Phytopathology Research

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A loop-mediated isothermal amplification assay for the rapid diagnosis of soybean rust caused by *Phakopsora pachyrhizi*

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Abstract

Soybean rust caused by the fungus *Phakopsora pachyrhizi* is one of the most destructive diseases to soybean production worldwide. For the rapid diagnosis and the control of soybean rust, we developed a loop-mediated isothermal amplification (LAMP) assay that targets a *P. pachyrhizi*-specific gene (*Phapa_6409908*) identified from a comparative genomic analysis of 61 *Pucciniomycotina* strains. Using a set of screened primers and the optimized reaction conditions of 62°C for 70 min, the LAMP assay can detect *P. pachyrhizi* while excluding other plant pathogens. The assay consistently showed high sensitivity in detecting low contents of *P. pachyrhizi* DNA (10 pg). We confirmed the practical application of this LAMP assay in diagnosing soybean rust using soybean leaf samples collected from fields in four cities of Sichuan Province, China, in 2020 and 2021. The developed LAMP assay provides a specific, sensitive, and rapid method for the diagnosis of soybean rust caused by *P. pachyrhizi*, which can assist in the prediction, prevention, and control of this major soybean disease.

Keywords Soybean rust, *Phakopsora pachyrhizi*, Molecular detection, LAMP

Background

Phakopsora pachyrhizi, a causal agent of soybean rust, causes significant economic damage worldwide and poses a potential threat to food security (Pennisi 2010; Godoy et al. 2016). *P. pachyrhizi* was first isolated in 1902 in Japan, after which it was reported in other soybean-producing countries, such as Brazil and the United States (Schneider et al. 2005; Yorinori et al. 2005). Leaf discoloration is a typical symptom of soybean rust, which ultimately causes plant premature senility, resulting in yield loss due to reduced grain size (Hartman et al. 2005). In Brazil, from 2001 to 2003, the cost of fungicides for controlling soybean rust reached up to $2 billion per year (Yorinori et al. 2005; Silva et al. 2020). In Asia, soybean rust has caused soybean yield losses about 20–80% (Miles et al. 2007), and it is also a major soybean disease in South China (Ye et al. 2023).

As known thus far, all commercial soybean cultivars are susceptible to *P. pachyrhizi*, and the most effective soybean rust control method is the application of fungicides, such as strobilurin, triazole, and benzimidazole (Levy 2005; Miles et al. 2007; Mueller et al. 2009). Decisions on fungicide use are mainly based on visual rust disease symptoms, and fungicides are applied when the

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symptoms appear (Hartman et al. 2011b). However, *P. pachyrhizi* is difficult to detect in early stages of infection. The symptoms are not visible until 5–8 days post-inoculation (dpi) when asexual urediniospores colonize leaves within a uredium, and progress further at 10–14 dpi when mature urediniospores are released (Saksirirat and Hoppe 1990). Moreover, many factors impact the disease process and impede predictions, such as the host susceptibility, environmental conditions, the pathogenicity of the strains, and coinfection by multiple pathogens. Lacking precise detection techniques, soybean rust is difficult to detect and control before visible symptoms appear.

Most assays developed for the detection of *P. pachyrhizi* are based on molecular technologies, including polymerase chain reaction (PCR) (Frederick et al. 2002; Villavicencio et al. 2007). However, the traditional PCR often requires nested steps to attain adequate sensitivity. By contrast, the loop-mediated isothermal amplification (LAMP) technique can rapidly amplify nucleic acids with high specificity, sensitivity, and efficiency under isothermal conditions. LAMP requires a set of two outer primers (F3 and B3), two inner primers (forward inner primer [FIP] and backward inner primer [BIP]), and one (loop backward [LB]) or two (LB and loop forward [LF]) loop primers. In combination with DNA polymerase, LAMP greatly increases the speed and sensitivity of detection assays (Mori et al. 2001; Nagamine et al. 2002; Hartman et al. 2011a). For the rapid diagnosis of crop diseases, LAMP assays have been developed to detect many pathogenic species, including *Fusarium asiaticum* (causing wheat head blight or scab), *Pythium spinosum* and other *Pythium* species (causing root rot and damping-off of soybean), and *Uromyces betae* (causing sugar beet rust) (Xu et al. 2017; Zeng et al. 2017; Feng et al. 2019, 2021; Kaczmarek et al. 2019). However, no LAMP assay has been developed to detect *P. pachyrhizi* yet. The database of the Joint Genome Institute has released the genome assemblies of three *P. pachyrhizi* strains (K8108, MT2006, and UFV02) collected from different locations, providing a useful data resource for gene functional studies and the screening of specific genes in *P. pachyrhizi* as a target for molecular detection.

In this study, we identified a *P. pachyrhizi*-specific gene as a molecular target for the LAMP assay, which is accurate, sensitive, and can rapidly detect *P. pachyrhizi*. We also assessed its application potential for soybean rust diagnosis in field samples.

Results

Identification of a target gene for LAMP detection

To identify a target gene for the specific detection of *P. pachyrhizi*, we performed a comparative genomic analysis among 61 genome-sequenced *Pucciniomycotina* strains, including the *P. pachyrhizi* strains K8108, MT2006, and UFV02 (Fig. 1). We assigned all of the predicted protein sequences to the OrthoFinder web server (Additional file 1: Table S1) and identified 53,887 orthologous groups (Additional file 1: Table S2). Then, we selected 869 orthologous groups that contain a single member (gene) and are present in the genomes of the three *P. pachyrhizi* strains (Additional file 1: Table S3). Of these, we identified 143 groups, in which all three *P. pachyrhizi* strains have an identical protein sequence (Additional file 1: Table S4). Among these groups, the gene *Phapa_6409908* (annotated from *P. pachyrhizi* strain MT2006) in group OG0033484 was finally selected as the candidate gene due to no sequence polymorphism among *P. pachyrhizi* strains (Additional file 2: Figure S1).

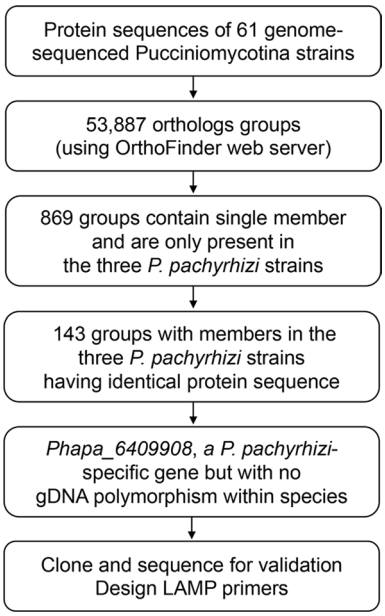

Fig. 1 Flow chart of the target gene identification and cloning process

We further confirmed the conservation of the genomic DNA sequence of *Phapa_6409908* among *P. pachyrhizi* species via PCR and the product sequencing in several *P. pachyrhizi* strains (Additional file 2: Figure S1). The genomic DNA sequence of *Phapa_6409908* is 1,011 bp, with 435 bp coding sequences and five exons. Then, based on the specific sequence of *Phapa_6409908*, we obtained multiple sets of candidate primers for the LAMP assay using PrimerExplorer version 4. After screening, we selected a set of primers, in which the BIP with B1c and B2, the FIP with F1c and F2, and the F3 and B3 outer primers as well as one loop primer (LB), were used for the LAMP assays (Fig. 2; Table 1).

Fig. 2 Gene sequences of *Phapa_6409908* and the loop-mediated isothermal amplification primers based on PrimerExplorer version 4 (http://primerexplorer.jp/e). The primers B3, F1c, and LF are the reverse complement sequences

Table 1 Primers used for the loop-mediated isothermal amplification assay

Primer name	Primer sequence (5′–3′)	Length (nt)
F3 (forward outer)	TCAGACTGATGGGTCATCT	19
B3(backward outer)	AGATGAAACGTTGATCAA GATAA	23
FIP (forward inner; F1c + F2)	TACAGAGGCAGTCCTGCT TG–TCAATAAATTAGCTTGAA CTGAGTG	45
BIP (backward inner; B1c + B2)	ACACAATCAATAACAGAG TGAGAGG–GCACTATTAAGA GAAGAAGACC	47
LB (loop backward)	GATGACGTAAGGTACTGA CACCT	23

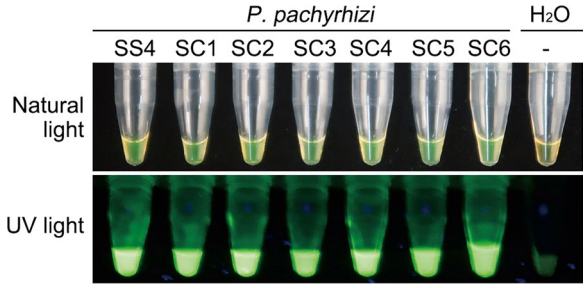

Fig. 3 Application of the loop-mediated isothermal amplification assay for the detection of *Phakopsora pachyrhizi* isolates. A positive reaction was indicated by a color change from orange to green-yellow under natural light and intense green fluorescence under ultraviolet light

Specificity of the LAMP assay

Using the SYBR Green I fluorochrome, the LAMP reaction results were visible under natural or ultraviolet (UV) light. Positive reactions changed color from orange to green-yellow under natural light or intense fluorescent green under UV light, whereas negative reactions exhibited the initial orange color under natural light or weak (or no) fluorescence under UV light. To optimize the reaction conditions (i.e., temperature and time) and improve the assay specificity, LAMP assays were performed using the pure genomic DNA of seven *P. pachyrhizi* strains, including SS4 from Fujian Province (Chen et al. 2015) and others from Sichuan Province, China, as templates. Specificity for the target DNA in the LAMP assay was assessed by optimizing the reaction at 62°C for 70 min, where the *P. pachyrhizi* strains yielded positive reactions compared to controls (Fig. 3).

To further assess the specificity of the LAMP assay, we used pure genomic DNAs of 14 additional plant pathogens, including nine commonly distributed species in diseased soybean (*Phytophthora sojae*, three *Pythium* species, two *Fusarium* species, *Macrophomina phaseolina*, *Calonectria ilicicola*, and *Colletotrichum gloeosporioides*) and the closely related rust fungi. There were

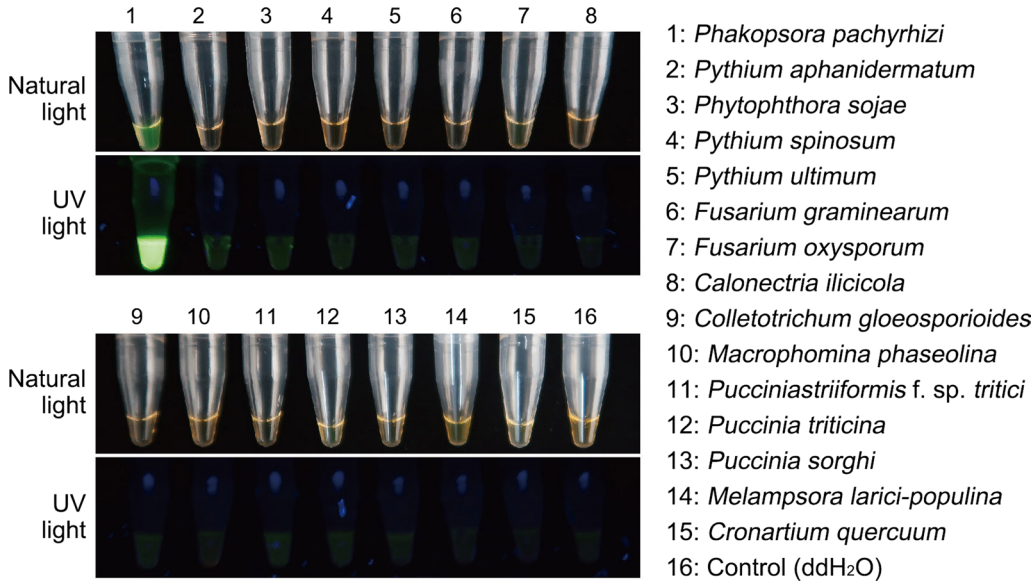

Fig. 4 Specificity of the *Phapa_6409908* loop-mediated isothermal amplification assay

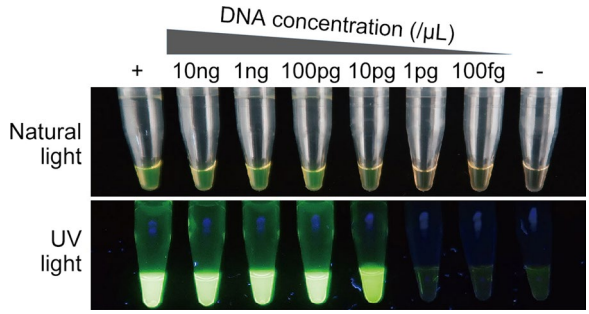

Fig. 5 Sensitivity of the loop-mediated isothermal amplification (LAMP) assay for the detection of *Phakopsora pachyrhizi*. Serial tenfold dilutions (from 10 ng to 100 fg) of genomic DNA of *P. pachyrhizi* SS4 were used for the LAMP assay. A positive reaction was indicated by a color change from orange to yellow-green under natural light and intense green fluorescence under ultraviolet light. Plus and minus signs indicate the positive and negative controls, respectively

positive reactions for *P. pachyrhizi* strain SS4 but not the other 14 non-*P. pachyrhizi* isolates (Fig. 4).

Sensitivity of the LAMP assay

Serial tenfold dilutions (from 10 ng to 100 fg) of the genomic DNA of *P. pachyrhizi* strain SS4 were used to evaluate the sensitivity of the LAMP assay. The result showed that the lowest concentration detected was 10 pg (Fig. 5).

LAMP detection for the diseased samples

To evaluate the practical applicability of the LAMP assay for the detection of *P. pachyrhizi* in diseased soybean plants, we extracted the genomic DNA of potted soybean leaves that were artificially inoculated with *P. pachyrhizi* strain SS4. The DNA extracted from the inoculated soybean leaves showed positive reactions, whereas the non-inoculated healthy soybean leaves showed negative reactions (Fig. 6a).

In a field investigation in Sichuan Province, China, we performed LAMP assays on 32 suspected soybean leaf samples collected in 2020 (samples 1–16) and 2021 (samples 17–32). All 16 samples in 2020 and 81% (13 of 16) of samples in 2021 (Fig. 6b) were positive. Importantly, *P. pachyrhizi* can be isolated from LAMP-positive leaves but not from LAMP-negative leaves (Additional file 2: Figure S2). These results support the utility of the LAMP assay for the detection of *P. pachyrhizi*-infected soybean leaves in the field.

Discussion

Specific, sensitive, and rapid detection of pathogens is the first and key step for the prevention and control of plant disease. Soybean rust is the most economically important soybean disease worldwide, and *P. pachyrhizi* is the major pathogen causing this disease. Here, we developed a LAMP assay for rapid detection of *P. pachyrhizi*, with a specific target identified from comparative genomic analysis of 61 *Pucciniomycotina* strains. The developed LAMP assay is optimized at 62°C for 70 min to specifically detect the *P. pachyrhizi* strains, and other soybean pathogens and closely related rust fungi cannot be detected. In addition, it can be used to successfully diagnose soybean rust in leaf samples from fields.

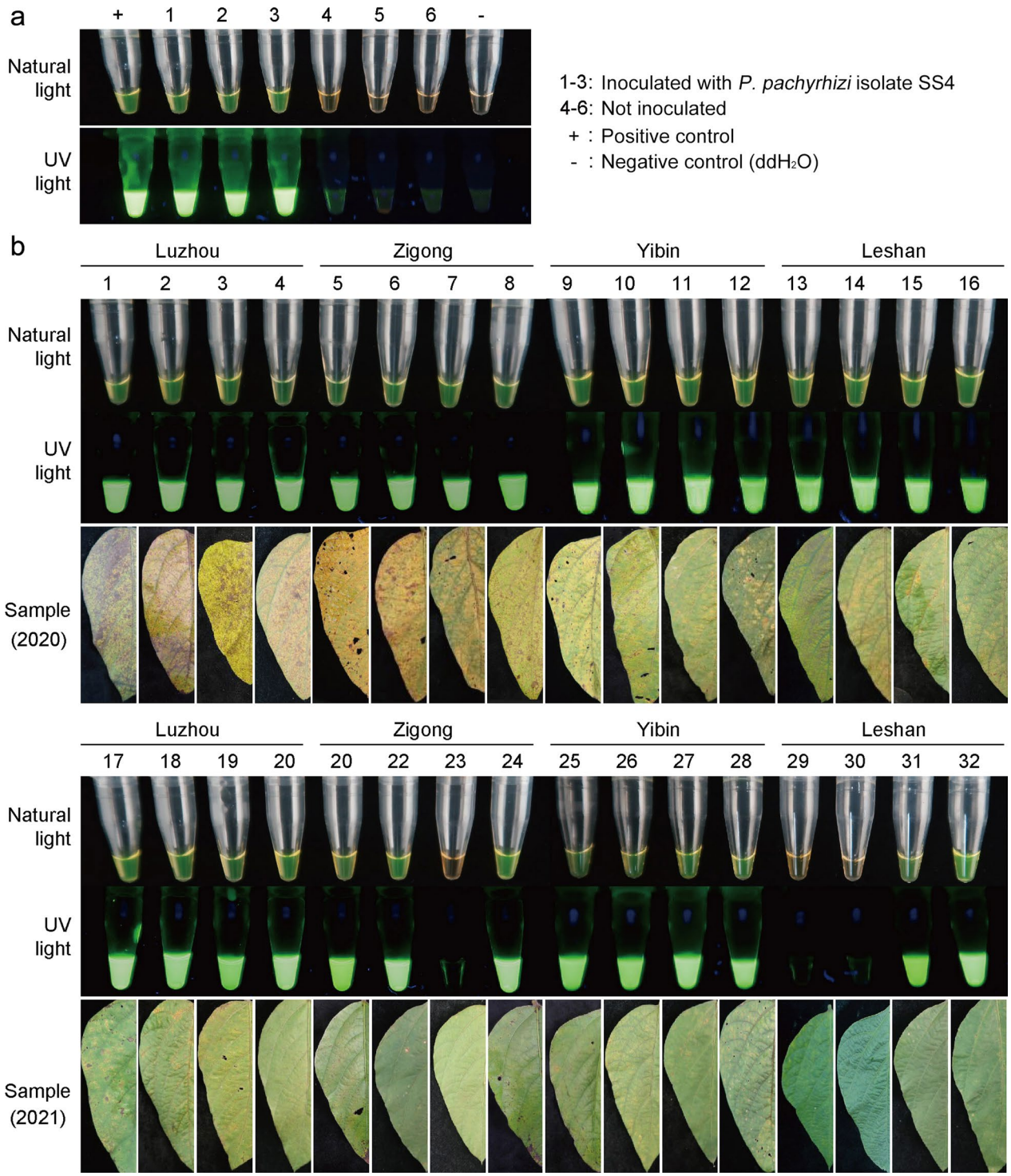

Fig. 6 Application of the loop-mediated isothermal amplification assay for the detection of *Phakopsora pachyrhizi* in inoculated leaves of potted soybean plants (**a**) and leaves of field-grown soybean plants (**b**). The plus and minus signs indicate the positive and negative controls, respectively. Samples 1–3 and 4–6 were collected from four different inoculated soybean leaves and three different healthy soybean leaves, respectively. In **b**, the detection results of 32 samples are shown as examples. Samples were randomly collected from Luzhou, Zigong, Yibin, and Leshan in the 2020 and 2021 growing seasons

Many reported targets for LAMP or molecular detection are based on the presence of internal transcribed spacer (ITS) sequences, housekeeping genes, singleton genes, or sequences determined via suppressive subtraction hybridization (Parkinson et al. 2009; Ballard et al. 2011). However, LAMP assays using such conventional targets often cannot readily distinguish the closely related species due to insufficient sequence polymorphisms (Feng et al. 2021). For example, in a previous study, a LAMP assay based on ITS sequences failed to distinguish *Pythium spinosum* and *Pythium huanghuaiense*, two major pathogens of soybean damping-off (Feng et al. 2019). However, in another study, the novel target *M90* identified via comparative genomic analysis accurately assists LAMP to distinguish *Pythium terrestris*, *Py. spinosum*, and *Py. huanghuaiense* (Feng et al. 2021). These results highlight the potential of bioinformatics and comparative genomics analyses in identifying molecular diagnostic targets for plant pathogens (Balodi et al. 2017). Rust fungi belong to an ancient lineage with many closely related species. The recent report for the genome sequences of three *P. pachyrhizi* strains and other rust fungi provides a valuable resource for the identification of molecular detection targets. In this study, we performed a comparative genomic analysis based on the genomes of 61 *Pucciniomycotina* strains and identified *Phapa_6409908*, an interspecific specific and intraspecific conserved gene, as a novel detection target of *P. pachyrhizi*.

Using soybean leaves collected from fields in the growing season over two years, the LAMP assay successfully detected all samples infected by *P. pachyrhizi*, including leaves without visible symptoms but from the field where *P. pachyrhizi* was isolated (e.g., samples 31 and 32) (Fig. 6; Additional file 2: Figure S2). This finding indicates that the LAMP assay can detect the presence of *P. pachyrhizi* in asymptomatic soybean leaves, which will be useful for guiding timely control of ASR. All 16 field samples in 2020 were positive, and 81% of 2021 samples were positive (Fig. 6), suggesting that soybean rust widely spread in Sichuan Province in 2020 than in 2021.

LAMP assays are faster, simpler, and more sensitive than conventional real-time PCR methods. They only require a heating block or water bath to incubate the reaction mixture rather than a thermal cycler. These assays can be completed in about 1 h under isothermal conditions, compared to nearly 2 h for a typical 30-cycle PCR. In addition, the results of LAMP assays are easier to interpret because the amplified products can be detected visually by adding SYBR Green I (Feng et al. 2021). Sensitivity assessment indicate that the limit of detection for LAMP assays was 10 pg, which is sufficient for disease detection. Compared to traditional PCR, LAMP assays require a relatively low purity of template DNA to detect the target from the total DNA of diseased leaves, indicating that LAMP assays are reliable and appropriate for diagnosing *P. pachyrhizi* infections in fields.

The present study may have some limitations which should be paid attention to. First, in such sensitive assays, DNA aerosol contamination among samples could lead to false-positive results. To inhibit the LAMP biochemical amplification process and increase sensitivity, the DNA-binding dye SYBR Green I is added after completion of the LAMP reaction. This step increases the risk of cross-contaminating amplicons when the reaction tube is opened to add the dye, and care should be taken. In addition, more plant pathogens, such as additional rust fungal species, should be tested in future studies to further assess the specificity of the current LAMP assay. For example, we were unable to test *Phakopsora meibomiae*, another pathogen that causes soybean rust, because its genome data were unavailable. In addition, it is distributed mainly in the tropical and subtropical regions of Americas, and has not been reported in Asia. Importantly, it is less harmful than *P. pachyrhizi*.

Conclusions

A LAMP assay targeting a *P. pachyrhizi*-specific gene (*Phapa_6409908*), identified from a comparative genomic analysis of 61 *Pucciniomycotina* strains, was developed for the detection of *P. pachyrhizi*. The assay is rapid, sensitive, and accurate, and can be applied for the early diagnosis of soybean rust in fields.

Methods

Strain sources

We obtained *P. pachyrhizi* strain SS4 from the Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan, China. It was previously isolated and purified from a soybean rust sample in Fujian Province, China, by Prof. Zhihui Shan (Chen et al. 2015). Six *P. pachyrhizi* strains (SC1–SC6) were isolated from soybean leaves collected in Zigong, Sichuan Province, and each of them was purified from a single spore, and stored in the authors' laboratory at Nanjing Agricultural University.

Plant growth conditions and inoculation assays

Disease-susceptible soybean (*Glycine max* cv. Williams 82) seeds were grown in a greenhouse at 25°C with a 14 h photoperiod until stage V2 (Fehr et al. 1971). Urediniospores of *P. pachyrhizi* were collected and resuspended in 0.01% (v/v) Tween 20 solution to a final concentration of approximately 1.0×10^5 spores/mL. This suspension was fully sprayed onto leaves. Plants were incubated in a dark chamber at 25°C for 24 h and then transferred to a

growth chamber at 25°C and 70% relative humidity under a 16 h photoperiod.

DNA extraction

Urediniospore samples were collected from inoculated soybean leaves 14 days after inoculation and then stored at −70°C. Leaves were harvested from either healthy plants or disease plants at 14 dpi inoculated with *P. pachyrhizi* and quickly placed in liquid nitrogen and stored at −70°C. DNA was extracted from urediniospores or plant leaf samples using the DNAsecure Plant Kit (Tiangen, Beijing, China). The DNA concentration was estimated using a spectrophotometer, and all samples were held at −20°C.

Target gene identification and cloning

To identify the *P. pachyrhizi*-specific gene as a candidate molecular detection target, the predicted protein sequences of 61 genome-sequenced *Pucciniomycotina* strains (Additional file 1: Table S1; downloaded from http://genome.jgi.doe.gov/programs/fungi) were assigned to orthologous groups using the OrthoFinder web server (https://orthomcl.org). Orthologous groups with a single member in each species were selected and further analyzed, as described in Fig. 1. The genomic DNA of *Phapa_6409908* in *P. pachyrhizi* was amplified via PCR with a forward primer (5′–ATGCTGAGTACT AGGGTTATC–3′) and a reverse primer (5′–GGGGCA GTAGGCACCTTTC–3′). The PCR products were sent to Sangon Biotech (Nanjing, China) for purification and sequencing. Sequence alignment was performed using MUSCLE (version 3.6) software integrated in SeqHunter version 2.0 (Ye et al. 2010).

LAMP primer design

Based on the *Phapa_6409908* sequences, candidate LAMP primers were designed using PrimerExplorer version 4 (http://primerexplorer.jp/e). The final set of primers is listed in Table 1, including FIP, BIP, F3, B3, and LB. All of the primers were synthesized by Sangon Biotech (Nanjing, China).

LAMP reaction and detection of the LAMP product

Each LAMP assay reaction was performed in a total volume of 25 μL containing 0.1 μL each of the F3 and B3 primers, 0.8 μM each of the FIP and BIP primers, 0.1 μM each of the LF and LB primers, 1.4 mM dNTPs, 0.8 M betaine, 20 mM Tris–HCl (pH 8.8), 10 mM $(NH4)_2SO_4$, 10 mM KCl, 6 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 8 U Bst DNA polymerase (New England BioLabs, Ipswich, MA, USA), and 4 μL target DNA sample. The reaction mixture was mixed and incubated in a PCR Thermal Cycler Dice (TP600; Takara Bio, Shiga, Japan) at 62°C for 70 min. Each reaction included a positive and a negative control, and each sample was reacted in triplicate. After adding 0.25 μL SYBR Green I solution to the PCR product, the LAMP reaction was observed. An unchanged orange reaction color under natural light and weak or lacking-fluorescence under UV light indicate a negative reaction (i.e., presence of target DNA below the limit of detection or an absence of target DNA). A change in the reaction color to yellow-green under natural light or intense green fluorescence under UV light indicate a positive reaction (i.e., the presence of the target DNA).

Specificity of the LAMP assay

The sets of candidate primers were tested separately. Each set was used to detect different isolates, and only the set that produced positive reactions for all isolates of the target species (*P. pachyrhizi*) was selected. To test specificity, the selected primer sets were used to detect other oomycete and rust fungi species (sample information for the specificity assay is listed in Additional file 1: Table S5). Only the primer sets that produced a positive reaction for all *P. pachyrhizi* isolates and negative reactions for all isolates of nontarget species were selected. The specificity of each LAMP reaction was assessed three times.

Sensitivity of the LAMP assay

To estimate the detection sensitivity of the LAMP assay, assays were executed using tenfold serial dilutions (from 10 ng to 10 fg) of genomic DNA of *P. pachyrhizi* isolate SS4. The sensitivity of each LAMP assay was tested at least three times. LAMP assays with high sensitivity (at a minimum to the picogram level) were chosen for further evaluation.

Detection of soybean leaves in inoculated potted plants and field samples

Soybean leaves were inoculated with urediniospores of *P. pachyrhizi* SS4 to evaluate the practical application of LAMP assays for detecting *P. pachyrhizi*-infected plant leaves. As a negative control, soybean leaves were inoculated with 0.01% (v/v) Tween 20 solution. DNA was extracted from the leaves for evaluation using the developed assay.

Leaf samples were also collected from four soybean fields in Luzhou (samples 1–4 and 17–20), Zigong (samples 5–8 and 21–24), Yibin (samples 9–12 and 21–24), and Leshan (13–16 and 29–32), Sichuan Province, China, in the growing seasons of 2020 (samples 1–16) and 2021 (samples 17–32). At each site, four fields were randomly selected from which four independent samples were collected. In each field, the leaves in the V2 growth stage were collected randomly from living

soybean plants and stored in sterile plastic bags. Each leaf sample was placed on an experimental table, and a 1 cm diameter hole punch was used to take samples. Each leaf was sampled three times independently (representing three replicates). DNA was extracted from the leaf discs using the DNAsecure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. DNA samples were stored at −20°C and then evaluated using the developed LAMP assay.

Abbreviations

ITS	Internal transcribed spacer
LAMP	Loop-mediated isothermal amplification
LB/LF	Backward/forward loop primer
UV	Ultraviolet

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00181-1.

Additional file 1: Table S1. Genome data used for the identification of candidate detection targets. **Table S2.** The 53,887 orthologous groups identified from the genomes of 61 *Pucciniomycotina* strains. **Table S3.** The 868 candidate orthologous groups used for the identification of interspecific specific, intraspecific conserved, and single-copy genes. **Table S4.** The 143 candidate orthologous groups with identical protein sequences in all three *Phakopsora pachyrhizi* strains. **Table S5.** DNA samples used for testing the specificity of the loop-mediated isothermal amplification assay.

Additional file 2: Figure S1. Sequence alignment for the genomic DNA of *Phapa_6409908*. **Figure S2.** Isolation and verification of *Phakopsora pachyrhizi* from the field samples of soybean leaves.

Acknowledgements

We thank Prof. Zhihui Shan from Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, China, for kindly providing the *P. pachyrhizi* strain SS4 and the suggestion on the pathogen inoculation.

Authors' contributions

YW, WY, and HOY conceived the experiments; ZZ and HOY performed the bioinformatics analysis. HOY and GS performed the experiments. HOY, HY, and YW collected the filed sample. HOY, WY, and GS wrote the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the China Agriculture Research System (CARS-004), the Key R&D Program of Hainan Province (ZDYF2022XDNY241), and the National Natural Science Foundation of China (32172374, 31721004).

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 18 February 2023 Accepted: 25 May 2023
Published online: 16 June 2023

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