Genomics-Based Marker Discovery and Diagnostic Assay Development for Wheat Blast

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Abstract

Wheat blast has emerged as a major threat to wheat production in South America. Although originally restricted to Brazil, the disease has since been observed in the neighboring countries of Argentina, Bolivia, and Paraguay and recently the pathogen, *Magnaporthe oryzae* *Triticum* pathotype, was isolated from infected wheat in Bangladesh. There is growing concern that the pathogen may continue to spread to other parts of the world, including the United States, where several *M. oryzae* pathotypes are endemic. *M. oryzae* pathotypes are morphologically indistinguishable and, therefore, must be characterized genotypically. Symptoms of wheat blast include bleaching of the head, which closely resembles the symptoms of *Fusarium* head blight, further complicating efforts to

monitor for the presence of the pathogen in the field. We used a genomics-based approach to identify molecular markers unique to the *Triticum* pathotype of *M. oryzae*. One of these markers, MoT3, was selected for the development of a polymerase chain reaction (PCR)-based diagnostic assay that was evaluated for specificity using DNA from 284 *M. oryzae* isolates collected from a diverse array of host species. Conventional PCR primers were designed to amplify a 361-bp product, and the protocol consistently amplified from as little as 0.1 ng of purified DNA. The specificity of the MoT3-based assay was also evaluated using *Fusarium* spp. DNA, from which no amplicons were detected.

Wheat blast, caused by the fungal pathogen *Magnaporthe oryzae* B. C. Couch (syn. *Pyricularia oryzae* Cavara) *Triticum* pathotype (Couch and Kohn 2002; Murakami et al. 2000; Zhang et al. 2016), has emerged as a serious threat to wheat production in South America. First discovered in the state of Paraná, Brazil in 1985 (Igarashi et al. 1986), the disease has since been reported in all wheat-producing regions of Brazil (Dos Anjos et al. 1996; Goulart and Paiva 2000; Goulart et al. 1990; Igarashi 1990; Maciel et al. 2014; Picinini and Fernandes 1990) and in the neighboring countries of Argentina (Cabrera and Gutiérrez 2007), Bolivia (Barea and

Toledo 1996), and Paraguay (Viedma 2005). More recently, wheat blast was reported for the first time outside of South America, during the 2015–16 cropping season in Bangladesh (Malaker et al. 2016). Yield loss attributable to wheat blast is strongly influenced by weather and, under optimal conditions, can reach nearly 100% (Kohli et al. 2011). Although *M. oryzae* pathotypes display a high degree of host specificity, isolates of the *Triticum* pathotype are known to infect other crop species, including black oat, rye, and barley, as well as noncultivated graminaceous species (Anjos and Charchar 2000; Kohli et al. 2011; Mehta and Baier 1998; Mehta et al. 2006). Due to the severity and scale of the disease during outbreaks in South America and Bangladesh, there is growing concern that the wheat blast pathogen may continue to spread to other wheat-growing regions throughout the world (Malaker et al. 2016; Valent et al. 2013). Based on the fact that other *M. oryzae* pathotypes are endemic in these regions, it is believed that *M. oryzae* *Triticum* strains may be well adapted and, therefore, have the potential to become established.

M. oryzae *Triticum* isolates can infect all aboveground parts of the plant but head infection is the most prevalent symptom in the field and the one most commonly associated with the disease (Cruz et al. 2015; Igarashi 1990; Kohli et al. 2011). When the rachis becomes infected, the portion of the spike above the point of infection becomes bleached. Bleached spikes produce seed that are small or malformed or have low test weight (Urashima et al. 2009), contributing to both yield losses and the spread of the disease via seed transmission. Spike infections also pose a problem from a diagnostic perspective because wheat blast can easily be confused with *Fusarium* head blight (FHB), which may mask the spread of the pathogen into areas where *Fusarium* spp. are common (Valent et al. 2013).

Currently, the diagnosis of wheat blast on suspect samples is reliant upon classical disease diagnostic methods, which include visual confirmation of the pyriform *M. oryzae* conidia. Molecular markers for polymerase chain reaction (PCR)-based assays are limited to confirmation at the species level and cannot be used to determine the pathotype of the isolate (Harmon et al. 2003). This limitation is problematic because opportunistic infection of wheat by endemic *M. oryzae* pathotypes could trigger unnecessary regulatory actions and unneeded mitigation measures by growers. Such an event occurred in 2011, when a single wheat plant displaying wheat blast symptoms

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This is contribution number 16-261-J from the Kansas Agricultural Experiment Station.

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Accepted for publication 9 September 2016.

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was discovered in Kentucky and *M. oryzae* was isolated from the infected head (Farman et al., in press). Although this isolate was later determined to be most closely related to an *M. oryzae* strain isolated from annual ryegrass, it required an intensive effort involving whole-genome sequencing and molecular characterization to rule out the occurrence of an exotic strain of the pathogen (Farman et al., in press).

PCR-based diagnostic assays are now firmly established in the methods employed by plant pathogen diagnostic laboratories and regulatory agencies. Such assays provide a rapid means for the detection of pathogens even when morphological features used for visual diagnoses are not present, such as during early stages of infection. Often, conserved genomic features such as the ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions 1 and 2 are utilized for the production of specific primers (Vincelli and Tisserat 2008). PCR assays based upon the ITS regions are favored because ITS regions 1 and 2 show considerable variation among individual fungal species and can be easily obtained using universal primers that anneal to the conserved portions of the rDNA that flank the ITS regions (White et al. 1990). Additionally, because rDNA is present in multiple copies in most fungal genomes, ITS-based PCR assays are more sensitive than those based on single-copy genes (Vincelli and Tisserat 2008). However, because the ITS regions typically show little variation within a species, they are not suitable for differentiation between pathotypes.

With the advent of new sequencing technologies, it has now become cost-effective to sequence entire genomes of closely related plant pathogens to rapidly obtain targets for diagnostic assay development (Lang et al. 2010). The advantages of this approach are that no a priori knowledge regarding target sites is required and that it allows for the simultaneous development of markers that can then be empirically tested for specificity. In this study, we utilized whole-genome draft sequences of *M. oryzae* strains and a bioinformatic pipeline to identify regions suitable for the development of a PCR-based assay that could differentiate between the *M. oryzae Triticum* strains present in South America from other *M. oryzae* pathotypes. The objectives of this study were to (i) sequence and assemble the genomes of multiple *M. oryzae Triticum* strains, (ii) perform whole-genome comparisons to identify sequences sufficiently unique to the *Triticum* pathotype for use as molecular markers, (iii) test a subset of potential markers against a panel of *M. oryzae Triticum* and *Lolium* isolates, and (iv) identify a marker for further verification of specificity against a large collection of *M. oryzae* isolates.

Materials and Methods

Fungal specimens and plant material. All fungal specimens used in this study are listed in Supplementary Table S1. South American *M. oryzae Triticum* isolates B2 and P3 and North American *Lolium* pathotype isolates LpKY97-1, PL3-1, and WBKY11-15 (Farman et al., in press) were used for preliminary screening of *M. oryzae Triticum*-specific primer pairs. 'Cavalier' wheat (World Wide Wheat L.L.C., Scottsdale, AZ) was used for pathogenicity testing and as the source for wheat genomic DNA.

Pathogen propagation and plant inoculations. *Magnaporthe* isolates were cultured on oatmeal agar (OMA) plates seeded with dried Whatman filter paper containing mycelia and conidia (Valent et al. 1986). The culture plates were grown under continuous light at room temperature for 7 to 12 days. For DNA extractions, the fungus was allowed to grow on 5-cm² pieces of sterile Whatman filter paper (number 007) placed on the agar surface. For plant inoculations, conidia were harvested in a sterile solution of 0.01% Tween-20 (vol/vol) by gentle agitation with a 1.3-cm flat, soft, nylon paint brush. Conidia suspensions were filtered through a 53- μ m sieve and the concentration was adjusted to 1×10^4 conidia/ml using a hemocytometer. Newly emerged spikes of wheat (Zadok's growth stage 59) were spray inoculated with an atomizer (Devilbiss Manufacturing Co. Toledo, OH) at 69 Kpa in the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) Foreign Disease-Weed Science Research Unit (FDWSRU) Biological

Safety Level 3 (BSL-3) Plant Pathogen Containment Facility (Melching et al. 1983). Individual heads were covered with a small, clear bag (7.5 by 13 cm) with a zipper closure (Uline, Coppell, TX), and placed in a shaded portion of the greenhouse. Bags were removed 24 h after inoculation and the plants were moved to a greenhouse bench with supplemental high-pressure sodium light to extend the day length to 16 h. Greenhouse temperature was 25°C ($\pm 4^\circ$ C) throughout the experiment. Plants were rated 14 to 16 days after inoculation based on the percentage of observed blasted florets per head. For the purpose of analysis, each individual spike was considered a replication. For liquid culture, a 1-cm² section of OMA culture containing fungal mycelia and conidia was used to inoculate minimal medium (3 g of Casamino acids, 3 g of yeast extract, and 6 g of sucrose in 1 liter of H₂O) and grown in an incubator at 24°C for 7 days.

DNA extraction. For genome sequencing and initial PCR screening, liquid-grown mycelial mats were harvested, blotted dry on paper towels, and lyophilized for 24 h. The lyophilized mycelium was ground to a fine powder in liquid nitrogen. For DNA extraction, the lyophilized powder (200 mg) was suspended in 4 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 50 mM EDTA, [pH 8.0], 0.5 M NaCl, and 1% sodium dodecyl sulfate) at 65°C and ground with a mortar and pestle. Phenol/chloroform/isoamyl alcohol (4 ml, 25:24:1) was added and mixed thoroughly. The mixture was transferred to a 30-ml Corex tube and centrifuged at 8,500 rpm at 4°C for 30 min. The aqueous phase was transferred to a clean tube and the DNA was precipitated by adding 0.54 volumes of isopropanol, mixed by inverting, and centrifuged at 8,500 rpm at 4°C for 10 min. The pellet was washed with 2 ml of 70% (vol/vol) ethanol, dried, and resuspended in 0.5 ml of Tris-EDTA (TE) with RNase A at 100 μ g/ml. The DNA concentration was quantified using a NanoDrop NO-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE). For additional PCR screening, DNA was extracted directly from Whatman filter paper (Jia et al. 2014). Briefly, a 1-cm² piece of dried filter paper containing fungal structures was placed in a 1.5-ml microcentrifuge tube containing 200 μ l of 10 $\times$ TE (100 mM Tris-HCl and 10 mM EDTA, pH 8.0), vortexed briefly, and incubated at 95°C for 10 min. Samples were centrifuged for 1 min at 3,000 rpm and stored at –20°C. The DNA solution (5 μ l) was used as template for PCR analysis. Genomic DNA isolated from *Fusarium graminearum* strain PH-1 and *F. oxysporum* f. sp. *lycopersici* strain 4287 was provided by Dr. Corby Kistler, USDA-ARS Cereal Disease Lab, St. Paul, MN.

Genome sequencing and assembly. High-quality genomic DNA (1 μ g) was fragmented with the Covaris S220 Focused-ultrasonicator (Covaris, Woburn, MA) using the manufacturer's recommended settings to obtain an average DNA fragment length of 500 bp, except isolates B2 and B51, which were fragmented to an average length of 300 bp. For sample library preparation, the NEBNext DNA Library Prep Master Mix set (New England BioLabs, Beverly, MA) for Illumina (San Diego, CA) and Illumina TruSeq indexed (bar-coded) adapters were used according to manufacturer's protocol. The DNA libraries were purified with the QIAquick PCR Purification Kit (Qiagen, Germantown, MD) followed by Agencourt AMPure XP Beads (Beckman Coulter, Danvers, MA) to ensure the removal of adapters and adapter dimers. The Pippin Prep System (Sage Science, Beverly, MA) was used to remove lower molecular weight content and to prepare the 500-bp fragment libraries for longer-read sequencing. The sample libraries were analyzed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA) and quantified with Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA). The sequencing was performed on the MiSeq personal sequencing system (Illumina) using the 500-cycle MiSeq reagent v2 kit (Illumina) for the 300-bp libraries or the 600-cycle MiSeq reagent v3 kit (Illumina) for the 500-bp libraries, according to the protocol provided.

Genome assembly conducted at USDA-ARS FDWSRU was performed in collaboration with the National Cancer Institute's Advanced Biomedical Computing Center (Ft. Detrick, MD) using CLC Genomics Workbench 7.0.3 (<http://www.clcbio.com>). Additional

M. oryzae genomic sequences were assembled at the University of Kentucky.

Identification of *M. oryzae* *Triticum*-specific loci and primer design.

A Perl script was used to split the assembled genomic sequence of *M. oryzae* *Triticum* isolate B2 into 500-bp fragments with a 250-bp overlapping sequence (Korf et al. 2003). To identify candidate loci unique to B2, a BLASTN (ver. 2.2.27) (Altschul et al. 1990) search was performed using default parameters to align the segmented B2 genome to a database containing the sequences of 16 non-*M. oryzae* *Triticum* isolates collected from 10 host species other than wheat (Table 1). B2 sequence fragments without significant similarity to the non-*M. oryzae* *Triticum* genomes were used for a second BLASTN search against a database containing three South American *M. oryzae* *Triticum* isolates collected from wheat. Sequences without significant similarity were removed. Primers were designed to the remaining sequences using Batch-Primer3 v 1.0 (You et al. 2008).

Conventional PCR. PCR was performed in a 50- μ l reaction containing 1 $\times$ ThermoPol buffer (New England BioLabs) 0.2 mM dNTP, 400 nM each primer (Table 2), 100 ng of template DNA (or 2.5 μ l of DNA extracted from filter paper or DNA extracted from plant samples), and 5 U of Taq DNA polymerase (New England BioLabs) using a Mastercycler Gradient Thermocycler (Eppendorf AG, Hamburg, Germany) with initial denaturation at 94°C for 90 s; followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 2 min. Amplified PCR products were analyzed on 2% agarose gels and stained with ethidium bromide. Alternatively, amplified PCR products were analyzed using a QIAxcel Advanced System running QIAxcel Screen Gel Software (Qiagen) equipped with a DNA high-resolution cartridge. The OM500 method was used with the following parameters: QX Alignment Marker injection at 4 kV and 20 s, sample injection at

5 kV for 10 s, and separation at 5 kV for 500 s. A 15-bp to 1-kb alignment marker was used and the PCR product size and concentration were determined using a 50- to 800-bp size marker (50 μ g/ μ l).

Real-time PCR. Real-time PCR assays were performed on an Applied Biosystems 7500 PCR instrument (Life Technologies). The reaction was performed in a 20- μ l volume containing 1 μ l of template DNA, 10 μ l of TaqMan Universal PCR Master Mix (Life Technologies), 400 nM each primer (Table 2), and 100 nM probe (6'-carboxyfluorescein [FAM] at the 5' end and black hole quencher-1 at the 3' end) (Eurofins MWG Operon LLC, Huntsville, AL) (Table 2). The real-time PCR assay consisted of 2 min of incubation at 50°C followed by 10 min of incubation at 95°C and 40 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed using the Applied Biosystems software, version 1.4.0. The threshold cycle (Ct) value was recorded as the cycle at which the fluorescent signal, associated with an exponential amplification of the PCR product, intersected the threshold set at 30. Every DNA sample was analyzed with triplicate real-time PCR, and three biological replicates were performed for each real-time PCR assay. Average Ct values with calculated standard deviations are reported.

Results

Using a BLASTN-based computational approach, 78 sequences with a length of 500 bp that were common to all four *M. oryzae* *Triticum* isolates but without significant similarity to the other sequenced *M. oryzae* isolates were identified (Supplementary Data S1). The genome sequence of *M. oryzae* *Triticum* isolate B2 was selected as the query genome because it was collected from a recent outbreak of wheat blast in Bolivia and, therefore, was assumed to be representative of current field isolates. To ensure that the markers identified were stable in the *Triticum* population, sequences from

Table 1. Sequenced *Magnaporthe oryzae* isolates used in this study

Isolate	Host ^a	Place of isolation	Year	Collector, source, or reference	Format of genome assembly	Role ^b	Reference
PL3-1	Annual ryegrass	Pulaski Co., KY	2002	J. Beale	454 assembly, paired end	Dev	Farman et al. (in press)
P-0028	Cheat grass	Paraguay	2014	M. Kohli	Illumina assembly	Conf	This study
P-0029	Cheat grass	Paraguay	2014	M. Kohli	Illumina assembly	Conf	This study
DsLIZ	Crabgrass	Lexington, KY	2000	M. Farman	Illumina assembly	Dev	This study
PH42	Finger millet	Philippines	1991	D. Tharreau	Illumina assembly	Dev	Farman et al. (in press)
B51	Goosegrass	Quirusillas, Bolivia	2012	J. Stack and C. Cruz	Illumina assembly	Dev	This study
Arcadia	Green foxtail	Lexington, KY	1998	M. Farman	Illumina assembly	Dev	Farman et al. (in press)
CHRF	Perennial ryegrass	Silver Spring, MD	1996	P. Dernoeden	Illumina assembly	Dev	This study
CHW	Perennial ryegrass	Severna Park, MD	1996	P. Dernoeden	Illumina assembly	Dev	This study
FH	Perennial ryegrass	Fountain Head, MD	1997	P. Dernoeden	454 assembly	Dev	Farman et al. (in press)
LpKY97	Perennial ryegrass	Lexington, KY	1997	P. Dernoeden	454 assembly, paired end	Dev	Farman et al. (in press)
ARB114 (IA1)	Rice	United States	2009	Y. Jia	Illumina assembly	Dev	This study
ZN61 (IB49)	Rice	United States	1992	J. Correll	Illumina assembly	Dev	This study
ZN57 (IC17)	Rice	United States	1992	J. Correll	Illumina assembly	Dev	This study
TM2 (IE1K)	Rice	United States	2003	T. Marchetti	Illumina assembly	Dev	This study
70-15	Rice	Laboratory cross	1991	Chao and Ellingboe 1991	Whole-genome shotgun assembly	Dev	Dean et al. 2005
SSFL02	St. Augustinegrass	Disneyworld, FL	2002	M. Farman	Illumina assembly	Dev	This study
Pg1213-2	Tall fescue	Georgia	2000	Tredway et al. 2005	Illumina assembly	Dev	This study
Pg1213-22	Tall fescue	Georgia	2000	Tredway et al. 2005	Illumina assembly	Dev	This study
B2	Wheat	Bolivia	2011	J. Stack and C. Cruz	Illumina and 454 assemblies	Dev	This study
B71	Wheat	Bolivia	2011	J. Stack and C. Cruz	Illumina assembly	Conf	Malaker et al., 2016
Br7	Wheat	Jagupita, Parana, Brazil	1990	Zhan et al. 2008	Illumina assembly	Dev	This study
Br80	Wheat	Brazil	1991	D. Tharreau	Illumina assembly	Dev	This study
P3	Wheat	Canindeyú, Paraguay	2012	G. Peterson	Illumina assembly	Conf	This study
PY5020	Wheat	Londrina, Brazil	2005	J. M. Fernandes	Illumina assembly	Conf	This study
PY22.1	Wheat	Parana, Brazil	2007	J. M. Fernandes	Illumina assembly	Conf	This study
T25	Wheat	Parana, Brazil	1988	S. Iragashi	Illumina assembly	Dev	This study
WBKY11-15	Wheat	Lexington, KY	2011	D. Hershman	454 assembly, paired end	Dev	Farman et al. (in press)

^a Annual ryegrass = *Lolium multiflorum*, Cheat grass = *Bromus tectorum*, Crabgrass = *Digitaria* spp., Finger millet = *Eleusine coracana*, Goosegrass = *E. indica*, Green foxtail = *Sataria viridis*, Perennial ryegrass = *L. perenne*, Rice = *Oryza sativa*, St. Augustinegrass = *Stenotaphrum secundatum*, Tall fescue = *Festuca arundinacea*, and Wheat = *Triticum* spp.

^b Role in study: Dev = marker development and Conf = marker confirmation.

archived isolates collected at least 20 years prior were used for comparisons. *M. oryzae* *Triticum* B2 candidate sequences that were not present in the draft genome sequences of isolates Br7, Br80, and T25 were removed.

Primer pairs were designed for a subset of the potential markers to test for specificity and validate the computational approach. Genomic DNA obtained from U.S. isolates PL3-1, LpKY97, and WBKY11-15 and South American isolates P3 and B2 was used as the templates for conventional PCR. The Pot2 transposon is known to be present in isolates of *M. oryzae* from various hosts and has been exploited for population studies and PCR-based diagnostics (Farman 2002; George et al. 1998; Harmon et al. 2003; Kachroo et al. 1994). Primers designed to amplify a region of the Pot2 transposon were used as a positive control and a 389-bp product was detected for all samples of the test panel (Supplementary Figure S1). For the putative *M. oryzae* *Triticum*-specific markers, amplification from one

or more of the U.S. isolates was observed for 6 of the 20 primer pairs tested; therefore, these were unsuitable for diagnostic purposes. However, 14 primer sets amplified target sequences exclusively from the South American isolates. Based on this analysis, we selected the MoT3 marker for further evaluation because the MoT3-specific primers produced a single amplicon of the same size for both isolates, and the relative intensity indicated that the sensitivity was greater than the Pot2 control. This was unexpected, because the MoT3 marker is located within a gene encoding a retinol dehydrogenase and appears to be single copy.

To evaluate the specificity of the MoT3 marker, a collection of 283 *M. oryzae* isolates was tested. These isolates were collected from an array of hosts, with the majority coming from *Triticum* and *Lolium* spp. Again, the Pot2a primer set was used as a positive control for all isolates, and all samples tested positive for this marker (Table 3). Amplification of the MoT3 marker was only observed

Table 2. Polymerase chain reaction primers and fluorescent probe used in this study

Primer, probe	Sequence	Target species	Seq ^a	Reference
MoT1F	5'-GCTGTTCTGGGCCACCTAC-3'	<i>Magnaporthe oryzae</i>	WB01	This study
MoT1R	5'-ATTCCTCCCCGATTTTCCTTT-3'	<i>M. oryzae</i>	WB01	This study
MoT2F	5'-ATCGACGCGAGTACGAAAAAC-3'	<i>M. oryzae</i>	WB04	This study
MoT2R	5'-CATGGGGTGAAAGGACTCAA-3'	<i>M. oryzae</i>	WB04	This study
MoT3F	5'-GTCGTCATCAACGTGACCAG-3'	<i>M. oryzae</i>	WB12	This study
MoT3R	5'-ACTTGACCCAAGCCTCGAAT-3'	<i>M. oryzae</i>	WB12	This study
MoT3_1F	5'-AGGATGTATGCCCTGACTGG-3'	<i>M. oryzae</i>	WB12	This study
MoT3_1R	5'-CTCGGCGATGCAAAAGTGAA-3'	<i>M. oryzae</i>	WB12	This study
MoT4F	5'-AGTTGATGGTTTCGATTTGG-3'	<i>M. oryzae</i>	WB05	This study
MoT4R	5'-AATCGTCCGCACCTTTGTAC-3'	<i>M. oryzae</i>	WB05	This study
MoT5F	5'-AGAATCCATGCAACGCCCTG-3'	<i>M. oryzae</i>	WB09	This study
MoT5R	5'-AGCAGCCGGTGACTAAGGAT-3'	<i>M. oryzae</i>	WB11	This study
MoT6F	5'-TGATGGGGTCCGGGAAAAGA-3'	<i>M. oryzae</i>	WB09	This study
MoT6R	5'-CAGGGCGTTGCATGGATTCT-3'	<i>M. oryzae</i>	WB09	This study
MoT7F	5'-TTGACTGTACGGGGATCTGC-3'	<i>M. oryzae</i>	WB07	This study
MoT7R	5'-ACAATTTCCAACCGTGACA-3'	<i>M. oryzae</i>	WB07	This study
MoT8F	5'-GATATGGGGCGATGGATTTT-3'	<i>M. oryzae</i>	WB39	This study
MoT8R	5'-CGACTCGATTTTCGACCTTACC-3'	<i>M. oryzae</i>	WB39	This study
MoT9F	5'-TTCGTGAGGTGCGTTCAAGT-3'	<i>M. oryzae</i>	WB21	This study
MoT9R	5'-AACGCTAGCGAACCCTTCTC-3'	<i>M. oryzae</i>	WB21	This study
MoT10F	5'-GTGACAAAGTGCGGACGATT-3'	<i>M. oryzae</i>	WB06	This study
MoT10R	5'-TGGAATGTTTCGCGTATCCT-3'	<i>M. oryzae</i>	WB06	This study
MoT11F	5'-ACCAATGTATGCGCGCAGAA-3'	<i>M. oryzae</i>	WB02	This study
MoT11R	5'-GCCAGATCAACTTCGAGGGC-3'	<i>M. oryzae</i>	WB02	This study
MoT12F	5'-ATAGCACCGGGAGTTTCGTT-3'	<i>M. oryzae</i>	WB77	This study
MoT12R	5'-TTGTGCAGAAATTAGCGGAAA-3'	<i>M. oryzae</i>	WB77	This study
MoT13F	5'-GCCACGAATTTTCGGGTAAT-3'	<i>M. oryzae</i>	WB78	This study
MoT13R	5'-CCACGCGAAATATTTTGGA-3'	<i>M. oryzae</i>	WB78	This study
MoT14F	5'-CAAAATTAGTCGGCGACCTG-3'	<i>M. oryzae</i>	WB08	This study
MoT14R	5'-CCTCCGAATCTTCTTGGTC-3'	<i>M. oryzae</i>	WB08	This study
MoT15F	5'-CAAATTGGCGGACAGAAAAGA-3'	<i>M. oryzae</i>	WB45	This study
MoT15R	5'-CGATATCGTCGAAACCATGC-3'	<i>M. oryzae</i>	WB45	This study
MoT16F	5'-TCGAAAGAAGGTGGCGAGTA-3'	<i>M. oryzae</i>	WB02	This study
MoT16R	5'-GCCAAAATCAGAGCCAGT-3'	<i>M. oryzae</i>	WB02	This study
MoT17F	5'-AGGCATTGAGATGGCGTTTA-3'	<i>M. oryzae</i>	WB03	This study
MoT17R	5'-CGGTTCTTAGCCAGTAGGG-3'	<i>M. oryzae</i>	WB03	This study
MoT18F	5'-GATCCGGAACCAACGTGTAA-3'	<i>M. oryzae</i>	WB11	This study
MoT18R	5'-AAGGATGAACGCAACATTGG-3'	<i>M. oryzae</i>	WB11	This study
MoT19F	5'-GGTGTTCGCGTTGGATTAAA-3'	<i>M. oryzae</i>	WB18	This study
MoT19R	5'-GGCCATTTTTCGAAGACACA-3'	<i>M. oryzae</i>	WB18	This study
MoT20F	5'-CGCAATGGACTGGGAGTTTA-3'	<i>M. oryzae</i>	WB40	This study
MoT20R	5'-GGCGAATTGAAATGGTTCG-3'	<i>M. oryzae</i>	WB40	This study
Pot2a-L2	5'-GCAATTTTCATGCAACCGAAA-3'	<i>M. oryzae</i>	...	This study
Pot2a-R2	5'-CGTACGCCAACCAGATTGAA-3'	<i>M. oryzae</i>	...	This study
ITS-Fu1f	5'-ACAATCATAACCTGTGAACAT-3'	<i>Fusarium</i> spp.	...	Arif et al. 2012
ITS-Fu1r	5'-CAGAAAGTTGGGTGTTTACGG-3'	<i>Fusarium</i> spp.	...	Arif et al. 2012
MoT3_FAM2	5'-[FAM]-TTCTAACGGTTTGCAATTGCACAAAACAAC-[BHQ1]-3' ^b	<i>M. oryzae</i>	WB12	This study

^a Target sequence, included as Supplementary Data S1.

^b FAM = 6'-carboxyfluorescein and BHQ1 = black hole quencher-1.

for a single isolate collected from a host other than wheat, and all but one isolate collected from wheat tested positive for MoT3 (Table 3). The wheat-derived isolate that did not test positive for the MoT3 marker was PY05010, and P-0029 was the only isolate not collected from wheat that tested positive. These two isolates were tested for pathogenicity on Cavalier wheat and virulence correlated with the presence of MoT3. Isolate P-0029 was highly virulent, whereas PY05010 was only mildly pathogenic. Genome sequencing of P-0029 confirmed the presence of the MoT3 marker in this isolate, and detected all 77 of the other wheat-specific marker candidates.

To test the sensitivity of the MoT3F and MoT3R primers, a 10-fold serial dilution of *M. oryzae Triticum* genomic DNA was prepared and used as the template in conventional PCR. Using 30 PCR cycles, the MoT3 primers consistently amplified the target sequence in reactions containing as little as 0.1 ng of DNA (Fig. 1A). Because of the greater sensitivity and rapidity offered by real-time PCR (Schaad and Frederick 2002), the MoT3 assay was modified for a Taqman 5' nuclease assay. Primers MoT3_1F and MoT3_1R were used together with a FAM-labeled probe, MoT3_FAM2 (Table 2). To determine the sensitivity of the assay, the same 10 serial dilutions of genomic *M. oryzae Triticum* DNA template tested by conventional PCR were used; 10 pg of DNA could be detected using real-time PCR (Fig. 1B). Reactions containing 1 pg of template DNA did not yield a positive Ct value, indicating no amplification of the target sequence. A negative linear relationship was found between the Ct value and the log of the diluted *M. oryzae Triticum* DNA ($R^2 = 0.995$). Control reactions containing no template DNA did not produce a positive Ct value through 35 cycles, indicating that no amplification occurred.

F. graminearum is a common pathogen of wheat, causing FHB or scab. The symptomology of FHB closely resembles that of wheat blast. As an additional test of specificity, the MoT3 primers were tested to ensure that nonspecific amplification did not occur in the presence of *F. graminearum* DNA. Conventional PCR using *F. graminearum* and *F. oxysporum* genomic DNA did not yield a detectable product with the MoT3F/MoT3R primers (Fig. 2). Integrity of the DNA was demonstrated by the amplification of a portion of the ITS region using primers specific for the genus *Fusarium* (Arif et al. 2012).

Discussion

Due to the spread and severity of wheat blast in South America and now Bangladesh, there is growing concern that *M. oryzae Triticum* strains may spread to other areas of the world (Malaker et al. 2016; Valent et al. 2013). Effective monitoring for such movement will require intensive scouting efforts and a means for rapidly and accurately identifying the pathogen in suspect samples. In the present

Table 3. *Magnaporthe oryzae* isolates tested for the presence of the MoT3 and Pot2 markers

Host species	Isolates tested	Number of isolates positive for marker	
		MoT3	Pot2a
<i>Brachiaria</i> spp.	3	0	3
<i>Bromus tectorum</i>	2	1	2
<i>Digitaria</i> spp.	30	0	30
<i>Eleusine</i> spp. ^a	4	0	4
<i>Eragrostis curvula</i>	1	0	1
<i>Festuca arundinacea</i>	11	0	11
<i>Lolium</i> spp. ^b	72	0	72
<i>Oryza sativa</i>	4	0	4
<i>Setaria</i> spp. ^c	40	0	40
<i>Stenotaphrum secundatum</i>	3	0	3
<i>Triticum</i> spp. ^d	115	113	115

^a *Eleusine* spp. includes *Eleusine coracana* and *E. indica*.

^b *Lolium* spp. includes *Lolium multiflorum*, *L. perenne*, and an unknown *Lolium* sp.

^c *Setaria* spp. includes *Setaria faberi*, *S. viridis*, and unknown *Setaria* sp.

^d *Triticum* spp. includes *Triticum aestivum* and *T. durum*.

study, two PCR-based diagnostic assays (end-point PCR and real-time PCR) were developed to detect isolates of the *Triticum* pathotype of *M. oryzae* using pathogen-specific primers derived from a newly discovered marker, MoT3.

The most important attribute of any diagnostic assay is a high degree of specificity for the target organism. The MoT3 marker was tested using DNA from 284 *M. oryzae* isolates obtained from 11 different host species and collected from widely spaced geographic locations. A deliberate effort was also made to test both archived material and more recently collected isolates to help ensure that the marker was stable in the genome over time. With the exception of two isolates—P-0029 collected from *Bromus tectorum* that tested positive for MoT3, and PY05010, an isolate from wheat that tested negative—the MoT3 marker was specific for the South American *Triticum* pathotype.

Unlike obligate biotrophic fungi such as the rusts that are often restricted to a single host species, *M. oryzae* occurs as a series of host-specialized pathogen populations capable of infecting multiple hosts (Couch et al. 2005). Isolate P-0029 was collected from diseased cheat grass (*B. tectorum*) growing within a cultivated field of wheat where wheat blast was evident at high levels. Laboratory tests confirmed that isolate P-0029 was virulent on wheat as well as *B. tectorum*. Additionally, a phylogenetic analysis based on whole-genome sequence data revealed that P-0029 groups with *M. oryzae* strains from wheat (M. Farman, unpublished). Taken together, these data suggest that P-0029 is a bona fide isolate of the *Triticum* pathotype that was growing on a different host species within a field of *M. oryzae Triticum*-infected wheat. The opposite appears to be the case for PY05010 because, although it was collected from wheat, it is quite different from the majority of *Triticum* pathotype isolates. PY05010 was previously characterized using a set of 11 microsatellite (simple-sequence repeat) loci that defined 48 distinct multilocus microsatellite genotypes

Fig. 1. Sensitivity of conventional polymerase chain reaction (PCR) and real-time PCR assay using diluted template DNA. **A**, Agarose gel images of conventional PCR assays. The size of the products is indicated to the left of each gel, and the primer combinations (MoT3F/MoT3R or Pot2a-L2/Pot2a-R2) used for each assay are indicated to the right. Amounts of DNA for each reaction are indicated. **B**, Correlation of the cycle threshold (Ct; number when fluorescence of sample exceeded background fluorescence) values to the log of template DNA concentration using real-time PCR with the *Magnaporthe oryzae Triticum* pathotype-specific primer set MoT3F/MoT3R and the 6'-carboxyfluorescein-labeled probe MoT3_FAM2.

(MLMG) within a collection of 148 wheat-infecting *M. oryzae* isolates from Brazil (Maciel et al. 2014). That analysis placed PY05010 in its own unique MLMG (Maciel et al. 2014). More recently, a whole-genome-based phylogenetic analysis revealed that PY05010 belongs to a small subset of wheat blast pathogens that group more closely with isolates of *M. oryzae* obtained from *Lolium arundinaceum* and *L. multiflorum* (M. Farman, unpublished data). Thus, although MoT3 appears to be specific for the wheat blast pathogen, it is unable to detect a small fraction of strains from within this population.

Another desirable attribute of a diagnostic assay is a high level of sensitivity. To this end, high-copy-number sequences such as the ITS region of the rDNA are often selected for diagnostic assay development as a convenient way to achieve a desired level of sensitivity. However, although the ITS region is often suitable for distinguishing between different species, it typically lacks the necessary variation for distinguishing intraspecific variation, thus making it unsuitable for wheat blast diagnostics. Likewise, *Magnaporthe*-specific repetitive elements such as the *M. oryzae* telomeric retrotransposon (MoTeR) and the Pot2 transposable element do not offer the level of specificity necessary for distinguishing between pathotypes of *M. oryzae*. Using purified DNA, the detection limit with the MoT3 primers was 0.1 ng with conventional PCR and 10 pg with real-time PCR assays. Although further optimization of the PCR conditions using the MoT3-specific primers to achieve greater levels of sensitivity is possible, it may be necessary to initially screen suspect wheat samples with primers targeting repetitive elements such as MoTeR or Pot2 to first confirm the presence of *M. oryzae*. Positive samples could then be rescreened with the more specific MoT3 primers to determine whether they belong to the *Triticum* pathotype.

The diagnostic assays developed in this study could prove to be valuable resources for combating the spread of the wheat blast pathogen by providing a rapid means for either confirming or ruling out wheat blast in suspect samples. Visual diagnosis of wheat spikes infected by *M. oryzae* is complicated by the fact that the symptoms of the disease resemble those caused by *Fusarium* spp. (Valent et al. 2013), the causal agent of FHB. Therefore, wheat blast could go unnoticed in areas where FHB is known to occur unless wheat producers and extension specialists are trained to anticipate and recognize the disease. The MoT3 primers were tested against *F. graminearum* and *F. oxysporum* genomic DNA to ensure that nonspecific amplification from *Fusarium*-infected wheat samples would not interfere with the assay.

Further spread of *M. oryzae* *Triticum* strains is likely to occur by either wind dispersal of conidia and possibly ascospores, or movement of infected seed. It has been reported that conidia can travel distances of at least 1,000 m (Urashima et al. 2007); thus, although wind dispersal is a concern for further spread in South America and South Asia, it is unlikely that wind alone will greatly contribute to the dissemination of the pathogen to other parts of the world. Therefore, man-mediated movement of infected seed remains the primary concern. Seed transmission of wheat blast has been established (Goulart and Paiva 2000; Maciel et al. 2014). One study determined that the

percentage of infected seed from fields where the disease was present reached levels as high as 83% (Urashima et al. 2009). The assay presented here could be used for testing seed for the presence of possible inoculum and could help prevent the spread of wheat blast into new areas. For local movement, the assay could be used in spore trap experiments for in-field disease monitoring and epidemiology studies. The MoT3-based assay could also be used to provide a rapid means for determining whether wheat blast discovered in new areas is caused by endemic or exotic isolates. The MoT3 marker was not detected in any of the North American *M. oryzae* isolates examined and, therefore, could be used to assist in determining the origin of *M. oryzae* isolates infecting wheat if the disease were to occur in the United States.

Because it is now known that some endemic strains of *M. oryzae* in the United States have the capacity to infect wheat under the appropriate conditions, it is imperative that a method for rapid determination of pathogen origin is available for future discoveries, because this information will affect how researchers and regulatory agencies respond. Likewise, because importation of the pathogen on infected seed is the most likely way for the disease to spread, surveillance of grain from infected areas is warranted. The MoT3-based PCR assays presented here have the demonstrated specificity to differentiate the *Triticum* pathotype from other *M. oryzae* pathotype populations and could prove useful for monitoring shipments of seed. However, further work to determine the appropriate sampling methods to assist in these control measures will be required.

Acknowledgments

This project is supported by Agriculture and Food Research Initiative Competitive Grant number 2013-68004-20378 from the USDA National Institute of Food and Agriculture and by the USDA-ARS Project number 8044-22000-041-00. M. L. Pieck was supported, in part, by a USDA-ARS postdoctoral fellowship awarded to K. F. Pedley. Isolates of *M. oryzae* collected in Brazil were kindly provided by J. L. Nunes Maciel under the terms of a material transfer agreement between Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) and the USDA-ARS. We thank A. Akhunova at the Kansas State University Integrated Genomic Facility, J. Shan at the Frederick National Laboratory for Cancer Research, and J. Lehr at the USDA-ARS FDWSRU for their contributions to this work.

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Fig. 2. Agarose gels of polymerase chain reaction (PCR) assays showing the specificity of the primers. The size of the products is indicated to the left of each gel, and the primer combinations (MoT3F/MoT3R or ITS-fu1f/ITS-fu1r) used for each assay are indicated to the right. Genomic DNA for each assay is indicated by lane number: 1 = *Fusarium graminearum*, 2 = *F. oxysporum*, and 3 = *Magnaporthe oryzae* *Triticum* pathotype isolate B2.

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