Quantification of *Magnaporthe grisea* During Infection of Rice Plants Using Real-Time Polymerase Chain Reaction and Northern Blot/Phosphoimaging Analyses

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ABSTRACT

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Rice blast, caused by *Magnaporthe grisea*, is a serious fungal disease of rice worldwide. Currently, evaluation of the fungal pathogenicity and host resistance is mainly based on a disease rating or measurement of blast lesion number and size. However, these methods only provide visual estimation rather than accurate measurement of fungal growth in rice plants. In this study, DNA-based real-time polymerase chain reaction (PCR) and RNA-based northern blot/phosphoimaging analyses were evaluated to quantify *M. grisea*. Both methods were sensitive, specific, and reproducible and could accurately measure the relative growth and

absolute biomass of *M. grisea*. The real-time PCR analysis showed that the growth of *M. grisea* in seedling leaves of susceptible cultivars (M201 and Wells) was ≈ 46 to 80 times higher than that of a resistant cultivar (Drew) at 4 and 6 days after inoculation. The data obtained from the real-time PCR assays also were consistent with that from northern blot/phosphoimaging analysis. However, the real-time PCR approach was much faster and more convenient in most cases. Therefore, it is an excellent tool for in planta quantification of *M. grisea* and can be used for reliable assessment of fungal pathogenicity and host resistance.

Additional keywords: disease resistance, *Oryza sativa*, *Pyricularia grisea*, virulence.

In the study of plant–fungus interactions, a rapid and accurate measurement of fungal growth in planta often is required to adequately evaluate fungal pathogenicity and host resistance. Various methods have been developed to detect and quantify fungal pathogens in plant and soil environments. For example, total fungal biomass has been estimated by measuring levels of fungal constituents such as ergosterol or chitin (10,11,26,32,33). This method often lacks specificity and does not always correlate with the fungal biomass due to fungal sporulation and interference from enzymatic activities in plants (5,40). Immunological methods also have been used for fungal biomass determination (8,14,21,30). However, it is generally difficult to obtain antibodies with the required specificity for particular fungi (45). In addition, immunoassays often are complicated by false-positive reactions (39,41). Many studies have used GUS (a bacterial β -glucuronidase gene)-transformed strains to quantify fungal biomass in plants (1,7,20,22,31,44). Although these genetically modified strains offer relatively easy and quick quantification of fungi in plants, the biggest disadvantage of this method is the requirement of transgenic fungi that are not available in most cases. There are also great concerns for using transgenic fungi in field trials because of environmental consideration. Furthermore, several researchers have reported that the GUS enzyme was not always an adequate marker for necrotrophic pathogens, because GUS activity did not correlate with the fungal biomass due to inactivation of GUS enzyme in the lesion (4,43).

During the past several years, the polymerase chain reaction (PCR) method has emerged as a major tool to detect and identify

fungal pathogens. Specific primers can be designed to distinguish closely related fungal species; therefore, it offers higher sensitivity and specificity than many other methods (16). A number of studies have used quantitative PCR to measure plant pathogenic fungi during fungus–plant interactions (12,18,19,24). However, such quantitative data is not directly derived from target DNAs but from the ratio between target and competitor DNAs. It also needs additional post-PCR confirmation steps such as running gels and image processing. More recently, the real-time PCR technique has been developed to detect and quantify DNAs (15). This technique combines thermal cycling with online fluorescence detection of the PCR amplification and records a specific cycle number, in term of cycle threshold (Ct), at which a statistically significant increase in the fluorescence (just greater than the background) can first be detected. The Ct is inversely proportional to the log of the initial template of DNA. The more templates that are initially present in PCR samples, the smaller the Ct it takes to get to a point where the fluorescent signal is detectable above background. The Ct value has been shown to be the most reliable parameter to detect and quantify target DNAs with real-time PCR (15). The quantitative data based on Ct is more accurate than data derived from endpoint determinations because Ct is a measurement taken during the exponential phase of PCR amplification when the PCR efficiency has yet to be influenced by limiting reagents, small differences in reaction components, or cycling conditions. As a result, real-time PCR technique has been increasingly used to detect or quantify viral, bacterial, and fungal plant pathogens (2,3,6,9,28,34,36,46).

Among RNA-based methods, northern blot analysis has been used to quantify fungal biomass in infected plants by measuring the constitutive expression of fungal genes such as actin, β -tubulin, and rRNA genes (4,23,27). During the infection, the amount of fungal biomass increases with time in infected tissues. Conse-

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quently, the proportion of certain fungal RNAs increase with time in total RNAs extracted from infected plant tissues. Thus, the level of constitutively expressed fungal genes could be used to calculate fungal biomass in infected plants.

Rice blast, caused by *Magnaporthe grisea* (T. T. Hebert) Yaegashi & Udagawa (anamorph *Pyricularia grisea* (Cooke) Sacc.), is a serious fungal disease that has a significant negative impact on rice production in the world. The blast fungus can penetrate directly through the outer cell wall into epidermal cells and grow intracellularly and intercellularly (17). Up-to-date evaluation of the fungal pathogenicity and host resistance is mainly based on disease ratings or measurements of blast lesion number and size (13,37,38). Although these methods are quick and easy to perform, they only provide a visual estimation rather than an accurate measurement of *M. grisea* growth in rice plants. In this study, we have evaluated DNA-based real-time PCR and RNA-based northern blot/phosphorimaging analyses for the quantification of *M. grisea* growth. A pair of DNA primers and a 330-bp probe were designed based on the 3' end of *M. grisea* 28S ribosomal gene (*rDNA*) and tested for detection sensitivity and specificity. The reproducibility and consistency of both methods have been verified and compared. Furthermore, DNA and RNA standard curves were constructed to facilitate the calculation of relative fungal growth or absolute biomass. We have demonstrated that both methods can be used to reliably quantify the growth of *M. grisea* in rice plants.

MATERIALS AND METHODS

Fungal isolate and plant materials. The fungal isolate used in this study belongs to the IC-17 pathotype of *M. grisea*. The blast fungus was grown at 24°C for 5 days in a previously described medium broth (13). Mycelia were harvested and washed twice with sterilized water by filtration through a Buchner funnel onto Whatman filter paper. Fungal mycelia then were blotted dry and weighed. For DNA extraction, fungal mycelia were freeze dried, weighed, and then stored at -80°C. For RNA extraction, fresh mycelia were immediately frozen with liquid nitrogen and stored at -80°C. To obtain conidia for fungal inoculation, the blast isolate was cultured on rice bran agar at room temperature (≈23°C) for 8 days before harvesting conidia.

The cultivars of rice (*Oryza sativa* subsp. *japonica*) used in the study were Drew, Wells, and M201. Whereas Drew is resistant, both Wells and M201 are susceptible to this blast isolate under greenhouse conditions.

Pathogen inoculation. Rice seedlings were grown in a computer-controlled greenhouse at temperatures of ≈28°C day and 25°C night. Two-week-old rice seedlings were spray inoculated with *M. grisea* at a concentration of 2.5×10^5 spores/ml. After inoculation, the seedlings were first incubated in a dew chamber at 22°C for 24 h at 100% relative humidity and then returned to the greenhouse. Leaf samples were collected at 0, 2, 4, and 6 days postinoculation (DPI), immediately frozen with liquid nitrogen, and then stored at -80°C freezer. Disease was scored in a scale of 0 to 9 at 6 DPI according to the disease rating method described previously (13).

DNA extraction. Fungal mycelia or infected leaf tissue was ground with liquid nitrogen and transferred into a Eppendorf tube containing 400 µl of extraction buffer (0.3 M NaCl, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, 2% sarkosyl, 0.5% sodium dodecyl sulfate, 5 M urea, and 5% Phenol). Subsequently, 400 µl of phenol/chloroform solution (1:1, pH 8.0) was added. After vortexing for 10 min, samples were centrifuged for 5 min at 14,000 rpm. Supernatants were transferred into a new tube and DNAs were precipitated with 0.7 volume of isopropanol by centrifugation (5 min at 12,000 rpm). Precipitated DNAs were washed with 70% ethanol, air dried, and dissolved in 40 µl of Tris-EDTA (TE) buffer containing RNase at 10 µg/ml.

Primer and probe design. A specific pair of DNA primers (forward, 5'-TAC GAG AGG AAC CGC TCA TTC AGA TAA TTA; reverse, 5'-TCA GCA GAT CGT AAC GAT AAA GCT ACT C) were designed based on the 3' end of the *M. grisea* 28S *rDNA* gene. PCR amplification with this pair of primers yielded a 330-bp fragment that was used as the probe to detect rRNA in northern blot analysis. The 28S *rDNA* gene is present at as many as 100 or more copies per genome in fungi and is constitutively expressed at very high levels; therefore, it would provide very good detection sensitivity at both the DNA and RNA levels (25,35).

Real-time PCR assay. Real-time PCR was performed using a MicroAmp Optical 96-well reaction plate and MicroAmp Optical Caps (PE Biosystems, Foster City, CA) in an ABI Prism 7700 Sequence Detection System (PE Biosystems). Each well contains a 25-µl reaction mixture that includes 12.5 µl of 2× SYBR Green PCR Master Mix (PE Biosystems) and 12.5 µl of DNA solution (1 pmole each of forward and reverse primers and 15 ng of rice DNA templates). To construct a standard curve for calculation of the fungal DNA amount and biomass, a series of known amounts of *M. grisea* DNA (1, 10¹, 10², 10³, or 10⁴ pg) were mixed with 15 ng of rice DNA isolated from uninfected leaves. Thermal cycling conditions consisted of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

RNA extraction and northern blot analysis. Total RNAs were extracted from the fungal mycelia or infected leaves using TRIZOL reagent according to the manufacturer's instructions (Life Technologies Inc., Rockville, MD). Total RNA (10 µg) was fractionated on a 1.2% agarose gel and blotted onto a nylon membrane (MSI, Westborough, MA). The blots were hybridized with the [α -³²P] dCTP-labeled 330-bp *rDNA* fragment of *M. grisea* using Perfect-Hybridization buffer (Sigma-Aldrich, St. Louis) at 68°C overnight, and washed at a high stringency as described by the manufacturer. The radioactive signals of hybridized 28S rRNA of *M. grisea* were determined by phosphorimaging (Molecular Dynamics, Sunnyvale, CA). In addition, sample loading was normalized based on rice 25S rDNA in the same northern blot. A series of known amounts of *M. grisea* RNAs (0.2, 2, 20, and 200 ng), together with 10 µg of rice RNA from uninfected leaves, were also loaded in RNA gels to construct RNA standard curves for calculation of the fungal RNA amount and biomass.

RESULTS

Primer specificity and DNA standard curves. The accuracy of PCR-based assays can be significantly affected by the specificity of DNA primers. To evaluate the specificity of the primer pair used in our assays, an agarose gel analysis was performed after 40 cycles of real-time PCR on ABI Prism 7700 Sequence Detection System. The expected 330-bp fragment was only observed with samples containing 10 pg or more of *M. grisea* DNA, but not with samples containing 1 pg of fungal DNA (likely due to low amount of the template) or rice DNA (15 ng) only (Fig. 1A). Furthermore, no additional fragments were visible on the gel. These results indicate that the primer pair was specific for the 28S *rDNA* of *M. grisea*, and not interfered by the presence of rice DNA.

Amplification profiles of the same samples generated with cycle-by-cycle collection of fluorescence are shown in Figure 1B. The ΔR_n values for the no-template control and rice DNA control remained at the baseline throughout 40 cycles; therefore, their Ct values were 40. In contrast, the Ct values for the 10⁴-, 10³-, 10²-, 10¹-, and 1-pg samples of *M. grisea* DNA were 17.78, 21.01, 26.61, 31.75, and 36.47, respectively. These results further confirmed that the primer pair was specific to 28S *rDNA* of *M. grisea* and not interfered by the presence of rice DNA.

Following the analysis of amplification profiles, a standard curve was constructed based on the starting amounts (10⁴, 10³,

10^2 , 10^1 , and 1 pg) of *M. grisea* DNA (in the presence of 15 ng of rice DNA) versus their Ct values obtained from real time PCR (Fig. 1C). A linear relationship was observed when Ct values were plotted against the logs of the initial amounts of *M. grisea* DNA. With this standard curve, each 10-fold difference in initial DNA amounts was represented by approximately five-cycle differences in Ct, and the linearity extended at least over 10^4 -fold range. The

correlation coefficient (r) for the standard curve was 0.99 (an average from three tests of different DNA sample preparations), indicating that the individual data points were highly correlated with the calculated linear regression. In addition, a standard curve was constructed for conversion of the fungal DNA amount to its biomass (dry weight, Fig. 1C). This was derived according to extractable DNA amounts (from the average of three carefully conducted extractions) in freeze-dried fungal mycelia. The standard curves allowed for the calculation of *M. grisea* DNA amount or biomass in infected rice plants based on the test sample's Ct values obtained from real-time PCR.

Probe specificity and RNA standard curves. In RNA-based northern blot/phosphoimaging analysis, the 330-bp *rDNA* fragment amplified by the above primer pair was used as a probe to hybridize RNA blots loaded with various amounts (0.2, 2, 20, and 200 ng) of *M. grisea* RNA in the presence of 10 μ g of rice RNA. A hybridizing band was observed in samples with *M. grisea* RNA, but not in the sample with rice RNA only (Fig. 2A). Under our experimental conditions, therefore, the probe was very specific to the 28S rRNA of *M. grisea* and was not interfered by rice RNA. Although the radioactive signal for the 0.2-ng *M. grisea* RNA sample was difficult to see on the northern blot (Fig. 2A), it was detectable using phosphorimaging. The average counts of radioactivity for 0.2-, 2-, 20-, and 200-ng *M. grisea* RNAs were 77, 180, 1,254, and 6,577, respectively. Following northern blot/phosphoimaging analysis, a standard curve was constructed based on the initial amounts (0.2, 2, 20, and 200 ng) of *M. grisea* RNA versus their radioactivity counts (Fig. 2B). The linearity of the standard curve extended at least over 10^3 -fold range with a correlation coefficient of 0.99 (an average from three tests of different RNA sample preparations), indicating a high correlation between the individual data points and the calculated linear regression. To convert the fungal RNA amount to biomass, a standard curve was derived according to the extractable RNA amounts (from the average of three carefully conducted extractions) in fungal my-

Fig. 1. Real-time polymerase chain reaction (PCR) analysis and construction of DNA standard curves. **A**, Visual evaluation of real-time PCR products (40 cycles) in a 1% agarose gel. All samples contained 15 ng of rice DNA in addition to different amounts of *M. grisea* DNA. **B**, Real-time amplification profiles of *Magnaporthe grisea* DNA samples generated from cycle-by-cycle collection of fluorescence. A ΔRn value represents a difference in fluorescence intensity between two cycles. A cycle threshold (Ct) value represents a specific cycle number at which the ΔRn value becomes greater than the base line. **C**, Standard curves for conversion of Ct values to the fungal DNA amount and biomass. The log of the initial amounts of *M. grisea* DNA was plotted versus the corresponding Ct values or fungal dry weights.

Fig. 2. Northern blot/phosphoimaging analysis and construction of RNA standard curves. **A**, *Magnaporthe grisea* RNA blots probed with the [α - 32 P] labeled 330-bp rDNA fragment. All samples contained 10 μ g of rice RNA in addition to different amounts of *M. grisea* RNA. **B**, Standard curves for conversion of radioactive counts to the fungal RNA amount and biomass. The log of the initial amount of *M. grisea* RNA was plotted versus the corresponding radioactive counts or fungal dry weights.

celia (Fig. 2B). Similar to real-time PCR assays, these RNA standard curves allowed for the calculation of *M. grisea* RNA amount or biomass in rice leaves based on radioactive counts obtained from northern/phosphoimaging analysis.

Reproducibility of real-time PCR and northern blot/phosphoimaging analyses. A reliable assay requires small variation and good reproducibility. In an effort to validate the reproducibility of the real-time PCR method, PCR amplifications were conducted using DNAs from three different preparations. The *M. grisea* DNAs obtained from three different preparations all were quantified with an ultraviolet spectrophotometer. Subsequently, real-time PCR was performed with 10^4 and 10^2 pg of *M. grisea* DNAs. Highly reproducible Ct values with very small standard deviation were observed among triplicate samples and three different preparations (Table 1). The standard deviation of Ct values within the triplicates is ≈ 0.02 to 0.12 and 0.06 to 0.08 for 10^4 and 10^2 pg of DNA samples, respectively. The standard deviation of Ct values among three different sample preparations was 0.23 and 0.72 for 10^4 and 10^2 pg of DNA samples, respectively. Relatively large variation at the lower concentration of DNA was probably due to stochastic effects that reduced the precise quantification of PCR. These results demonstrate that amplifications by real-time PCR are not only highly reproducible within replicates of the same sample preparation but also very reproducible among different sample preparations. We suggest that at least 10^2 pg of total DNA should be used to reduce potential stochastic effects and to obtain more reliable results.

To verify the reproducibility of northern blot/phosphoimaging analysis, total RNAs were extracted separately from three different samples of *M. grisea* and quantified with an ultraviolet spectrophotometer. Northern blot/phosphoimaging analysis was then performed using 200 and 2 ng of *M. grisea* RNAs from each sample preparation. The standard deviation of radioactivity counts

within triplicates of the same sample preparation was 270 to 437 and 11 to 19 for 200 and 2 ng of *M. grisea* RNA samples, respectively (Table 2). The standard deviation among three different sample preparations was 356 and 14 counts for 200 and 2 ng of *M. grisea* RNA, respectively. Therefore, data from RNA blot/phosphoimaging analysis have a good reproducibility within replicates from the same preparation or from three different preparations. However, it has a relatively larger standard deviation (5.7 to 8.1% of the mean) than that of the real-time PCR method (1.3 to 2.7% of the mean).

Determination of *M. grisea* growth in infected rice leaves. In order to demonstrate and compare these two methods for quantification of the fungal growth in planta, a blast isolate belonging to the IC-17 pathotype was inoculated onto two susceptible (M201 and Wells) and one resistant (Drew) rice cultivars. Six days after inoculation, broad spindle-shaped expanding lesions developed on the M201 and Wells cultivars. Both M201 and Wells were rated 6.0 on the 0-to-9 scale rating for their disease severity. In contrast, small reddish lesions developed on the Drew cultivar. The hypersensitive reaction on Drew was rated 2.0 on the 0-to-9 scale rating. To quantify the fungal infection, total DNAs were extracted from infected rice leaves at 0, 2, 4, and 6 DPI. The fungal DNA amount (or fungal biomass) in infected leaves was calculated using DNA standard curves (Fig. 1C) after determining the Ct values by real-time PCR. The infection was very restricted in resistant cv. Drew up to 6 DPI (Fig. 3). However, infection was detectable in susceptible cvs. M201 and Wells at 2 DPI. At 4 and 6 DPI, the growth of *M. grisea* was ≈ 46 and 66 times higher in M201 than in Drew, respectively.

Northern blot/phosphoimaging analysis was also used to quantify the infection of *M. grisea* in rice plants. The total RNAs extracted from infected leaves were blotted onto a nylon membrane and probed with a [α - 32 P]-labeled 330-bp rDNA fragment of *M.*

TABLE 1. Amplification reproducibility of the real-time polymerase chain reaction (PCR) from three independent DNA preparations^a

Sample preparations	10 ng DNA			0.1 ng DNA		
	Ct	Mean	Standard deviation	Ct	Mean	Standard deviation
1	17.50	...	...	25.81	...	...
	17.52	...	...	25.83	...	...
	17.53	17.52	0.02	25.93	25.86	0.06
2	18.00	...	...	26.92	...	...
	17.79	...	...	27.01	...	...
	17.99	17.93	0.12	26.85	26.93	0.08
3	17.84	...	...	27.21	...	...
	17.86	...	...	27.19	...	...
	17.89	17.86	0.03	27.32	27.24	0.07
	...	17.77	0.23	...	26.68	0.72

^a Three DNA samples were independently prepared from *Magnaporthe grisea* mycelia and analyzed by the real-time PCR in triplicate using 10 ng and 0.1 ng DNAs. The standard deviations in the last line of the table represent variations between samples. The real-time PCR assays were repeated once using exactly the same DNA samples and replicates and yielded nearly identical results. Ct = cycle threshold.

TABLE 2. Reproducibility of the northern blot/phosphoimaging analysis from three independent RNA preparations^a

Sample preparations	200 ng RNA			2 ng RNA		
	Count	Mean	Standard deviation	Count	Mean	Standard deviation
1	6,296.33	...	...	166.85	...	...
	6,835.87	...	...	152.74	...	...
	6,598.67	6,576.96	270.42	178.97	166.18	13.13
2	5,814.13	...	...	146.65	...	...
	6,642.58	...	...	136.65	...	...
	5,987.58	6,148.09	436.93	158.99	147.13	11.19
3	6,109.39	...	...	192.30	...	...
	5,513.45	...	...	154.10	...	...
	5,986.56	5,869.80	314.66	178.60	174.90	19.40
	...	6,198.28	356.24	...	176.20	14.20

^a Three RNA samples were independently prepared from *Magnaporthe grisea* mycelia and analyzed in triplicate by the northern blot/phosphoimaging assay using 200- and 2-ng RNAs. The standard deviations in the last line of the table represent variations between samples. The northern blot/phosphoimaging analyses were repeated once using the exactly same RNA samples/replicates and yielded nearly identical results.

grisea (Fig. 4A). After determining radioactive counts by phosphoimaging analysis, the fungal RNA amounts (or fungal growth) in infected leaves were readily calculated using RNA standard curves (Fig. 4).

To validate and compare these two methods, the DNA and RNA amounts of *M. grisea* in rice were converted to fungal biomass (dry weight) using the standard curves in Figures 1C and 2B. Both

Fig. 3. Growth of *Magnaporthe grisea* in seedling leaves of resistant (Drew) and susceptible (M201 and Wells) rice cultivars as measured by the fungal DNA amounts using the real-time polymerase chain reaction analysis. Each data point represents the average and standard deviation of three DNA samples from a single cultivar at a specific time (0, 2, 4, or 6 days post-inoculation). Leaf samples were collected from rice seedlings grown in three large trays (total nine trays for three cultivars). Each sample contained pooled leaves collected from six infected seedlings grown in the same tray. The experiment was repeated once with similar results.

Fig. 4. Determination of *Magnaporthe grisea* growth in infected rice leaves using northern blot/phosphoimaging analysis. **A**, RNA blots (10 μ g total RNA per lane) probed with radiolabeled *M. grisea* rDNA and rice 25S rDNA, respectively. **B**, Growth of *M. grisea* in seedling leaves of resistant (Drew) and susceptible (M201 and Wells) cultivars as measured by the fungal RNA amounts. Each data point represents the average and standard deviation of three RNA samples from a single cultivar at a specific time (0, 2, 4, or 6 days postinoculation). Leaf samples were collected from rice seedlings grown in three large trays (total nine trays for three cultivars). Each sample contained pooled leaves collected from six infected seedlings grown in the same tray. The experiment was repeated once with similar results.

real-time PCR and northern blot/phosphoimaging analysis gave very similar results with regard to the fungal biomass or growth of *M. grisea* in rice leaves in nearly all the measurements, indicating that these two methods are compatible and corroborate each other (Fig. 5). The discrepancy of the fungal biomass measured by the two methods at 4 DPI in M201 is likely due to sampling variations because not exactly the same leaves (but very similar samples from the same pots) were used in the real-time PCR and northern blot/phosphoimaging analyses. The efficiency of DNA and RNA extractions also may contribute to the discrepancy.

DISCUSSION

During the past several decades, the *O. sativa*-*M. grisea* interaction has been extensively studied because of its significant impact on worldwide rice production. As an excellent model system for studying cereal pathology, the entire genomes of both rice and *M. grisea* are now being sequenced. Although attempts have been made to detect and quantify the rice blast fungus using Southern hybridization and monoclonal antibody-based immunoassay (29, 42), to our knowledge, no adequate method has been developed to accurately quantify the growth of *M. grisea* in planta. In this study, we have evaluated the real-time PCR and northern blot/phosphoimaging analyses for quantification of *M. grisea* in rice plants. Our results show that both methods yielded comparable data and provide a sensitive and reliable measurement of *M. grisea* biomass in vitro or in planta. Most recently, the real-time PCR approach has been employed to quantify the growth of *Phytophthora* spp. in host plants (6) and to monitor *Pyrenophora* infection in barley seed (2,3). However, this is the first time that both DNA-based real-time PCR and RNA-based northern blot/phosphoimaging analyses were utilized and directly compared for the quantification of a fungal plant pathogen.

In comparison to the traditional disease rating that is based on blast lesion size and number, the DNA-based real-time PCR approach allows a more accurate calculation of relative growth and absolute biomass of *M. grisea*. However, the amount of fungal growth in planta may not always be proportional to the development of disease symptoms elicited by different blast isolates. Therefore, both quantification of the fungal growth and phenotypic assessment of disease symptoms are needed to more precisely evaluate virulence or aggressiveness of different blast isolates or mutants. Using our methods, blast resistance levels of different rice cultivars also can be more accurately evaluated (Figs. 3 to 5). When lesions just became visible at 4 DPI, the growth of *M. grisea* was 80 times higher in the susceptible cv. Wells than in the resistant cv. Drew, which carries a *Pita* resis-

Fig. 5. Comparison between the real-time polymerase chain reaction and northern blot/phosphoimaging analyses for quantification of *Magnaporthe grisea* growth based on the calculated fungal biomass in rice seedlings.

tance gene. Due to its high sensitivity and reproducibility, the real-time PCR method will be ideal for detecting minor changes of resistance or susceptibility in particular transgenic plants or mutants. Real-time PCR can be rapidly performed (usually within 3 h); therefore, it is more amenable for high-throughput analysis of a large number of samples. In contrast, northern blot/phosphoimaging analysis is relatively slow and more labor intensive. However, it may have an advantage to the investigators who study the expression of defense-related genes during disease resistance response. In these cases, both levels of defense gene expression and disease resistance (e.g., relative growth of *M. grisea*) can be accurately quantified and correlated using the same RNA blots prepared from same leaf samples.

To enhance the detection sensitivity, our PCR primers were designed based on the 3' region of 28S *M. grisea* rDNA that exists in multiple copies in the fungal genome. In addition, the 330-bp rDNA fragment amplified by the same pair of primers was used as a probe for the northern blot/phosphoimaging analysis in order to minimize potential variations between two assay methods. Consistent results were obtained with both methods using these rDNA primers and probe. Under our experimental conditions, we have not observed any complications (e.g., cross-reaction or cross-hybridization) with the leaf samples from growth chamber and greenhouse tests. The rDNA sequences are well conserved within closely related species and genera; therefore, the primers and probe may detect and quantify other related fungi that coexist in rice plants. Thus, extra care must be taken if the above primers and probe are to be used for analyzing leaf samples from rice fields. It will be more prudent to design highly specific primers for the real-time PCR detection and quantification of *M. grisea* in field samples. The protocol utilizes the double-stranded DNA dye SYBR Green I for detection of amplified products, it should be readily adaptable to any existing primer sets that are more specific for the blast fungus.

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