Quick and Accurate Detection and Quantification of *Magnaporthe oryzae* in Rice Using Real-Time Quantitative Polymerase Chain Reaction

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Abstract

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Rice blast, caused by *Magnaporthe oryzae*, is one of the most severe fungal diseases in rice worldwide. In this study, we developed methods to quickly and accurately detect and quantify *M. oryzae* in the pure cultures of the fungus, rice plants, and rice seed by using SYBR Green I of the real-time quantitative polymerase chain reaction (qPCR). Results of absolute qPCR show that *Magnaporthe oryzae* can be detected at as low as 6.9×10^{-5} ng of genomic DNA. Results also show

that all 10 varieties of rice seed examined in this study contain this fungus, indicating that *M. oryzae* is generally widespread in rice seed. We report the quantification of DNA of *M. oryzae* in rice leaves collected in the field, instead of in the lab, using relative qPCR by using rice *actin* gene as a housekeeping gene. Our results show great practical significance because we would know the potential fungal infection even before planting.

Rice blast, caused by *Magnaporthe oryzae* B.C. Couch (formerly *M. grisea* (T.T. Hebert) M.E. Barr) (6), is a significant disease that affects rice worldwide (16). This fungus damages the entire rice plant by infecting both the aboveground parts and roots (19), causing the reduction of rice production by 10 to 30% or sometimes up to almost 100% (15). This disease is extremely difficult to control, and its outbreaks and recurrence in large areas are major problems all over the world (8). Therefore, early and quick detection of this fungus is particularly important in order to control its infection and damage.

Several commonly used methods (e.g., capturing spores, recording the incidence of infections, and applying the disease index) have been used to detect the fungal pathogen *M. oryzae* (13,20). However, these methods are well known to be extremely labor and time consuming. More important, the results derived from these methods show varied levels of accuracy. Moreover, the molecular techniques polymerase chain reaction (PCR) and quantitative (q)PCR have also been applied to detect *M. oryzae*. For example, PCR methods are used to quickly detect *M. oryzae* in perennial ryegrass (11) and in rice seed (5).

Real-time qPCR has been widely used in detection and quantification of many kinds of viruses, bacteria, and fungi. For example, Poon et al. (17) have applied the qPCR technology to examine the pandemic potential of the novel human influenza virus H1N1. This technology is also used to detect and quantify the *Citrus tristeza virus* in plant tissues (2), the bacteria *Holophagae* and *Verrucomicrobia* (7), and fungal pathogens *Fusarium solani* in soybean roots (10) and *Puccinia striiformis* causing wheat stripe rust (22).

Furthermore, the qPCR methods are used to detect this fungus in infected rice plants (18). Qi and Yang (18) demonstrated that results of qPCR are consistent with those of Northern blot or phosphoimaging analyses, and the qPCR methods are much faster and more convenient than the latter. Berruyer et al. (1) used qPCR to examine the varied effects of different inoculation methods. Methods of detecting *M. oryzae* by using the TaqMan probe were also developed (12,14).

In previous studies, PCR-based (11) and qPCR-based (12) methods had been developed for detection of the pathogen. However, PCR has low sensitivity and cannot accurately quantify the amount of pathogen in rice plants, while the methods using qPCR to detect the pathogen are still limited to the lab instead of being available in the field. In this study, our goal was to develop a novel method to quickly and accurately detect and quantify *M. oryzae* in both rice leaves and seed by using qPCR before symptoms of rice blast appear on the host in the field, instead of being limited in the lab.

Materials and Methods

DNA extraction. The fungal species isolates used in this study (Table 1) were first cultured on potato dextrose agar (PDA) medium (200 g of potato, 18 g of dextrose, and 18 g of agar per liter) at 25°C. After 3 to 5 days, three plugs (5 mm in diameter) of fungal mycelia were aseptically transferred to 25-ml aliquots of liquid potato dextrose broth in 50-ml Erlenmeyer flasks. The cultures were constantly shaken at 25°C for 3 to 5 days. Mycelia were collected for fungal DNA extraction. Needles were used to pick fungal mycelia, which were washed two to three times using sterile water and dried using filter paper. To obtain plant samples, rice seed were first disinfected in 10% sodium hypochlorite for 3 min and washed three times using sterile water. The seed were then cultured in petri dishes with sterile water at 25°C for 1 to 2 weeks; the water in petri dishes was changed once a day. *Escherichia coli* DH5 α was cultivated in 20 ml of Luria-Bertani broth in 50-ml Erlenmeyer flasks at 37°C and shaken at 200 rpm for at least 8 h; then, the bacteria were collected for DNA extraction. The bacterial suspension was kept in 1.5-ml centrifuge tubes and centrifuged for 1 min at $10,000 \times g$; then, the supernatant was discarded and the precipitate was used to extract DNA. Due to the common presence of *E. coli* in the rice field, we chose *E. coli* as the candidate to ex-

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amine the specificity of PCR primers (see below). The fungus, *E. coli*, and rice tissues were ground separately with liquid nitrogen and DNA was extracted by using the Classic Genomic DNA Isolation Kit (Bio Basic Inc.), following the protocol provided by the manufacturer.

Primers and qPCR system. The sequences of qPCR primers, designed with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), targeting the 18S-28S region of ribosomal DNA (rDNA; GenBank accession number DQ493955), of *M. oryzae* were forward, 5'-GGCATCGTTAGCGGTCTTC-3', and reverse, 5'-CTACGAGGCTGCATAACGAC-3'. The selection of ribosomal gene is based on the study of Qi and Yang (18). The ribosomal gene is present at as many as 100 or more copies per genome in fungi and is expressed at high levels, thus providing sensitive detection at both DNA and RNA levels. Therefore, the minor variation in the number of copies will not largely affect the detection (3,4). This is an advantageous property that the single copy markers do not have. Primer sequences of a housekeeping gene of rice (*Oryza sativa*), the *actin* gene (GenBank accession number BT130427), were designed as forward, 5'-ATGGCAACA TTGTGCTCAGTG-3', and reverse, 5'-CCTCCGATCCAGACG CTGTA-3'. To determine whether the DNA extracts from the fresh rice plant samples and the rice seed in this study were free of PCR-inhibitors, the housekeeping gene *glutathione S-transferase* in *Aphis gossypii* Glover was used (9).

The qPCR reactions were performed by using the optical eight-tube strip with the ABI PRISM 7500 Real-Time PCR system and its software. The SYBR *Premix Ex Taq* used in this study was made by Takara BIO CATALOG and the reactions were performed in a 20- μ l volume based on the manufacturer's instructions: 10 μ l of SYBR *Premix Ex Taq*, 0.4 μ l of PCR forward primer (10 μ M), 0.4 μ l of PCR reverse primer (10 μ M), 0.4 μ l of ROX Reference Dye II, 2 μ l of DNA template, and 6.8 μ l of double-distilled (dd)H₂O. The thermal cycling program contained 1 cycle of 95°C for 30 s and 40 cycles of 95°C for 5 s and 62°C for 40 s.

Specificity and reproducibility. In order to establish the specificity of the qPCR primers of rDNA of *M. oryzae*, we applied qPCR to amplify the DNA samples of rice, bacteria, and fungus using primers designed based on a unique region of *M. oryzae* genome (i.e., 18S-28S region in rDNA). We also conducted BLAST analyses at the National Center for Biotechnology Information to confirm that the primers could not generate any nonspecific amplification and no similar sequences detected in any other species.

Table 1. Specificity and reproducibility of the primers in *Magnaporthe oryzae*^a

Sample	Ct ^b	NegRep ^c
<i>M. oryzae</i> 060104-2	17.663	None
<i>M. oryzae</i> 060302	16.756	None
<i>M. oryzae</i> 060307-2	17.432	None
<i>M. oryzae</i> 060308	17.929	None
<i>M. oryzae</i> 061409-1	16.029	None
<i>M. oryzae</i> 061412-1	16.565	None
<i>M. oryzae</i> 062203-1	17.819	None
<i>M. oryzae</i> 062802-1	17.061	None
<i>M. oryzae</i> 062806-2	17.775	None
<i>M. oryzae</i> 063201-1	17.991	None
<i>Escherichia coli</i> DH5 α ^d	35.431	1
<i>Fusarium solani</i> ^d	37.551	1
<i>Oryza sativa</i> var. Li-Jiangxintuanhegu ^d	37.654	1
<i>Rhizoctonia solani</i> ^d	38.216	2
Negative control (=pure water)	—	3

^a All strains of *M. oryzae* were collected from fields. Strain numbers of *M. oryzae* established in this study and isolate names of other species are given after the species names.

^b Cycle threshold.

^c Negative repetition (NegRep) indicates no fluorescence of the three repetitions in a sample.

^d Ct values are more than 35 cycles for these organisms.

The absolute qPCR was also used to determine the specificity and reproducibility of the primers of *M. oryzae* using genomic DNA templates of *M. oryzae* (10 strains), *E. coli* DH5 α , *F. solani*, *O. sativa* var. Li-Jiangxintuanhegu, and *Rhizoctonia solani*, the common organisms found in rice fields (Table 1). All fungal DNA extracts were diluted to 0.25 ng/ μ l by using the ultraviolet spectrophotometer. Diluted fungal DNA (2 μ l) was used as template and triplicate PCR reactions were performed for each run.

Sensitivity and DNA standard curve. Strain 040101-3 of *M. oryzae* was selected to conduct an absolute qPCR to determine the sensitivity of the primers of this fungus and to make the standard curve of these primers. DNA extract was diluted to 345 ng/ μ l by using the ultraviolet spectrophotometer. A 10-fold dilution series of the total DNA, ranging from 3.45×10^1 to 3.45×10^{-6} ng/ μ l, was incorporated into each absolute qPCR assay (2 μ l in each reaction well) with triplicates, which were used to generate the standard curve. A simple PCR was also carried out to evaluate the sensitivity of the qPCR and to determine the optimal conditions for amplification. Primers were the same for both the simple PCR and qPCR. Each of the simple PCR reactions contained 2 μ l of genomic DNA, 2 μ l of 10 $\times$ buffer, 2 μ l of Mg²⁺, 1 μ l of forward primer (10 μ M), 1 μ l of reverse primer (10 μ M), 1 μ l of dNTP (2.5 mM), and 0.5 μ l of *Taq* E, with ddH₂O added to a total volume of 20 μ l. The PCR program consisted of 94°C for 5 min; 30 cycles of 94°C for 30 s, 62°C for 45 s, and 72°C for 45 s; and a final extension of 72°C for 10 min.

Inhibition effects of DNA extracts on an insect gene amplification. Genomic DNA samples extracted from fresh rice plant samples and rice seed were diluted to 25 ng/ μ l and used in an absolute qPCR to determine whether PCR inhibitors were present in the DNA extracts from the pure cultures of *M. oryzae*. The feasibility of this method determines future experimental methods as well. For example, a single experiment of inhibition effect will be sufficient when a change of reagents or experimental procedures occurs. Primers of the insect *A. gossypii* on the gene *glutathione S-transferase* instead of the primers of *M. oryzae* were used. Each reaction contained 2 μ l of plasmid with 2 μ l of sample. Positive and negative control reactions were also conducted; they contained 2 μ l of plasmid and 2 μ l of sterile water, respectively. Each PCR reaction was run in triplicate.

The relative qPCR. The $\Delta\Delta$ Cycle threshold (Ct) method was used to conduct the relative qPCR (10). In this method, Ct values obtained for the target (i.e., genomic DNA of *M. oryzae*) and the internal control (i.e., rice *actin* gene), test sample, and a calibrator (i.e., the control sample; the $\Delta\Delta$ Ct is defined as 1) were used to calculate two parameters, Δ Ct and $\Delta\Delta$ Ct, as follows: Δ Ct = Ct_(target) – Ct_(internal control), while $\Delta\Delta$ Ct = Δ Ct_(test sample) – Δ Ct_(calibrator). The quantity of the target, which was normalized based on the internal control and relative to the calibrator, was given by the value of $2^{-\Delta\Delta$ Ct}. Relative qPCR was used to determine the relative quantities (RQs) of the genomic DNA in *M. oryzae* and the rice DNA relative to a control sample known as a calibrator.

A positive validation experiment was done prior to the $\Delta\Delta$ Ct experiment. The absolute value (M value) of the slope difference between the housekeeping gene and the target gene must be less than 0.1 in order to use the $\Delta\Delta$ Ct method (12). M value is defined as the absolute difference between the slopes of the standard curves of the housekeeping gene and the target gene. We modified the method of Gao et al. (10) to calculate the M value. Specifically, on the basis of the results of the validation experiment, we chose to use a subset of 10-fold dilution series of the total genomic DNA extracted from pure culture of *M. oryzae*, ranging from 6.9×10^1 to 6.9×10^{-2} ng and the same concentrations of the total genomic DNA of rice. These assays were run in triplicate. The slopes were calculated for both *M. oryzae* and rice, and the M value was calculated. Because the M value (= 0.086) calculated based on these experiments was less than 0.1, the $\Delta\Delta$ Ct method was validated to conduct the relative qPCR.

Detection and quantification of *M. oryzae* in rice plants by artificial inoculation. Relative qPCR assays were used to detect

and quantify genomic DNA of *M. oryzae* in rice plants, and seven strains (i.e., S18, N66, M247, L275, 2162, 3112, and 3117) of this fungus were inoculated on leaves of rice ‘Li-Jiangxintuanheigu’ in lab. Rice seedlings were grown in greenhouse at 28°C for about 2 weeks. Conidia of *M. oryzae* were harvested and resuspended to 10^5 conidia/ml in sterile water. Briefly, PDA mycelium disks, 8 mm in diameter were made of the activated fungi and were seeded in 9-cm petri dishes with medium containing oatmeal (30 g oat and 20 g agar per liter). They were cultured for 7 days at 25°C until mycelia covered the entire petri dish. The mycelia were collected using an SS-Spreader and cultured in moisturized cheese cloth until the conidia were generated. The conidia were washed off and collected using sterile water and the concentration was calculated by using a hemocytometer. The bacterial suspension was diluted to 10^5 conidia/ml and sprayed to the rice plants. After inoculation, the rice plants were kept in dark at 22°C for at least 1 day and then kept in greenhouse at room temperature with high moisture. One week later, leaf samples were collected and washed in sterile water, and 0.03 g of leaves of each sample was used for DNA extraction. Extracted DNA was dissolved in 30 μ l of sterile water, and a 50- or 100-fold dilution was prepared for later use. The relative qPCR reactions were conducted as described above using the extracted DNA.

The disease severity of samples N66, S18, M247, and L275 was assessed to be at levels 0, 1, 3 to 5, and 7 to 9, respectively. The affected levels of rice leaves are defined by the State Standard of the People’s Republic of China GB/T 17980.19-2000 (i.e., Guidelines for the Field Efficacy Trials [I]—Fungicides Against Leaf Disease of Rice). According to these standards, disease severity is categorized from level 0 (designated as not being affected) to level 9 (designated as 50 to 100% of leaf area affected).

Relative qPCR assays were run to detect and quantify the genomic DNA of *M. oryzae* inoculated on leaves of another nine rice varieties (i.e., Li-Jiangxintuanheigu, ‘jj88’, ‘Nipponbare’, ‘3’, ‘7’, ‘8’, ‘11’, ‘12’, and ‘15’) collected in both the greenhouse and the field. Three leaves were collected from each sample rice plant. The first leaf was cleaned in sterile water for three times, then stored as backup at –80°C. The second or the third leaves were chosen and 0.03 g of leaf tissue was used for DNA extraction. Extracted DNA was dissolved in 30 μ l of sterile water, and a 50- or 100-fold dilution was prepared for late use.

Detection and quantification of *M. oryzae* from rice seed. DNA was extracted from 10 samples of rice seed (i.e., ‘01-124’, ‘hejiang18’, ‘jida3’, ‘jida6’, jj88, Li-Jiangxintuanheigu, ‘y3-15’, ‘y3-21’, ‘y3-31’, and ‘y3-9’), which were harvested at the farm in Changchun, Jilin Province, China. For each sample, four to five seeds were used. Relative qPCR assays were run to detect and quantify *M. oryzae* from the rice seed. All of the qPCR reactions of *M. oryzae* were run as described above for rice plants and in triplicates.

Results

Specificity and reproducibility. Results showed that only the 10 strains of *M. oryzae* had Ct values of 16 to 18 cycles. There was no amplification of rDNA from other genomic DNA (i.e., *E. coli*, *F. solani*, *O. sativa*, and *R. solani*; Table 1), indicating that the primer pairs were specific for the genomic rDNA of *M. oryzae* and were not cross-reacted by other examined DNA samples (Table 1). Level of amplification was over the limit of detection (see standard curve below).

Sensitivity and DNA standard curve. The results of primer sensitivity, the DNA standard curves of qPCR, and the results of the simple PCR are shown in Figure 1. These results indicated that we reproducibly detected and quantified the total DNA of *M. oryzae* at as low as 6.9×10^{-5} ng in an individual reaction well (Fig. 1A). Following the analysis of amplification profiles, a standard curve was constructed based on the starting amounts of DNA of *M. oryzae* (6.9×10^1 ng, 6.9×10^0 ng, 6.9×10^{-1} ng, 6.9×10^{-2} ng, 6.9×10^{-3} ng, 6.9×10^{-4} ng, and 6.9×10^{-5} ng) versus their Ct values obtained from real-time PCR (Fig. 1B). The standard curve con-

tained the slope of –3.478 and Y_Inter 18.809, with an R^2 of 0.996. These results showed that, in 30 cycles, qPCR (Fig. 1A; 40 cycles) was about 10 times more sensitive than simple PCR (Fig. 1C) in amplifying these DNA samples; that is, sensitivity increased from

Fig. 1. Sensitivity and DNA standard curve of quantitative polymerase chain reaction (PCR). **A**, Results of seven dilution series. The average cycle threshold (Ct) values of A to G (H is not amplified) are 12.793, 15.814, 19.095, 22.542, 26.276, 30.106, and 33.238, respectively. **B**, DNA standard curve based on the starting amounts of DNA of *Magnaporthe oryzae* (6.9×10^1 , 6.9×10^0 , 6.9×10^{-1} , 6.9×10^{-2} , 6.9×10^{-3} , 6.9×10^{-4} , and 6.9×10^{-5} ng) versus their Ct values obtained from real-time PCR. **C**, Simple PCR in 30 cycles. Lanes A to G indicate DNA of 6.9×10^1 , 6.9×10^0 , 6.9×10^{-1} , 6.9×10^{-2} , 6.9×10^{-3} , 6.9×10^{-4} , and 6.9×10^{-5} ng, respectively. Lane M contains the known DNA markers (bands from top to bottom indicate 2,000, 1,000, 750, 500, 250, and 100 bp, respectively) and lane N indicates the negative control.

6.9×10^{-3} ng (Fig. 1C) to 6.9×10^{-4} ng (Fig. 1A). We conducted 40 cycles of qPCR and 30 cycles of simple PCR with the goal to identify the optimal number of cycles in order to compare the performance of qPCR and simple PCR. These results demonstrated the advantage of using qPCR over simple PCR in further conducting the detection and quantification of this fungus in rice plants and seed.

Inhibition effects of DNA extracts on an insect gene amplification. The Ct value of the positive control reaction was 20.072, while the Ct values of four rice plants ranged from 20.101 to 20.230, with their Δ Ct (i.e., the difference between the Ct value of a sample and the positive control) ranging from 0.029 to 0.158 (Table 2). The Ct values of four rice seeds ranged from 20.097 to 20.405, and their Δ Ct ranged from 0.025 to 0.333. These results demonstrated clearly that there were no inhibitors in these qPCR reactions.

Detection and quantification of *M. oryzae* in rice plants by artificial inoculation. The results showed that, by using rice sample N66 as the calibrator, the $RQ = 2^{-\Delta\Delta Ct}$ values varied from 1.35×10^1 to 1.64×10^3 (Table 3). The calculation of RQ value is explained in the section “The relative qPCR” in Materials and Methods. For example, N66 is used as the calibrator (Table 3) to calculate the $\Delta\Delta Ct$ of sample S18: $\Delta\Delta Ct_{S18} = \Delta Ct_{S18} - \Delta Ct_{N66} = 3.457 - 7.211 = -3.754$, then the RQ is $2^{-(-3.754)} = 13.49$, which is rounded up to 1.35×10 (Table 3). The RQ values were also correlated closely with the disease severity assessed based on affected leaf area (Table 3). Therefore, these results validated the use of rice *actin* gene as the housekeeping gene in the subsequent relative qPCR to quantify the fungal DNA. After inoculation, even

though the disease severity of N66 was assessed to be level 0, the fluorescence signal was detected by using its genomic DNA (Table 3).

Results from the above assays established the criteria to be used to determine whether a genomic DNA sample contained *M. oryzae* or not: (i) if $Ct < 32.00$, then the genomic DNA sample contains *M. oryzae*; (ii) if $32.00 \leq Ct \leq 35.00$, then the melt curve should be used to determine whether the DNA sample contains the fungus or not; and (iii) if $Ct > 35.00$, then it indicates that the DNA sample does not contain *M. oryzae* (Table 4). The selecting of these cut-off values is based on the following rational. At Ct value of 32, multiple peaks were observed on the melt curve; therefore, this was set as the starting point to observe the melt curve. On the standard curve, the signals were observed at Ct of 33. Furthermore, the presence of the fungus *M. oryzae* in Nipponbare rice was observed on the melt curve at Ct of 35, which was set as the ending point of examination. In other words, Ct values of 35 or less indicated the presence of the fungus, while Ct values of 36 or above indicated the absence of the fungus.

Relative qPCR assays were run to detect and quantify the genomic DNA of *M. oryzae* inoculated on leaves of another nine rice varieties collected in both the greenhouse and the field. Based on the above Ct cut-off values, the results (Table 4) indicated that six out of these nine samples contained the fungus (i.e., with Ct values of 35 or less).

Relative qPCR assays were run to detect and quantify *M. oryzae* from the rice seed. The results (Table 5) indicated that the Ct values of the 10 rice seeds ranged from 23.570 to 30.122, which are less than 32, the Ct cut-off value, indicating the presence of the fungus. In other words, all 10 rice seeds contained the fungus *M. oryzae*, with hejiang18 containing the least amount of this fungus

Table 2. Absolute quantitative polymerase chain reaction (PCR) assays testing for the presence of PCR inhibitors in DNA extracts from plants or seed of eight rice varieties

Sample ^a	Origin ^b	Ct ^c	Δ Ct ^d
z101	Seed	20.405	0.333
101	Plant	20.230	0.158
z126	Seed	20.386	0.314
126	Plant	20.181	0.109
z117	Seed	20.097	0.025
117	Plant	20.223	0.151
zjida6	Seed	20.277	0.205
jida6	Plant	20.101	0.029
Positive control	—	20.072	—
Negative control	—	—	—

^a Positive control contains pure insect plasmid DNA and negative control contains pure water.

^b Plant samples were cultured in the lab and Seed samples were collected in the field.

^c Cycle threshold.

^d Δ Ct is calculated as $\Delta Ct = Ct_{(sample)} - Ct_{(positive\ control)}$.

Table 3. Relative real-time quantitative polymerase chain reaction assays quantifying *Magnaporthe oryzae* inoculated in plants of Li-Jiangxintuanheigu rice in the lab

Sample ^a	Cycle threshold (Ct)		Δ Ct ^b	RQ
	Rice	<i>M. oryzae</i>		
<i>M. oryzae</i> 3112	22.717	19.245	-3.472	-1.64×10^3
<i>M. oryzae</i> 3117	23.461	20.315	-3.146	-1.31×10^3
<i>M. oryzae</i> 2162	23.257	25.011	1.754	4.39×10
<i>M. oryzae</i> N66	22.145	29.356	7.211	1.00
<i>M. oryzae</i> S18	20.778	24.235	3.457	1.35×10
<i>M. oryzae</i> M247	22.667	21.873	-0.794	-2.57×10^2
<i>M. oryzae</i> L275	21.575	19.416	-2.159	-6.62×10^2

^a Strain numbers of *M. oryzae* established in this study are given after the species name. Strain N66 is used as a calibrator in the calculations of relative quantity (RQ) values (i.e., the values of $2^{-\Delta\Delta Ct}$; see text for an example of calculating the RQ values) of *M. oryzae*. Negative control contains pure water and shows no amplification.

^b Δ Ct is calculated as the difference between $Ct_{(M. oryzae)}$ and $Ct_{(rice)}$.

Table 4. Relative real-time quantitative polymerase chain reaction assays quantifying *Magnaporthe oryzae* from rice plants grown in greenhouse and collected in the field^a

Sample	Source	Cycle threshold (Ct)		Δ Ct ^b	RQ
		Rice	<i>M. oryzae</i>		
Li-Jiangxintuanheigu	Greenhouse	22.621	36.049	13.428	—
JJ88 ^c	Greenhouse	22.308	34.708	12.400	2.74×10^{-2}
Nipponbare ^c	Greenhouse	22.923	35.076	12.153	3.25×10^{-2}
<i>M. oryzae</i> N66	Greenhouse	22.145	29.356	7.211	1.00
3 ^c	Field	23.030	32.012	8.982	2.93×10^{-1}
7	Field	22.084	36.900	14.816	—
8	Field	23.402	36.243	12.841	—
11 ^c	Field	24.420	32.136	7.716	7.05×10^{-1}
12 ^c	Field	24.508	33.480	8.972	2.95×10^{-1}
15 ^c	Field	24.575	31.939	7.364	8.99×10^{-1}

^a Strain N66 is used as a calibrator in the calculations of relative quantity (RQ) values of *M. oryzae*. RQ values are not reported in three samples (i.e., Li-Jiangxintuanheigu, 7, and 8) when the fungus is not detected. Negative control contains pure water and shows no amplification in 40 cycles.

^b Δ Ct is calculated as the difference between $Ct_{(M. oryzae)}$ and $Ct_{(rice)}$.

^c Fungus is detected in these samples.

(ΔC_t of 4.569) while jida3 rice harbored the highest amount of this fungus (ΔC_t value of -3.271).

Discussion

In our study, we developed a novel method by using qPCR to quickly and accurately detect and quantify *M. oryzae* in rice plants and seed prior to its causing significant damage to crop production (Fig. 1; Tables 3 and 4). Results of absolute qPCR show that *M. oryzae* can be detected at as low as 6.9×10^{-5} ng of genomic DNA. This method enables us to quantify the fungus *M. oryzae* based on the quantity of DNA in the rice genome, instead of the quantity of the fungus DNA itself. Our study is the first report of detection and quantification of *M. oryzae* by using qPCR in rice seed instead of in rice leaves (Table 5). Results show that *M. oryzae* is generally widespread in rice seed, as demonstrated in this study, where all 10 examined varieties of rice seed contained this fungus (Table 5). The detection of this fungus is determined by the C_t values (Tables 4 and 5). This method has great practical significance because we would know and predict the potential fungal infection in the same year even before the seed are planted. These results largely agree with the fungal contamination observed in the local (i.e., northeastern China) rice planting. For example, jj88 rice has been locally known to have high fungal infection, and this is supported by our data (i.e., jj88 contains high amount of fungus DNA in our study, with an RQ of 1.91×10^2 ; Table 5). Meanwhile, the nonlocal variety hejiang18 is cultivated only in the greenhouse, instead of in the field, and contains the least amount of fungus (RQ = 1.00) among all of the examined rice varieties (Table 5). These results would be supported by further study to include more varieties of rice seed.

We realized that it is important to detect the fungus in seed and attempted to recover the fungus from the rice seed. For example, we planted the rice seed and observed the development of rice blast. We also washed the seed and cultured the water used to wash the seed to observe the colonies grown on the PDA medium. However, we understand that, due to the various causes of rice blast and the inhibition of *M. oryzae* by other pathogens, these results cannot directly verify our results based on the qPCR. Future experiments on detection of fungal infection in seed will further improve our methods. Nevertheless, we believe that it is practically significant to demonstrate that our methods can quickly and accurately detect and quantify the fungus in the seed before they are planted, thus saving time and labor for farmers.

Previous studies (18) have focused on the detection and quantification of *M. oryzae* by inoculating the rice plants with *M. oryzae* in lab. The significance of our study is that, to our knowledge, this is the first report of using qPCR to detect and quantify *M. oryzae* in its host collected in the field instead of in lab. Furthermore, our method can quantify the pathogenic fungus and is more accurate than those using simple PCR to detect the fungus (5,11). Compared

with the TaqMan method of detecting this fungus (12,14,21), our methods are easier to operate and economically cheaper, making it more convenient to apply in the field on a large scale. The TaqMan method requires the design of two primers and probes and the specificity of both of the primers and the probes, whereas our method requires only two primers. Furthermore, PCR inhibitor or DNA from other organisms does not interfere with our method. Therefore, we believe that our method is appropriate and can be applied as a high-throughput technique for monitoring the development of this fungus in the field in order to provide accurate and timely theoretical support for fungal disease prevention and control.

Su'udi et al. (21) used TaqMan real-time PCR to quantify *M. oryzae* in rice leaves by measuring the gene *MHP1*, which is not commonly found in many other fungus pathogens. In our studies, we examined not only the rice leaves inoculated in the greenhouse but also the leaves collected from the field, demonstrating the capability of our methods to detect samples collected in the field. This application is further supported by our comparative studies of PCR inhibitory factors, sensitivity of the detection, and disease severity. Furthermore, we used SYBR Green I to detect the fungus in uninfected rice samples and several fungi and bacteria commonly found in the field. These results, together with those derived from BLAST analyses, revealed that the genetic markers are not found in these samples and established the high specificity of the genetic marker we have chosen in our study. Overall, our method, using SYBR Green I to detect the fungus in rice plants and seed in the field, is well established and reliable, thus presenting significant practical value.

Because rice blast is an extremely severe disease and may cause significant damage to rice production worldwide, the quantitative detection and prediction of the development of this disease is biologically and economically important. Although the detection of the disease would be accurate after the infection symptoms are observed, it would be a waste of pesticide, labor, and time because the damage is already done and subsequently causing reduction of crop production due to delayed treatment of the infection. Furthermore, it is generally misleading or inaccurate to use a previous year's infection in the field to predict the current year's infection incidence. Therefore, the significance of our method is to detect and quantify the fungus before the infection symptoms are observed in the current year. Furthermore, practical prediction based on these results will be more accurate, reliable, and timely. We strongly believe it is a major improvement to integrate infection predication with molecular biology in the field.

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Table 5. Relative quantitative polymerase chain reaction assays quantifying *Magnaporthe oryzae* in seed of 10 rice varieties collected in the field^a

Sample ^b	Cycle threshold (C_t)			RQ
	Rice	<i>M. oryzae</i>	ΔC_t^c	
01-124	27.047	25.595	-1.452	6.49×10
hejiang18	25.553	30.122	4.569	1.00
jida3	26.841	23.570	-3.271	2.29×10^2
jida6	29.358	30.581	1.224	1.02×10
jj88	27.433	24.429	-3.005	1.91×10^2
Li-Jiangxintuanheigu	26.092	25.095	-0.996	4.73×10
y3-15	27.493	26.288	-1.206	5.47×10
y3-21	27.119	29.201	2.082	5.61
y3-31	26.548	29.503	2.955	3.06
y3-9	26.613	27.041	0.427	1.77×10

^a Sample hejiang18 is used as a calibrator in the calculations of relative quantity (RQ) values of *M. oryzae*. Negative control contains pure water and shows no amplification in 40 cycles.

^b Fungus was detected in all of these samples.

^c ΔC_t is calculated as the difference between $C_t(M. oryzae)$ and $C_t(\text{rice})$.

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