

Detection of Avirulence Gene *AvrPi9* in *Magnaporthe oryzae*, a Rice Blast Fungus, Using a Combination of RPA and CRISPR-Cas12a Techniques

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ABSTRACT

Rice blast disease is one of the most devastating diseases of rice production worldwide, which causes by an ascomycete fungus, *Magnaporthe oryzae*. The virulence of the rice blast fungus is determined by avirulence genes (*Avr* genes). Therefore, the identification of *Avr* genes is important for rice resistance variety improvement. *Avr* genes are currently identified using the pathogenicity assay with rice near-isogenic lines (NILs) or PCR amplification and gene sequencing, both of which are time-consuming and labor-intensive methods. This study aims to develop a simple method for *Avr* gene identification using *AvrPi9* as a model. A recombinase polymerase amplification (RPA) technique was carried out to amplify *AvrPi9* by incubating rice blast fungus genomic DNA with gene-specific primers at 37°C for 20 min. Cas12a-based *AvrPi9* detection was performed by incubating at 37°C for 5 min. The fluorescence signal was visualized by the naked eye under an LED transilluminator. The study found that *AvrPi9* can be amplified and detected using RPA and a Cas12a-based method. *AvrPi9_crRNA2* has a higher efficiency than *AvrPi9_crRNA1*. The sensitivity of the method was 3.8 ng of DNA target for *AvrPi9_crRNA1* and 1.9 ng of DNA target for *AvrPi9_crRNA2*. This RPA and Cas12a combination technique is a newer method for *Avr* gene detection in plants and has several advantages over traditional methods. It is considered easier to use and more efficient in terms of time and labor, making it a potentially useful tool for plant breeders and pathologists.

1. Introduction

Rice is the most important food crop for mankind, which is consumed by more than 50% of the world's population. The United States Department of Agriculture-Economic Research Service reported that Thailand is the second largest rice exporter between 2020 to 2022, especially the two world-famous jasmine rice varieties, KDML105 and RD6. However, both KDML105 and RD6 rice varieties are highly susceptible to rice blast disease (Chaipanya *et al.* 2017; Childs 2022).

Rice blast disease caused by the fungus *Magnaporthe oryzae* is a major threat to rice production worldwide. The fungus can cause

severe damage to the leaves, stems, and grain-filled panicles of the rice plant, leading to reduced yields and decreased grain quality. Effective management strategies are necessary to minimize the impact of this disease (Miah *et al.* 2017). According to the gene for gene concept, rice blast resistance occurs only when the rice resistance gene (*R* gene) can recognize the avirulence gene (*Avr* gene) of rice blast fungus resulting in the triggering of effector-triggered immunity or ETI response (Flor 1971; Glowacki *et al.* 2011; Keller *et al.* 2000; Wang *et al.* 2017). Twelve *Avr* genes of rice blast fungus have been cloned, including *AvrPi9*. *AvrPi9* is an avirulence gene located on chromosome 7 that expresses strongly at early stages of blast disease. It encodes a small secreted effector that is localized in the biotrophic interfacial complex of the rice blast fungus and is translocated

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to rice cells during infection. The *AvrPi9* gene product is recognized by the Pi9 resistance protein at the *Pi2/9* locus of rice (Wang *et al.* 2017). *AvrPi9* is reported to be widely distributed among Thai rice blast isolates. The detection of this gene can facilitate the monitoring of rice blast population and data can be useful for the rice blast resistance breeding programs (Sutthiphai *et al.* 2022).

Identifying *Avr* genes is crucial for rice blast disease resistance breeding initiatives. Currently, breeders and plant pathologists determine *Avr* genes by conducting pathogenicity assays on rice near-isogenic lines (NILs) (Parinthawong and Tansian, 2020) or the PCR amplification and *Avr* gene sequencing (Sutthiphai *et al.* 2022). Both methods, however, are time-consuming and require a lot of effort.

The isothermal amplification method was developed to replace the PCR thermocycling step by conducting it at a single temperature, i.e., loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), helicase-dependent amplification (HDA), nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), and multiple strand displacement amplification (MDA) (Moon *et al.* 2022). RPA technique can be operated at temperatures ranging from 37–42°C in a heat box. RPA process begins when a recombinase enzyme binds to primers, resulting in the formation of a recombinase-primer complex. The complex is then bound to a primer-specific site on the DNA template. Furthermore, single-stranded binding proteins stabilize the displaced DNA strand to prevent the inserted primer from being ejected. To begin DNA synthesis, the DNA polymerase binds to the 3' end of the primer (Daher *et al.* 2016).

CRISPR-Cas system is a prokaryotic adaptive immunity mechanism used to degrade invading nucleic acids, becoming a new generation of genome-engineering tools. CRISPR-Cas12a (formerly known as *cpf1*) is an RNA-guided endonuclease that belongs to CRISPR-Cas class 2 type V. This system is formed by the interaction of crRNA and Cas12a nuclease. When the Cas12a enzyme recognizes the PAM sequence of TTTV on the DNA and crRNA binds to the target sequence at a position next to 3' of PAM on the opposite DNA strand, the Cas12a enzyme is activated and the target DNA is degraded. Furthermore, after degrading the target DNA, Cas12a degrades non-

target ssDNA, a process known as collateral cleavage activity. CRISPR-Cas12a has recently been purposed as a detection tool for its collateral cleavage activity by providing a quenched ssDNA reporter (Figure 1A) (Makarova *et al.* 2020; Pickar-Oliver and Gersbach 2019).

The coupled isothermal amplification by recombinase polymerase amplification with CRISPR-Cas12a was first developed and used for the diagnosis of the human papillomavirus (Chen *et al.* 2018). Since then, it was applied as a plant pathogen detection tool based on the pathogen's conserved gene, i.e., *Xanthomonas*, *Fusarium*, and *Magnaporthe* (Buddhachat *et al.* 2022; Li *et al.* 2022; Zhang *et al.* 2020). However, the detection of pathogens alone is inadequate for developing plant resistance in breeding programs. This study aims to simplify the detection of avirulence genes in the rice blast fungus by using RPA-Cas12a techniques using *AvrPi9* as a model.

2. Materials and Methods

2.1. Fungus Materials

Three *Magnaporthe oryzae* isolates, consisting of two with *AvrPi9* (10100 and 70-15) and one without (NYK55003), were grown on rice flour agar (RFA: 20 g/L rice flour, 2 g/L yeast extract, and 20 g/L agar) at 28°C for 7 days, and later the active mycelium was transferred to new RFA plates and continued to be cultured at 28°C for 7–10 days.

2.2. DNA Extraction

Genomic DNA was extracted from the mycelium of each *M. oryzae* isolate using the cetyltrimethylammonium bromide (CTAB) method. The mycelium was ground into powder in liquid nitrogen and transferred into a microcentrifuge tube with 700 µL CTAB (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, and 2% SDS) and 2 µL β-mercaptoethanol. After incubation at 65°C for 1 hour and cooling down to room temperature, 500 µL of chloroform:isoamyl alcohol (24:1 v/v) was added into the sample, mixed by slight inversion, and centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous phase was transferred into the new microcentrifuge tube. Then, 500 µL of isopropanol was added and mixed by gentle inversion. After overnight incubation at -20°C, the sample was centrifuged at 12,000 rpm for 10 min at 4°C, discarded

supernatant, washed DNA pellet with 500 µL of 95% ethanol, centrifuged at 12,000 rpm for 10 min at 4°C, and discarded 95% ethanol. Then, the DNA sample was washed with 500 µL of 70% ethanol, centrifuged at 12,000 rpm for 10 min at 4°C, and discarded 70% ethanol, repeated this step two times. After that, the DNA pellet was dried at room temperature for 15 min. Finally, the DNA pellet was resuspended with 30 µL TE buffer and incubated at 37°C for 15 min. The DNA quality was determined by 1% agarose gel electrophoresis and DNA quantity was quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The purified genomic DNA was diluted with sterile distilled water to a concentration of 50 ng/µL before use.

2.3. *AvrPi9* Amplification

Polymerase chain reaction (PCR) and Recombinase polymerase amplification (RPA) were performed to amplify *AvrPi9* using gene-specific primers (Table 1 and Figure 1B). The PCR was performed with a total volume of 50 µL containing 5 µL of 10X PCR buffer, 2 µL of 50 mM MgCl₂, 1 µL of 10 mM dNTP mixture, 2 µL of 5 µM of each primer, 0.5 µL of vivantis Taq (Vivantis Technologies, Malaysia), and 50 ng of fungal DNA using an Eppendorf Mastercycler® nexus gradient thermal cycler (Eppendorf Co, USA) under the following conditions: initial denaturation at 95°C for 5 min followed by additional denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min (these three steps were repeated for 35 cycles); a final extension at 72°C for 5 min. The PCR product was determined by 1% agarose gel electrophoresis and purified using a PCR purification kit (BIO-HELIX, Taiwan). The purified PCR product quality was determined by 1% agarose gel electrophoresis, quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and sequenced with a commercial sequencing service provider (U2Bio, Thailand).

The RPA was performed using a slightly modified manufacturer protocol. The 46.5 µL of reaction mix was prepared in a microcentrifuge tube, containing 2.4 µL of 10 µM of each primer, 29.5 µL of primer-free rehydration buffer, and 12.2 µL of ultrapure water. Then, the reaction mixture was added to the lyophilized TwistAmp® Basic reaction, and 46.5 µL of the mixture was divided by four and transferred to the PCR tubes. Finally, 50 ng of fungal DNA and 0.6 µL of 280 mM Magnesium Acetate were added to the mixture in each PCR tube before incubating at 37°C for 20 min. The RPA product was determined by 2% agarose gel electrophoresis.

2.4. crRNAs Design and ssDNA Fluorophore-Quencher Reporter (FQ Reporter)

The nucleotide sequence of *AvrPi9* was downloaded from NCBI (GenBank accession numbers: MW288317). The crRNAs were manually designed to target the *AvrPi9* gene at a position next to 3' of PAM sequence (TTTV) on the opposite DNA strand. The oligonucleotide of each crRNA was chemical synthesized with IDT (Integrated DNA Technologies, USA). The ssDNA fluorophore-quencher reporter (FQ reporter) was synthesized as follows: 5'-/56-FAM/TTATT/3BHQ_1/-3' via IDT (Integrated DNA Technologies, USA).

2.5. Examination of Cas12a-based *AvrPi9* Detection in Genomic DNA

To examine the CRISPR-Cas12a assay for *AvrPi9* detection in genomic DNA of rice blast fungus without gene amplification. The 50 ng/µL genomic DNA of three rice blast isolates, consisting of two with *AvrPi9* (10100 and 70-15) and one without (NYK55003), were used to test in this experiment and 30 ng/µL *AvrPi9* PCR product of each rice blast isolate was used as a positive control in Cas12a-based detection assay. The CRISPR-Cas12a reaction was prepared with a

Table 1. Nucleotide sequences of PCR primers, RPA primers, and crRNAs

Reaction	Name	Sequence (5'-3')	Product size (bp)	Reference
PCR	<i>AvrPi9</i> _Fw	CAGCCCAACATGCAGTTCTC	342	Wu <i>et al.</i> (2015)
	<i>AvrPi9</i> _Rv	CTACCAGTGGCTCTTTTCGAC		
RPA	<i>AvrPi9</i> _RPA-F1	AGGAAGCGCCCGAGGCCGAAGGTGACGCCAA	342 to 115	This study
	<i>AvrPi9</i> _RPA-R1	TGGGGGGTTGCTTATTTACCCATCATCGCAGCC		
Cas12a detection	<i>AvrPi9</i> _crRNA1	UAAUUUCUACUAAGUGUAGAUCGCCCGCUACACCUGCCCCAA		
	<i>AvrPi9</i> _crRNA2	UAAUUUCUACUAAGUGUAGAUCGCCGCUUGUGGCAGUUGGGG		

*Underlined sequence represents a target-specific sequence

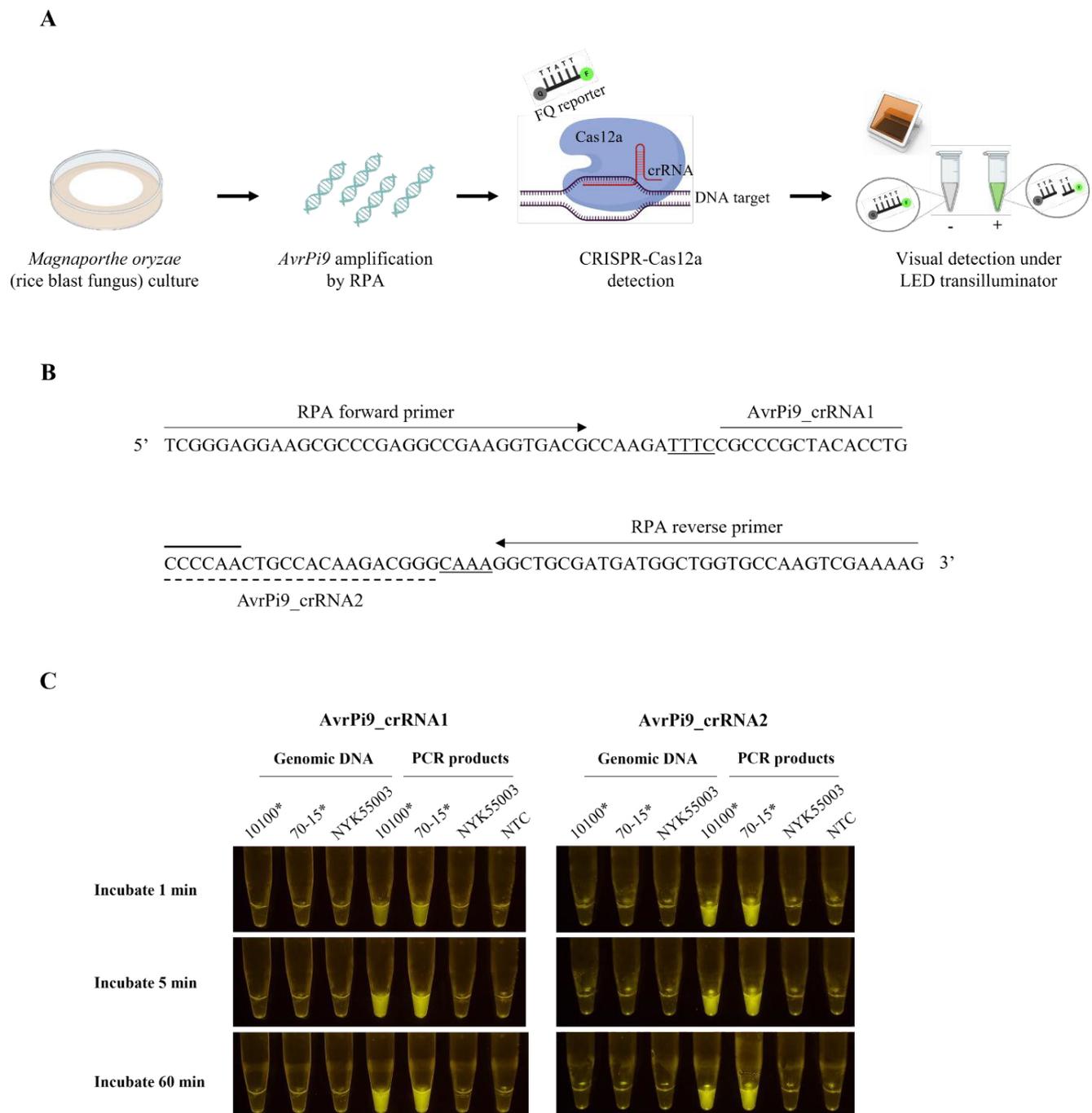


Figure 1. Cas12a-based *AvrPi9* detection method (A) Schematic diagram of RPA/CRISPR-Cas12a detection. (B) Nucleotide sequence of *AvrPi9*, RPA primer design region, PAM sequence (underlined sequence), and crRNAs design region. (C) Cas12a-based *AvrPi9* detection of genomic DNA. The asterisk represents rice blast isolate, which contains *AvrPi9*. PCR products of *AvrPi9* were used as a positive control. NTC is a non-template control

total volume of 20 μL containing 2 μL of 10X NEBuffer r2.1 Reaction Buffer, 2 μL of 1 μM crRNA, 2 μL of 1 μM LbCas12a enzyme (New England, USA), 1 μL of 10 μM FQ reporter, 12 μL of nuclease-free water and 1 μL of genomic DNA or 1 μL of PCR product and incubated at 37°C for various time intervals (1, 5, and 60 min). The green fluorescence signal was visualized by eyes under an LED transilluminator (MaestroGen, Taiwan).

2.6. Optimization of PCR/CRISPR-Cas12a Detection Assay

To optimize of CRISPR-Cas12a assay for *AvrPi9* detection, the experiment was conducted using various concentration ratios of crRNAs and LbCas12a, ranging from 15:15 to 100:100 nM in a 1:1 ratio. The 30 ng/ μL *AvrPi9* PCR product of three rice blast isolates, consisting of two with *AvrPi9* (10100 and 70-15) and one without (NYK55003) were used to test in the experiment. CRISPR-Cas12a reaction was prepared with a total volume of 20 μL containing 2 μL of 10X NEBuffer r2.1 Reaction Buffer, 1 μL of 10 μM FQ reporter, 1 μL of PCR product, [15 nM] crRNA:[15 nM] LbCas12a or [25 nM] crRNA:[25 nM] LbCas12a or [50 nM] crRNA:[50nM] LbCas12a or [100 nM] crRNA:[100 nM] LbCas12a, and nuclease-free water up to 20 μL . The reaction was incubated at 37°C for 5 min. The green fluorescence signal was visualized by eyes under an LED transilluminator (MaestroGen, Taiwan).

2.7. Optimization of RPA/CRISPR-Cas12a Detection Assay

To optimize an optimal volume of *AvrPi9* RPA product for the CRISPR-Cas12a detection assay, two volumes (1 μL and 2 μL) of *AvrPi9* RPA product were used for testing in this experiment. Three rice blast isolates, consisting of two with *AvrPi9* (10100 and 70-15) and one without (NYK55003), were used as models. The CRISPR-Cas12a reaction was prepared with a total volume of 20 μL containing 2 μL of 10X NEBuffer r2.1 Reaction Buffer, 1 μL of 1 μM crRNA, 1 μL of 1 μM LbCas12a enzyme (New England, USA), 1 μL of 10 μM FQ reporter, 1 μL or 2 μL of PRA product, nuclease-free water up to 20 μL and incubated at 37°C for 5 min. The green fluorescence signal was

visualized by eyes under an LED transilluminator (MaestroGen, Taiwan).

2.8. Examination of the Limit of Detection (LoD)

The sensitivity of the detection was examined through a 2-fold serial dilution of *AvrPi9* PCR product of rice blast isolate (10100), starting from an initial concentration of 30 ng/ μL . The concentration of *AvrPi9* ranged from 30 ng/ μL to 0.1 ng/ μL . The reaction was prepared with a total volume of 20 μL containing 2 μL of 10X NEBuffer r2.1 Reaction Buffer, 1 μL of 1 μM crRNA, 1 μL of 1 μM LbCas12a enzyme (New England, USA), 1 μL of 10 μM FQ reporter, 1 μL of diluted PCR product, and 14 μL of nuclease-free water and incubated at 37°C for 5 min in CFX96 Touch Real-Time PCR machine (Bio-Rad Laboratories, USA). The kinetic of fluorescence signal was measured by recording the mean relative fluorescence unit (RFU) of three replicates for each sample in the FAM channel using a Real-Time PCR machine. Additionally, the green fluorescence signal was visualized by eyes under an LED transilluminator (MaestroGen, Taiwan).

2.9. Validation of RPA/CRISPR-Cas12a Detection Assay

To validate CRISPR-Cas12a assay for *AvrPi9* RPA products, fifteen rice blast isolates from Sutthiphai *et al.* (2022) contain different *Avr* gene, including *AvrPi9* were used to test in this experiment (Supplementary 1). Genomic DNA was extracted from the mycelium of each rice blast isolate using CTAB method. The modified RPA method in this study was performed to amplify *AvrPi9* in 50 ng/ μL genomic DNA by incubating at 37°C for 20 min. RPA products was determined by gel electrophoresis and 1 μL of RPA products was used to test with CRISPR-Cas12a assay. The CRISPR-Cas12a reaction was prepared with a total volume of 20 μL containing 2 μL of 10X NEBuffer r2.1 Reaction Buffer, 1 μL of 1 μM crRNA, 1 μL of 1 μM LbCas12a enzyme (New England, USA), 1 μL of 10 μM FQ reporter, 1 μL of RPA product, and 14 μL of nuclease-free water and incubated at 37°C for 5 min. The green fluorescence signal was visualized by eyes under an LED transilluminator (MaestroGen, Taiwan).

3. Results

3.1. Genomic DNA Detection of *AvrPi9* Using Cas12a Method

Two crRNAs, *AvrPi9_crRNA1* and *AvrPi9_crRNA2*, were designed to target *AvrPi9* in rice blast fungus. *AvrPi9_crRNA1* is designed to have a binding site on the reverse DNA strand, while *AvrPi9_crRNA2* is designed to have a binding site on the forward DNA strand (Figure 1B). Each crRNA comprises a 21-base loop sequence, is an important part of crRNA secondary structure formation or pseudoknot-type hairpin structure formation, along with a 21 base target-specific sequence (Table 1).

crRNAs were used to detect *AvrPi9* in 50 ng/ μ L genomic DNA, utilizing 100 nM crRNA and 100 nM LbCas12a. The results showed that green fluorescence was not observed in genomic DNA test of rice blast isolates containing *AvrPi9* for all incubation times, in

comparison to the PCR products of *AvrPi9* (positive control) and the non-template control (NTC). The results indicate that Cas12a-based detection method was not effective in identifying *AvrPi9* in the fungal genomic DNA (Figure 1C).

3.2. Optimization of Cas12a-based *AvrPi9* Detection

To optimize the Cas12a-based *AvrPi9* detection, the various concentration ratios of crRNAs and Cas12a were performed in a concentration ratio of 1:1, ranging from 15:15 to 100:100 nM, and incubated with 30 ng/ μ L of *AvrPi9* PCR products of three rice blast isolates (10100, 70-15, and NYK55003) (Supplementary 2). The results showed that 50 nM of crRNAs and 50 nM of Cas12a were the minimal concentration that produced a green fluorescence signal, which was suitable condition for detecting PCR products of *AvrPi9*

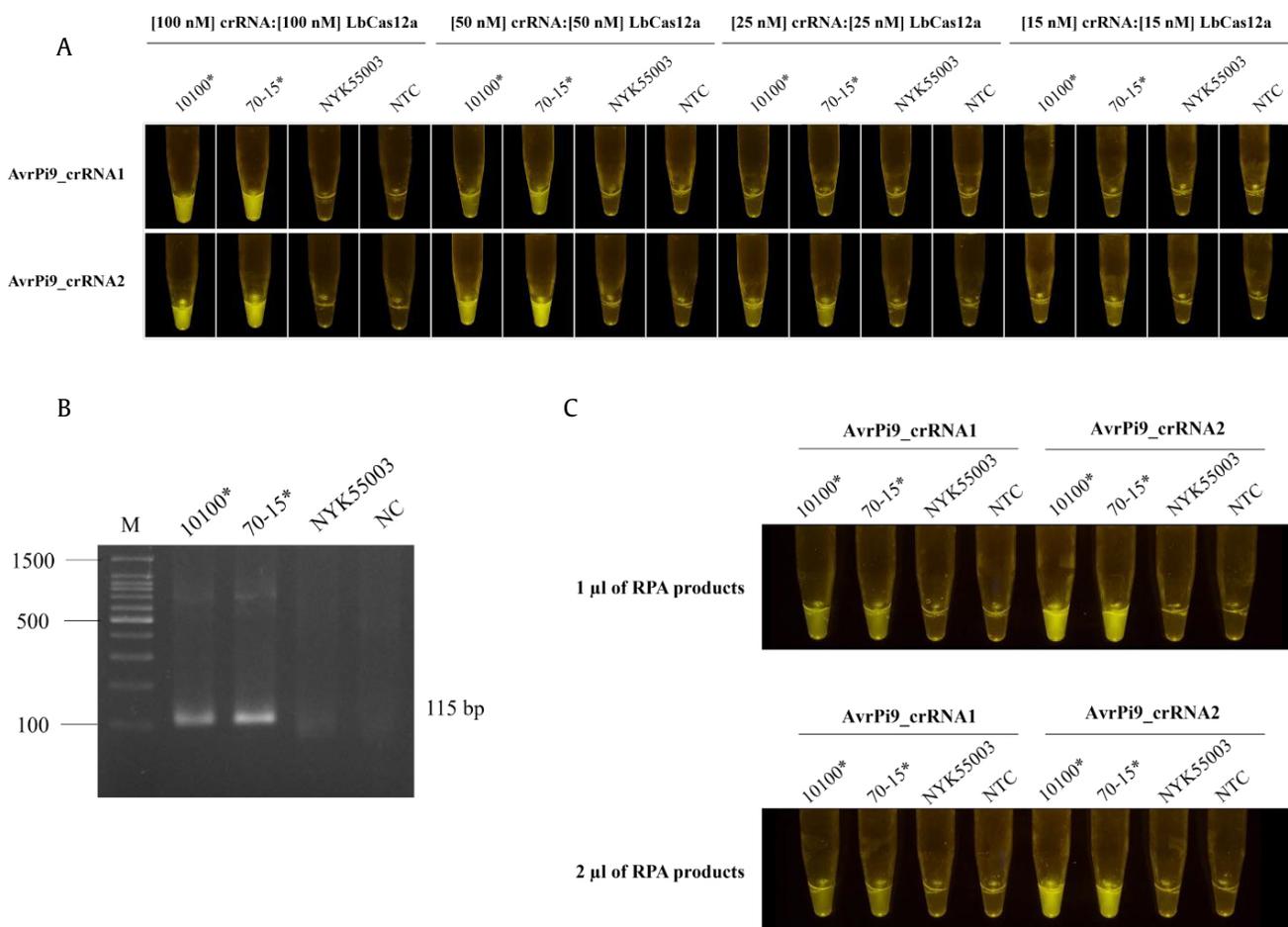


Figure 2. Optimization of CRISPR-Cas12a detection assay (A) Optimization of Cas12a-based *AvrPi9* detection of PCR products. The asterisk represents rice blast isolate containing *AvrPi9*; NTC is a non-template control. (B) RPA products of *AvrPi9*. The asterisk represents rice blast isolate, which contains *AvrPi9*; NC is a negative control. (C) Cas12a-based *AvrPi9* detection of RPA products using 50 nM of crRNAs and 50 nM of Cas12a. The asterisk represents rice blast isolate containing *AvrPi9*; NTC is a non-template control

(Figure 2A). Therefore, this condition was selected to detect RPA products of *AvrPi9*.

To optimize an optimal volume of RPA products for detecting by CRISPR-Cas12a, 1 or 2 μL of RPA products of three rice blast isolates (10100, 70-15, and NYK55003) were incubate with an optimal concentration, 50 nM, of crRNAs and Cas12a at 37°C for 5 min. Agarose gel of *AvrPi9* amplification using the RPA method was shown in Figure 2B. The results reveal that both 1 and 2 μL of *AvrPi9* RPA products can be used to successfully detect by CRISPR-Cas12a detection method (Figure 2C). In conclusion, Cas12a-based *AvrPi9* detection assay can be used to detect either PCR products or RPA products of *AvrPi9* and a minimal concentration of crRNAs and Cas12a is 50 nM.

3.3. Limit of Cas12a-based *AvrPi9* Detection

The sensitivity of Cas12a-based *AvrPi9* detection was examined by incubating a 2-fold serial dilution of *AvrPi9* PCR product with each crRNA at 37°C for 5 min, the kinetics of fluorescence signal was measured, and the green fluorescence was observed under LED transilluminator. The concentration of *AvrPi9* was diluted from 30 ng/ μL to 0.1 ng/ μL . The results showed that the limit of detection was at 3.1 ng of DNA target for *AvrPi9_crRNA1* and 1.9 ng of DNA target for *AvrPi9_crRNA2* and it was confirmed by the kinetics of fluorescence signal results, indicating that *AvrPi9_crRNA2* has higher sensitivity than *AvrPi9_crRNA1* in Cas12a-based *AvrPi9* detection (Figure 3A and B).

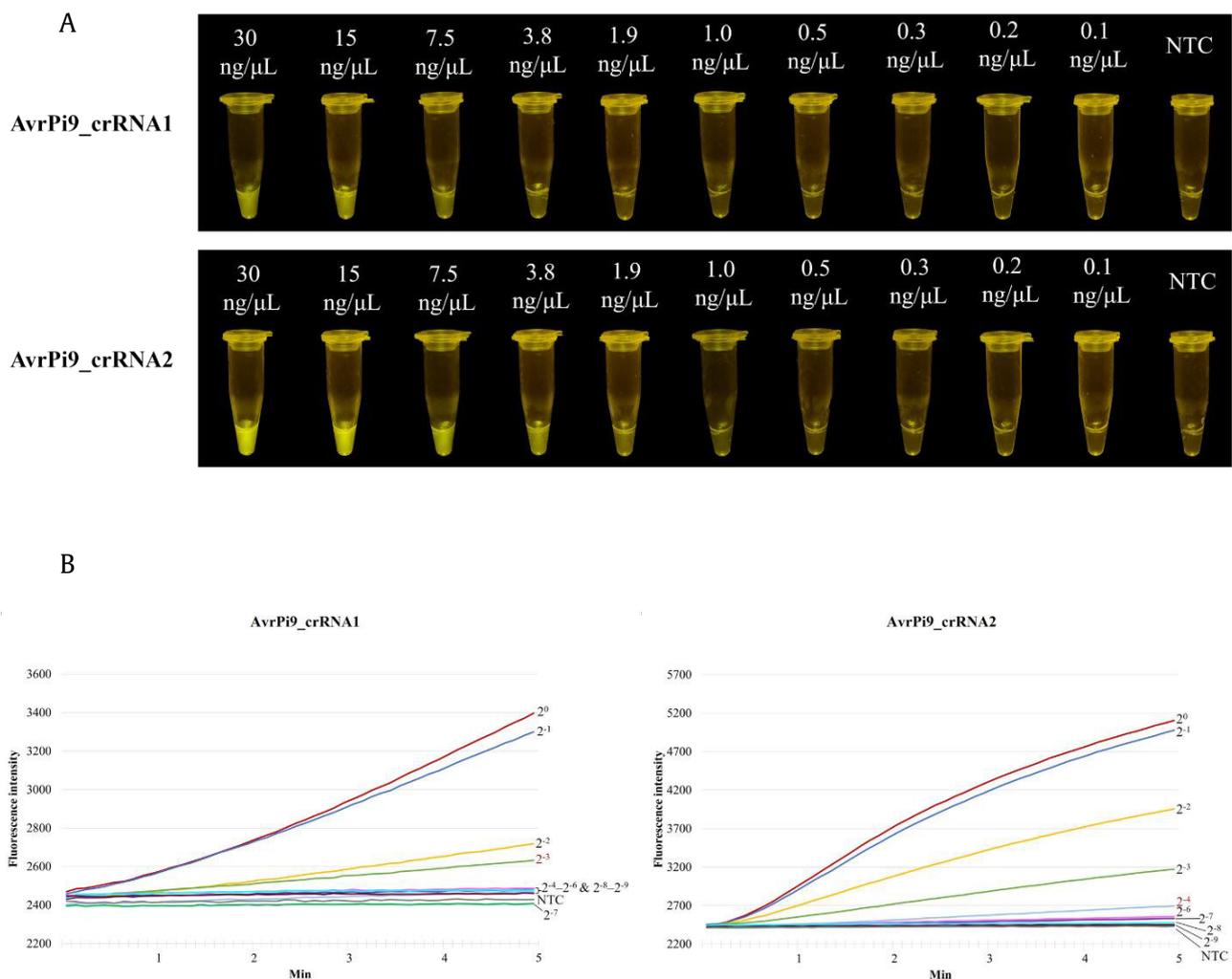


Figure 3. Limit of Cas12a-based *AvrPi9* detection (A) Limit of Cas12a-based *AvrPi9* detection of *AvrPi9_crRNA1* and *AvrPi9_crRNA2* under LED transilluminator. NTC is a non-template control. (B) Kinetics of the fluorescence signal generate over 5 min of *AvrPi9_crRNA1* and *AvrPi9_crRNA2* using different serial dilutions of *AvrPi9* PCR products. Each graph line was determined by the RFU mean of three replicates of each sample.

3.4. Validation of RPA/Cas12a-based *AvrPi9* Detection

In order to validate the detection of Cas12a-based *AvrPi9* in RPA products, fifteen isolates of rice blast fungus (Table S1) were amplified using RPA and then incubated with the CRISPR-Cas12a reaction. The agarose gel of the RPA products revealed a consistent target size of 115 bp for *AvrPi9* across all fifteen rice blast isolates. The results from Cas12a-based detection revealed that both *AvrPi9_crRNA1* and *AvrPi9_crRNA2* reactions exhibited green fluorescence in all fifteen rice blast isolates, consistent with the result of the RPA agarose gel. This outcome suggests the efficacy of RPA/Cas12a-based detection in identifying *AvrPi9* within the RPA product of rice blast fungus (Figure 4).

4. Discussion

The combination of RPA and CRISPR-Cas12a has been used as a plant pathogen detection tool, i.e., *Magnaporthe* detection, *Fusarium* detection, and *Xanthomonas* detection, based on the pathogen's conserved gene (Buddhachat *et al.* 2022; Li *et al.* 2022; Zhang *et al.* 2020). Our study was the first report that used the coupled RPA and CRISPR-Cas12a techniques to detect an avirulence gene of the rice blast fungus. The Cas12a-based *AvrPi9* detection cannot be performed without target gene amplification may be due to the low copy number of target gene, as the 40 Mb of fungal genome contains only one copy of *AvrPi9* (Dean *et al.* 2015) and our findings showed that the limit of Cas12a-based *AvrPi9* detection is 1.9 ng of target DNA, which is consistent with previous Cas12a-based plant pathogen detection research that requires to amplify the target gene before detecting by CRISPR-Cas12a

(Aman *et al.* 2020; Buddhachat *et al.* 2022; Wheatley *et al.* 2021; Zhang *et al.* 2020).

This study successfully performed a binary complex of crRNA and Cas12a at a concentration ratio of 1:1, 50 nM crRNA:50 nM Cas12a, as previously suggested by Zetsche *et al.* (2015). Similarly, several studies have shown that a concentration ratio of 1:1 (crRNA:Cas12a) is an optimal concentration to form a binary complex of crRNA and Cas12a (Buddhachat *et al.* 2022; Shin *et al.* 2021; Zhu *et al.* 2022). In addition, an optimal concentration of 50 nM crRNA:50 nM Cas12a in this study is much lower than that in previous reports, Shin *et al.* (2021) used 1 μ M crRNA:1 μ M Cas12a, Buddhachat *et al.* (2022) used 165 nM crRNA:165 nM Cas12a, and Zhu *et al.* (2022) used 250 nM crRNA: 250 nM Cas12a. Our finding suggested that the cost of the detection can be reduced significantly by using less amount of crRNA and Cas12a enzyme.

Our study discovered that *AvrPi9_crRNA2*, which is designed in the opposite direction to *AvrPi9_crRNA1* and has its binding site on the reverse strand of target DNA, is more efficient than *AvrPi9_crRNA1*. Our study used the CTAB method in the process of fungal genomic DNA extraction, which is a time-consuming approach. We suggest utilizing rapid fungal DNA extraction methods from infected plant tissue, as reported in previous studies (Shin *et al.* 2021; Zhang *et al.* 2020). Alternatively, the commercial genomic DNA extraction kit can be coupled with the RPA/Cas12a-based *AvrPi9* detection method.

This study was successful in simplifying a rice blast fungus avirulence gene detection using a combination of RPA and Cas12a-based techniques. The RPA/Cas12a-based *Avr* detection process can be performed at 37°C and completed in less than 60 min, including

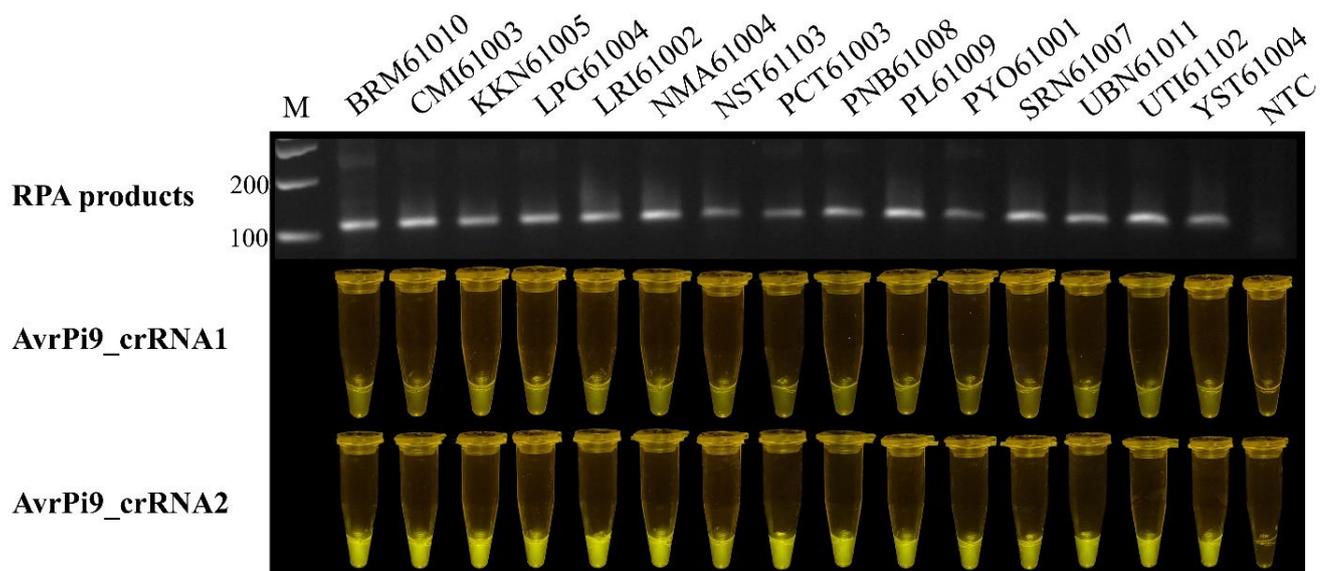


Figure 4. Validation of Cas12a-based *AvrPi9* detection in RPA products of fifteen rice blast isolates in both crRNAs (*AvrPi9_crRNA1* and *AvrPi9_crRNA2*). NTC is a non-template control

20 min for RPA incubation, 5 min for CRISPR-Cas12a incubation, and 5 min for visual evaluation under an LED transilluminator. The cost of RPA/Cas12a-based *Avr* gene detection is approximately \$10 per sample, depending on reagent prices in different countries. In conclusion, this Cas12a-based detection has advantages over the traditional *Avr* gene detection methods, pathogenicity assay, and PCR amplification and sequencing in terms of ease of use, an instrument-free method, and reduced labor and time. The application of RPA/CRISPR-Cas12a detection method for detecting avirulence gene in plant pathogens will benefit plant pathologists by providing a simple, sensitive, and fast tool for monitoring avirulence gene of rice blast fungus.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

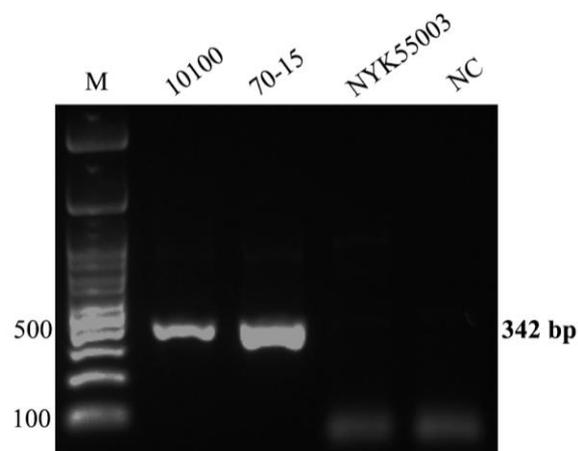
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Supplementary 1. Table of *Avr* gene information in rice blast fungus isolates used in this study. The data was obtained from Sutthiphai et al. (2022)

Isolate	<i>Avr</i> gene		
	<i>AvrPi9</i>	<i>AVR-Pik</i>	<i>AVR-Pita</i>
10100	+	+	+
70-15	+	+	+
NYK55003	-	-	-
BRM61010	+	+	+
CMI61003	+	+	+
KKN61005	+	+	+
LPG61004	+	+	+
LRI61002	+	+	+
NMA61004	+	+	+
NST61103	+	+	+
PCT61003	+	+	+
PNB61008	+	+	+
PL61009	+	+	-
PYO61001	+	+	+
SRN61007	+	+	-
UBN61011	+	+	+
UTI61102	+	+	-
YST61004	+	+	+



Supplementary 2. Agarose gel of *AvrPi9* PCR products; 10100 and 70-15 are rice blast isolate with *AvrPi9*; NYK55003 is rice blast isolate without *AvrPi9*; NC is negative control