Quantitative and Qualitative Influence of Inoculation Methods on In Planta Growth of Rice Blast Fungus

Romain Berruyer, Stéphane Poussier, Prasanna Kankanala, Gloria Mosquera, and Barbara Valent

Department of Plant Pathology, 4024 Throckmorton Plant Sciences Center, Kansas State University, Manhattan 66506.

Current address of S. Poussier: Institut National d'Horticulture, 2, rue Le Nôtre, 49045 Angers Cedex 01, France.

Accepted for publication 5 December 2005.

ABSTRACT

Berruyer, R., Poussier, S., Kankanala, P., Mosquera, G., and Valent, B. 2006. Quantitative and qualitative influence of inoculation methods on in planta growth of rice blast fungus. *Phytopathology* 96:346-355.

Molecular analyses of early disease events require infected plant tissue in which the pathogen is present in high quantities and interacts with the plant in a way found in the field. In this study, a quantitative polymerase chain reaction (Q-PCR) assay was developed to determine an "infection ratio" of fungal to plant cells in infected tissues. This assay was used to evaluate four inoculation methods (spray, mist, dip, and sheath) as well as use of whole plants or excised parts. Fluorescence stereomicroscopy was used to follow individual lesions developing from appressoria to macro-

scopic symptoms. Disease progression and outcomes were documented from 24 to 96 h postinoculation (hpi), as well as effectiveness of *Pi-ta*-mediated resistance. Even at 96 hpi, fungus proliferated well ahead of visible plant damage, especially in veins. Developing lesions sometimes were surrounded by greener areas in detached leaves. Spray inoculation was not sufficient for detecting fungal gene expression in planta before 96 h. Alternatively, a leaf sheath assay produced infected tissues containing 10 to 30% fungal DNA by 34 h. Used together, Q-PCR quantification and fluorescence stereomicroscopy will facilitate studies of early plant invasion because infection density and fungal growth stages are directly observed, not assumed from incubation time.

The rice (*Oryza sativa* L.)–*Magnaporthe oryzae* B. Couch (formerly *Magnaporthe grisea* (Hebert) Barr) (6) pathosystem has become a model for studying plant–fungus interactions. This fungus is being intensively studied due to the economic importance of the threat it poses to world rice production (28), and rapid progress has been made in understanding the fungal infection process (38). *M. oryzae* is able to infect all aboveground parts of the rice plant (28), and it has been shown to infect roots in laboratory studies (9,35). After spore germination, the germ tube differentiates into a penetration structure, the appressorium (15). Once penetration is achieved, the first hypha to enter the epidermal cell differentiates into specialized multicellular bulbous invasive hyphae (13). After filling the initial epidermal cells, longer, more conventionally cylindrical hyphae ramify out into adjacent tissue and the leaf tissue is colonized rapidly (38,41). The hemibiotrophic blast fungus initially uses a biotrophic strategy to enter and overcome living host cells. However, cellular and molecular details of fungal proliferation strategies inside the plant tissue remain to be described.

Most of the genetic and molecular studies of the plant–fungus interaction have focused on how the fungus gains entrance into host plant tissue (i.e., appressorium formation and function) (38). Appressoria use a mechanical penetration mechanism based on the build up of high turgor pressure inside the appressorial cell and focusing of this pressure to force a penetration peg through the outer plant surface (15). Environmental sensing and signal transduction pathways leading to appressorium formation and turgor pressure generation have been studied extensively (38). These studies were facilitated by various methods to stimulate appressorium formation in vitro. It has been possible to induce appressorial differentiation on artificial supports such as dialysis

membrane (16), mylar (15), polyester membrane (GelBond from Cambrex Bioproducts) (27), or leaf wax coated glass (22) and to extract fungal RNA (16), proteins (22), or other metabolites without contaminating them with rice compounds.

On the other hand, relatively few genetic determinants of tissue colonization have been identified. First, most nonpathogenic mutants that have been obtained identify genes involved in appressorium formation and function and not tissue colonization (38). Moreover, in vitro study of bulbous invasive hyphae has been limited. Although it has been possible to produce similar-appearing hyphae in an artificial medium (cellophane) (2), it is unknown if these hyphae physiologically resemble invasive hyphae formed in planta. As a consequence, the tissue colonization phase must be studied in planta. With this premise, studying the gene expression, protein production, and physiology of the fungus means having to cope with mixed materials, in which plant and fungus components are combined. Expression of individual genes can be studied by transformation with chimerical constructs bearing fluorescent protein domains (1), because the stability and activity of different fluorescent proteins in *M. oryzae* cytoplasm has been proven (3,7). General patterns of in planta fungal gene expression can be studied using the microarray technique if one is careful to design the microarray in a way that prevents cross-hybridization with rice nucleic acids. Two necessary characteristics of infected plant tissue for molecular study of early stages of plant–fungus interactions are high levels of fungal infestation and a plant–fungus interaction that is as relevant as possible to what occurs in the field.

Cytological studies have shown that most of the major blast resistance (*R*) genes prevent disease by blocking fungal growth after the pathogen penetrates and begins to grow within rice cells (23,28). Therefore, studies of the early stages of tissue invasion are critical for understanding how *R* genes recognize and respond to fungal avirulence (*AVR*) genes. Molecular and cellular studies on the cloned *Pi-ta* blast resistance gene and its corresponding *AVR-Pita* gene (5,17) suggest that these genes trigger resistance

Corresponding author: B. Valent; E-mail address: bvalent@ksu.edu

DOI: 10.1094/PHYTO-96-0346

© 2006 The American Phytopathological Society

during the early colonization stage. Indeed, current evidence suggests that the AVR-Pita metalloprotease triggers the hypersensitive resistance response in rice by binding the Pi-ta resistance protein inside rice cells (18). These results also suggest that understanding the other role for fungal AVR genes, in promoting disease in the absence of their cognate R gene, also will require a better understanding of early biotrophic colonization strategies.

The infection assay used in laboratory studies is a critical factor in determining whether these studies will relate to control of the disease in the field. Spray inoculation methods became a standard for the study of the rice-*M. oryzae* interaction (28). Among other interesting characteristics, spray inoculation is a reasonably good mimic of what occurs in the field and, thus, resistance or susceptibility responses observed in the laboratory can be expected to be observed in the field (42). Moreover, this method of inoculation can produce isolated lesions for assessment of plant resistance using a visual disease scale (42). This method is being used for microarray gene expression analysis experiments (25), but samples used are from late stages of infection (more than 4 days). In-depth studies of early infection events will require enrichment for numbers of penetration sites so that the events in the first few invaded rice cells can be detected against the background of plant cells that are not yet interacting with the fungus. Independent measurements of plant/fungus ratios in spray-inoculated tissues already have been performed using different methods (quantitative Southern, quantitative northern, semiquantitative and quantitative polymerase chain reaction [Q-PCR] and reverse transcriptase [RT]-PCR), although there is a large variation in the reported results (29,39).

New tools were needed to facilitate studies of early biotrophic invasion of the rice blast fungus, and for studies of the dual roles of fungal AVR genes in causing disease and in triggering hypersensitive resistance. The objectives of this study were to (i) develop a simple and reliable technique to document the proportion of fungus to plant components represented in particular infected tissue samples; (ii) develop a reliable technique to connect initial infection events with the eventual disease outcome (ranging from fully susceptible lesion to resistance) for individual infection sites; and (iii) apply these techniques to assess the impact of inoculation method on infection density, on fungal development in planta, and on maintenance of *Pi-ta*-mediated resistance.

MATERIALS AND METHODS

Plasmids, strains, and cultivars. The plasmid pBV13 was produced by insertion of the enhanced yellow fluorescent protein (*EYFP*) gene from pEYFP (BD Biosciences Clontech, Palo Alto, CA) into the *M. oryzae* vector pSM324 as described by Bourett et al. (3). This vector contains a bialaphos resistance gene for selection and the RP27 *M. oryzae* constitutive ribosomal promoter.

O-137 is a field rice blast isolate collected at the China National Rice Research Institute (Hangzhou) in 1985 by Y. Chen and B. Valent. This strain contains the avirulence gene *AVR-Pita* and, thus, is avirulent on *Pi-ta*-containing cultivars.

KV1 is a strain obtained by transformation of O-137 with pBV13 using a transformation protocol described previously (37). This strain constitutively expresses the *EYFP* gene. Yashiro-mochi is a *japonica* rice cultivar bearing the resistance gene *Pi-ta* (5). This cultivar is resistant to strains O-137 and KV1. YT16 is a doubled haploid rice line derived from a cross between the *japonica* rice cvs. Yashiro-mochi and Tsuyuake (44). This line is susceptible to both the O-137 and KV1 strains.

Plant cultivation and inoculation methods. Five rice seed per pot were planted in SunGrow Metromix 200 potting soil and incubated in a Conviron BDW120 growth chamber. The chamber was programmed to water the plants automatically four times per day with a 0.14% (wt/vol) solution of Jack's professional water-

soluble fertilizer 20-10-20 peat-Lite. Plants were grown under a combination of Standard Frost incandescent lamps (100 W) and ED37 (400 W) metal halide lamps (both from Philips Lighting Company, Sommers, NJ). At pot height, 1 m from the bulbs, light ranged in intensity from 600 to 700 $\mu\text{mol}/\text{m}^2/\text{s}$. Plants were grown at 80% relative humidity under a daily cycle of 12 h of light at 28°C and 12 h of darkness at 24°C. Plants were inoculated ≈ 3 weeks after germination.

The inoculum was a suspension of 10^5 conidia/ml (unless otherwise specified) in a water solution containing 0.25% gelatin (type B from bovine skin, Sigma G-6650) and 0.02% Tween 20 (polyoxyethylene sorbitan monolaurate; Qbiogene Inc., Carlsbad, CA), as optimized by Jia et al. (19). Conidial suspensions were prepared as described (42). For negative controls, the same gelatin and Tween 20 solution was used for mock inoculation.

Spray inoculation was performed as described by Valent et al. (42). Mist inoculation was performed on batches of four to six pots placed in 32-gal cylindrical plastic cans containing 200 ml of water. Inoculum was sprayed downward from a height of 0.5 to 0.7 m above the aperture of the can using an artist's airbrush (Paasche H No. 1) connected to compressed air at 4.1 bar (60 psi). This resulted in the formation of a turbulent mist of tiny droplets at the plant level. Inoculation was performed until leaf surface saturation (≈ 50 ml of inoculum). Cans subsequently were sealed for 24 h in order to maintain the high humidity required for the fungus to penetrate the cuticle of the host (15). For both inoculation methods, 1% red food coloring (Crown Colony; distributed by Safeway Stores Incorporated, Oakland, CA) was added to the inoculum to facilitate the selection of effectively inoculated leaf areas.

Dip inoculation was performed by immersing 7-cm segments of detached leaves in 50 ml of inoculum for 15 min. The leaves selected for dip inoculation were the youngest fully developed leaves from the primary stem of the plant. After inoculation, leaves were incubated in petri dishes over a wet sheet of filter paper (Whatman 90-mm qualitative filter paper disks, catalog number 1001 090) in order to maintain the high humidity needed for survival of the excised leaves.

Leaf sheath inoculation was based on the method described by Sakamoto (32) and Koga et al. (23). Briefly, leaf sheaths were detached from the plant and cut in ≈ 7 -cm segments. Inoculum was injected in the space delineated by the inner epidermis. The leaf sheaths were held horizontally in a petri dish over a wet sheet of filter paper until sampling.

Drop inoculation was performed as described by Jia et al. (19) on detached leaves selected as for dip inoculation, using 20- μl drops of a 10^3 conidia/ml inoculum (unless otherwise specified).

Material sampling and DNA extractions. For spray and mist inoculations, ≈ 0.5 cm² of leaf tissue was harvested from effectively inoculated parts of the youngest fully developed leaf from the primary stem of the plant. Sampling of dip-inoculated detached leaves also was done by harvesting ≈ 0.5 cm² of leaf tissues. Wounded tissue at the extremities of the detached leaves was not sampled. Leaf sheath samples were prepared as described by Kankanala and Valent (21). Immediately before analysis, the leaf sheath pieces were trimmed with a razor blade to produce inner epidermal cell sections that were highly enriched in infection sites. DNA extractions from infected tissue samples were performed using the Extract-N-Amp DNA extraction kit (Sigma-Aldrich, St. Louis) as described by Harmon (12).

Primers and concentration standards. The primer sequences SP6F (ACGACCCGTCTTTACTTATTGG) and SP6R (AAGT-ACGCTTGTTTTGTTGGAT) were based on the sequence of the repetitive element *Pot2* (European Molecular Biology Laboratory [EMBL] accession Z33638), an inverted repeat transposon found in *M. oryzae* (20). Those of the primers RB19F (CTCC-CTTGTTTCGGTCAAAAA) and RB19R (GACATTAAACAA-GAGGCAGCA) were based on the sequence of the monocopy

rice resistance gene *Pi-ta* (5) (EMBL accession AY196754). Both of these primer pairs were designed manually, considering the requirements for Q-PCR with the iCycler Real-Time PCR system from Bio-Rad. Particularly, the amplicon sizes (99 and 132 bp, respectively) were in the 50- to 150-bp range needed for consistent results in real-time PCR. Moreover, the absence of secondary structure in both the primers and amplified sequences was checked using the Mfold web server (46). Melting temperatures of the primers were determined as described by Breslauer et al. (4). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

PCR products from *M. oryzae* strain O-137 and rice cv. YT16 obtained with the primer pairs SP6F/SP6R and RB19F/RB19R, respectively, were cloned in the pGEM-T easy vector (Promega Corp., Madison, WI), yielding the plasmids pBV42 and pBV43, respectively. Lengths of pBV42 and pBV43 were 3,111 and 3,144 bp. Plasmids were extracted using the plasmid extraction kit Qiaprep spin (Qiagen, Venlo, The Netherlands) and diluted to a concentration of 2×10^9 molecules/ μ l. From these two solutions, different standard dilutions and mixes were made containing pBV42 and/or pBV43 at 10^2 to 10^7 molecules/ μ l.

Q-PCR assays. Q-PCR principles are explained in Raeymaekers (30) and Suzuki et al. (36). Briefly, a reactive (in the case of this study, SYBR Green) added in the PCR mixture becomes fluorescent when combined with double-stranded amplified DNA. During each cycle, fluorescence level is measured by the Q-PCR apparatus. The cycle at which the fluorescence level reaches a significant value above background (threshold cycle [C_T]) is in a linear relationship with the logarithm of the target DNA concentration. After amplification, a melt curve is generated by measuring fluorescence (i.e., proportion of double-stranded DNA) of the sample at various temperatures between 50 and 90°C. From this curve, it is possible to determine the melting temperature (T_m) of the amplified product, and to verify that primer dimers and non-specific amplification products did not occur.

Q-PCR was conducted in a 25- μ l volume using iCycler iQ 96 PCR plates and iCycler iQ optical quality tape with the iCycler thermocycler and the iCycler iQ Real-Time PCR detection system (Biorad). The C_T measurement for each sample was performed in three replicates. DNA template (1 μ l) was added to 24 μ l of master mix containing 12.5 μ l of iQ SYBR Green Supermix (Bio-Rad), 0.5 μ l (5 pmol) of each primer, 1.25 μ l (0.625 μ g) of acetylated bovine serum albumin (BSA) (Promega Corp., Madison, WI), and 9.75 μ l of autoclaved filter-sterilized double distilled water.

The Q-PCR amplification cycle was performed in four steps as follow: initial denaturation (95°C, 3 min), amplification (50 cycles consisting of 30 s at 59°C with fluorescence data acquisition and 30 s at 95°C), final elongation (3 min at 72°C), and melting curve (fluorescence acquisition after 10-s incubations at temperatures increasing by 0.5°C increments between 55 and 95°C).

Fluorescence data analysis, C_T calculation, and statistical data analysis were performed using iCycler iQ software (version 3), excel version 10 (Microsoft), and R statistical analysis software version 2.0.1 (31).

Microscopy. Microscopy of infected rice leaf sheath tissues was performed with an Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany) using an EC plan neofluar $\times 20$ objective. Fluorescence of the EYFP protein was observed using a fluoArc lighting system (Zeiss) and a YFP-specific filter (excitation 500 $\pm$ 20 nm, emission 535 $\pm$ 30 nm, filter set 46; Zeiss). Images were acquired using an Axiocam HRC camera and analyzed with Axiovision software (version 3.1; Zeiss).

Fungal growth and lesion development on rice leaves was observed using a MZ-FLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany). Fluorescence of the EYFP protein was detected using a 106Z-100W (Leica) lighting system and two

green fluorescent protein (GFP)-specific filter sets: GFPplus (excitation 460 to 500 nm, emission >510 nm) and GFPplant (excitation 450 to 490 nm, emission 500 to 550 nm).

RESULTS

Validity of the Q-PCR method. In order to test the validity, sensitivity, and reproducibility of the Q-PCR protocol, samples with known concentrations of DNA targets (fungus *Pot2* in pBV42 and rice *Pi-ta* in pBV43), and each of the two primer pairs (SP6F/SP6R or RB19F/SP19R) were used. In the first step, results were analyzed by linear regression analysis of the C_T data using the decimal logarithm ($\log_{10}$) of the number of cognate targets as the only quantitative variable (Table 1). For each primer pair, a good correlation ($r^2 > 0.989$) was observed between the concentration of target DNA and the observed C_T values when using the cognate primers.

The effect of mixtures of the *Pot2* and *Pi-ta* plasmids on C_T values was assessed by two quantitative factor analysis of variance using $\log_{10}(N_{pBV42})$ and $\log_{10}(N_{pBV43})$ as variation factors. The same good correlation was found between C_T and corresponding target DNA concentration, and no significant effect was found between C_T and alternate target DNA concentration (Table 1).

In order to assess the effect of mixtures of fungus and rice DNAs as well as the effect of adding BSA to the reaction mix, amplification of the *Pot2* target was performed in the presence and absence of BSA and with two kinds of standards, one in which pBV42 DNA was diluted in water, as described previously, and one in which pBV42 DNA was diluted in DNA extracts from an uninfected YT16 leaf sample. In these experiments, the regression coefficients were always consistent with $r^2 > 0.998$. Although the amplification was less efficient from leaf extracts than with pure samples, BSA had a positive effect on both efficiency and correlation coefficient that counteracted most detrimental effects of the impurities (Table 2).

Calculation of an infection ratio. For DNA samples from infected rice tissue, six Q-PCR amplifications, three with primers SP6F and SP6R (*Pot2*, *M. oryzae*) and three with RB19F and RB19R (*Pi-ta*, rice), were performed. During each independent Q-PCR experiment, amplification was performed on standards containing known concentrations of pBV42 and pBV43. From these standards, linear regression models were established, linking observed C_T s with target concentration $\log_{10}$. These models were used to calculate the number of copies of the target DNA in each sample from the observed C_T s. They also were used to calculate correlation coefficients and efficiencies.

The sequence used for quantifying rice DNA is a single-copy sequence from the rice *Pi-ta* gene, which has no homologous sequences in the *M. oryzae* genome. The *Pot2* transposon sequence used to quantify fungus has ≈ 100 copies in the *M. oryzae* genome (20). An infection ratio of $I = N_{Pot2}/(N_{Pi-ta} \times 100)$ was calculated from the determined number of target sequences of *Pi-ta* (N_{Pi-ta}) and *Pot2* (N_{Pot2}) in each sample. This infection ratio can be seen as a fungus cell per plant cell ratio (because *M. oryzae* hyphae are septate with a single nucleus per cell) (14), if it is assumed that the extraction efficiency is the same for fungal hyphae and plant cells.

Inoculation method greatly influences the rate of accumulation of fungal biomass in both susceptible and resistant tissues. In order to assess fungal growth, the susceptible cv. YT16 and the resistant cv. Yashiro-mochi were inoculated with spores of strain KV1 using four inoculation methods: spray, mist, dip, and sheath. Sampling from infected plants was done at five time points: 24, 34, 48, 72, and 96 h postinoculation (hpi). Mock-inoculated plants were sampled at two time points, 48 and 96 hpi. This experimental design was repeated three times for biological replication. DNA was extracted and I ratios were calculated from

two to five different leaf or leaf sheath samples for each sampling, which is at each time point and for each combination of repetition, cultivar, inoculum, and inoculation method.

I ratio data were analyzed by making a linear model from $\log_{10}(I)$ considering repetition as a block treatment. For KV1-infected samples, each cultivar–inoculation method–time combination was considered as a treatment. The block treatment estimate was not significantly different than zero ($P > \alpha = 5\%$). In most of the uninoculated samples (92%), no significant amplification occurred with the *Pot2* primers. Nevertheless, very high C_T s (corresponding to very low N_{pot2} figures) sometimes were recorded from these samples, and were considered as background noise. A mean $\log_{10}(I)$ ($= -3.109 \pm 0.13 = \log_{10}(I_0)$, $I_0 = 10^{-3.109} = 0.000778$) was calculated from those negative controls from which a $\log(N_{pot2})$ figure was calculable. Treatments with mean $\log_{10}(I)$ not significantly different than $\log_{10}(I_0)$ were considered not to be significantly infected. Because 40 different treatments were tested, in order to take a general primary risk $\alpha = 5\%$, samples were considered to be significantly infected only when $P < 0.132\%$ (Binomial law), with P being the probability of overcoming the t value associated with one treatment effect estimate. When $1\% > P > 0.132\%$, indicative data points are given between parenthesis. When $P > 1\%$, I is set to I_0 (Fig. 1).

Samples from spray-inoculated plants did not show significant amounts of fungus before 96 h, when lesions first were becoming visible (Fig. 2). At that time, the I ratio reached 0.17 (that is, ≈ 17 fungal nuclei for every 100 plant nuclei) in susceptible YT16 plants, whereas it did not show significant values in resistant Yashiro-mochi plants (Fig. 1A and B). This result is comparable with fungal RNA versus plant RNA ratio observed in similar conditions in other studies (25,40).

The tracking of fungal growth in susceptible rice inoculated using three other protocols (mist, dip, and sheath inoculations) showed that fungal biomass in inoculated tissue was strongly influenced by the type of inoculation (Fig. 1A). All three methods gave higher I ratios than observed after spray inoculation. I ratios were higher by two orders of magnitude in plant samples inoculated using the leaf sheath method. Intermediate ratios, ≈ 10

times higher than with spray inoculation, were obtained using dip and mist inoculation methods.

In the case of mist inoculation, numerous early symptoms were observed in susceptible plant leaves 96 h after inoculation (Fig. 2), whereas some tiny brown hypersensitive spots (type 1 resistance) (42) appeared on resistant Yashiro-mochi leaves. Eight days (192 h) after inoculation, susceptible leaves were covered completely with coalescing lesions, whereas symptoms did not develop in the incompatible interaction (Fig. 2). In some instances, susceptible plants inoculated using the mist technique eventually died.

Quantification of fungal growth in resistant plant tissues also showed strong differences depending on inoculation method (Fig. 1B). As expected, no significant fungal growth was seen 96 h

TABLE 2. Effect of plant extracts and bovine serum albumin (BSA) on the efficiency and the repeatability of the quantitative polymerase chain reaction (Q-PCR)^a

Dilutant mix	Water	Leaf extract
With BSA		
r^2	0.999322	0.999314
Intercept	38.42	39.66
Slope	-3.42	-3.535
Efficiency	96.03%	91.80%
Without BSA		
r^2	0.998264	0.998657
Intercept	38.953	39.995
Slope	-3.507	-3.627
Efficiency	92.80%	88.69%

^a In order to determine the effect of impurities in plant extracts and of BSA on the Q-PCR efficiency and reliability, Q-PCR experiments were conducted with primers SP6F and SP6R (*Pot2*, fungus) and known quantities of the *Pot2*-containing plasmid pBV42. The plasmid was diluted either in water or in a crude extract from uninoculated rice leaves. Analyses were performed with 0.625 μ g of BSA or without BSA added to the reaction mix. The values presented are the result of a linear regression analysis using $\log_{10}(N_{pot2})$ as factor and threshold cycle (C_T) as value. The efficiency of the Q-PCR is calculated from the slope (s). Here, $C_T = \text{intercept} + s \times \log_{10}(N_{pot2})$; thus, efficiency = $10^{-(1/s)} - 1$.

TABLE 1. One-factor and two-factor analysis of variance (ANOVA) of the threshold cycle (C_T) observed when amplifying *Pot2* or *Pi-ta* separately or from mixtures of both targets

Intercept, model	Coefficient ^a	SE ^b	t Stat	P^c
One-factor ANOVA				
Factor = $\log_{10}(N_{pBV42})$				
Variable = C_T with SP6F and SP6R as primers				
Intercept	41.09648	0.155919	263.5764	4.33×10^{-118}
$\log_{10}(N_{pBV42})$	-3.5535	0.034267	-103.701	3.44×10^{-86}
Model: $C_T = 41.096 - 3.553 \times \log_{10}(N_{pBV42})$				
$r^2 = 0.992707$; efficiency = 91.16%				
Factor = $\log_{10}(N_{pBV43})$				
Variable = C_T with RB19F and RB19R as primers				
Intercept	42.40032	0.162091	261.5832	4.40×10^{-119}
$\log_{10}(N_{pBV43})$	-3.79981	0.043742	-86.869	6.06×10^{-81}
Model: $C_T = 42.4 - 3.8 \times \log_{10}(N_{pBV43})$				
$r^2 = 0.98951$; efficiency = 83.30%				
Two-factor ANOVA				
Factors = $\log_{10}(N_{pBV42})$ and $\log_{10}(N_{pBV43})$				
Variable = C_T with SP6F and SP6R as primers				
Intercept	41.3069	0.266858	154.7899	7.8×10^{-99}
$\log_{10}(N_{pBV42})$	-3.55496	0.034312	-103.607	2.66×10^{-85}
$\log_{10}(N_{pBV43})$	-0.05789	0.059573	-0.97179	0.334
Factors = $\log_{10}(N_{pBV42})$ and $\log_{10}(N_{pBV43})$				
Variable = C_T with RB19F and RB19R as primers				
Intercept	42.36646	0.191699	221.005	4.71×10^{-112}
$\log_{10}(N_{pBV42})$	0.008193	0.024415	0.335563	0.73
$\log_{10}(N_{pBV43})$	-3.79956	0.043993	-86.3677	5.72×10^{-80}

^a Coefficient of the factor effect on the variable. All the factors presented here are quantitative.

^b Standard error of the coefficient.

^c Probability of overcoming the t value associated with the factor t variable to reach such a value by chance.

after spray, mist, or dip inoculations. Detached leaves inoculated using the dip method showed significant fungal growth until 48 h, at a level that can be compared with the susceptible interaction. At 72 and 96 h, the I ratio from these tissues dropped back to non-significant values. In mist-inoculated leaves, such early growth perhaps occurred, although it was not significant at the chosen level (i.e., $\alpha = 5\%$, $P < 0.132\%$). P values for mist inoculation of

Yashiro-mochi plants were 1% at 34 h, 0.2% at 48 h, and 0.7% at 72 h. Leaf sheath samples inoculated at 10^5 spores/ml showed heavy fungal growth that was delayed and reduced compared with what it was in susceptible plants. This breakdown of resistance did not occur in leaf sheath assays inoculated with 10^3 spores/ml (data not shown).

Interestingly, whatever the method of inoculation, the *Pot2*/*Pi-ta* ratio was not systematically lower in resistant than in susceptible plants until after 48 h. Starting at 72 h, *Pot2*/*Pi-ta* was lower (sheath inoculation method) or nonsignificant (other inoculation protocols) in resistant plants.

Real-time macroscopic overview of fungal development in planta by fluorescence stereomicroscopy. It previously has been difficult to connect early microscopic observations at individual infection sites with the type of macroscopic symptom developed days after initial observation of those sites. We demonstrated the feasibility of directly following macroscopic lesion development from individual appressoria in both compatible and incompatible interactions using a fungal strain that strongly expresses a constitutive EYFP transgene. Development of individual infection sites over 4 days is shown in Figure 3, comparing sites resulting from drop inoculation of susceptible YT16 or resistant Yashiro-mochi. Appressoria appeared as tiny green spots on the leaves under the fluorescent light source by 24 hpi (Fig. 3A). At 34 hpi, cell invasion continued at most infection sites in susceptible tissue, and at some infection sites in resistant tissue (Fig. 3B). At 48 h, pronounced differences were seen between susceptible and resistant interactions. Fungal growth in the resistant plant had drastically slowed, and a resistance reaction from the plant became visible as autofluorescence. Fluorescent fungus was still present at 48 hpi in some Yashiro-mochi infection sites (Fig. 3C). No EYFP fluorescence was seen at 72 and 96 hpi in the resistant interaction. On susceptible leaves, growth accelerated and lesions became visible. Sporulation began at the center of developing lesions 96 hpi under high humidity conditions in the petri dish.

Interestingly, the drop inoculation results (Fig. 3) were consistent with what was observed by Q-PCR in the other inoculation methods: Some fungal growth was visible in resistant interaction up until 48 h. Nevertheless, fungal hyphae did not grow on Yashiro-mochi after 48 h and their fluorescence disappeared. This can be interpreted as the death of the fungus due to plant resistance.

Stereoscopic imaging of fungal growth and early etiological observations. Visible lesion development always lagged behind fungal growth (Fig. 4). At 96 hpi, sporulation was visible in the

Fig. 1. Impact of inoculation method on the relative fungal content over time as measured by quantitative polymerase chain reaction in **A**, susceptible YT16 (*pi-ta/pi-ta*) or **B**, resistant Yashiro-mochi (*Pi-ta*) rice. The infection level of strain KV1 was evaluated by measuring the numbers of the single copy *Pi-ta* gene and an ≈ 100 -copy sequence from the *Magnaporthe oryzae* transposon *Pot2*. From these data, an infection ratio (*I*) that represents the relative number of fungus cells per plant cell is calculated. Each point corresponds to the estimated effect of time after inoculation and inoculation method, combined as one treatment. This estimate was calculated using a GLM model, from three blocks, corresponding to three separate repetitions of the experiment, and each consisting of two to five samples. I_0 is the mean *I* ratio observed in some of the uninoculated samples with the *M. oryzae* *Pot2* primers. When *I* ratio from a treatment is not significantly ($\alpha = 5\%$, $P = 0.132\%$) different from I_0 , the symbol is either put in parenthesis ($0.132\% < P < 1\%$) or brought to I_0 ($P > 1\%$).

Fig. 2. Visible symptoms 4 and 8 days after spray and mist inoculations on whole rice plants. Susceptible YT16 and resistant Yashiro-mochi rice plants were inoculated with *Magnaporthe oryzae* strain KV1, which contains the *AVR-Pita* avirulence gene and the enhanced yellow fluorescent protein transgene. These pictures were taken 4 days (1 and 2) and 8 days (3 and 4) after spray (1 and 3) or mist (2 and 4) inoculation with the same inoculation concentration of 10^5 spores/ml. Mist-inoculated susceptible leaves did not show typical individual lesions but, instead, developed dense, rapidly coalescing lesions.

lesions of drop-inoculated detached leaf pieces. Developing lesions often were surrounded by dark green areas. On spray-inoculated leaves, lesions visible 96 hpi were small, whitish spots, and sporulation did not begin when plants were incubated in the growth chamber (due to humidity $\leq 80\%$).

Although detached leaf or leaf sheath inoculation assays facilitate visualization of fungal growth, it long has been known that detached plant parts show enhanced susceptibility to rice blast

Fig. 3. Visualization of fungal growth in the same leaf over time. Growth of the enhanced yellow fluorescent protein (EYFP)-expressing strain KV1 was observed in detached rice leaves from susceptible (YT16) and resistant (Yashiro-mochi) cultivars. Leaves were inoculated using the drop method (19), with a 10^3 conidia/ml suspension. Pictures of the same area of the leaf were made under white light (White) or a green fluorescent protein plant (excitation 450 to 490 nm, emission 500 to 550 nm) filter (GFP), at **A**, 24, **B**, 34, **C**, 48, **D**, 72, and **E**, 96 h postinoculation (hpi). Although their shapes are not identifiable at this scale, appressoria are visible 24 hpi (a). As also can be seen on sheath-inoculated samples (data not shown), some appressoria (red arrowhead) already have penetrated the leaf tissue (a'). At 34 hpi, the first rice cell was invaded in some of the infection points in both the susceptible and resistant interactions (b). In the susceptible interaction, the fungus invaded neighboring rice cells (c). In the resistant interaction (c'), autofluorescence appeared around infection point by 48 h, whereas EYFP fluorescence from KV1 progressively disappeared after this time point, indicating fungal death. In the susceptible interaction, late plant cell reaction was visible as brown areas. Although less fast, fungal growth nevertheless continued around the outline (d) of the reacting area. Sporulation began by 96 hpi. White arrows show some of the most isolated spores. Red arrow in **D** shows faster growth along conductive tissue. Magnification $\times 25$, bar = 1 mm.

disease (23). In order to separate effects of inoculation method from effects due to use of excised plant pieces, spray-inoculated YT16 leaves were observed 96 hpi. Normal disease development in our whole leaf spray assay is shown in Figure 4A. In contrast, 96-h symptoms resulting from the same spray inoculation as in Figure 4A (except that these leaves were excised from the plant after 24 h and incubated in a petri dish as in the cut leaf assay) are shown in Figure 4B. This experiment showed changes in lesion color that result from excision of the leaf tissue. Green areas often are visible surrounding developing lesions as the rest of the leaf senesces. In all cases, the fungal growth front remained asymptomatic for a period before visible symptoms developed (highlighted in Fig. 4C and D). Moreover, we routinely observed that *M. oryzae* hyphae grew faster in conductive tissue than in adjacent leaf cells (Figs. 3 and 4, especially 4D).

Imaging and quantifying fungal growth in the same sample.

The Q-PCR assay has utility for characterization and standardization of infection assay conditions, but it is not practical to perform this procedure with each infection assay. Therefore, correlation between I ratios and visible fungal growth in the same samples was investigated. Leaf sheath-inoculated samples, when

Fig. 4. *Magnaporthe oryzae* grows ahead of the visible lesion. Stereomicroscopic images were taken of KV1 growing under different conditions in susceptible YT16 rice leaves 96 h postinoculation (hpi). Pictures in the left column were taken under white light, pictures in the right column correspond to the same rice leaf area but were taken using a green fluorescent protein-plus filter (excitation 460 to 500 nm, emission > 510 nm). Under this filter, healthy plant cells showed red fluorescence due to chlorophyll. Conditions were: **A**, standard spray inoculation on whole plants; **B**, spray inoculation as in **A** except that the leaf was detached from the plant 24 hpi and incubated in the same condition as dip inoculated leaves; **C**, dip inoculation on detached leaves; **D**, zoom in picture of the same leaf area as **C**. Red arrow shows faster fungal growth along conductive tissue. Dotted white lines in GFP pictures outline margins of some visible lesions. Magnification $\times 10$ (**A** and **B**), $\times 25$ (**C**), and $\times 63$ (**D**); bar = 1 mm.

prepared as described by Kankanala and Valent (21), were transparent enough for light microscopy without chemical treatments. Thus, it was possible to capture micrographs and measure I ratio by Q-PCR from the same tissue. This was done with all the leaf sheath samples used for this study. A correlation was observed between EYFP fluorescence and measured I ratio. Although the entire leaf sheath sample used for DNA extraction could not be documented with photomicrographs, representative images were recorded (Fig. 5). Not only does this analysis record relative density of fungus sites, it documents the morphological types of hyphae present as well as visible plant responses.

In general, there was good correlation between fungal growth as documented by photomicrographs and the measured I ratios. For example, sample 2 seemed to have fewer infection points than sample 1, but had a higher I ratio (Fig. 5). This can be explained by the fact that sample 2 was collected 10 h later than sample 1, providing more time for fungal growth at each penetration point. Nevertheless, this analysis documents inherent biological variability in this complex interaction. Different samples collected at the same time point sometimes gave different numeric results, and this diversity was reflected when imaging. An example of this can be seen by comparing samples 2 and 3 (Fig. 5), which show two independent 34-hpi samples of KV1 infecting Yashiro-mochi. Infection points are more numerous and the infection process is more advanced in sample 3 than in sample 2. The correlation between the visualized growth of the pathogen and the determined I ratio suggests that, once the assay is characterized, rapid visual scanning by fluorescence microscopy allows one to approximate the level of fungal infection.

Another observation in Figure 5 is that morphological types of the fungal hyphae are more homogeneous at early stages when bulbous invasive hyphae predominate (samples 1 to 3) than at late stages (samples 4 to 6) when both bulbous and classically cylindrical hyphae are visible (especially in sample 4). Plant reactions also may be visible at these later time points, such as in sample 6, where several epidermal cells have become dark brown due to a resistance response.

DISCUSSION

Use of Q-PCR as a counting tool. Numerous applications have been found for real-time Q-PCR in plant research. They range from detecting genetically modified organism contamination in food to research on gene expression analysis (11). Q-PCR also has been used for quantifying insert number in transgenic plants (26). In the domain of plant pathology, Q-PCR has been used for quantifying the pathogen in planta, generally for a diagnostic purpose (34). Here, we present a Q-PCR-based method for quantifying *M. oryzae* infection that is fast, specific, reproducible, and sensitive, and that permits measurement of plant infection in barely infected to heavily infected samples. Compared with bacterial pathogens, Q-PCR is needed more for fungal quantification. Bacterial growth in planta can be monitored by diluting and

Fig. 5. Correlation between I ratio and visual images of *Magnaporthe oryzae* growth in leaf sheaths. The I ratio, as calculated in this study, represented a relative ratio of fungus to plant cells. It is possible to both observe fungal growth and measure I ratios in the same samples. Thus, one determines the morphological form of the hyphae (and the degree of synchronous growth) together with the relative numbers of fungus and plant cells. The I ratios shown here are not means, but correspond to the sample from which the picture was taken. These photomicrographs were taken using the Axioplan 2 compound microscope at various times after inoculation of both resistant Yashiro-mochi and susceptible YT16 with the avirulent, enhanced yellow fluorescent protein-labeled KV1 strain, as follows: 1 = Yashiro-mochi, 24 h; 2 = Yashiro-mochi, 34 h; 3 = Yashiro-mochi, 34 h; 4 = YT16, 48 h; 5 = YT16, 72 h; and 6 = YT16, 72 h. Note that the EYFP fluorescence is shown here as white. Magnification $\times 200$, bar = 100 μm .

plating bacteria released from ground plant samples on selective media (10). Such a biological quantification method does not work for fungi that grow as cohesive mycelial hyphae that are not separated into single, viable cells from plant tissue.

Indirect methods of growth measurement are being used in order to assess cultivar resistance or strain aggressiveness. Such methods generally are based on later stages of fungal growth, such as symptom size, symptom number, or sporulation intensity (45). Alternatively, direct methods analyzing specific DNA or RNA content can give a chronologic view of fungal growth and, thus, earlier or more detailed information about quantitative aspects of the interaction.

A simple and reliable protocol. For molecular analyses, the relative ratio of fungus and plant nucleic acids is more important than the absolute biomass of the fungus. We developed a procedure for quantifying copies of fungus and plant genes in the same infected tissue samples. This method is faster and more sensitive than Southern-based quantification reported by Talbot et al. (39). Qi and Yang (29) reported Northern and real-time PCR quantification methods for monitoring the degree of infection of rice leaves by *M. oryzae*. Their method relies on quantitative DNA extraction from plant tissue in order to assess fungal DNA quantity per microgram of plant biomass. This DNA content is translated into fungal biomass by extrapolating a DNA extraction yield from fungus grown in liquid culture. Our method, assessing both fungus and plant DNA content in the same samples, does not require quantitative extraction of sample DNA, and does not require weighing the sample.

We also demonstrated the possibility of obtaining higher amplification efficiency from an easily made plant extract. Schaad et al. (33) used crude plant extracts and showed that it decreases the sensitivity of PCR-based pathogen detection. In our study, sensitivity and efficiency of the amplification were restored by using extracting chemicals that counteract the PCR inhibitors and the addition of BSA in the PCR reaction mix. BSA was used because it had been shown to enhance efficiency of numerous DNA-modifying enzymes (43), including DNA polymerases (24).

Comparison of quantification data with what has been observed in other studies. Fungal growth has been measured in infected plant tissues in other experiments (29,39) using spray-inoculated 15-day-old plants. Fungal nucleic acid quantities were measured using different methods (quantitative Southern, quantitative northern, and Q-PCR) from weighed infected tissues. Meanwhile, carefully conducted nucleic acid extractions were performed from fungus grown in liquid cultures. From the yield of these extractions, biomass/DNA ratios were calculated, which then were used to calculate fungus/plant biomass ratios. These different steps of calculations and conversions require several assumptions, including (i) the nucleotide extraction protocol has similar yields when conducted from hyphae growing in planta and those growing in liquid media; (ii) fungal DNA content is the same in hyphae grown in liquid media and the morphologically different biotrophic intracellular hyphae; and (iii) plant DNA content and extraction yield is the same in infected and uninfected tissue, especially at latter stages of infection when lignification processes take place in infected plant cells (8).

In this article, both plant and fungus DNA quantities were measured and the fungus DNA/plant DNA ratios were calculated in terms of fungus genome copies per plant genome copies (I ratio). We do make the assumption that the yield is the same for infected plant cells and for fungal cells infecting them. If one accepts that the I ratios are not absolutes and just compares them, then this assumption becomes: the DNA extraction yield difference between the infected plant cells and the fungal cells infecting them is always the same.

If one makes the same assumption as the previous authors, it is possible to use extraction yield data already established previ-

ously to recalculate infection ratios in comparable units. We expected that the experiments from Talbot et al. (39) and Qi and Yang (29) would give somewhat higher infection levels than in our experiment, because their infected plants were younger and, thus, more susceptible than ours. Three days after spray inoculation, infection level was roughly 300 times higher in Talbot et al. (39) than in this article. Four days after inoculation, infection level was ≈ 100 times higher in this article than in Qi and Yang (29).

Utility of different inoculation methods. We demonstrated that, using the same inoculum, the type of infection method has an effect of several orders of magnitude on the quantity of fungus growing in the plant at the earliest time points. For each infection method, we also determined how closely the fungal infection process resembled disease development seen in the field. Each inoculation method has its own value for molecular and cellular studies of fungal growth and plant–fungus interactions at different stages.

Spray inoculation is used routinely for studying rice–*M. oryzae* interaction (28). This inoculation method produces both susceptible and resistant interactions that closely resemble the situation in the field. However, fungus was not detected in spray-inoculated leaf tissues until 96 hpi, even using the repetitive *Pot2* sequence to maximize potential for detection of fungus genes, and this limits the possibilities for studying early infection stages.

Mist inoculation, inspired by assays mentioned by Ou (28), gave both larger I ratios and denser infection points resulting in rapidly coalescing symptoms. This method of inoculation probably results in more infection sites due to the higher density of smaller drops of inoculum deposited on the plant surface. This method does not result in the development of typical, individual eye-shaped blast symptoms that commonly are seen in the field. Nevertheless, the mist inoculation method is a good complement to the spray method, especially at intermediate stages (72 to 96 hpi), because it produces measurable I ratios, early symptoms similar to spray assays, and the possibility to use tissue from intact plants.

The highest fungus/plant ratio during early, biotrophic stages occurred in the leaf sheath assay because this infected tissue can be trimmed easily to exclude lower plant cell layers not yet invaded by the fungus. In our study, a mean I ratio of 0.232 (i.e., 23 fungal nuclei per every 100 plant nuclei) was found in the susceptible excised sheath tissue 34 hpi, showing a very high level of fungus content, by far the largest one at this time point. In preliminary experiments, total RNAs isolated independently from infected leaf sheaths at 34 hpi contained from 20 to 30% fungal RNAs (G. Mosquera and B. Valent, *unpublished data*). These two results combined suggest that leaf sheath inoculation is a good method for studying fungal gene expression during the early biotrophic stages of infection. Additionally, the infected sheath tissue was optically clear, allowing detailed visualization of the fungus–plant interactions before attempting further studies on the sample. As can be seen when comparing samples 2 and 3 or 5 and 6 (Fig. 5), fungal growth and resistance reactions from plant cells can vary from sample to sample, even when assay conditions are the same. Rapid microscopic observation of fluorescent fungus allowed selection of samples depending on actual in planta fungal growth stage, and other conditions such as the level of plant cell reaction. This can be especially interesting when performing RNA extractions for gene expression analysis, because expression level can be correlated with a specific stage of growth and morphological behavior of the fungus.

Nevertheless, the leaf sheath assay diverged slightly from nature in the later stages of infection, especially at higher inoculum concentrations. Koga et al. (23) made morphological comparisons of fungal growth between excised and intact leaf sheath. Higher plant susceptibility was observed with the excised sheath inoculation method. We had similar observations (data not

shown). This difference was seen 48 h after inoculation, suggesting that only the early infection process mimics what occurs in the field. In this study, vigorous—although delayed—growth also was observed in the resistant interaction at inoculum concentrations of 10^5 spores/ml.

Dip inoculation of detached leaves resulted in a more natural infection outcome than inoculation of detached sheaths. In compatible interactions, the I ratio increased exponentially to reach comparable values with what was seen in whole-plant inoculation methods. In the resistant interaction, although plant resistance was not overcome by the fungus, a significant I ratio ($\approx 6\%$) was observed 48 h after inoculation. This might allow studies of the fungus trying to grow in adverse conditions of plant cells engaging in a hypersensitive reaction, although it is not clear what accounts for this increased growth.

Outcomes of infection assays can differ under different growth conditions due to the effect of plant health on the fungal disease development. Normal fungus behavior was seen in the resistant interaction when using drop inoculation with conidial concentrations of 10^3 conidia/ml (Fig. 3). Similar observations were made at 10^2 and 10^4 conidia/ml, but resistance, although visible, was overcome and sporulation occurred when using an inoculum concentration of 10^5 conidia/ml. The ability to overcome the resistance seemed to be directly proportional to the inoculum concentration as it was observed on leaf sheaths inoculated with 10^5 conidia/ml but not at 10^3 conidia/ml (data not shown). Under conditions used by Jia et al (19), resistance specificity was maintained at 10^5 conidia/ml. This suggests that caution is required, together with careful characterization of particular assay conditions. The Q-PCR and stereoscopic techniques reported here will aid in assay characterization.

Conclusive remarks. In this article, we have presented a fast, reliable, and reproducible method for quantifying *M. oryzae* nucleic acids in infected rice tissues. Using this protocol together with fluorescence microscopy of EYFP-labeled fungus, we surveyed various inoculation methods for impact on in planta fungal growth. Classical spray inoculation remains the standard for predicting pathogenic potential in the field. Nevertheless, spray-inoculated plants showed low levels of fungal infection at early time points, impairing precise and effective study of the plant–fungus interaction because most of the plant cells were free of fungus. We recommend mist inoculation of whole plants for molecular analysis of intermediate disease stages (72 to 96 h), and the detached leaf sheath assay for molecular analyses of early in planta growth stages (24 to 48 h). A significant finding in this study is documentation that fungal growth always precedes visible lesion formation and that asymptomatic infected areas exist on rice leaves even in late stages of infection, whatever inoculation method is used. At no time did we detect a disease stage in which the fungus killed rice cells ahead of its growth front, suggesting that there may not be a switch to a distinct necrotrophic phase in rice blast disease. Details of hemibiotrophic growth in blast disease remain to be elucidated. Late stages (96 h and later) of fungal growth are characterized by heterogeneity of the infected samples, containing both asymptomatic and necrotic infected plant cells, bulbous and cylindrical fungal hyphae, and fungal growth in different tissues, such as epidermal, mesophyll, and conductive tissues. In these late stages, as shown in this study and by Koga et al. (23), the inoculation method, inoculum concentration, and state of the plant tissue had major effects on the disease outcome. Biological heterogeneity was less apparent during earlier in planta growth (up to 36 h), when biotrophic invasive hyphae predominated. The use of carefully devised inoculation methods and sampling times will permit the more precise study of different growth stages as the blast fungus proliferates throughout its host tissue. These results give a new importance to the inoculation method and sampling time choice when studying in planta growth physiology and gene expression of *M. oryzae*.

ACKNOWLEDGMENTS

This research was supported by the Kansas State Agricultural Experiment Station and by USDA-NRI grant no. 2002-35319-12567. We thank T. Todd for critical reading of the manuscript.

LITERATURE CITED

- Böhnert, H. U., Fudal, I., Dioh, W., Tharreau, D., Notteghem, J. L., and Lebrun, M. H. 2004. A putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. *Plant Cell* 16:2499-2513.
- Bourett, T. M., and Howard, R. J. 1990. In vitro development of penetration structures in the rice blast fungus *Magnaporthe grisea*. *Can. J. Bot.* 68:329-342.
- Bourett, T. M., Sweigard, J. A., Czymmek, K. J., Carroll, A., and Howard, R. J. 2002. Reef coral fluorescent proteins for visualizing fungal pathogens. *Fungal Genet. Biol.* 37:211-220.
- Breslauer, K. J., Frank, R., Blocker, H., and Marky, L. A. 1986. Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci. USA* 83:3746-3750.
- Bryan, G. T., Wu, K.-S., Farrall, L., Jia, Y., Hershey, H. P., McAdams, S. A., Faulk, K. N., Donaldson, G. K., Tarchini, R., and Valent, B. 2000. A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene *Pi-ta*. *Plant Cell* 12:2033-2046.
- Couch, B. C., and Kohn, L. M. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* 94:683-693.
- Czymmek, K. J., Bourett, T. M., Sweigard, J. A., Carroll, A. M., and Howard, R. J. 2002. Utility of cytoplasmic fluorescent proteins for live cell imaging of *Magnaporthe grisea* in planta. *Mycologia* 94:280-289.
- Dixon, R. A., and Harrison, M. J. 1990. Activation, structure, and organization of genes involved in microbial defense in plants. *Adv. Genet.* 28:165-234.
- Dufresne, M., and Osbourn, A. E. 2000. Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. *Mol. Plant-Microbe Interact.* 14:300-307.
- Feys, B. J., Benedetti, C. E., Penfold, C. N., and Turner, J. G. 1994. *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* 6:751-759.
- Gachon, C., Mingam, A., and Charrier, B. 2004. Real-time PCR: What relevance to plant studies? *J. Exp. Bot.* 55:1445-1454.
- Harmon, P. F., Dunkle, L. D., and Latin, R. 2003. A rapid PCR-based method for the detection of *Magnaporthe oryzae* from infected perennial ryegrass. *Plant Dis.* 87:1072-1076.
- Heath, M. C., Valent, B., Howard, R. J., and Chumley, F. G. 1990. Interactions of two strains of *Magnaporthe grisea* with rice, goosegrass and weeping lovegrass. *Can. J. Bot.* 68:1627-1637.
- Horino, O., and Akai, S. 1965. Comparison of the electron micrograph of conidia of *Helminthosporium oryzae* Ito & Kurib. and *Pyricularia oryzae* Cavara. *Trans. Mycol. Soc. Jpn.* 6:41-46. (In Japanese and English)
- Howard, R. J., and Valent, B. 1996. Breaking and entering: Host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annu. Rev. Microbiol.* 50:491-512.
- Irie, T., Matsumura, H., Terauchi, R., and Saitoh, H. 2003. Serial Analysis of Gene Expression (SAGE) of *Magnaporthe grisea*: Genes involved in appressorium formation. *Mol. Genet. Genomics* 270:181-189.
- Jia, Y., Bryan, G. T., Farrall, L., and Valent, B. 2003. Natural variation at the *Pi-ta* rice blast resistance locus. *Phytopathology* 93:1452-1459.
- Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., and Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J.* 19:4004-4014.
- Jia, Y., Valent, B., and Lee, F. N. 2003. Determination of host responses to *Magnaporthe grisea* on detached rice leaves using a spot inoculation method. *Plant Dis.* 87:129-133.
- Kachroo, P., Leong, S. A., and Chattoo, B. B. 1994. *Pot2*, an inverted repeat transposon from the rice blast fungus *Magnaporthe grisea*. *Mol. Gen. Genet.* 245:339-348.
- Kankanala, P., and Valent, B. 2005. Molecular and cellular biology of biotrophic interactions in rice blast disease. *Fungal Genet. Newsl.* 582(Suppl.):123.
- Kim, S. T., Yu, S., Kim, S. G., Kim, H. J., Kang, S. Y., Hwang Du, H., Jang, Y. S., and Kang, K. Y. 2004. Proteome analysis of rice blast fungus (*Magnaporthe grisea*) proteome during appressorium formation. *Proteomics* 4:3579-3587.
- Koga, H., Dohi, K., Nakayachi, O., and Mori, M. 2004. A novel inoculation method of *Magnaporthe grisea* for cytological observation of the

- infection process using intact leaf sheaths of rice plants. *Physiol. Mol. Plant Pathol.* 64:67-72.
24. Kreader, C. A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.* 62:1102-1106.
 25. Lagorce, A., Darchis, A., Munier, F., Morel, J.-B., De Rose, R., Beffa, R., and Lebrun, M.-H. 2005. Transcriptional analysis of the pathogenic fungus *Magnaporthe grisea* during rice infection. *Fungal Genet. Newsl.* 52(Suppl.):90.
 26. Mason, G., Provero, P., Vaira, A. M., and Accotto, G. P. 2002. Estimating the number of integrations in transformed plants by quantitative real-time PCR. *BMC Biotechnol.* 2:20.
 27. Mitchell, T. K., and Dean, R. A. 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* 7:1869-1878.
 28. Ou, S. H. 1985. *Rice Diseases*. 2nd ed. Commonwealth Agricultural Bureau, U.K.
 29. Qi, M., and Yang, Y. 2002. Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and northern blot/phosphoimaging analyses. *Phytopathology* 92:870-876.
 30. Raeymaekers, L. 2000. Basic principles of quantitative PCR. *Mol. Biotechnol.* 15:115-122.
 31. R Development Core Team 2004. R: A language and environment for statistical computing. Published online by the R Foundation for Statistical Computing, Vienna, Austria.
 32. Sakamoto, M. 1949. On the new method of sheath-inoculation on rice plants with blast fungus, *Pyricularia oryzae* Cav. for the study of the disease-resistant nature of the plant. *Bull. Inst. Agric. Res. Tohoku Univ.* 1:120-129.
 33. Schaad, N. W., and Frederick, R. D. 2002. Real-time PCR and its application for rapid plant disease diagnostics. *Can. J. Plant Pathol.* 24:250-258.
 34. Schaad, N. W., Frederick, R. D., Shaw, J., Schneider, W. L., Hickson, R., Petrillo, M. D., and Luster, D. G. 2003. Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annu. Rev. Phytopathol.* 41:305-324.
 35. Sesma, A., and Osbourn, A. E. 2004. The rice leaf pathogen undergoes developmental process typical of root infecting fungi. *Nature* 431:582-586.
 36. Suzuki, M. T., Taylor, L. T., and DeLong, E. F. 2000. Quantitative analysis of small subunit rRNA genes in mixed microbial populations via 5' nuclease assays. *Appl. Environ. Microbiol.* 66:4605-4614.
 37. Sweigard, J. A., Carroll, A. M., Kang, S., Farrall, L., Chumley, F. G., and Valent, B. 1995. Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus. *Plant Cell* 7:1221-1233.
 38. Talbot, N. J. 2003. On the trail of a cereal killer: Exploring the biology of *Magnaporthe grisea*. *Annu. Rev. Microbiol.* 57:177-202.
 39. Talbot, N. J., Ebbole, D. J., and Hamer, J. E. 1993. Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 5:1575-1590.
 40. Tucker, S. L., Donofrio, N., Dean, R., Henderson, D. A., and Orbach, M. J. 2005. Investigating the interaction between *Magnaporthe grisea* and rice through a comprehensive timecourse experiment using microarray analysis. *Fungal. Genet. Newsl.* 52(Suppl.):143.
 41. Valent, B., and Chumley, F. G. 1991. Molecular genetic analysis of the rice blast fungus *Magnaporthe grisea*. *Annu. Rev. Phytopathol.* 29:443-467.
 42. Valent, B., Farrall, L., and Chumley, F. G. 1991. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127:87-101.
 43. Williams, R., Kline, M., and Smith, R. 1996. BSA and restriction enzyme digestions. *Promega Notes Mag.* 59:46.
 44. Wu, K.-S., Martinez, C., Lentini, Z., Tohme, J., Chumley, F. G., Scolnik, P. A., and Valent, B. 1996. Cloning a blast resistance gene by chromosome walking. Pages 669-674 in: *Rice Genetics III*. Proc. Third Int. Rice Genet. Symp. G. S. Khush, ed. International Rice Research Institute, Manila, Philippines.
 45. Yeh, W. H., and Bonman, J. M. 1986. Assessment of potential resistance to *Pyricularia oryzae* in six rice cultivars. *Plant Pathol.* 35:319-323.
 46. Zuker, M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31:3406-3415.