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Rapid Detection of Wheat Blast Pathogen *Magnaporthe oryzae Triticum* Pathotype Using Genome-Specific Primers and Cas12a-mediated Technology

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ABSTRACT

Wheat blast, caused by the fungus *Magnaporthe oryzae Triticum* (MoT) pathotype, is a devastating disease persistent in South America and Bangladesh. Since MoT generally fails to cause visual symptoms in wheat until the heading stage when the infection would have advanced, disease control by fungicide application solely based on the detection of visual symptoms is ineffective. To develop an accurate and sensitive method to detect MoT at the seedling and vegetative stages for disease control, we sequenced the genomes of two MoT isolates from Brazil and identified two DNA fragments, *MoT-6098* and *MoT-6099*, that are present in the MoT genome but not in the genome of the rice-infecting *Magnaporthe oryzae Oryzae* (MoO) pathotype. Using polymerase chain reaction (PCR), we confirmed the specificity of the two markers in 53 MoT and MoO isolates from South America and Bangladesh. To test the efficiency of the two markers, we first established a loop-mediated isothermal amplification (LAMP) method to detect MoT at isothermal conditions, without the use of a PCR machine. Following this, we used the Cas12a protein and guide RNAs (gRNAs) to target the *MoT-6098* and *MoT-6099* sequences. The activated Cas12a showed indiscriminate single-stranded deoxyribonuclease (ssDNase) activity. We then combined target-dependent Cas12a ssDNase activation with recombinase polymerase amplification (RPA) and nucleic acid lateral flow immunoassay (NALFIA) to develop a method that accurately, sensitively, and cost-effectively detects MoT-specific DNA sequences in infected wheat plants. This novel technique can be easily adapted for the rapid detection of wheat blast and other important plant diseases in the field.

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1. Introduction

The hemibiotrophic fungal pathogen, *Magnaporthe oryzae* (*M. oryzae*), affects many cereals including the two most important staple crops, rice and wheat. Rice blast, caused by *M. oryzae Oryza* (MoO) pathotype, was reported nearly three centuries ago in China and Japan and is now found in over 85 countries [1]. Wheat blast, caused by *M. oryzae Triticum* (MoT) pathotype, was initially identified in six municipalities of the Parana State in Brazil in 1985 [2], and its distribution gradually expanded in South America to about 3×10^6 hm^2, in the early 1990s [3]. For the first time, the disease was spotted outside of South America in eight districts in Bangladesh in 2016, where it rapidly became a serious threat to wheat production [4]. In early 2017, various newspapers reported that symptoms resembling those of wheat blast were observed for the first time in Indian wheat fields close to the India–Bangladesh border [5]. Between 2016 and 2019, the disease spread to a total of 20 districts in Bangladesh, and it continues to pose a serious threat to the food nutritional security of Bangladesh [6]. Based on the

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agro-climatic conditions of the region where wheat blast was first identified in Bangladesh in 2016, a recent study reported that up to 7×10^6 hm^2 of wheat fields in India, Pakistan, and Bangladesh are vulnerable to MoT infection [7]. With a conservative estimate of 5%–10% yield loss, the annual potential economic loss could be 132 million–264 million USD. Therefore, an integrated approach is urgently required to control the disease in Bangladesh and to prevent this disease from spreading to other regions of the world.

It remains unclear how MoT infects wheat plants, the identity of its alternative hosts, and those climate factors that favor an epidemic. Although MoT is capable of infecting both leaves and spikes of the wheat plant, spike infections are of greater economic significance. Infection in the rachis or peduncle can block the translocation of photosynthates, killing the upper parts of the spike. The disease can cause yield losses of up to 100% under pathogen favorable conditions [8]. However, as spike blast is seen developing in fields without visual symptoms of leaf blast, the correlation between the two is unclear. Therefore, controlling spike blast through application of fungicides can be difficult as farmers cannot always use lesions on leaves before the heading stage to warrant fungicide application. Additionally, wheat blast is also a seed-transmittable disease [9]. Screening seeds and certifying them for MoT will reduce the risk of further spread of the disease to other countries. Therefore, development of a rapid, sensitive, and low-cost method for MoT detection in wheat seedlings in the field, in alternative hosts in nearby areas, and in wheat seeds in commercial shipments, is critical for the control of wheat blast disease in South America and Bangladesh.

Pieck et al. [10] identified DNA markers that appeared to be unique to MoT strains. One of the markers, *MoT3*, showed specificity when tested on DNA from 284 *M. oryzae* strains from 11 host species, collected from several countries. A 360- base pair (bp) fragment of the MoT3 marker was amplified in the MoT strains but not in the MoO strains. However, the fragment is located within a single-copy gene encoding a retinol dehydrogenase, which is present in MoT, MoO, and *M. oryzae* Lolium (MoL) strains. A recent study analyzed the polymorphism among 81 previously assembled *Magnaporthe* genomes and found that the MoT3 sequence is not present in all the MoT strains [11]. A new DNA marker, *C17*, was identified that is specific to the *Triticum* lineage with high sensitivity [11]. In quantitation real-time (qRT)-polymerase chain reaction (PCR) reactions, it successfully amplified DNA from 100% of the 30 wheat-borne strains used in the study. However, it is not clear whether both *MoT3* and *C17* are useful for rapid MoT detection in infected wheat plants or not in the field.

In recent years, several new technologies have been developed to rapidly detect plant diseases in both laboratory and field conditions. For example, the loop-mediated isothermal amplification (LAMP) [12,13] and recombinase polymerase amplification (RPA) [14] are being widely used for plant disease detection. It is worth mentioning that a revolutionary detection method for human diseases, which integrates the programmable nucleotide-target carried by Cas proteins, such as Cas12a and Cas13a, with the nucleic acid rapid lateral flow immunoassay (NALFIA) method, was recently developed [15–17]. This method is an accurate, rapid, specific, and cost-effective alternative that can be easily adapted for plant disease detection.

In this study, we aimed to develop a novel method for the rapid and accurate detection of MoT on field, using RPA, Cas12a, and NALFIA. First, we *de novo* sequenced the genome of two wheat blast strains from Brazil using next generation sequencing (NGS) strategy. Second, we performed a pair-wise gene sequence alignment analysis between the MoO and MoT genomes using the BLAT software [18], which led to the identification of two MoT-specific fragments: *MoT-6098* and *MoT-6099*. Third, we designed a few sets of primers based on the *MoT-6098* and *MoT-6099* sequences and verified the sensitivity of the primers via PCR and LAMP. Finally, we combined the Cas12a-based detection method with RPA and NALFIA technologies and developed a sensitive and low-cost method for detecting MoT in infected wheat plants in the field. Our study provides a new detection method for the devastating wheat blast disease.

2. Materials and methods

2.1. Fungal materials

The MoO strains used in this study (RB22, R01-1, P131, Guy11, N60, N63, and N71) were cultured in the Institute of Plant Protection, Chinese Academy of Agricultural Science, China. The DNA samples of all of the MoT strains were prepared at the Bangabandhu Sheikh Mujibur Rahman Agricultural University; the Foreign Disease-Weed Science Research Unit of United States Department of Agriculture-Agricultural Research Service at Ft. Detrick, USA; and the Centro de Ciências Agrárias, Universidade Federal de São Carlos, Brazil.

2.2. Primers used in this study

All primers used in this study are listed in Table 1.

2.3. Genome sequencing and comparative genomic analysis

The genomic DNA of MoT strains PR01-37.V.1 and PR01-37.V.3 was sequenced using the Illumina sequencing platform (Hisep X_ten, BioMarker Co., Ltd., China). In brief, DNA of PR01-37.V.1 and PR01-37.V.3 were randomly sheared into ~500 bp fragments and cloned to the sequencing vector for library construction, before the library was sequenced using the Illumina system. The clean sequence read data were obtained after sequence quality control. A *de novo* assembly of the two genomes was performed using SPAdes-3.13.0 software (St. Petersburg State University, Russia). The Augustus.2.5.5 software (University of Gottingen, Germany) was used for gene prediction of MoO and MoT genomes. A pair-wise gene alignment pipeline, which utilized BLAT [18] as the core alignment tool, was then developed. The potential MoT-specific genes were then identified by analyzing the gene presence/absence from the global pair-wise gene sequence alignment among the two MoT strains (PR01-37.V.1 and PR01-37.V.3) and three MoO strains. The potential specific genes were then aligned to the additional MoO and MoT genomes [4,19]. Specifically, a two-step approach was used to identify the MoT specific genes. First, we aligned the 378 candidate genes to National Center for Biotechnology Information Search database nr databases to remove the MoO genes. Second, we aligned the 100 candidate MoT genes to more sequenced MoT and MoO genomes to test whether they were present in all of the MoT genomes but absent in all of the MoO genomes. Finally, two MoT-specific genes were identified in the analysis. The detailed bioinformatic analysis procedure is shown in Fig. 1.

2.4. Loop-mediated isothermal amplification

The LAMP reaction mixtures were prepared to a final volume of 20 μL and contained 1 μL of template DNA (20 $\mu mol \cdot L^{-1}$), 2.0 $\mu mol \cdot L^{-1}$ of the forward and backward inner primers (FIP and BIP), 0.5 $\mu mol \cdot L^{-1}$ F3 and B3 primers (Table 1), 1 $mmol \cdot L^{-1}$ of deoxy-ribonucleoside triphosphate (dNTP), 8 U (1U = 16.67 nkat) of Bst DNA polymerase (New England Biolabs, USA), and 1× ThermoPol reaction buffer (20 $mmol \cdot L^{-1}$ Tris-HCL, 10 $mmol \cdot L^{-1}$ KCl, 2 $mmol \cdot L^{-1}$ $MgSO_4$, 10 $mmol \cdot L^{-1}$ $(NH_4)_2SO_4$, and 0.1% Triton X-100, pH 8.8). The mixture was kept at 65 °C for 1 h before the reaction was terminated by moving the mixture to 85 °C for 5 min.

Table 1
List of primers and small guide RNA (sgRNA) sequences used in this study.

Primer	Sequence (from 5′ to 3′)	Target	Reference	T_m (°C)
Mo.T6098 F	ACCAATATCACCTGAACGCAGACAT	MoT	For PCR	58.4
Mo.T6098 R	GATTCCAGATTCACCACCAAAACAG	MoT	For PCR	56.4
Mo.T6099 F	TCTGTATTTCACACTTGGGCTTTGG	MoT	For PCR	57.5
Mo.T6099 R	AACGTCATGTAGTGCGTCTTGTTGA	MoT	For PCR	59.1
Pot2 F	CGTCACACGTTCTTCAACC	MoT & MoO	For PCR	53.6
Pot2 R	CGTTTCACGCTTCTCCG	MoT & MoO	For PCR	53.1
Mo.T6098-SF[a]	TTTCTGCTCGTTGGGGAGAC	MoT	For RT-PCR	56.4
Mo.T6098-SR[a]	ATGTACCCCCGTACCCATCA	MoT	For RT-PCR	56.3
Mo.T6099-SF[a]	ATGTTGGTAACCACCGGTCC	MoT	For RT-PCR	56.3
Mo.T6099-SR[a]	CTTCGTATGCCTGCCGTTTG	MoT	For RT-PCR	56.4
Pot2-SF[a]	ACGACCCGTCTTTACTTATTTGG	MoT & MoO	For RT-PCR	54.8
Pot2-SR[a]	AAGTAGCGTTGGTTTTGTTGGAT	MoT & MoO	For RT-PCR	55.4
Fam-2229-F	5′-[FAM-dT]CGTCACATAGGGTATATCTTTTCACAACAG	ssDNA	For RPA	57.6
2229-nof-prob	AACCTACACTCGACTCACCGCAGTTCCCC AG (THF) CGCAGTATCGTAGCCG, 3′, C3 Spacer	ssDNA	For RPA	74.6
5B-2229-R	5′-BIOTIN-TTGTCATTCGCGGTCTTAGAA GCACTAACTA	ssDNA	For RPA	61.3
P6098_370-gRNA	AAUUUCUACUGUUGUAGAU CCGAUUUGCUGGUCGAAGCAUGU	Cas12a-binding	For Cas12a	–
Mo.T6098F3	GCCCTGCATCATGTACCAAT	MoT	For LAMP	54.7
Mo.T6098B3	ACTCGTCGGCATCTGTCA	MoT	For LAMP	55.1
Mo.T6098 FIP	AGCCCTGTCTCCGCTTGTGTCACCTGAAC GCAGACATCG	MoT	For LAMP	72.4
Mo.T6098 BIP	ATGTCATTGACGGCGTCGCCTGACGTCGA TTCCCCAGT	MoT	For LAMP	72.5
Mo.T6099F3	CGCATCTGGAGGGGAACT	MoT	For LAMP	55.4
Mo.T6099B3	CTATCACCTGGCGGAGCT	MoT	For LAMP	55.2
Mo.T6099 FIP	AATCGGTGGCTGGCAGAGACGTCTCTGCA CACGGTCGA	MoT	For LAMP	73.3
Mo.T6099 BIP	GGTCGCTAACGTGGTGACGGTTCTCGGTG GCGAGGATG	MoT	For LAMP	72.8

[a] Primers used for sensitivity assays in real time PCR (RT-PCR). ssDNA: single-stranded DNA; THF: tetrahydrofuran; FAM-dT: 5-carboxyfluorescein attached to 5′ thymine nucleoside; BIOTIN: biotin label; SF: shorter forward primer; SR: shorter reverse primer; FIP: forward inner primer; BIP: backward inner primer.

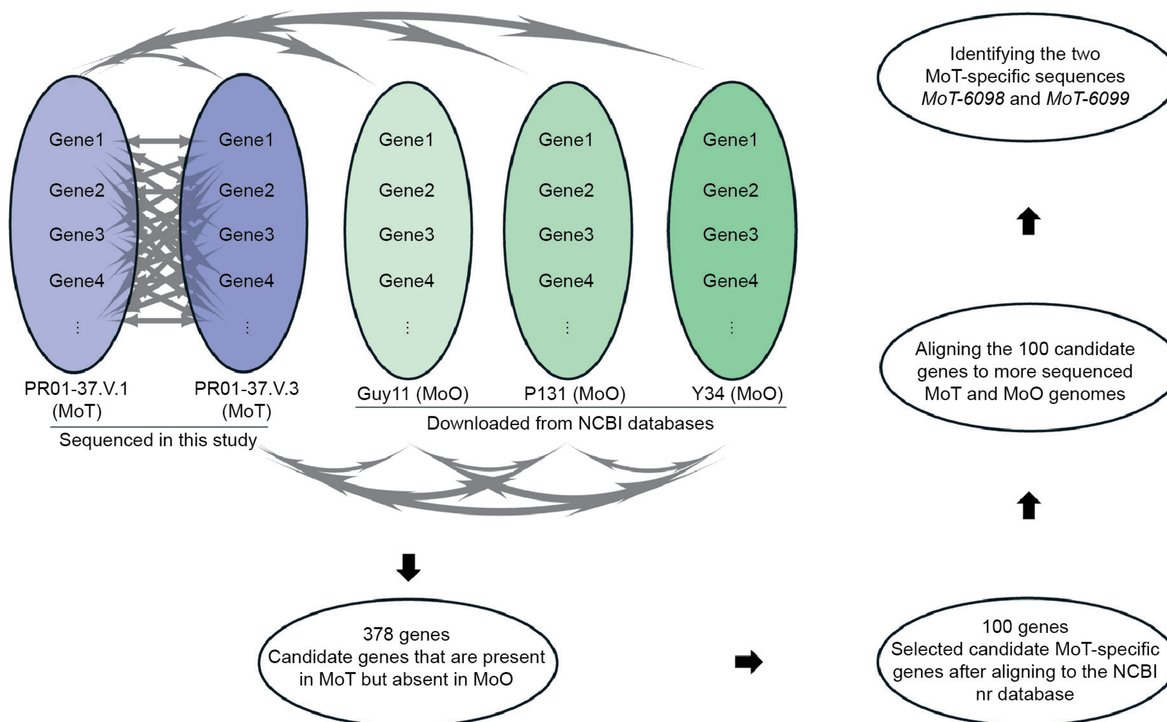

Fig. 1. Pipeline for the identification of two MoT-specific sequences. The ellipses at the upper-left side of the figure represent different *M. oryzae* genomes and Gene1, 2, 3, 4,…, *N* represent all of the predicted genes in the sequenced genomes. The arrows between two genes in two genomes indicate the bi-directional pairwise gene sequence alignments, the arched arrows between the strains represent the sequence alignments of individual genes in the two genomes. nr: non-redundant protein sequence.

2.5. Detection of LAMP reaction products

A 2 μL volume of 1:10 diluted SYBR Green I (10 000×, Invitrogen, USA) was added to the LAMP products. Reactions that turned green were recorded as positive, and those that remained brown were recorded as negative. The amplified LAMP reactions were also analyzed on a 2% agarose gel that was stained with ethidium bromide. The gel was photographed under ultraviolet light using a Gel Doc XR+ Imager system (Bio-Rad, USA).

2.6. Recombinase polymerase amplification

RPA was performed as previously described by Piepenburg et al. [14] using the kit (#WLN8203KIT) from AMP-Future Biothech Co. Ltd. (China). For the first RPA, *MoT-6098*/*MoT-6099*-specific primer pairs were designed, and a tetrahydrofuran (THF) probe was added as the Nfoase recognition site. The amplicon should contain at least one protospacer adjacent motif (PAM) site (four-base domain of "TTTA") for Cas12a recognition. For the second RPA,

single-stranded DNA (ssDNA)-specific primer pairs were designed and labeled with 5-carboxyfluorescein (5-FAM) at the 5′ end and with biotin at the 3′ end. For the RPA, the instructions in the RPA kit were followed. In brief, the primers were mixed with the RPA solution containing the recombinase, polymerase, and the buffers, following which 1 μL of sample DNA (or of Cas12a-digested solution) was added; the preparation (total volume = 50 μL) was then kept at 37–39 °C for 8–12 min.

2.7. ssDNA design

ssDNA design has two requirements: ① The ssDNA should be short (to facilitate rapid PCR detection) and ② there should be no identical sequence between the ssDNA and the target genome sequence. To meet these requirements, a computer program was used to randomly design 10 000 120-bp DNA sequences. These 120-bp sequences were then aligned to the genomes of wheat, MoT, and MoO genomes using the BLAST software [20], and the sequences with an *E*-value $\leq e^{-10}$ or more than 10 bp identical to the target were discarded. The following sequence was randomly selected among sequences that had no detectable similarity with wheat, MoT, and MoO sequences: "ACGTCGTCACA TAGGGTATATCTTTTCACAACAGTAACCTACACTCGACTCACCGCAGTT CCCCAGA CGCAGTATCGTAGCCGCGGATAGTTAGTGCTTCTAAGACCG CGAATGACAACA."

2.8. Cas12a-based detection

For the RPA with Cas12a, a previously described method was used [16]. The Cas12a protein was purchased from New England BioLabs (NEB#M0653S, USA). In brief, Cas12a was mixed with small guide RNA (sgRNA) (equimolar volume) and reaction buffer, and the Cas12a mixture was incubated for ~10 min at room temperature. The RPA-amplified target DNA solution was then incubated in the Cas12a mixture at 37 °C for about 10 min to activate the Cas12a ssDNA digestion activity. Finally, the designed ssDNA and the activated Cas12a protein were combined for ssDNA digestion.

2.9. NALFIA detection using PCRD strips

Abingdon Health PCRD test cassettes (#FD51673, USA) were used for DNA visualization. In brief, 5 μL of the amplicon from the RPA reaction was mixed with 70 μL of PCRD extraction buffer, and the total volume (75 μL) was added to the sample well of the PCRD text cassette. The result was assessed after 3–5 min.

3. Results

3.1. Identification of the MoT-specific sequences through comparative genomics

To investigate the genomic differences between MoT and MoO, we extracted DNA from two MoT strains, PR01-37.V.1 and PR01-37.V.3, which were collected from infected wheat plants in Brazil. We then re-sequenced the two strains using the Illumina sequencing platform (USA) with a genome coverage of about 150 coverage. After genome assembly, we obtained a ~38.5 megabase (Mb) genome sequence for PR01-37.V.1 and a ~40.0 Mb genome sequence for PR01-37.V.3. We also downloaded the genome sequences of three MoO strains, Guy11, P131, and Y34, from GenBank [21]. Through pair-wise gene sequence alignments, we identified 378 MoT genes that were absent in the three MoO strains. Alignment of these 378 genes with the GenBank protein database (nr) led to the identification of 289 genes with high homology with those in the databases. Among them, 189 had hits of MoO genes, and the other 100 were MoT-specific candidate genes (Table S1 in Appendix A). Except for a few genes that were highly homologous with bacterial genes, most of the genes' hits are fungal genes, and more than half (57 of 100) of those hits belonged to six fungal species (Table S2 in Appendix A). When we aligned the 57 genes to other ~20 sequenced MoT and MoO genomes [4,19], we identified two genes, *MoT-6098* and *MoT-6099*, that were specific to MoT. *MoT-6098* encodes a putative acid trehalase protein, while *MoT-6099* encodes a duf341 family protein. Because *MoT-6098* and *MoT-6099* were present in all of the sequenced MoT genomes but not in any of the sequenced MoO genomes, we chose these two MoT-specific genes to develop MoT-specific markers.

3.2. Sensitivity of the MoT-6098 and MoT-6099 primer pairs for distinguishing MoT from MoO

To determine the specificity of the *MoT-6098* and *MoT-6099* sequences in the MoT and MoO strains, we designed a set of primers (MoT-6098F and MoT-6098R, and MoT-6099F and MoT-6099R, in Table 1), and used them in PCR reactions with DNA samples isolated from 53 diverse *M. oryzae* strains. The 53 strains were collected from 14 grass species in nine countries (Tables 2 and 3), and consisted 15 strains from rice, 19 strains from wheat, and 19 strains from possible alternative hosts, such as barley, oat, *Setaria, Brachiaria, Lolium, Bromus, Eleusine, Eragrostis, Festuca*, *Setaria viridis*, and *Stenotaphrum* (Tables 2 and 3). We PCR tested the 25 strains that were collected from Bangladesh and Brazil (Table 2) in the Urashima and Islam laboratories. Among them, two DNA samples from *Fusarium graminearum* (*F. graminearum*), which causes the *Fusarium* head blight of wheat, isolated in the Urashima laboratory were included to test the primer specificity between fungal species. In addition, we included four DNA samples from isolates infecting rice and *Lolium* as the controls, which were available in the Wang laboratory. The PCR analysis indicated that the two genes were present in all eight MoT strains, one barley strain, and one US *Lolium* strain, but not in any of the strains from rice, *Setaria*, *Brachiaria*, oat, and two *F. graminearum* (Fig. 2 and Table 2). Following this, we tested the 24 *M. oryzae* strains that were deposited in the USDA-ARS's Foreign Disease-Weed Science Research Unit (Table 3). Except for two strains collected from *Brachiaria* species, all other strains contained the two genes. Among the 22 MoT-positive strains, 11 were collected from wheat and the rest were isolated from alternative hosts of MoT, consisting *Lolium, Eleusine, Bromus, Eragrostis, Stenotaphrum secundatum,* and *Festuca*, collected in Brazil, Bolivia, Paraguay, Japan, and the USA (Fig. S1 in Appendix A and Table 3).

We then conducted a sensitivity assay to detect the efficiency of the two primer pairs. Five concentrations (0.01, 0.1, 1, 10, and 50 ng·μL^{-1}) of the DNA from a culture of MoT strain BO12TA301 were used in the PCR reactions with the MoT-6098F/R and MoT-6099F/R primer pairs. The Pot2R/F primer pair was used as the control. The PCR results indicated that MoT-6099F/R generated a DNA band with 0.1 ng·μL^{-1} DNA and MoT-6098F/R generated a DNA band with 10 ng·μL^{-1} DNA (Fig. 3(a)). We also tested the primer sensitivity using real-time PCR with the same DNA dilutions that were used in the conventional gel-based PCR. Reactions containing only 0.01 ng·μL^{-1} of template DNA yielded a positive cycle threshold (Ct) value for both primer pairs, indicating that amplification of the target sequence by these primers is very sensitive (Figs. 3(b)–(d)).

3.3. Detection of MoT in infected plants

To determine whether the MoT-6098F/R and MoT-6099F/R primer pairs could be used to detect MoT in infected wheat plants, we isolated DNA from leaves and panicles of wheat plants infected with the MoT strain BTJP4-5, in a growth chamber in the Bangabandhu Sheikh Mujibur Rahman Agricultural University in

Table 2
M. oryzae isolates from Bangladesh and Brazil used for the detection of *MoT-6098* and *MoT-6099* fragments using PCR.

Well #	Isolate	Host	Origin	Mo.T6098	Mo.T6099	Pot2
1	BTGS-4-C	Wheat	Bangladesh	+[a]	+	+
2	BTJP 4-1	Wheat	Bangladesh	+	+	+
3	BTJP 4-5	Wheat	Bangladesh	+	+	+
4	BTGP	Wheat	Bangladesh	+	+	+
5	BTMP 182-2	Wheat	Bangladesh	+	+	+
6	BTMP 1839-2	Wheat	Bangladesh	+	+	+
7	BTMP 1845-3	Wheat	Bangladesh	+	+	+
8	PR01 83	Wheat	Brazil	+	+	+
9	CVPNO2-17-C	Barley	Brazil	+	+	+
10	RBSA 18-B-1	Rice	Bangladesh	–[b]	–	+
11	RBCH 1814-2	Rice	Bangladesh	–	–	+
12	RBME 18-16-2	Rice	Bangladesh	–	–	+
13	RBME 1819-3	Rice	Bangladesh	–	–	+
14	RBDI 1831-5	Rice	Bangladesh	–	–	+
15	RBRA 18 34-1	Rice	Bangladesh	–	–	+
16	RBRA1836-3	Rice	Bangladesh	–	–	+
17	RBTA 1847-3	Rice	Bangladesh	–	–	+
18	RBTA 1711-1	Rice	Bangladesh	–	–	+
19	Rb-13-B	Rice	Bangladesh	–	–	+
20	SgCCA2a	*Setaria geniculate*	Brazil	–	–	+
21	WhGi02-01	*Fusarium* from wheat	Brazil	–	–	+
22	FS	*Fusarium* from wheat	Brazil	–	–	+
23	VP03-17	Rice	Brazil	–	–	+
24	BpCCA3d	*Brachiaria plantaginea*	Brazil	–	–	+
25	TrAv01-17	Oat	Brazil	–	–	+
A	BN-0050	Rice	Africa	–	–	+
B	KJ201	Rice	Republic of Korea	–	–	+
C	RB22	Rice	Republic of Korea	–	–	+
D	Cl-6	Rice	Colombia	–	–	+
E	PL-2	*Lolium*	USA	+	+	+
F	Water			–	–	–

[a] Positive amplification.
[b] Negative amplification.

Table 3
M. oryzae isolates from USDA-ARS used for the detection of *MoT-6098* and *MoT-6099* fragments using PCR.

Well #	Isolate	Host	Origin	Mo.T6098	Mo.T6099	Pot2
1	T-17	Wheat	Brazil	+[a]	+	+
2	BZ-45	Wheat	Brazil	+	+	+
3	BZ-19	Wheat	Brazil	+	+	+
4	T-42	Wheat	Brazil	+	+	+
5	BZ-6	Wheat	Brazil	+	+	+
6	T-2	Wheat	Brazil	+	+	+
7	T-25	Wheat	Brazil	+	+	+
8	BO12TA301	Wheat	Bolivia	+	+	+
9	B-2	Wheat	Bolivia	+	+	+
10	P-13	Wheat	Paraguay	+	+	+
11	P-3-1	Wheat	Paraguay	+	+	+
12	P-28	*Bromus* spp.	Paraguay	+	+	+
13	P-29	*Bromus* spp.	Paraguay	+	+	+
14	EC4J	*Eleusine coracana*	Japan	+	+	+
15	U-203	*Eragrostis curvula*	USA	+	+	+
16	U-199	*Festuca arundinacea*	USA	+	+	+
17	U-116	Green foxtail	USA	+	+	+
18	U-69	*Lolium multiflorum*	USA	+	+	+
19	S-3	*Setaria*	USA	+	+	+
20	U-100	*Setaria faberi*	USA	+	+	+
21	PL-2	*Lolium*	USA	+	+	+
22	A-2	*Stenotaphrum secundatum*	USA	+	+	+
23	P-26	*Brachiaria* spp.	Paraguay	–[b]	–	+
24	P-30	*Brachiaria* spp.	Paraguay	–	–	+
A	BN-0050	Rice	Africa	–	–	+
B	KJ201	Rice	Republic of Korea	–	–	+
C	RB22	Rice	Republic of Korea	–	–	+
D	Cl-6	Rice	Colombia	–	–	+
E	Water			–	–	–

[a] Positive amplification.
[b] Negative amplification.

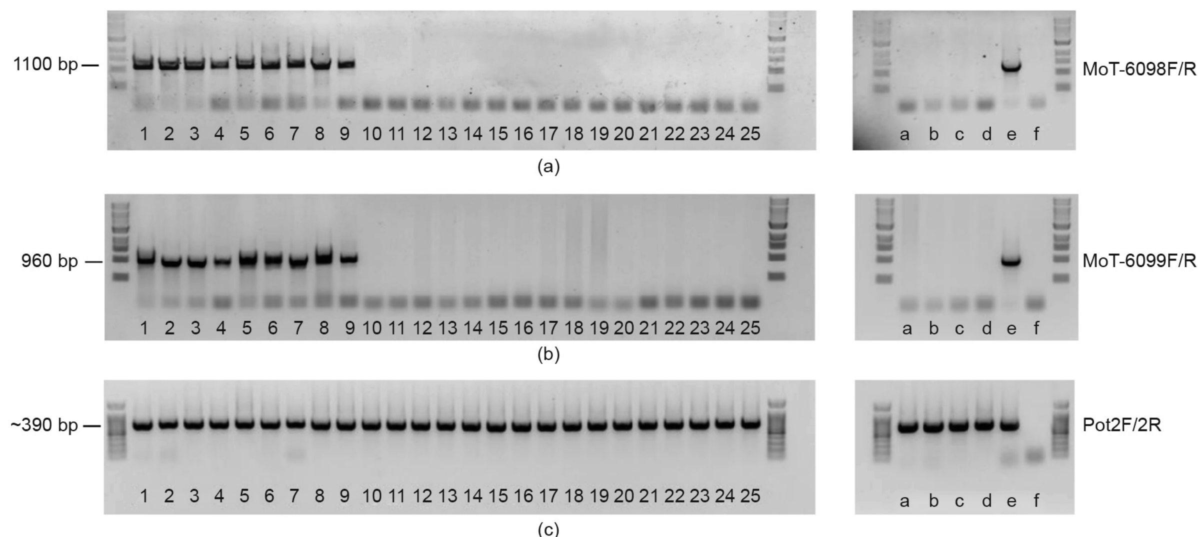

Fig. 2. *MoT-6098* and *MoT-6099* sequences are presence in all tested MoT strains but absence in all tested MoO strains detected by PCR. Photographs of gels (1%) obtained with MoT and MoO samples that were amplified with (a) the MoT-6098F/R primer pair, (b) the MoT-6099F/R primer pair, and (c) the control Pot2F/2R primer pair. DNA sample information (lane 1–25 and a–f in the gels) is listed in Table 2.

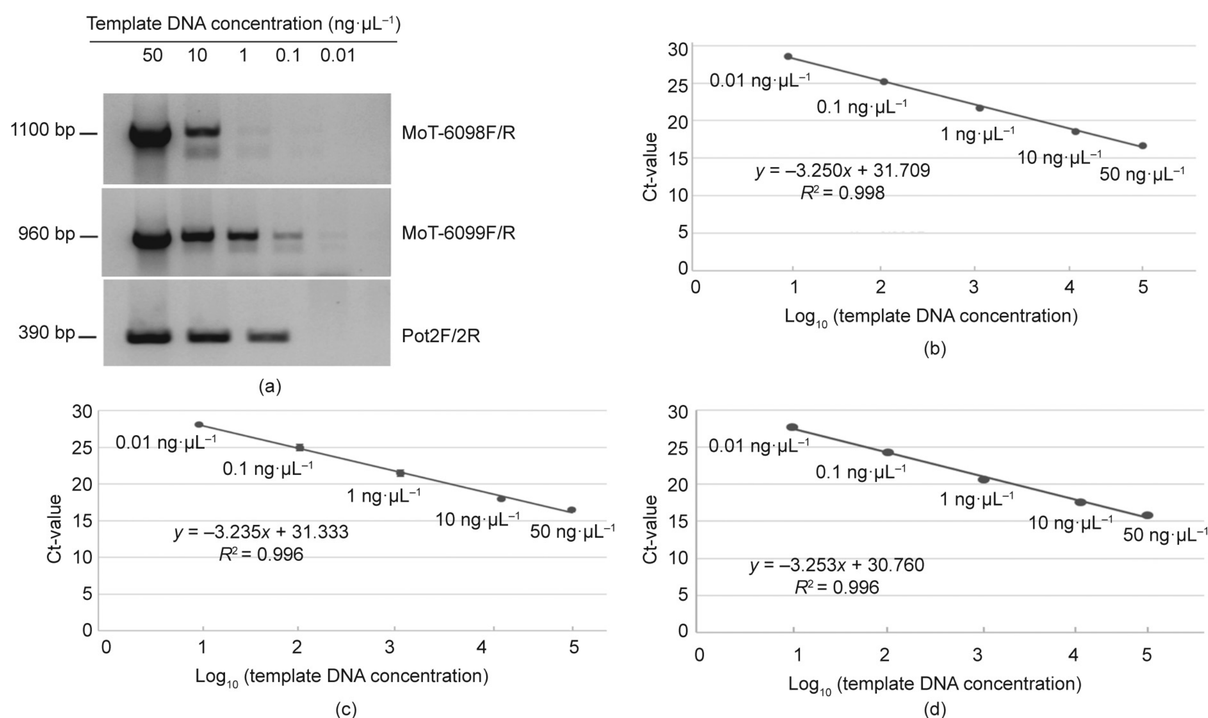

Fig. 3. Sensitivity assays of the primer pairs using conventional PCR and RT-PCR DNA from the MoT strain BO12TA301. (a) Sensitivity assays using conventional PCR. Photograph of 1% agarose gels was from primer combinations MoT-6098F/R, MoT-6099F/R, and Pot2F/2R. Specific dilutions of each DNA sample and size of amplicons are indicated. (b–d) Sensitivity assays using RT-PCR. Relationship between the Ct-value (where fluorescence of the sample surpassed background fluorescence) and the log of the DNA template concentration is depicted via RT-PCR using primer combinations of (b) MoT-6098F/R, (c) MoT-6099F/R, and (d) Pot2F/2R, respectively. The primers are listed in Table 1. R^2: coefficient of determination; y: linear model of the PCR data.

Bangladesh. As controls, we also isolated DNA from naturally infected panicles of rice and mycelia of MoT isolate BTJP4-5 and MoO isolate of RB-13b. The PCR results indicated that the *MoT-6098* and *MoT-6099* related bands were amplified with DNA obtained from MoT-infected wheat leaves and panicles and MoT mycelia, but not from the DNA isolated from MoO-infected rice leaves or MoO mycelia (Fig. 4). These results demonstrated that the MoT-6098 and MoT-6099 primers can effectively detect MoT in infected wheat plants.

3.4. Use of LAMP to detect MoT

Since LAMP is a specific, rapid, and efficient method for DNA detection under isothermal conditions, we used LAMP to detect the *MoT-6098* and *MoT-6099* fragments in MoT. The amplified DNA was visualized in both agarose gels and 1000× SYBR Green dye solutions. In the agarose gel assay, amplified bands were present in the reactions with the DNA from three MoT strains but not from five MoO strains (Fig. 5(a)). Consistent with the gel-based

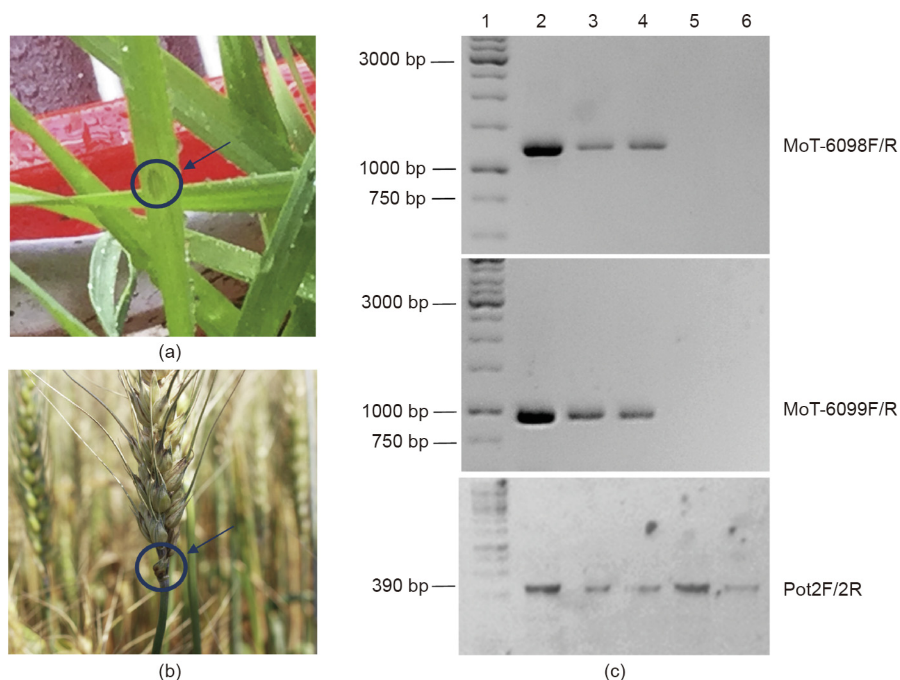

Fig. 4. Detection of MoT in infected wheat plants. (a) Early stage of leaf infection (circle indicates a water-soaked lesion in a leaf 14 days after sowing). (b) Panicle infection (circle shows a gray spot in the neck of a spike 105 d after sowing). (c) PCR detection of MoT sequences in infected young and adult plants using agarose gel electrophoresis. Lane 1 is the DNA marker lane. Lanes 2 to 6 represent DNA isolated from the following samples: mycelia of MoT strain BTJP4-5 (lane 2), wheat leaves artificially inoculated with MoT strain BTJP4-5 (lane 3), the panicle neck of a naturally infected wheat plant in an MoT-infected wheat field (lane 4), mycelia of MoO strain RB-13b (lane 5), and the panicle neck of a naturally infected rice plant in an MoO-infected rice field (lane 6).

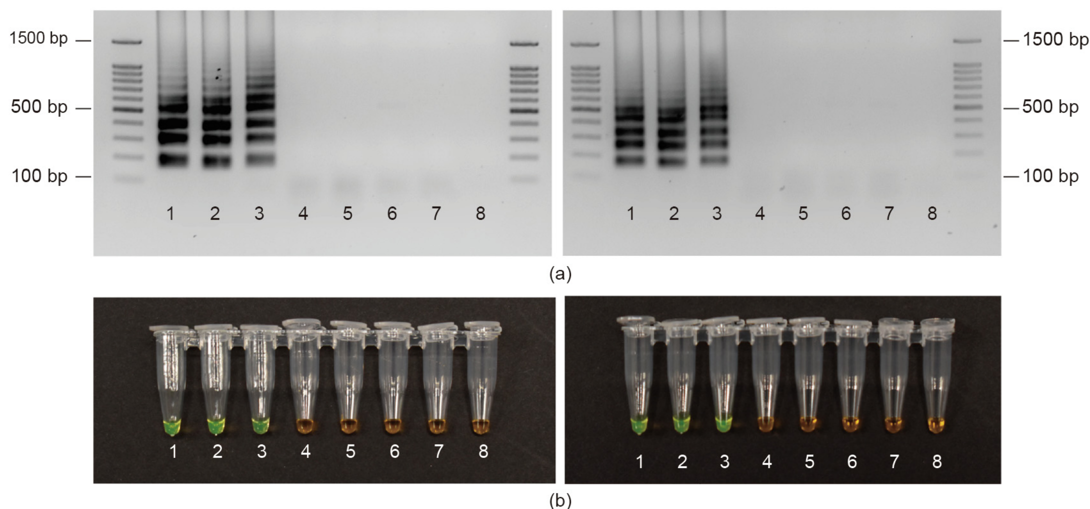

Fig. 5. LAMP detection of MoT sequences. The LAMP assay was conducted with eight samples (labeled 1 to 8) containing MoT genomic DNA (samples 1 to 3) and MoO genomic DNA (samples 4 to 8). (a) LAMP-amplified genomic DNA visualized on a 2% agarose gel; the left side and the right side of the gel indicate results obtained with LAMP primers that targeted *MoT-6098* and *MoT-6099*, respectively. (b) LAMP-amplified genomic DNA visualized with SYBR Green dye. The left side and the right side indicate the results obtained with LAMP primers that targeted *MoT-6098* and *MoT-6099*, respectively.

results, green color was obtained with the SYBR Green dye solutions containing the DNA of three MoT strains but not with the solutions containing the DNA of five MoO strains (Fig. 5(b)). These results showed that LAMP could be used to detect MoT rapidly and efficiently under isothermal conditions.

3.5. Integrating the Cas12a protein with RPA and NALFIA technologies for the rapid detection of MoT in the field

The application of the LAMP technology in field is limited because it requires high temperature conditions (60–65 °C). Cas12a is a recently identified nuclease that can nonspecifically digest ssDNA or single-stranded RNA (ssRNA) after sgRNA-mediated DNA binding of the target sequence [12]. Based on Cas12a's sequence recognition specificity and ssDNA nuclease activity, we used this protein in combination with the RPA and NALFIA technologies for rapid MoT detection (Figs. 6(a) and (b)). This process first uses RPA to amplify the MoT-specific sequences in MoT, and the amplified product is then incubated with sgRNA that targets *MoT-6098* and *MoT-6099* sequences and the Cas12a protein. If the DNA sample of MoT contains the target sequences, the nuclease activity of Cas12a will be activated and the Cas12a protein will

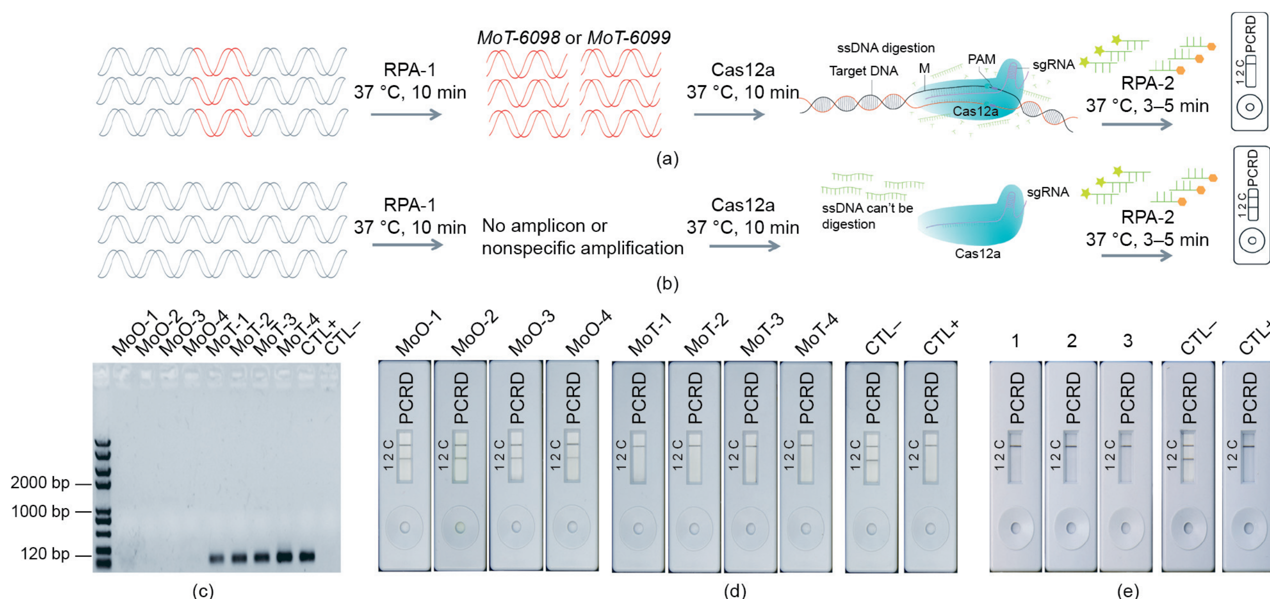

Fig. 6. Rapid detection of MoT by integrating the Cas12a protein with RPA and NALFIA technologies. (a) The Cas12a-mediated detection method when a DNA sample contains the *MoT-6098* or *MoT-6099* sequence. (b) The Cas12a-mediated detection method when a DNA sample does not contain the *MoT-6098* or *MoT-6099* sequence. (c) Detection of the amplified DNA from the first RPA on an agarose gel using the *MoT-6098* primer pair. (d) NALFIA detection of the *MoT-6098* sequence in MoO and MoT strains after the second RPA on the strips. (e) NALFIA detection of the *MoT-6098* sequence using DNA isolated from mycelia of the MoT strain BTJP4-5 (lane 1), a wheat leaf artificially inoculated with MoT strain BTJP4-5 (lane 2), and the panicle neck of a wheat plant that was naturally infected with MoT in the field (lane 3). CTL-: negative control; CTL+: positive control.

nonspecifically digest the randomly designed ssDNA probes in the reaction. A second RPA then uses the FAM-labeled primer (left primer) and the biotin-labeled primer (right primer) for the ssDNA fragments, and the amplicon is loaded onto lateral flow strips (PCRD) for the detection of the ssDNA fragment. If the MoT sample contains the sgRNA target sequences, the activated Cas12a will digest the ssDNA, and thus, the biotin- and FAM-labeled fragment of ssDNA will not appear on the PCRD strip (Fig. 6(a)). In contrast, the ssDNA will be amplified in the second RPA and a biotin- and FAM-labeled ssDNA band will appear on the PCRD strip (Fig. 6(b)). We selected four MoT and four MoO strains for the RPA and NALFIA assays and included a 120-bp randomly designed DNA fragment (see experimental procedures in Appendix A) for Cas12a digestion. The agarose gel analysis confirmed the amplification of the *MoT-6098* and *MoT-6099* fragments in the first RPA reaction for the four MoT samples, but not for the four MoO samples (Fig. 6(c)). After the second RPA, the reaction solutions were loaded onto the PCRD strips for NALFIA detection. The results showed that the ssDNA band (the second band from top) was clearly evident in all four MoO samples and in the positive control, but not in the four MoT samples or in the negative control (Fig. 6(d)).

Furthermore, when used with the DNA isolated from MoT-infected wheat leaves, the NALFIA assay detected MoT in both artificially infected leaves and naturally infected panicles (Fig. 6(e)). To further test the sensitivity of the Cas12a-based NALFIA method, we compared it with the conventional PCR method using different concentrations of DNA from fungal mycelia (Fig. 7). As shown in Figs. 7(a) and (c), a week band was observed when the fungal

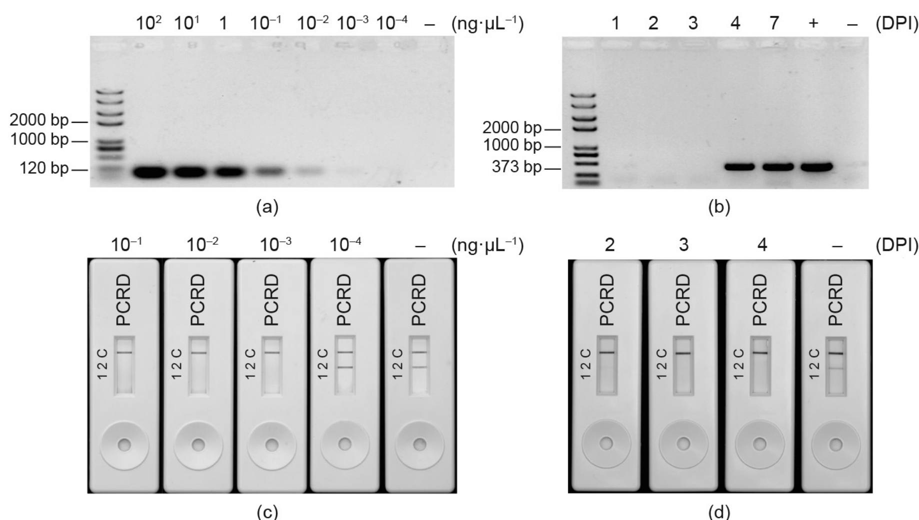

Fig. 7. Sensitivity assays of the Cas12a-RPA-NALFIA-mediated method and its comparison with traditional PCR. (a) Sensitivity of PCR to detect the *MoT-6098* sequence using different concentration of template DNA from mycelia on an agarose gel. (b) Sensitivity of PCR to detect the *MoT-6098* sequence in the inoculated leaves on an agarose gel. (c) Sensitivity of the Cas12a-RPA-NALFIA-mediated method to detect the *MoT-6098* sequence from mycelia on PCRD strips. (d) Sensitivity of the Cas12a-RPA-NALFIA-mediated method to detect the *MoT-6098* sequence in the inoculated leaves on PCRD strips. DPI: days post inoculation.

DNA concentration was 0.01 μg·μL^{-1}. In contrast, the Cas12a-based NALFIA method clearly detected the target sequence in as little as 0.001 μg·μL^{-1} of the fungal DNA. When DNA from the inoculated leaves was used, PCR detected the target sequence four days post-inoculation (Fig. 7(b)). However, the Cas12a-based NALFIA method detected the target sequence as early as two days post-inoculation (Fig. 7(d)). These results demonstrate that the new detection method can rapidly, accurately, and efficiently identify MoT in wheat fields, thereby providing a simple and economical platform for the detection of the devastating wheat blast disease, in South American and South Asian countries.

4. Discussion

With the development of NGS technology, researchers can now rapidly and inexpensively sequence an individual genome. Although NGS technology enables researchers to identify genomic differences among closely related organisms, identifying sequences that are unique to a specific species or pathotype remain challenging. To identify sequences in MoT that are not present in MoO, we re-sequenced two MoT strains from Brazil. Using a pairwise, two-step global alignment method, we aligned the potentially different genes in the two MoT strains with sequenced MoO and MoT strains to identify MoT-specific genes. Among the 57 MoT-unique candidate genes, we found two sequences that were present in MoT and other *M. oryzae* strains collected from alternative hosts, but not in any MoO strains. Our PCR analysis with 53 *M. oryzae* strains confirmed the results and demonstrated that the combination of NGS sequencing with a two-step global alignment method swiftly enables accurate identification of unique sequences when comparing two highly similar genomes.

Rapid and accurate diagnostic assays of emergent and re-emergent diseases are required to control crop diseases. In some cases, disease control depends on the immediate identification of the causal pathogens in the field. Immunological-based detection (IBD) techniques, such as enzyme-linked immunosorbent assay, have been used for on-site pathogen detection since the 1970s. As the antibodies used in the conventional IBD method are not stable, the method now uses modified colloidal gold or other substitute particles that are activated and therefore visualized by specific antibodies. The limitation of the IBD method is that specific antibodies are unavailable for most plant pathogens. Over the last four decades, PCR and RT-PCR have been used extensively to detect pathogen DNA or RNA sequences in infected plants [22]. The disadvantages of the PCR method are the requirement of a laboratory with a PCR machine and the relatively long time (4–8 h) required to obtain the diagnostic result. In contrast, NALFIA is a paper-based platform for the detection and quantification of analytes in complexes, providing results within 5–30 min [23]. The low cost and ease of the assay have resulted in the expansion of the NALFIA application in multiple fields, including plant pathogen detection. The combination of NALFIA with nucleotide detection methods, such as LAMP and RPA, has recently become a popular way to detect plant pathogens [24]. Another diagnostic method uses the Cas12a protein, which has indiscriminate ssDNA cleavage activity that completely degrades ssDNA molecules after binding to the target sequence through sgRNA [16]. By combining Cas12a single-stranded deoxyribonuclease activation with isothermal amplification, a new method called DNA endonuclease-targeted clustered regularly interspaced short palindromic repeats trans reporter was developed for the rapid and specific detection of a virus in human samples [16].

In this study, we first determined the sensitivity of primers with PCR and LAMP at isothermal conditions. Following which, we included the Cas12a protein and a random DNA fragment in the first RPA reaction to amplify the target sequences in MoT with *MoT-6098* or MoT-6099 primer pairs, at 37 °C. In the second RPA reaction, biotin- and FAM-labeled primers were used to amplify the random DNA fragment for PCRD strip detection. Positive results were obtained with DNA isolated from MoT mycelial tissues and from MoT-infected wheat leaves. The entire procedure required only about 0.5 h and was conducted at 25–40 °C without the need for any major laboratory equipment. The total cost of the assay per sample was about 6 USD when commercial PCRD strips were used. The cost per sample can be reduced to less than 4 USD if the strips are manufactured on a large scale using non-patented methods. Therefore, the simple method developed in this study can be easily adapted by plant pathologists, quarantine specialists, and agriculture extension workers to rapidly detect MoT in infected plants, seed lots, and alternate hosts.

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Data availability statement

The data that support the findings of this study have being submitted to GenBank and the accession numbers are JAAXMV000000000 and JAAXMU000000000.

Compliance with ethics guidelines

Houxiang Kang, Peng Ye, Kangyu Hua, Yufei Deng, Maria Bellizzi, Dipali Rani Gupta, Nur Uddin Mahmud, Alfredo S. Urashima, Sanjoy Kumar Paul, Gary Peterson, Yilin Zhou, Md Tofazzal Islam, Xueping Zhou, and Guo-Liang Wang declare that they have no conflict of interest or financial conflicts to disclose

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2020.07.016.

References

[1] Talbot NJ. On the trail of a cereal killer: exploring the biology of *Magnaporthe grisea*. Annu Rev Microbiol 2003;57(1):177–202.
[2] Igarashi S, Utiamada CM, Igarashi LC, Kazuma AH, Lopes RS. Pyricularia em trigo. 1. Ocorrência de Pyricularia sp. no estado do Paraná. Fitopatol Bras 1986;11:351–2. Portuguese.
[3] Tufan HA, McGrann GRD, Magusin A, Morel JB, Miché L, Boyd LA. Wheat blast: histopathology and transcriptome reprogramming in response to adapted and nonadapted *Magnaporthe* isolates. New Phytol 2009;184(2):473–84.
[4] Islam MT, Croll D, Gladieux P, Soanes DM, Persoons A, Bhattacharjee P, et al. Emergence of wheat blast in Bangladesh was caused by a South American lineage of *Magnaporthe oryzae*. BMC Biol 2016;14(1):84.
[5] Mottaleb KA, Singh PK, Sonder K, Kruseman G, Tiwari TP, Barma NCD, et al. Threat of wheat blast to South Asia's food security: an ex-ante analysis. PLoS ONE 2018;13(5):e0197555.

[6] Gupta DR, Avila CSR, Win J, Soanes DM, Ryder LS, Croll D, et al. Cautionary notes on use of the MoT3 diagnostic assay for *Magnaporthe oryzae* wheat and rice blast isolates. Phytopathology 2019;109(4):504–8.

[7] Mottaleb KA, Singh PK, Sonder K, Kruseman G, Erenstein O. Averting wheat blast by implementing a 'wheat holiday': in search of alternative crops in West Bengal, India. PLoS ONE 2019;14(2):e0211410.

[8] Castroagudín VL, Moreira SI, Pereira DAS, Moreira SS, Brunner PC, Maciel JLN, et al. *Pyricularia graminis-tritici*, a new *Pyricularia* species causing wheat blast. Persoonia 2016;37:199–216.

[9] Sadat MA, Choi J. Wheat blast: a new fungal inhabitant to Bangladesh threatening world wheat production. Plant Pathol J 2017;33(2):103–8.

[10] Pieck ML, Ruck A, Farman ML, Peterson GL, Stack JP, Valent B, et al. Genomics-based marker discovery and diagnostic assay development for wheat blast. Plant Dis 2017;101(1):103–9.

[11] Thierry M, Gladieux P, Fournier F, Tharreau D, Loos R. A genomic approach to develop a new qPCR test enabling detection of the *Pyricularia oryzae* lineage causing wheat blast. Plant Dis 2019;104(1):60–70.

[12] Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000;28(12):E63.

[13] Dhama K, Karthik K, Chakraborty S, Tiwari R, Kapoor S, Kumar A, et al. Loop-mediated isothermal amplification of DNA (LAMP): a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. Pak J Biol Sci 2014;17(2):151–66.

[14] Piepenburg O, Williams CH, Stemple DL, Armes NA. DNA detection using recombination proteins. PLoS Biol 2006;4(7):e204.

[15] Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 2015;163(3):759–71.

[16] Chen JS, Ma E, Harrington LB, Da Costa M, Tian X, Palefsky JM, et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science 2018;360(6387):436–9.

[17] Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ, Zhang F. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. Science 2018;360(6387):439–44.

[18] Kent WJ. BLAT— the BLAST-like alignment tool. Genome Res 2002;12(4):656–64.

[19] Kang H, Zhu D, Lin R, Opiyo SO, Jiang N, Shiu SH, et al. A novel method for identifying polymorphic transposable elements via scanning of high-throughput short reads. DNA Res 2016;23(3):241–51.

[20] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25(17):3389–402.

[21] Xue M, Yang J, Li Z, Hu S, Yao N, Dean RA, et al. Comparative analysis of the genomes of two field isolates of the rice blast fungus *Magnaporthe oryzae*. PLoS Genet 2012;8(8):e1002869.

[22] Fang Y, Ramasamy RP. Current and prospective methods for plant disease detection. Biosensors 2015;5(3):537–61.

[23] Koczula KM, Gallotta A. Lateral flow assays. Essays Biochem 2016;60(1):111–20.

[24] Zhao G, Hou P, Huan Y, He C, Wang H, He H. Development of a recombinase polymerase amplification combined with a lateral flow dipstick assay for rapid detection of the *Mycoplasma bovis*. BMC Vet Res 2018;14(1):412.