detached leaves were placed immediately in square petri dishes (245 mm²; Corning, Corning, NY) containing a 3 MM Whatman filter paper (225 mm²) moistened with 25 ml of deionized H₂O. Treatment leaves were arranged in the petri dish in a randomized block design with petri dish as block. *R. solani* strain RR0140 was grown for 3 days at 25°C on

Corresponding author: Y. Yang
E-mail: yuy3@psu.edu

Current address of Y. Yang: Department of Plant Pathology, Pennsylvania State University, University Park 16802.

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Difco potato dextrose agar (PDA) supplemented with tetracycline at 15 mg/liter to prevent bacterial contamination. A 0.7-mm-diameter PDA plug was excised from the periphery of a 3-day-old actively growing culture with a 1-ml pipette tip. The plug was placed in the middle of the abaxial surface of the leaf segment with a sterile toothpick. Petri dishes with inoculated leaves were maintained on the bench at 22°C under continuous fluorescent light (10 to 20 EM⁻² s⁻¹) for 72 h. The entire lesion length was measured 3 days after inoculation. This inoculation method was used solely to facilitate the rapid generation of infected rice leaves for the development of the real-time PCR quantification assay. Analysis of variance of lesion length was performed using JMP software (5.1.2; SAS Institute Inc. Cary, NC). The standard error of the mean was calculated.

DNA extraction. Approximately 300 mg of fungal mycelia or infected leaf tissue was ground with liquid nitrogen and transferred into an Eppendorf tube containing 400 µl of extraction buffer (0.3 M NaCl, 50 mM TrisHCl, pH 7.5, 20 mM EDTA, 2% sarkosyl, 0.5% sodium dodecyl sulfate, and 5 M urea). Subsequently, phenol/chloroform at 400 µl solution (1:1, pH 8.0) was added. After vortexing for 10 min, samples were centrifuged for 5 min at 14,000 rpm. The supernatant was transferred into a new tube and 2 µl of RNase at 10mg/ml was added. The sample was vor-

texed a few seconds and incubated at 37°C for 1 h. The DNA was precipitated with 0.7 volumes of isopropanol followed by centrifugation for 5 min at 14,000 rpm. Precipitated DNA was washed with 70% ethanol, air dried, and dissolved in 100 µl of Tris (10 mM)-EDTA (1 mM). DNA concentration was determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE).

Primer design. PCR primers specific for *R. solani* AG-1 IA were selected by the comparison of sequence alignments from the 18-28S ribosomal (r)DNA ITS region of 17 *R. solani*, four *R. oryzae*, and two *R. oryzae sativa* isolates (Table 1). Sequence alignments were performed with Vector NTI 6 software (Informax, North Bethesda, MD). Based on this analysis, the following pair of specific primers was designed for real-time PCR: forward primer Rs1F (65 to 92), 5'-GCCTTT TCTACCTTAATTTGGCAG-3' and reverse primer Rs2R (202 to 234) 5'-GTGTGTAAATTAAGTAGACAGCAA ATG-3'. Primer sequences were verified to have low homodimer and heterodimer potential as well as a low probability of forming a hairpin loop, using the OligoAnalyzer 3.0 web software (IDT, Coralville, IA). These primers amplified a fragment of 137 to 140 bp suitable for both conventional and real-time PCR detection. Fragment size varied from 137 to 140 bp, as determined by DNA sequence analysis,

depending on the specific isolates tested. The specificity of Rs1F and Rs2R was tested against the DNA of *R. solani* isolates from anastomosis groups AG 1-IA (rice), 1-IB (bean), 2, 4, 7, 8, and 11, as well as *R. oryzae* and *R. oryzae sativa*, using conventional PCR. PCR fragments were separated on a 1.5% agarose gel and stained with ethidium bromide.

Primers specific to the rice ubiquitin gene (accession number AF184279) were used to assess the quality of extracted DNA, allow the comparison DNA amount measured spectrophotometrically to the cycle threshold (Ct) values, and allow the potential for relative QPCR. Ubiquitin primer sequences were as follows: RUBQF 5'-GTG GCC AGT AAG TCC TCA GC and RUBQR 5'-GAA ACG GGA CAC GAC CAA GG (35). The inclusion of the rice ubiquitin primers allows the potential for relative quantification as well as absolute *R. solani* DNA quantification.

Real-time PCR assay. Real-time PCR was performed with a Thermo-Fast 96-well reaction plate and Thermo-Fast Caps (ABgene, Epsom, Surrey, UK) in the Mx3000P real-time PCR machine from Stratagene Corporation (La Jolla, CA). Each well contains a 25-µl reaction mixture that includes 12.5 µl of 2× SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and 12.5 µl of primer (1 pmole of each forward and reverse primer). Extracted DNA (1 µl) at 100 ng/µl was added

Table 1. *Rhizoctonia* spp. referenced in this study^a

Genus species	Host	Origin	Isolate code	AG subgroup	Accession number
<i>Rhizoctonia solani</i>	<i>Oryza sativa</i> , rice	Arkansas	RR0135	AG-1 IA	AY185115
<i>R. solani</i>	Rice	Arkansas	RR0134	AG-1 IA	AY185114
<i>R. solani</i>	Rice	Arkansas	RR0129	AG-1 IA	AY185113
<i>R. solani</i>	Rice	Arkansas	RR0107	AG-1 IA	AY185108
<i>R. solani</i>	Rice	Arkansas	RR0103	AG-1 IA	AY185106
<i>R. solani</i>	Rice	Arkansas	RR0101	AG-1 IA	AY185104
<i>R. solani</i>	Rice	Arkansas	RR0102	AG-1 IA	AY185105
<i>R. solani</i>	Rice	Arkansas	RR0113	AG-1 IA	AY185109
<i>R. solani</i>	Rice	Ivory Coast	T62	AG-1 IA	AJ000200
<i>R. solani</i>	Rice	Vietnam	T5	AG-1 IA	AJ000199
<i>R. solani</i>	Rice	Philippines	T68	AG-1 IA	AJ000197
<i>R. solani</i>	<i>Lolium multiflorum</i>	Japan	na	AG-1 IA	AB122134
<i>R. solani</i>	<i>Phaseolus vulgaris</i>	Nebraska	PR5	AG-1 IB	AF308629
<i>R. solani</i>	na	na	na	AG-1 IB	AB122136
<i>R. solani</i>	na	na	na	AG-1 IB	AB122140
<i>R. solani</i>	na	Brazil	AIC	AG-1 IC	AY154300
<i>R. solani</i>	<i>Solanum tuberosum</i>	Alaska	AG-2 1	AG-2 1	na
<i>R. solani</i>	<i>Gossypium hirsutum</i>	Arkansas	AG-4	AG-4	na
<i>R. solani</i>	<i>G. hirsutum</i>	Arkansas	AG-7	AG-7	na
<i>R. solani</i>	<i>Hordeum distychum</i>	Washington	AG-8	AG-8	na
<i>R. solani</i>	Soil	Arkansas	AG-11	AG-11	na
<i>R. solani</i>	Leaf litter	Australia	WDa	AG-12	AF153807
<i>R. oryzae</i>	Rice	Japan	na	na	AJ000195
<i>R. oryzae</i>	Rice	Japan	na	na	AJ000196
<i>R. oryzae</i>	Rice	Arkansas	HP1P42A	na	na
<i>R. oryzae</i>	Rice	Arkansas	1	na	na
<i>R. oryzae</i>	Rice	Arkansas	2	na	na
<i>R. oryzae</i>	Rice	Arkansas	4	na	na
<i>R. oryzae sativae</i>	Rice	Arkansas	na	na	na
<i>R. oryzae sativae</i>	Rice	Côte d'Ivoire	C2	na	AJ000191
<i>R. oryzae sativae</i>	Rice	West Malaysia	C1	na	AJ000192
<i>R. oryzae sativae</i>	Rice	Japan	C3	na	AJ000193
<i>R. oryzae sativae</i>	Rice	Japan	C6	na	AJ000194

^a Abbreviation: na = not available.

to the reaction mix. Every DNA sample was analyzed with duplicate real-time PCR reactions. Amplification of extracted *R. solani* DNA was performed under the following conditions: (i) 95°C for 15 min; (ii) 40 cycles of 95°C for 15 s, 60°C annealing for 30 s, and 72°C extension for 15 s; (iii) 95°C for 1 min; and (iv) 55°C for 30 s. The annealing temperature was lowered to 52°C when using both the *R. solani* primers Rs1F and Rs2R and the rice ubiquitin primers RUBQF and RUBQR on samples during the same run. Absolute QPCR was employed to determine the quantities of *R. solani* DNA in detached rice leaves. A subset of 10-fold dilution series of *R. solani* DNA (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg) from a pure culture was analyzed in each absolute QPCR assay in duplicate to generate a standard curve. A real-time PCR assay typically was performed with three biological replications. The standard error of the mean was calculated accordingly. In addition to the data presented in this study, the assay has been

tested successfully with different tissues of *R. solani*-infected rice plants from several other experiments.

RESULTS

Selection and testing of primer specificity. A nucleotide sequence comparison of the 18-28S rDNA ITS region revealed significant differences between *R. solani* AG-1 IA, *R. solani* from other intraspecific groups within AG-1, other AG groups, *R. oryzae*, and *R. oryzae sativa*. Based on these differences in sequence, PCR primers Rs1F and Rs2R were designed to be specific to the ITS of *R. solani* AG-1 IA isolates (Fig. 1). Primer sequences Rs1F and Rs2R matched all *R. solani* AG-1 IA sequences shown in Figure 1, as well as numerous ITS sequences of AG-1 IA isolates in the National Center for Biotechnology Information GenBank database identified using the Basic Local Alignment Search Tool (BLAST; 1). These primer sequences did not match the sequences of *R. solani* AG-1 IB (accession numbers

AF308629, AB122136, and AB122140), AG-1 IC (accession number AY154300), *R. oryzae*, or *R. oryzae sativa* (Fig. 1).

The specificity of Rs1F and Rs2R was tested against the DNA of *R. solani* isolates from anastomosis groups AG 1-IA (rice), 1-IB (bean), 2, 4, 7, 8, and 11, as well as *R. oryzae* and *R. oryzae sativa*. The specific PCR fragment was amplified from the DNAs of three AG-1 IA isolates but not from the DNA of other *R. solani* AG groups, *R. oryzae*, or *R. oryzae sativa* (Fig. 2). The predicted diagnostic amplicon generated by conventional PCR of *R. solani* AG-1 IA isolates ranged in size from 137 to 140 bp depending on the isolate tested.

Sensitivity of real-time PCR assay. To determine the assay sensitivity, DNA from *R. solani* AG 1-IA was used to prepare 10-fold serial dilutions from 100 ng/μl to 1 pg/μl. Dilution of known quantities of purified *R. solani* AG-1 IA DNA revealed that as little as 1 pg can be readily detected by real-time PCR (Fig. 3A). Although the

Fig. 1. DNA sequence comparison of the ribosomal (r)DNA internal transcribed spacer (ITS) region of *Rhizoctonia solani*, *R. oryzae*, and *R. oryzae sativa* along with their GenBank accession numbers. Arrows indicate forward and reverse primers selected in this study for conventional and real-time polymerase chain reaction. The sequence of *R. solani* rDNA ITS regions begins at base 60 and finishes at base 212.

no-template control (NTC) was detected at a Ct of approximately 37 (Fig. 3A), analysis of the dissociation curve revealed that the amplification was due to primer dimer (data not shown). The dissociation temperature of the NTC amplicon was much lower than the experimental samples, consistent with an amplicon produced by

primer dimer. A negative linear relationship was found between Ct value and the log of diluted pathogen DNA ($R^2 = 0.9911$). The technical variation of the QPCR assay was low when using purified *R. solani* DNA (Fig. 3B). *R. solani* DNA was quantifiable from 1 pg to 100 ng (Fig. 3B). It may be possible to detect less than

1 pg of pathogen DNA, although dilutions of less than 1 pg were not tested.

Quantification of *R. solani* DNA from infected rice leaves by real-time PCR. Real-time PCR analysis revealed that *R. solani* DNA increased over time as symptoms developed in artificially inoculated rice leaves (data not shown). Fungal growth appeared to follow a sigmoidal curve. The difference between the partially resistant and susceptible cultivars in the detached leaf assay, as determined by visible symptoms, occurred on day 3 post inoculation. Day 3 post inoculation also appeared to be the most appropriate time for measuring disease by real-time PCR quantification of pathogen DNA. Lesions produced by *R. solani* on Nipponbare were more than twofold longer than those on Jasmine 85 (Fig. 4A). Likewise, concentrations of *R. solani* DNA were threefold higher on Nipponbare than on Jasmine 85 (Fig. 4B). Dissociation curve analysis of amplicons produced by real-time PCR revealed two distinct peaks corresponding to the amplicons produced using primers specific to *R. solani* AG-1 IA (79.5°C) and to the rice ubiquitin gene (81.5°C) (Fig. 4C). The comparison of disease measurements by either lesion length (Fig. 4A) or *R. solani* DNA quantification (Fig. 4B) differentiated resistant Jasmine 85 and susceptible Nipponbare rice cultivars.

DISCUSSION

Early, rapid, and reliable detection of the sheath blight pathogen, *R. solani* AG-1 IA, will improve the timing and implementation of control measures for the disease, especially fungicide application. Application of fungicides at the initiation of disease may be more effective than after sheath blight has become established, making early detection of the pathogen critical for disease control. Detection of *R. solani* AG-1 IA by the real-time PCR method presented here is both rapid and reliable. The pathogen may be detected in infected rice tissue before fungal structures such as mycelia and sclerotia are visible, and even before disease symptoms appear. Although detection of the sheath blight pathogen by conventional PCR has been reported (17), these primer sequences for *R. solani* are not suitable for real-time PCR because the amplicon they produce is too long. Another advantage of real-time PCR detection is that it can be performed in the field using portable machines, providing the capacity for onsite diagnosis (30), which is not possible with conventional PCR.

Results of this study demonstrate that primers Rs1F and Rs2R are highly specific, amplifying DNA from *R. solani* AG-1 IA but not from the most closely related intraspecific group, AG-1 IB, or from the other AG groups of *R. solani* tested here. Although the ITS sequences of AG-1 IB isolates are most similar to those of AG-1 IA (32), sequence alignments further con-

Fig. 2. Primer specificity test for *Rhizoctonia solani* anastomosis group 1 (AG-1) intraspecific group IA. Lane 1, *R. solani* RR0102; lane 2, *R. solani* RR0113; lane 3, *R. solani* RR0107; lane 4, *R. solani* AG-1 intraspecific group IB; lane 5, *R. solani* AG-2 1; lane 6, *R. solani* AG-4; lane 7, *R. solani* AG-7; lane 8, *R. solani* AG-8; lane 9, *R. solani* AG-11; lane 10, *R. oryzae* HP1P42A; lane 11, *R. oryzae* sativa; lane 12, *R. oryzae* #1; lane 13, *R. oryzae* #2; lane 14, *R. oryzae* #4; and lane 15, negative control.

Fig. 3. *Rhizoctonia solani* DNA dilutions measured by real-time polymerase chain reaction (PCR) cycle threshold (Ct) value. **A**, Representative real-time PCR fluorescent curves for *R. solani* DNA concentrations from left to right are 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and no-template control (NTC). **B**, Correlation of Ct value to the log of *R. solani* DNA concentration.

firm that our primers are specific to AG-1 IA but not to AG-1 IB (Fig. 1). The rDNA-ITS sequence analysis of *R. solani* AG-1 revealed 99% sequence identity within AG-1 IA but lower similarity (91 to 95%) between subgroups IA, IB, IC, and ID (32). Sequence similarity was even lower for AG-2 1 (89%) and AG-4 (81%) (32). Furthermore, the primers do not amplify DNA from the sheath spot pathogen *R. oryzae* or the aggregate sheath spot pathogen *R. oryzae-sativa*, providing evidence for their specificity. Although TaqMan probes offer an additional level of specificity over SYBR green primers alone, the specificity was sufficient (Figs. 1 and 2). In addition, TaqMan probes are more expensive than the SYBR green dye. The primers were specific to the rDNA ITS region sequences representing 80 other *R. solani* AG-1 IA groups from Arkansas, Vietnam, and the Philippines (Fig. 1). Restriction fragment length polymorphism (RFLP) analysis of the genetic diversity of *R. solani* AG-1 IA populations from India and Texas revealed only moderate population subdivision, despite being very distant geographically. The relatively small genetic variation in *R. solani* isolates AG-1 IA detected by ITS sequence (32) and RFLP analysis (20) suggests that the real-time PCR technique described here may be useful for the detection of *R. solani* isolates from a wide geographic area.

Resistance to sheath blight in rice is polygenic, requiring plant breeders to search for QTLs to improve the efficiency of cultivar selection. Locating QTLs for resistance to sheath blight in rice has been a challenging endeavor met with limited success (18,19,23), and many of the reported QTLs remain to be verified by independent studies (23). In fact, one of the main goals of the current multi-institutional Rice Coordinated Agricultural Project from the United States Department of Agriculture is to identify QTLs for resistance to sheath blight (<http://www.uark.edu/ua/ricecap/>). Identification of QTLs for resistance to sheath blight has been difficult because of the variation in disease pressure between locations and years, variation in the visual rating of disease between scientists, and variation in experimental conditions between different studies. Quantification of *R. solani* DNA in infested rice tissue by real-time PCR may provide breeders and other researchers with an unbiased method for measuring fungal growth in rice plants. The use of primers specific to the rice ubiquitin gene allows the user to employ relative QPCR, often used in gene expression studies (25), in addition to the absolute QPCR presented in this study. The ubiquitin primers also allow the user to compare the accuracy of DNA quantification levels determined spectrophotometrically to the Ct values obtained by QPCR. The analysis of amplicon dissociation temperature generates

additional evidence for primer specificity. Real-time PCR accurately quantified pathogen DNA in numerous other systems (8,14,31,33,34,38), including the *Mag-*

naporthae grisea in the rice–rice blast interaction (24). In summary, the real-time PCR technique described here may be applicable for detecting and quantifying *R. solani*

Fig. 4. Quantification of *Rhizoctonia solani* infection of detached rice leaves 3 days after pathogen inoculation on a moderately resistant cultivar (Jasmine 85) and a susceptible cultivar (Nipponbare). **A**, Mean lesion length caused by *R. solani*. **B**, Quantification of *R. solani* DNA by real-time polymerase chain reaction from 100 ng of total DNA extracted from the same rice leaves. Variation among three biological replications is represented by the standard error of the mean. **C**, Dissociation curve analysis of amplicons produced by *R. solani* primers Rs1F and Rs2R and rice ubiquitin primers RUBQF and RUBQR.

AG-1 IA isolates from around the world, improving disease control and providing an unbiased method for quantifying *R. solani* growth in rice plants.

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