Short Communication

A Rapid, Equipment-Free Method for Detecting *Phytophthora infestans* in the Field Using a Lateral Flow Strip-Based Recombinase Polymerase Amplification Assay

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Abstract

Late blight, caused by the oomycete *Phytophthora infestans*, is a major constraint on the production of potatoes and tomatoes as well as a constant threat to global food security. An early diagnostic tool is important for the effective management of late blight in the field. Here, in combination with a simplified DNA extraction method, we developed a lateral flow strip-based recombinase polymerase amplification (LF-RPA) assay for the rapid, equipment-free detection of *P. infestans*. This assay targets the Ras-related protein (*Ypt1*) gene and can be performed over a wide range of temperatures (25 to 45°C). All 12 *P. infestans* isolates yielded positive detection results using the LF-RPA assay, and no cross-reaction occurred with related oomycetes or fungal species. With this assay, the detection limit was 500

fg of genomic DNA in optimized conditions. Furthermore, by combining a simplified polyethylene glycol-NaOH method for extracting DNA from plant samples, the entire LF-RPA assay enabled the detection of *P. infestans* within 30 min with no specialized equipment. When applied to field samples, it successfully detected *P. infestans* in naturally diseased potato plants from eight different fields in China. Therefore, the LF-RPA assay is simple, rapid, and cost-effective and has potential for further development as a kit for diagnosing late blight in resource-limited settings or even on-site.

Keywords: diagnostics, isothermal amplification, oomycetes, RPA, visualization

Late blight, which is caused by *Phytophthora infestans*, is very destructive to potato and tomato production globally (Fry 2008; Kamoun 2006). The most famous outbreak of this disease was in Europe, leading to the Irish Potato Famine in 1845 (Fry et al. 2015). Late blight is still a major threat to human food security, causing annual losses of over \$3 billion worldwide (Fry 2008). China is the largest producer and consumer of potatoes in the world, with 5.8 million cultivated hectares (Birch et al. 2012; Haverkort et al. 2008; Li et al. 2019a). The prevention and control of late blight disease is crucial for potato production.

Because this pathogen has unique biological characteristics, multiple transmission routes, and rapid population divergence, it is difficult to manage and control (Haverkort et al. 2008). Early diagnosis is critical for managing late blight, but it can be challenging for farmers to recognize late blight symptoms. Therefore, rapid and convenient

diagnosis methods are required for the control of the disease. Various methods are available for detecting *P. infestans*, such as single-spore isolation of pathogens (Tumwine et al. 2000), conventional polymerase chain reaction (PCR) (Appel et al. 2001; Judelson and Tooley 2000), real-time PCR (Atallah and Stevenson 2006; Hussain et al. 2014), nested PCR (Khan et al. 2017), loop-mediated isothermal amplification (LAMP) (Hansen et al. 2016; Khan et al. 2017), and enzyme-linked immunosorbent assays (Harrison et al. 1990). However, these detection methods have some limitations: they are time-consuming, labor-intensive, technically complex, and usually have low sensitivity. Thus, the development of new, time-saving, cost-effective detection methods to overcome these limits is a topic of increasing interest.

A novel isothermal DNA amplification technology called recombinase polymerase amplification (RPA) was recently developed (Piepenburg et al. 2006). RPA uses three core factors: a recombinase, a DNA-binding protein, and a polymerase. Recombinases pair oligonucleotide primers with the homologous sequence in duplex DNA. Simultaneously, DNA-binding proteins bind to displaced strands of DNA, and the polymerase begins to synthesize complete double-stranded DNA where the primer has bound to the target DNA. The enzyme mixture amplifies the target region within the template exponentially. The RPA assay can be performed within 20 min without thermal cycling at temperatures between 25 and 45°C (Kersting et al. 2014; Yang et al. 2017b). RPA amplification products can also be detected using lateral flow strips (Ma et al. 2017). The results are easily read without specialized equipment, suggesting that combining RPA with lateral flow strips (LF-RPA) is suitable for developing a simple, rapid, specific method that can detect plant pathogens in resource-limited settings (Ma et al. 2017; Yang et al. 2017a). The LF-RPA assay has been used to detect diverse pathogens, including bacteria (Du et al. 2018; Ma et al. 2017), viruses (Miao et al. 2019;

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Wang et al. 2019), nematodes (Li et al. 2019b), mycoplasmas (Liu et al. 2019), and rickettsiosis (Qi et al. 2018). However, most of these are human pathogens, and only a few publications have reported the application of RPA to detect plant pathogens. Recently, a LF-RPA method was developed for detecting *P. capsici* in pepper (Yu et al. 2019) and *P. sojae* in soybean (Dai et al. 2019). In addition, the use of LF-RPA in increasingly large-scale clinical and field applications has led to the need for an effective sample-processing method. Unfortunately, DNA extraction based on the cetyl trimethylammonium bromide method or a plant genomic DNA purification kit is time-consuming and not easily adaptable for practical application (Silva et al. 2018). Chomczynski and Rymaszewski (2006) found an alkaline lysis reagent that allowed a wide variety of biological samples to be used directly in molecular amplification, without further neutralization or DNA isolation steps.

The purpose of this study was to develop a LF-RPA assay, in combination with a simplified DNA extraction method, for the rapid, equipment-free detection of *P. infestans*. To achieve this goal, various elements of LF-RPA including primer specificity and efficiency were analyzed and optimized. Moreover, *P. infestans* cultures and greenhouse-grown potato plants inoculated with *P. infestans* were used for the method development, whereas field potato leaf samples were employed for the method validation.

P. infestans isolates (Table 1) were cultured in the dark on rye mixed with 10% vegetable (V8) juice agar medium at 18°C. Other

Phytophthora and *Pythium* species (Table 1) were cultured in the dark on 10% V8 agar medium at 25°C. Pure cultures of different fungal isolates were routinely maintained on potato dextrose agar medium at 25°C in the dark (Tian et al. 2019). The genomic DNA of different isolates and infected samples was extracted using the DNA-secure Plant Kit (DP320) (Tiagen, Beijing, China), according to the manufacturer's instructions. DNA was quantified using the Qubit 3 Fluorometer and Qubit 1× dsDNA HS Assay Kit (Thermo Fisher Scientific, Wilmington, DE). Ten-fold serial dilutions of *P. infestans* genomic DNA ranging from 50 ng to 500 fg per microliter were used as DNA templates for a subsequent sensitivity test. According to the equation of Wilhelm et al. (2003), the number of *P. infestans* genomes present in 50 ng of genomic DNA was $\sim 1.9 \times 10^5$ copies, and 500 fg of DNA contains approximately two genome copies.

The target Ras-related protein (*Ypt1*) gene contains sufficient variation across a wide range of *Phytophthora* species (Schena and Cooke 2006; Schena et al. 2008) and thus was used to detect *P. infestans* in this study. The *Ypt1* sequences of 16 *P. infestans* isolates and 57 other closely related *Phytophthora* species were obtained from GenBank. Based on multiplex sequence alignment analysis, three RPA primer pairs were designed for *P. infestans*-specific gene regions (Supplementary Table S1) following the design principles in the RPA guidelines (TwistAmp DNA amplification kits combined instruction manual; TwistDx, Cambridge, U.K.). The probabilities of the formation of hairpins, self-dimers,

Table 1. Isolates tested for specificity of the LF-RPA assay

Species	Isolate	Origin	Host	LF-RPA ^a
<i>Phytophthora infestans</i>	T30-4	America	<i>Solanum tuberosum</i>	+
<i>P. infestans</i>	88069	America	<i>S. tuberosum</i>	+
<i>P. infestans</i>	AH-14-03	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	AH-15-06	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	AH-16-07	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	FJ-14-22	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	FJ-15-02	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	FJ-16-02	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	FJ-16-17	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	HN-16-02	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	B13	China	<i>S. tuberosum</i>	+
<i>P. infestans</i>	NL07434	China	<i>S. tuberosum</i>	+
<i>P. ipomoeae</i>	PIC99167	England	<i>Ipomoea longipedunculata</i>	–
<i>P. ipomoeae</i>	TL85	England	<i>I. longipedunculata</i>	–
<i>P. mirabilis</i>	PIC99114	England	<i>Mirabilis jalapa</i>	–
<i>P. mirabilis</i>	3010	England	<i>M. jalapa</i>	–
<i>P. sojae</i>	P6497	China	<i>Glycine max</i>	–
<i>P. sojae</i>	P7064	China	<i>G. max</i>	–
<i>P. capsici</i>	PC35	China	<i>Capsicum annuum</i>	–
<i>P. capsici</i>	LT263	China	<i>C. annuum</i>	–
<i>P. capsici</i>	LT1534	China	<i>C. annuum</i>	–
<i>P. parasitica</i>	PP025	China	<i>Nicotiana tabacum</i>	–
<i>P. nicotianae</i>	33C3	China	<i>N. tabacum</i>	–
<i>P. nicotianae</i>	DB 11032009	China	<i>N. tabacum</i>	–
<i>P. palmivora</i>	P80	China	<i>Hevea brasiliensis</i>	–
<i>P. palmivora</i>	PPM8	China	<i>H. brasiliensis</i>	–
<i>P. cryptogea</i>	St1	China	<i>Gerbera jamesonii</i>	–
<i>P. cryptogea</i>	SIND07	China	<i>G. jamesonii</i>	–
<i>P. cactorum</i>	T506D1	China	<i>Malus pumila</i>	–
<i>P. drechsleri</i>	MG 41-4	China	<i>Beta vulgaris</i>	–
<i>P. megasperma</i>	P45	China	<i>Medicago sativa</i>	–
<i>Peronophythora litchii</i>	MG 33-3	China	<i>Litchi chinensis</i>	–
<i>Pythium ultimum</i>	CHP13	China	<i>Solanum lycopersicum</i>	–
<i>Py. ultimum</i>	P6	China	<i>S. lycopersicum</i>	–
<i>Py. aphanidermatum</i>	Py15-7	China	<i>Cucumis sativus</i>	–
<i>Py. aphanidermatum</i>	PA1	China	<i>C. sativus</i>	–
<i>Fusarium solani</i>	MRC 2565	China	<i>Phaseolus vulgaris</i>	–
<i>F. solani</i>	NRRL 22678	China	<i>P. vulgaris</i>	–
<i>Rhizoctonia solani</i>	RHRG14	China	<i>S. tuberosum</i>	–
<i>R. solani</i>	Rol222	China	<i>S. tuberosum</i>	–

^a LF-RPA = lateral flow strip-based recombinase polymerase amplification; + = positive amplification; and – = negative amplification.

false priming, and cross-dimers of primers were evaluated using the Multiple Primer Analyzer online website (<https://www.thermofisher.com/>). The designed RPA primers were 35 or 36 nt long, and the amplification product lengths ranged from 212 to 243 bp (Supplementary Table S1). Biotin was added at the 5' end of the reverse primer for visualization in the lateral flow detection system. A specific internal probe was designed to add a 5' fluorescein (FAM), an abasic furan (dSpacer), and a C3 spacer (SpC3) to the 3' end (Supplementary Table S1). Primers and probes were synthesized by Sangon Biotech (Shanghai, China).

The LF-RPA assay was performed according to the quick guide of the TwistAmp nfo kit (TwistDx). Briefly, each reaction contained 29.5 μ l of rehydration buffer (supplied with the kit), 2.1 μ l of each primer (10 μ M), 0.6 μ l of probe (10 μ M), 12.2 μ l of nuclease-free water (not DEPC-treated) (Thermo Fisher Scientific), and 1 μ l of template. The solution was mixed by vortexing and spinning briefly, and then 2.5 μ l of 280 mM magnesium acetate (supplied with the kit) was added to each well. Reactions were performed at 39°C in a SimpliAmp thermal cycler (model A24812; Thermo Fisher Scientific) for 30 min. For the lateral flow strip (Milenia HybriDetect dipsticks, Milenia Biotec, Giessen, Germany) analysis, the reaction products were diluted 1/20 with HybriDetect assay buffer (supplied in the kit). Strips were placed vertically in 100 μ l of solvent at room temperature for 2 min. If the test and control lines were visible simultaneously, the result was positive; if only the control line was visible, the result was negative. Conventional PCR was performed in a 25- μ l total volume containing 12.5 μ l of 2 $\times$ Taq Master Mix (Vazyme Biotech, Nanjing, China), 10 μ M of each primer, 1 μ l of template DNA, and sterilized ddH₂O. All reactions were completed in a SimpliAmp thermal cycler (model A24812; Thermo Fisher Scientific) with an initial 3-min denaturation at 95°C, followed by 35 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s, with a final 5-min extension at 72°C. The PCR products were then electrophoresed on 2% gels stained with ethidium bromide. All tests were run under the same conditions and performed in triplicate.

Conventional PCR was initially used to evaluate the sensitivities and specificities of detection with three primer pairs. The result showed that the detection limit of all three primer pairs was 5 pg for conventional PCR (Supplementary Fig. S1A). However, the PiRPA-F2/R2 primer pair was excluded because its product contained obvious primer dimer bands that would affect the amplification efficiency or result in false-positive results. The primer pairs PiRPA-F1/R1 and PiRPA-F3/R3 were used to examine the specificity of conventional PCR, and the results revealed that both primer pairs were specific for *P. infestans* (Supplementary Fig. S1B). PiRPA-F3/R3 was selected for the subsequent RPA assay, and the corresponding PiProb probe was designed. To obtain the optimal primer combination for the LF-RPA assay, the primer combinations PiRPA-F1/R3', PiRPA-F2/R3', and PiRPA-F3/R3' were tested using

the RPA reaction system recommended in the TwistAmp RPA nfo kit. The primer combination PiRPA-F2/R3' had the highest sensitivity based on the LF-RPA assay (Fig. 1A). Therefore, this primer combination was selected for further analysis.

The optimum amplification temperature was evaluated by running the LF-RPA assay at different temperatures (20, 25, 30, 35, 40, 45, and 50°C) for 30 min. Results showed that test bands were obvious on the lateral flow strips after 5 min of incubation at temperatures from 25 to 45°C (Fig. 1B). The RPA amplification was best at 35 and 40°C, as shown by the presence of brighter test bands (Fig. 1B). Therefore, the reaction temperature was set to 39°C in the subsequent LF-RPA assays. Next, the detection time of the LF-RPA assay was evaluated. RPA amplification was performed at 39°C for different amounts of time (0, 5, 10, 20, 30, 40, and 50 min). A faint test band was detected with an amplification time of 10 min, and much brighter bands appeared between 20 and 50 min (Fig. 1C).

Fig. 2. Evaluation of the sensitivity of the lateral flow strip-based recombinase polymerase amplification (LF-RPA) assay. **A**, Sensitivity of the LF-RPA assay. **B**, Sensitivity of the conventional polymerase chain reaction assay. Serial dilutions of *Phytophthora infestans* T30-4 genomic DNA were used to evaluate the detection limit. NTC = no template control. At least three replicate tests were performed.

Fig. 1. Optimization of the lateral flow strip-based recombinase polymerase amplification (RPA) assay. **A**, Evaluation of the optimum primer combination. Different primer combinations are shown at the top of each pair of images. **B**, Optimization of the RPA amplification temperature. **C**, Optimization of RPA reaction time. At least three replicate tests were performed.

Accordingly, the reaction time was set to 20 min in the subsequent LF-RPA tests.

A total of 12 *P. infestans* isolates, 20 closely related *Phytophthora* isolates, four *Pythium* isolates, and four fungi were tested to evaluate the specificity of the LF-RPA primer using the above conditions. This primer combination yielded positive reactions for the 12 *P. infestans* isolates (Table 1), suggesting that this primer combination has the ability to amplify a wide range of *P. infestans* isolates. No amplification signals were obtained for the other related oomycetes or fungal isolates tested. Notably, there was no cross-reactivity with DNA samples from *P. mirabilis* or *P. ipomoeae*, which belong to the same 1c clade as *P. infestans* (Table 1). Therefore, this LF-RPA primer combination was highly specific for *P. infestans*. Next, the sensitivity of the LF-RPA assay was estimated using 10-fold serial dilutions of *P. infestans* genomic DNA ranging from 50 ng to 500 fg. A faint test band was detected visually for 500 fg of DNA (approximately two genome copies), and the band became brighter with increasing DNA amount (Fig. 2A). Meanwhile, the detection limit was 5 pg of DNA for the conventional PCR assay (Fig. 2B).

To determine the detection practicability of the developed LF-RPA assay, the leaf detached assay was performed. The 6-week-old potato plants (cv. Desiree) grown in a greenhouse under the 16-h 25°C/8-h 20°C day/night regime were prepared, and the terminal leaflet was cut from each potato plant ($n = 6$), followed by inoculating with 10 μ l of *P. infestans* T30-4 zoospore suspensions

(15,000 sporangia/ml). The inoculated leaflets were placed in the sealed box with wet filter papers, and the petioles were moisturized with wet cotton wools. This experiment was replicated three times. Slight symptoms began to appear at the inoculation site 60 h postinoculation (Fig. 3A), and then the terminal leaflet was divided into four regions: region I included the inoculation site, whereas region IV was farthest from the inoculation site (Fig. 3A). Trypan blue staining of the terminal leaflet, followed by microscopic examination, revealed that the plant tissues in regions I and II contained visible *P. infestans* mycelia, whereas no obvious mycelia were observed in regions III and IV (Fig. 3A). Next, the total genomic DNA in each of the four regions was extracted and detected by LF-RPA. Leaf tissues from mock-inoculated plants were used as negative controls. As shown in Figure 3B, test bands were clearly observed in regions I, II, and III, but not in region IV or healthy plants. The experiments were repeated three times with consistent results, suggesting that the LF-RPA assay can detect *P. infestans* in infected plants.

For the developed LF-RPA method to be useful in the field or low-resource settings, we used a simple polyethylene glycol (PEG)-NaOH to extract total plant leaf DNA using alkaline PEG lysis buffer. This method takes ~5 min and has been successfully applied to various plant leaf samples (Hwang et al. 2013). In this study, a 3 $\times$ 3-mm specimen of the leaf was ground in a 1.5-ml tube with a TissueLyser (Jingxin Industrial Development, Shanghai, China). The crushed pieces were lysed in 500 μ l of 6% PEG200 reagent (Sigma-Aldrich, Gillingham, U.K.) with 0.08% NaOH. The tubes were

Fig. 3. Early detection of *Phytophthora infestans* in infected plants. **A**, Representative potato terminal leaflet infected by *P. infestans* T30-4 zoospores at 60 h postinoculation (hpi). Artificial division of different areas of the terminal leaflet represents the varying stages of infection. After trypan blue staining of the terminal leaflet, microscopic examination was used to observe *P. infestans* mycelia in each region. Bars indicate 500 μ m. **B**, Lateral flow strip-based recombinase polymerase amplification (LF-RPA) assay of genomic DNA extracted from different regions of infected potato leaves. Healthy = healthy plant tissues. **C**, Flow chart of DNA extraction combined with the LF-RPA assay, demonstrating the entire equipment-free detection process, including simplified DNA extraction and LF-RPA assay.

shaken by hand for 2 min at room temperature and left standing for 1 min. Thereafter, 2 µl of the lysate was added to 50 µl of LF-RPA mixture. By utilizing the simplified PEG-NaOH method, the entire LF-RPA assay enabled detection in 30 min with no specialized equipment (Fig. 3C).

In May 2019, 72 naturally infected potato leaf samples with visible symptoms of late blight were collected from eight different fields in Chongqing ($n = 15$, cultivar Favorita), Guizhou ($n = 22$, cultivar Xuanshu 2), and Hunan ($n = 35$, cultivar Xingjia 2) provinces, China, where potatoes are planted in February (Supplementary Table S2). DNA was extracted from each sample using the above-mentioned PEG-NaOH method, and analyzed using both LF-RPA and conventional PCR methods. Of the 72 field samples, 52 were identified as positive using both LF-RPA and conventional PCR assays (Supplementary Table S2). Meanwhile, two healthy potato leaf samples collected from each of the eight fields were also tested, and all of these healthy samples produced negative results based on the LF-RPA assay. Therefore, the LF-RPA assay is a simple, accurate method for the practical detection of *P. infestans* in the field.

In conclusion, a novel method for rapid, equipment-free, visual diagnosis of late blight disease was developed in this study. The LF-RPA assay combined with the simplified PEG-NaOH method of DNA extraction could detect *P. infestans* at temperatures from 25 to 45°C within 30 min. The amplicons generated bands directly on the lateral flow strip, which can be read by the naked eye. The sensitivity assay revealed that the LF-RPA assay detected a minimum amount of 500 fg of *P. infestans* DNA. Meanwhile, the LF-RPA assay detected crude DNA extracted from plant tissues and enabled the practical diagnosis of late blight in field samples. The LF-RPA assay for *P. infestans* had high sensitivity and specificity, and it could be used as a routine laboratory test or even on-site where expensive diagnostic instruments are not available. Moreover, the developed method can also be applied to other economically important plant pathogens.

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