Article

PHYCI_587572: An RxLR Effector Gene and New Biomarker in A Recombinase Polymerase Amplification Assay for Rapid Detection of *Phytophthora cinnamomi*

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Abstract: *Phytophthora cinnamomi* is a devastating pathogen causing root and crown rot and dieback diseases of nearly 5000 plant species. Accurate and rapid detection of *P. cinnamomi* plays a fundamental role within the current disease prevention and management programs. In this study, a novel effector gene PHYCI_587572 was found as unique to *P. cinnamomi* based on a comparative genomic analysis of 12 *Phytophthora* species. Its avirulence homolog protein 87 (Avh87) is characterized by the Arg-Xaa-Leu-Arg (RxLR) motif. Avh87 suppressed the pro-apoptotic protein BAX- and elicitor protein INF1-mediated cell death of *Nicotiana benthamiana*. Furthermore, a recombinase polymerase amplification-lateral flow dipstick detection assay targeting this *P. cinnamomi*-specific biomarker was developed. While successfully detected 19 *P. cinnamomi* isolates of a global distribution, this assay lacked detection of 37 other oomycete and fungal species, including *P. parvispora*, a sister taxon of *P. cinnamomi*. In addition, it detected *P. cinnamomi* from artificially inoculated leaves of *Cedrus deodara*. Moreover, the RPA-LFD assay was found to be more sensitive than a conventional PCR assay, by detecting as low as 2 pg of genomic DNA in a 50-μL reaction. It detected *P. cinnamomi* in 13 infested soil samples, while the detection rate was 46.2% using PCR. Results in this study indicated that PHYCI_587572 is a unique biomarker for detecting *P. cinnamomi*. Although PHYCI_587572 was identified as an effector gene based on the RxLR motif of Avh87 and the avirulence activity on *Nicotiana*, its exact genetic background and biological function on the natural hosts of *P. cinnamomi* warrant further investigations.

Keywords: plant destroyers; disease diagnosis; RxLR-dEER; soil-borne pathogen; exclusivity; inclusivity

1. Introduction

Phytophthora cinnamomi is a destructive pathogen of nearly 5000 plant species including many economically important crops [1–3]. For example, root rot caused by *P. cinnamomi* is a widely spread and most severe disease of avocado with an estimated annual loss of \$40 million in California, USA

alone [4]. *Phytophthora cinnamomi* also causes root and crown rot, canker and dieback diseases on a wide range of woody ornamental crops such as azalea, boxwood, camellia, cedar, and rhododendron [5]. In addition to its impact on agriculture and horticulture, *P. cinnamomi* is a notorious invasive pathogen in natural plantations worldwide. In concert with chestnut blight, *P. cinnamomi* was responsible for the near extinction of wild American chestnut trees in the eastern United States [6,7]. In the forests of south-west Western Australia, *P. cinnamomi* has permanently altered the composition of plant communities and caused drastic decline of biodiversity by killing over 3500 susceptible endemic plant species and threatening macrofungal and bird species [3,8–10]. In China, *P. cinnamomi* has been found in many provinces and municipalities such as Fujian [11–13], Hainan [14], Jiangsu [13], Shaanxi [15], Shanghai [16], and Zhejiang [13], threatening the production of avocado, blueberry, chestnut, kiwifruit, ornamental trees, and the health of natural forests.

Phytophthora species secrete a wide range of effector proteins that overcome host immunity and facilitate the infection of plants [3,17] such as elicitors [18], small cysteine-rich toxins, transglutaminases, and RxLR effectors [3]. RxLR effectors, characterized by an Arg-Xaa-Leu-Arg (RxLR) motif, N-terminal signal peptide, and often Glu-Glu-Arg (EER) motif, are responsible for the translocation of effector proteins from plant apoplast into cytoplasm [19–21]. Although the exact number and pathogenesis roles of *P. cinnamomi* RxLR effectors remain to be determined, a comparative genomic analysis of *P. infestans* and *P. cinnamomi* has identified the presence of at least 171 RxLR effector genes in the *P. cinnamomi* genome [3]. Furthermore, only three out of 10 RxLR effector homologues that have known functional characters are present in the *P. cinnamomi* genome [3], implying the presence of *P. cinnamomi*-unique RxLR effector proteins and genes.

Early detection is the first and arguably most effective step within the hierarchy of plant disease control strategies. Although chemical application and soil sanitation could reduce disease severity and inoculum level [5], regular treatments could be costly and ineffective once *P. cinnamomi* has already widely spread in agricultural settings, and conceivably even more challenging in natural forests due to the scale of the problem. A different approach of disease management is early detection followed by the eradication of the pathogen. It is relatively inexpensive, sustainable, and effective if the pathogen is removed at an early stage. Nevertheless, its success relies on the accuracy and speed of the detection assay.

Detection assays of *P. cinnamomi* have advanced with molecular techniques, while their accuracy has been challenged by emerging new pathogens. Previous molecular detection assays of *P. cinnamomi* were developed based on conventional PCR [22–26], real-time PCR [27–29], and isothermal amplification assays [28,30]. However, many of these assays have failed in differentiating *P. cinnamomi* from *P. parvispora* [31], the species most closely related to *P. cinnamomi* within the genus *Phytophthora* [32–34].

Recombinase polymerase amplification (RPA) is an emerging technique that has been applied in detection assays of *Phytophthora* species [28,35–38]. The RPA process employs three core factors, including recombinase, DNA-binding proteins and polymerase. Recombinases perform to pair oligonucleotide primers with homologous sequence in duplex DNA. Simultaneously, DNA-binding proteins bind to displaced strands of DNA and polymerase begins to synthesize complete double-stranded DNA where the primer has bound to the target DNA. The enzyme mixture conducts exponential amplification of the target region within the template. Compared to PCR and loop-mediated isothermal amplification (LAMP) assays, RPA assays require shorter time span and less complex procedures and equipment for amplifying DNA templates of *Phytophthora* species [36–40]. In addition, RPA amplified products can be detected by lateral flow strips and the results can be easily read without any specialized equipment, suggesting that combination of RPA with lateral flow dipsticks (RPA-LFD) is suitable for developing a simple, rapid, and specific method for the detection of plant pathogens in resource-limited settings. The aims of this study were to identify a RxLR effector gene *PHYCI_587572* using a comparative genomic approach and develop an RPA-LFD assay targeting this *P. cinnamomi*-specific biomarker.

2. Materials and Methods

2.1. Isolate Selection and DNA Extraction

Phytophthora cinnamomi isolates were obtained from diseased roots of *Pinus sp.*, *Rhododendron simsii*, and *Camellia oleifera* from various provinces in China. The roots were surface disinfected, dried, and placed on Phytophthora selective agar PARP-V8 agar [5]. Surface disinfestation of the diseased roots were accomplished incubation in 70% ethanol. When using 70% ethanol, infected roots were given single dips and then dipped in three vials containing autoclaved distilled water for 5–10 min per vial. Following surface disinfestation, roots were surface dried by placing them on filter paper. A total of 76 isolates were used in this study including 19 of *P. cinnamomi*, 40 samples representing 21 *Phytophthora* species, one of *Globisporangium ultimum*, and 16 other fungal species (Table 1). For DNA extraction, each isolate was cultured in 10% clarified V₈ juice (oomycetes) or potato dextrose broth (fungi) at 20–25 °C in the dark for 4–5 days. Genomic DNA (gDNA) was extracted from the harvested mycelia using a E.Z.N.A.[®] HP Fungal DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) following the manufacturer's instructions. Concentrations of gDNA extractions were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) then adjusted as needed. Extractions were stored at −20 °C until use.

Table 1. List of oomycete and fungal isolates used in this study and their detection results in the recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) assay targeting the PHYCL_587572 biomarker.

(Sub) Clade	Species	Isolate	Host or Substrate	Location ^a	RPA-LFD ^b
7c	<i>Phytophthora cinnamomi</i>	Pci1	<i>Pinus sp.</i>	AH	+
		Pci2	<i>Rhododendron simsii</i>	JS	+
		Pci3	<i>Cedrus deodara</i>	JS	+
		Pci4	<i>Camellia oleifera</i>	JS	+
		Pci5	<i>Pinus sp.</i>	JS	+
		Pci6	<i>Rhododendron simsii</i>	AH	+
		Pci7	<i>Rhododendron simsii</i>	SD	+
		Pci8	<i>Cedrus deodara</i>	SD	+
		Pci9	<i>Cedrus deodara</i>	AH	+
		Pci10	<i>Pinus sp.</i>	SD	+
		JP-07-0035	<i>Pieris sp.</i>	Oregon, USA	+
		PCN-18-001	<i>Castanopsis sp.</i>	Taiwan	+
		PCN-18-078	soil	Taiwan	+
		JP-08-313	<i>Pieris sp.</i>	Oregon, USA	+
		JP-07-0376	N/A	Oregon, USA	+
		JP-09-325	N/A	Oregon, USA	+
		ATCC 15400	<i>Camellia japonica</i>	South Carolina, USA	+
		ATCC 15401	<i>Persea americana</i>	Puerto Rico	+
		CBS 144.22	<i>Cinnamomum burmannii</i>	Indonesia	+
		CBS132771	<i>Arbutus unedo</i>	Italy	–
		CBS132772	<i>Arbutus unedo</i>	Italy	–
7a	<i>P. cambivora</i>	CBS 248.60	<i>Castanea sativa</i>	USA	–
		CBS 209.46	<i>Fragaria × ananassa</i>	England, UK	–
7b	<i>P. rubi</i>	CBS 967.95	<i>Rubus idaeus</i>	Scotland, UK	–
		PMNJHG1	<i>Cucumis sativus</i>	JS	–
7b	<i>P. melonis</i>	PMNJHG2	<i>Cucumis sativus</i>	JS	–
		PMNJHG3	<i>Cucumis sativus</i>	JS	–
		PMNJDG1	<i>Benincasa hispida</i>	JS	–
		PMNJDG2	<i>Benincasa hispida</i>	JS	–
		PMNJDG3	<i>Benincasa hispida</i>	JS	–
		PMFJHL1	<i>Lagenaria siceraria</i>	FJ	–
		IMI 325917	<i>Cucumis sp.</i>	FJ	–
		P6497	<i>Glycine max</i>	Mississippi, USA	–
		Peng-R3	<i>Glycine max</i>	FJ	–
		Pcac1	<i>Malus pumila</i>	JS	–
1	<i>P. cactorum</i>	Pcac2	<i>Malus pumila</i>	JS	–
		Pcac3	<i>Rosa chinensis</i>	JS	–
		Pin1	<i>Solanum tuberosum</i>	FJ	–
		Pin2	<i>Solanum tuberosum</i>	YN	–
	<i>P. nicotianae</i>	Pni1	<i>Nicotiana tabacum</i>	YN	–

Table 1. Cont.

(Sub) Clade	Species	Isolate	Host or Substrate	Location ^a	RPA-LFD ^b
2	<i>P. capsici</i>	Pni2	<i>Lycopersicum</i> sp.	JS	–
		Pni3	<i>Sophora sinensis</i>	JS	–
		Pni4	<i>Citrus</i> sp.	JS	–
		Pcap1	<i>Capsicum annuum</i>	JS	–
		CBS 114348	<i>Ilex aquifolium</i>	The Netherlands	–
		Ppa1	Iridaceae	YN	–
		15C7	Soil	South Carolina, USA	–
		CBS 587.85	Soil	Taiwan	–
		CBS 305.36	<i>Matthiola incana</i>	California, USA	–
		57J3	Irrigation water	Mississippi, USA	–
8	<i>P. drechsleri</i>	CBS 292.35	<i>Beta vulgaris</i> var. <i>altissima</i>	California, USA	–
		ATCC 56353	<i>Citrus sinensis</i>	Australia	–
		CBS 270.31	<i>Citrus sinensis</i>	Setúbal, Portugal	–
		ATCC 34002	<i>Citrus</i> sp.	California, USA	–
		ATCC	<i>Quercus agrifolia</i>	California, USA	–
		MYA-2949			
		Pbo1	<i>Boehmeria nivea</i>	JS	–
		Pbo2	<i>Gossypium</i> sp.	JS	–
		Pbo3	<i>Boehmeria nivea</i>	JS	–
		Pbo4	<i>Gossypium</i> sp.	JS	–
Oomycete	<i>Globisporangium ultimum</i>	Gu1	Irrigation water	JS	–
Fungi	<i>Alternaria alternata</i>	LH1401	<i>Cucumis melo</i>	JS	–
	<i>Aspergillus oryzae</i>	Ao1	<i>Glycine max</i>	JS	–
	<i>Cercospora kikuchii</i>	Ck1	<i>Glycine max</i>	JS	–
	<i>Colletotrichum gloeosporioides</i>	Cg1	<i>Glycine max</i>	JS	–
	<i>C. truncatum</i>	Ct1	<i>Glycine max</i>	JS	–
	<i>Diaporthe phaseolorum</i> var. <i>caulivora</i>	DPC	<i>Glycine max</i>	JS	–
	<i>Fusarium oxysporum</i>	Fo1	<i>Pinus</i> sp.	JS	–
	<i>Fusarium solani</i>	Fs1	<i>Gossypium</i> sp.	JS	–
		Fs2	<i>Glycine max</i>	JS	–
	<i>Fusarium equiseti</i>	Fe1	<i>Pinus</i> sp.	JS	–
	<i>Magnaporthe grisea</i>	Guy11	<i>Oryza sativa</i>	French Guiana	–
	<i>Nigrospora sphaerica</i>	Ns1	<i>Glycine max</i>	JS	–
	<i>Phakopsora pachyrhizi</i>	Pa1	<i>Glycine max</i>	JS	–
	<i>Phomopsis asparagi</i>	Pas1	<i>Asparagus officinalis</i>	JS	–
	<i>Rhizoctonia solani</i>	Rs1	<i>Gossypium</i> sp.	JS	–
	<i>Verticillium dahliae</i>	Vda1	<i>Gossypium</i> sp.	JS	–

^a Abbreviations of provinces in China: AH, Anhui province; JS, Jiangsu province; SD, Shandong; FJ, Fujian province; YN, Yunnan province; ^b Positive (+) or negative (–) reaction result in the RPA-LFD assay for detecting *P. cinnamomi*.

2.2. Genomic Sequences

Genomic sequences and gene models of *P. capsici*, *P. cinnamomi*, *P. infestans*, *P. ramorum*, and *P. sojae* were retrieved from the MycoCosm portal [41]. Those of *P. asiatica*, *P. cactorum*, *P. cambivora*, *P. hibernalis*, *P. parvispora*, *P. syringae*, and *P. vignae* were also included in the subsequent comparative genomic analysis.

2.3. Identification of A *P. cinnamomi*-Unique RxLR Effector Gene

To identify RxLR effectors, the genome sequence of *P. cinnamomi* was translated to open reading frames (ORFs). ORFs encoding < 50 amino acids were excluded. SignalP v3.0 was used to identify the N-terminal signal peptide [42] within remaining ORFs. Proteins with Hidden

Markov Model (HMM) scores > 0.9 and without predicted transmembrane domain were predicted as secreted proteins. A regular expressions (REGEX) method was performed by script for identifying the $(^{\wedge}\backslash w\{1,40\}\backslash w\{1,96\}R\backslash wLR\backslash w\{1,40\}[ED][ED][KR])$ motif [43,44] within the predicted secreted proteins. The RxLR-EER domains of the candidates were aligned and then used to construct a HMM profile to search within the predicted secreted proteins using HMMER. Matches with a bit score of >0 were manually validated.

2.4. Construction of Binary Potato Virus X (PVX) Vectors

As no introns were predicted within *PHYCI_587572*, this gene was directly amplified using the gDNA of isolate Pci1 (Table 1). Each 25- μ L PCR reaction include 1 μ L of gDNA (100 ng per μ L), 1 μ L (10 μ M) of each of primers *PHYCI_587572*-PVX-HA(infusion)-F and *PHYCI_587572*-PVX-HA(infusion)-R (Table 2), 1 μ L of PrimeSTAR HS DNA Polymerase (Takara Bio, Kusatsu, Shiga, Japan), 4 μ L of dNTP Mixure (2.5 mM), 10 μ L of 5 $\times$ PrimeSTAR Buffer, and 32 μ L of nuclease-free water (nH₂O). PCR was carried out using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Singapore) following the program: an initial denaturation step at 95 °C for 3 min, 35 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s, plus a final extension at 72 °C for 5 min. Each set of PCR reactions included a NTC. PCR products were examined in 1% agarose gel electrophoresis at 120 V for approximately 25 min. Agarose gel was stained by ethidium bromide and visualized on a transilluminator. The PCR assay was carried out three times. PCR products were digested with the *Sma*I restriction enzyme (NEB, R0141S) and cloned into the PVX vector pGR107 [45] to form pGR107:*PHYCI_587572*. The constructs were confirmed by sequencing at Genscript (Nanjing, China) using the primers LBA and LBB (Table 2). Recombinant binary plasmids were maintained and propagated in *Escherichia coli* strain JM109 grown in Luria-Bertani (LB) media amended with kanamycin (50 mg per mL) and tetracycline (12.5 mg per mL).

Table 2. Primers used in the construction of binary potato virus X (PVX) vectors, infiltration of *Agrobacterium tumefaciens*, and the recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) assay targeting the *PHYCI_587572* biomarker.

Assay	Name	Sequence (5'–3')
PVX construction	<i>PHYCI_587572</i> -PVX-HA(infusion)-F	CTAGCATCGATTCCCGGGATGCTCTCGATGACCACAGCCTCC
	<i>PHYCI_587572</i> -PVX-HA(infusion)-R	CTCTAGAGGATCCCCGGGAAATTCTCCTCGCGCGTG
<i>Agrobacterium</i> infiltration	LBA	CAATCACAGTGTGGCTTGC
	LBB	GACCCATATGGGCTGTGTG
RPA	PciRL587572F	GCGAGGCCCTCTCGATGACCACAGCCTCCAACCA
	PciRL587572R	[Biotin]TTGCTGCAGATATGTGCTGCTTGCTGACCATC
	PciRL587572P	[FAM]GAGGCAGTCGACGATGATGA[THF]TCCTCCGAAGATTCC[C3-spacer]

2.5. *Agrobacterium Tumefaciens* Infiltration

Constructs were introduced into *A. tumefaciens* strain GV3101 by electroporation [46,47]. Recombinants (pGR107:*PHYCI_587572*) were selected with tetracycline (12.5 mg per mL) and kanamycin (50 mg per mL). Individual colonies were verified in PCR using the primers LBA and LBB (Table 2).

Nicotiana benthamiana plants were grown in a greenhouse at a day/night temperature of 25/16 °C and a 16 h photoperiod for 4–6 weeks. Recombinant GV3101 cells were grown in LB media amended with kanamycin (50 mg per mL) for 48 h, harvested, washed with 10 mM MgCl₂ three times, resuspended in 10 mM MgCl₂ to a final OD₆₀₀ of 0.4, then incubated at room temperature for 1 to 3 h prior to infiltration. For pressure infiltration, 100 mL of recombinant GV3101 cell suspension was infiltrated through a small nick into each of three infiltration sites per adaxial side of a *N. benthamiana* leaf using a syringe. After 0, 12, or 24 h, *Agrobacterium tumefaciens* cells carrying the *Bax* gene (pGR107:*Bax*) or *inf1* gene (pGR107:*inf1*) were infiltrated into each of the three filtration sites. *Agrobacterium tumefaciens* cells carrying the enhanced green fluorescent protein (eGFP) gene in place of pGR107:*Bax* or pGR107:*inf1* were infiltrated into three parallelly designated filtration sites of the same leaf after 0, 12, or 24 h as controls. Symptom development was monitored daily. Photographs were taken on 5 and 6 days

after filtration of pGR107:*Bax* and pGR107:*infl*, respectively. This experiment was conducted twice with three replicate leaves in each repeat.

2.6. RPA-LFD Assay

A set of RPA primers and probe targeting the *P. cinnamomi*-specific biomarker *PHYCI_587572*, including a forward primer PciRL587572F, a 5'-biotin-labeled reverse primer PciRL587572R, and a probe PciRL587572P labeled by fluorescein amidite (FAM), tetrahydrofuran (THF), and C3 spacer (Table 2), was designed according to the instruction for the nfo kit in the TwistAmp® DNA Amplification Kits Assay Design Manual. Primers and probe were synthesized by Sangon Biotech (Shanghai, China).

The RPA-LFD assay was performed following the procedures in the TwistAmp® nfo Kit Quick Guide (TwistDx Ltd., Cambridge, UK). Each RPA reaction in a total volume of 50 µL contained 1 µL of DNA template, 29.5 µL of rehydration buffer, 2.1 µL of each of primers PciRL587572F and PciRL587572R (10 µM), 0.6 µL of probe PciRL587572P (10 µM), 12.2 µL of *nfH₂O*, and 2.5 µL of magnesium acetate (280 mM). These reagents were added into reaction microtubes and mixed using a pipette. Freeze-dried pellet magnesium acetate was pre-added to the cap of the microtubes. After brief vortex and centrifugation, the reaction microtubes were incubated in a heating block set at 39 °C for 20 min. To visualize amplicons, 10 µL of RPA product was mixed with 90 µL of phosphate buffered saline with Tween 20 (PBST) running buffer. Then 5 µL of the RPA product per each reaction was added to the sample pad of a Milenia Genline HybriDetect 1 LFD (Milenia Biotec GmbH, Giessen, Germany). The LFD was then vertically immersed into 100 µL of PBST (Milenia Biotec GmbH, Giessen, Germany) in a sterile centrifuge tube for up to 5 min at room temperature (aver. 22 °C) until a clear control line was observed. The dipsticks yielded visible control lines, indicating valid tests. Test lines were visible on all dipsticks correlating to positive RPA reactions. A negative detection or NTC in contrast should not result in any signal or color at the position of the test line.

2.7. Evaluation of RPA Specificity and Sensitivity

The specificity of the RPA-LFD assay was evaluated by testing against all isolates listed in Table 1. One µL of gDNA (10 ng per µL) of each isolate was included in each 50 µL reactions. Each isolate was tested in triplicate.

To evaluate the sensitivity of the RPA-LFD assay, 10-fold serial dilution of gDNA of *P. cinnamomi* isolate Pci1 ranging from 100 ng to 100 fg (0.0001 ng) per µL were used as templates. Each concentration was evaluated in triplicate. Each set of reactions in the specificity and sensitivity evaluations included an NTC (*nfH₂O* in place of DNA template).

2.8. Detecting *P. cinnamomi* in Artificially Inoculated Pine Needles Using RPA-LFD

Pine needles were collected from a healthy deodar cedar tree (*Cedrus deodara*) at a landscape site of Nanjing Forestry University (Nanjing, China). They were artificially inoculated by *P. cinnamomi*. A 2 × 2 mm mycelial plug of *P. cinnamomi* isolate Pci1 was mixed with approximately 100 g of pine needles and incubated in each of triplicate sterile 250 mL flasks at 22 °C, 100% relative humidity, and a 12-h photoperiod for 5 days. Sterile agar plugs were mixed with pine needles to produce three non-inoculated control samples. Total DNAs were extracted from *P. cinnamomi*-colonized and non-inoculated samples using an NaOH lysis method. Briefly, 20 mg of plant tissues collected from the wound site of each Pine needle were placed into a 1.5-mL microtube containing 200 µL of NaOH (0.5 N). They were grinded for approximately 1 min until no large pieces of plant tissues were visible using a sterile tissue grinder pestle. Then, 5 µL of grinded tissues in NaOH were transferred to a new microtube containing 495 µL of Tris buffer (100 mM, pH 8.0). Two µL of the mixture were used as templates in the RPA-LFD assay. Purified gDNA (10 ng per µL) of isolate Pci1 and *nfH₂O* were used as a positive control and NTC, respectively, in two repeats of the experiment.

(100 fg) of gDNA or no template control (NTC). These results were consistent across three repeats of the evaluation. In the comparison PCR assay, ≥ 1 ng of gDNA was required for consistent positive results using primers PciRL587572F and PciRL587572R (Supplementary Figure S1). The result indicates that RPA-LFD assay was more sensitive than PCR by using LFD strips.

Figure 2. Suppression of BAX- and elicitor protein INF1-mediated cell death of *Nicotiana benthamiana* leaves infiltrated with *Agrobacterium tumefaciens* strains containing a PVX vector carry the *PHYCI_587572* gene (Avh87) or a control gene (eGFP). (A): 1, Avh87→eGFP; 2, Avh87→12h→eGFP; 3, Avh87→24h→eGFP; 4, Avh87→BAX; 5, Avh87→12h→BAX; 6, Avh87→24h→BAX. (B): 1, Avh87→eGFP; 2, Avh87→12h→eGFP; 3, Avh87→24h→eGFP; 4, Avh87→INF1; 5, Avh87→12h→INF1; 6, Avh87→24h→INF1.

Figure 3. Detection results of the recombinase polymerase amplification-lateral flow dipstick assay targeting the *PHYCI_587572* biomarker. Genomic DNAs (10 ng per 50- μ L reaction) of *Phytophthora cinnamomi* isolates and other oomycete and fungal species. Nuclease-free water was used in place of DNA templates in no template controls (NTC).

Figure 4. Evaluation results of sensitivity of the recombinase polymerase amplification-lateral flow dipstick assay targeting the *PHYCI_587572* biomarker using a 10-fold serial dilutions of genomic DNA (gDNA) of *Phytophthora cinnamomi* isolate Pci1. Nuclease-free water was used in place of DNA templates in no template controls (NTC).

3.4. Detecting *P. cinnamomi* in Artificially Inoculated Pine Needles

All LFDs had a visible control line indicating valid tests. *Phytophthora cinnamomi* was detected in the total DNAs extracted from three *P. cinnamomi*-infested pine needle samples and the positive control, whereas not detectable in three non-inoculated samples or NTC (Figure 5). Identical results were recorded in both repeats of the experiment.

Figure 5. Detection of *Phytophthora cinnamomi* in artificially inoculated pine needles of *Cedrus deodara* using the recombinase polymerase amplification-lateral flow dipstick assay. Genomic DNA (10 ng per μ L) of *P. cinnamomi* isolate Pci1 was used as the positive control (PC). Nuclease-free water was used in place of DNA templates in the no template control (NTC). Dipsticks of the first repeat are shown. Identical detection results were observed in the second repeat of the experiment.

3.5. Detection of *P. cinnamomi* in Infested Soil Samples

The RPA-LFD assay was applied to 13 *P. cinnamomi*-infested soil samples. Detection results were consistent among three repeats of the experiment. Using DNA samples directly extracted from soil samples, *P. cinnamomi* was detected in all 13 samples using the RPA-LFD assay, while it was detected in only six samples using PCR (Table 3). Using DNAs extracted from *C. deodara* leaf baits, both RPA-LFD and PCR assays detected *P. cinnamomi* in all 13 samples.

Table 3. Detection results of *Phytophthora cinnamomi* in 13 infested soil samples using the recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) and PCR assays.

Sample No.	Location ^a	Vegetation	Year	Detection of Pcin			
				DNAs from Soil		DNAs from Leaf Baits	
				RPA-LFD	PCR	RPA-LFD	PCR
1	Hefei, AH	<i>Pinus</i> sp.	2014	+	+	+	+
2	Huaibei, AH	<i>Rhododendron simsii</i>	2014	+	+	+	+
3	Liuan, AH	<i>Cedrus deodara</i>	2016	+	-	+	+
4	Suzhou, AH	<i>Camellia oleifera</i>	2016	+	-	+	+
5	Nanjing, JS	<i>Pinus</i> sp.	2015	+	+	+	+
6	Yancheng, JS	<i>Rhododendron simsii</i>	2015	+	-	+	+
7	Xuzhou, JS	<i>Cedrus deodara</i>	2016	+	-	+	+
8	Tianan, SD	<i>Pinus</i> sp.	2016	+	+	+	+
9	Jinan, SD	<i>Rhododendron simsii</i>	2017	+	-	+	+
10	Xiamen, FJ	<i>Pinus</i> sp.	2016	+	+	+	+
11	Zhangzhou, FJ	<i>Rhododendron simsii</i>	2016	+	-	+	+
12	Kunming, YN	<i>Pinus</i> sp.	2015	+	+	+	+
13	Anning, YN	<i>Rhododendron simsii</i>	2015	+	-	+	+

^a Province abbreviations: AH, Anhui; JS, Jiangsu; SD, Shandong; FJ, Fujian; YN, Yunnan.

4. Discussion

Phytophthora cinnamomi is one of the most invasive plant pathogens with a global impact [3,6]. Early and accurate detection of *P. cinnamomi* is paramount for implementing disease management promptly and avoiding unnecessary costs due to misdiagnosis. The present study describes PHYCI_587572, a potentially *P. cinnamomi*-specific RxLR effector gene identified using a comparative genomics approach, and a rapid and equipment-free RPA-LFD detection assay for *P. cinnamomi* that targets this novel biomarker. The findings in this study have important implications on the *P. cinnamomi*-hosts interactions and the management of this aggressive pathogen.

Despite the potential importance of RxLR proteins in the infection of plants by *P. cinnamomi*, to date, the molecular function of only a few RxLR effectors and genes has been determined [3]. Identification of oomycete pathogenicity effector genes has been facilitated by the increasing availability of genome sequences and the recognition of the RxLR-dEER motif. In this study, the PHYCI_587572 effector gene encoding Avh87 was identified from the genome sequence of *P. cinnamomi*. During this process, we also identified 265 other RxLR candidate genes. Efforts to identify their molecular functions are underway. Additionally, a comparative analysis of 12 *Phytophthora* genomes indicated the specificity of PHYCI_587572 to *P. cinnamomi* genome. Furthermore, the inhibition of Avh87 on BAX- and INF1-mediated cell death indicated the potentially fundamental role of Avh87 in the pathogenicity of *P. cinnamomi*. Further investigations are warranted on the biological function of PHYCI_587572 (Avh87) on the natural hosts of *P. cinnamomi*.

Given the important role of effector genes in oomycete pathogenicity, they are unique candidates for developing novel biomarkers. The RxLR effector gene PHYCI_587572 identified in this study was found lack of homolog in other reference *Phytophthora* species. This implies its unique function in the pathogenicity of *P. cinnamomi*. However, because many effector genes have been found rapidly evolving [21,48], thus have shown a higher degree of intra-specific polymorphism than universal housekeeping genes [49], utilizing a novel effector gene as a diagnostic biomarker may bear the risk of false-negative detections against mutated isolates of the target *Phytophthora* species. In this study, the novel RPA-LFD assay targeting the RxLR effector gene PHYCI_587572 accurately detected all 19 *P. cinnamomi* isolates recovered from at least eight host species in three provinces of China, two U.S. states, Puerto Rico, and Indonesia, including the ex-type isolate CBS 144.22 (Table 1). This finding indicates that the novel RPA-LFD assay has a high degree of inclusivity within *P. cinnamomi* isolates. Nevertheless, additional isolates should be tested in the future to confirm its inclusivity.

Rapid pathogen detection is extremely important to the management of aggressive diseases. The key advantage of RPA-based assays is their short reaction time span and requirement for less complex instrumentation. The RPA-LFD assay in this study could be finished within 30 min, which is only half of the time taken by a LAMP assay [30] and a quarter of PCR reactions. Also, it does not require a thermal cycler or gel electrophoresis and imaging system, as its results could be visualized on LFDs. Compared to the LAMP assay for *P. cinnamomi* [30], the RPA-LFD assay also needed fewer primers and lower incubation temperature. All these advantages make the RPA-LFD assay an ideal method for detecting *P. cinnamomi* under field conditions and in diagnostic laboratories.

The development of this novel RPA-LFD assay has further improved the sensitivity of isothermal detection assays for *P. cinnamomi*. As determined in this study, this RPA-LFD assay detected 0.001 ng of *P. cinnamomi* gDNA (~12 copies of nuclei) in a 50- μ L reaction system. This is at least 200 times more sensitive than our previously developed LAMP assay and 2000 times more sensitive than a PCR assay using primers F3 and B3 [30]. However, this sensitivity appears lower than a previously reported qPCR-based assay [29]. Although the RPA-LFD assay has the advantages of short time span and less instrumentation requirement against qPCR, future studies are warranted to further improve the sensitivity of isothermal assays for *P. cinnamomi*.

5. Conclusions

A novel RxLR effector *PHYCI_587572* was bioinformatically identified from the genome sequence of *P. cinnamomi*. A comparative analysis of genome sequences of 12 *Phytophthora* species indicated that this effector gene is unique to *P. cinnamomi*, thus an ideal biomarker for developing diagnostic assays. This assay was specific to *P. cinnamomi*, while it lacked detection against 37 other oomycete and fungal species, including *P. parvispora*, a sister taxon of *P. cinnamomi*. It was also found to be 200 times more sensitive than a previously developed LAMP assay. In addition, it detected *P. cinnamomi* in artificially inoculated leaves of *Cedrus deodara*. Results in this study indicated that the RPA-LFD assay is an ideal method for detecting *P. cinnamomi* under field conditions and in diagnostic laboratories, while understanding the precise molecular and biological functions of *PHYCI_587572* and its avirulence homolog protein Avh87 requires further investigation.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/11/3/306/s1>.

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