

Development of a loop-mediated isothermal amplification method for the rapid detection of *Pythium ultimum*

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Abstract *Pythium ultimum* is an important soil-borne pathogen that causes damping-off and root rot in a large number of plant species. We developed a loop-mediated isothermal amplification (LAMP) method for the rapid and accurate detection of *P. ultimum*. A target gene encoding a spore wall protein was identified from the *P. ultimum* genome using a comparative genomics approach. The LAMP assay efficiently amplified the target DNA at 64 °C for 60 min, and its specificity and sensitivity were evaluated. Of the pathogen isolates tested, positive results were obtained only with *P. ultimum* isolates. The detection limit of the LAMP method was 1 pg μL^{-1} DNA, which is 1000 times more sensitive than conventional PCR. Moreover, this method was successfully used to detect *P. ultimum* DNA extracted from infected plant tissues, and the detection limit for *P. ultimum* in plants was nearly the same as for in vitro samples. Consequently, our LAMP assay enables the rapid, specific, and sensitive detection of *P. ultimum* DNA from pure cultures, as well as from infected plant tissues.

Keywords Genomics · LAMP · *Pythium ultimum* · Diagnosis

Introduction

The genus *Pythium* is a harmful genera of plant pathogens found worldwide. More than 120 *Pythium* species have been identified, and most of them are soil-borne and saprophytic plant pathogens with broad host ranges (Hendrix and

Campbell 1973; Martin and Loper 1999). They usually attack young feeder roots and seedlings, and cause a variety of diseases, including pre- and post-emergence damping-off, and seed, root, and fruit rot (Larkin et al. 1995).

Pythium ultimum is a ubiquitous soil-borne pathogen and one of the most pathogenic *Pythium* species (Martin and Loper 1999). It is common in forests, grasslands, ponds, and agricultural ecosystems worldwide. *P. ultimum* attacks a large number of plant species, including soybean, wheat, corn, potato, and many other economic crops. It can infect the seeds, seedlings, roots, and lower stem parts of plants, resulting in damping-off and root rot. *P. ultimum* is also homothallic, meaning that a single isolate can mate with itself to complete the sexual stage (Francis and St Clair 1993). *P. ultimum* oospores can survive for extended periods in soil, initiating infections in roots or seeds. Control measures for *P. ultimum* caused diseases remain insufficient.

Pathogen detection is a fundamental part of disease control. The traditional method used for the identification and discrimination of *Pythium* species is the microscopic inspection of morphological characteristics (Pettitt et al. 2002). However, this method requires professional skills and is time-consuming, making it impractical for large-scale sample analysis. In recent years, molecular detection methods based on the polymerase chain reaction (PCR) have been developed. Conventional and real-time PCR assays have been widely used for the specific diagnosis of more than 20 plant-pathogenic *Pythium* species (Klemsdal et al. 2008; Schroeder et al. 2013). For example, Cullen et al. (2007) designed specific primers that target the internal transcribed spacer (ITS) region and detected *P. ultimum* using a conventional PCR assay (Cullen et al. 2007). However, PCR-based methods require experienced technicians and expensive special equipment (Morisset et al. 2008). Consequently, they may not be suitable in the field or resource-limited laboratories.

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Loop-mediated isothermal amplification (LAMP) is a relatively novel technique that amplifies target DNA with high specificity, rapidity and simplicity (Notomi et al. 2000). This technique uses *Bst* DNA polymerase, which uses strand displacement activity to achieve amplification. LAMP assays use four to six primers that recognize the corresponding regions of target DNA (Notomi et al. 2000). The advantage of LAMP is that amplification occurs in approximately 1 h under isothermal conditions. Therefore, simple incubators such as a heating block or water bath are sufficient for DNA amplification. LAMP amplification results in the accumulation of 10^9 copies of target DNA, making it more effective than PCR. The amplified products can be detected using multiple methods, of which gel electrophoresis is the most accurate and generates typical ladder-like banding patterns. The reaction products can also be detected visually by adding different dyes, including SYBR green, hydroxy naphthol blue (HNB), or calcein, which can be seen with the naked eye (Mori et al. 2001; Goto et al. 2009). These advantages make LAMP appealing for use in rapid, easily performable pathogen detection assays compared with other diagnostic methods. To date, this technique has been successfully applied to the detection of fungi (Niessen and Vogel 2010), bacteria (Rigano et al. 2010), oomycetes (Dai et al. 2012), and viruses (Fukuta et al. 2003).

This study developed a LAMP assay for the detection of *P. ultimum* and evaluated it using inoculated plant samples. This method should be useful for the early and accurate diagnosis of *P. ultimum* so that timely plant protection measures can be instituted.

Materials and methods

Isolates used in the LAMP assay

The isolates of *Pythium ultimum*, other *Pythium* spp., *Phytophthora* spp., and fungi (Table 1) used in this study were originally collected from different provinces in China, and were maintained at the Department of Plant Pathology, Nanjing Agricultural University (Nanjing, China). *Pythium* and *Phytophthora* isolates were grown on V8 agar, whereas the fungal isolates were cultured on potato dextrose agar medium at 25 °C for at least 3 days before extracting DNA. Mycelia were harvested, collected and stored at -20 °C. Mycelial DNA extraction was performed using a modified hexadecyl trimethylammonium bromide (CTAB) method (Murray and Thompson 1980).

Target selection and LAMP primer design

The rDNA ITS sequences of *P. ultimum* (GenBank accession number: D86515), *P. splendens* (AB796308), *P. spinosum* (AJ233457), and *P. helicoides* (AB108026) were obtained from

Table 1 Isolates used to test the specificity of the LAMP method and the assay results

Species	Clade ^a	Origin	LAMP detection ^b
<i>P. aphanidermatum</i>	A	Tomato	-
<i>P. aphanidermatum</i>	A	Pumpkin	-
<i>P. inflatum</i>	B1	Corn	-
<i>P. myriotylum</i>	B1	Weed	-
<i>P. catenulatum</i>	B1	Carrot	-
<i>P. dissotocum</i>	B2	Soybean	-
<i>P. coloratum</i>	B2	Soil	-
<i>P. hydnosporum</i>	D	Soil	-
<i>P. acanthicum</i>	D	Tea	-
<i>P. marsipium</i>	E2	Soil	-
<i>P. spinosum</i>	F	Corn	-
<i>Pythium ultimum</i>	I	Soybean	+
<i>Pythium ultimum</i>	I	Strawberry	+
<i>Pythium ultimum</i>	I	Tomato	+
<i>Pythium ultimum</i>	I	Cucumber	+
<i>P. splendens</i>	I	Weed	-
<i>P. perplexum</i>	J	Soil	-
<i>P. vexans</i>	K	Soil	-
<i>P. helicoides</i>	K	Soybean	-
<i>P. falciforme</i>		Soil	-
<i>P. guiyangense</i>		Mosquito	-
<i>Phytophthora sojae</i>		Soybean	-
<i>Ph. parasitica</i>		Tobacco	-
<i>Ph. capsici</i>		Pepper	-
<i>Ph. infestans</i>		Potato	-
<i>Valsa pyri</i>		Pear	-
<i>Botrytis cinerea</i>		Grape	-
<i>Fusarium solani</i>		Wheat	-

^a Phylogenetic clades of *Pythium* species from Levesque and de Cock (2004)

^b LAMP specificity detection using primer SW-1. '+' and '-' indicate positive and negative results, respectively

the GenBank database. The genome sequences of six publically available *Pythium* species, including *P. ultimum*, *Pythium aphanidermatum*, *Pythium arrhenomanes*, *Pythium irregulare*, *Pythium iwayamai*, and *Pythium vexans*, were obtained from the *Pythium* Genome Database (<http://pythium.plantbiology.msu.edu>), and used to perform a comparative genomics analysis. Using all of the *P. ultimum* genes as the query, BLAST searches against another five *Pythium* genomes were performed (E-value cutoff: 1E-5), and genes with no hits were treated as unique to *P. ultimum*. Subsequently, these unique genes were compared with *P. ultimum* genes using the BLAST tool (E-value cutoff: 1E-5) to obtain single-copy genes. Due to the limited *Pythium* genome sequences available, these unique single-copy genes were compared against the NCBI database, and genes without significant similarities (E-value

cutoff: 1E-20) to any known sequence in the NCBI database were retained as candidate diagnostic targets for *P. ultimum*. The LAMP primers were designed using PrimerExplorer V4 software (<http://primerexplorer.jp/elamp4.0.0>). The design of the target for the SW-1 primer set is illustrated in Fig. 1. Five primers, F3 (5'-CAACTGGAAAAGCAAGCGG-3'), B3 (5'-CCGAAGAAGTGTGTCCGC-3'), FIP (5'-GAGC CAGACGGGCCAGTATCAAGTTACAGTGGCGTTGTCA-3'), BIP (5'-TCTCTGTTGCTCGACTGGAGGGTTCC ACCTCCTGTAAGACCT-3'), and F-Loop (5'-GCTT GCTCCAGTACGAATGC-3'), were used to detect *P. ultimum*.

LAMP reaction

The LAMP reaction was performed in a 25 μ L mixture that included 2.5 μ L of 10 \times ThermoPol buffer, 1.6 μ M each of FIP and BIP primers, 0.2 μ M each of F3 and B3 primers, 0.8 μ M F-Loop primer, 0.8 M betaine, 6 mM MgSO₄, 8 U of *Bst* DNA polymerase, 1.4 mM dNTPs, 0.2 mM HNB, and 2 μ L of target DNA. The reaction mixture was incubated at 64 $^{\circ}$ C for 60 min in a standard water bath. The LAMP products were visualized by adding HNB before amplification. Samples that turned blue were considered to be positive, while samples that remained violet were negative. In addition, the LAMP products were electrophoresed in 2% agarose gels and then stained with ethidium bromide for visualization, and positive reactions resulted in a ladder-like banding pattern. The specificity and sensitivity tests were repeated at least three times.

PCR reaction

PCRs were conducted using the method of Kong et al. (2003). The PCR reaction mixture contained 2 μ L of DNA templates, 2.5 μ L of the 10 \times PCR buffer, 2.5 μ L each of the 10 μ M forward and reverse primers, 2 μ L of the 2 mM dNTP solutions, 0.1 μ L (5 U/ μ L) of Taq polymerase, and 13.4 μ L sterile nanopure water in a total volume of 25 μ L. PCR was carried out in a DNA thermal cycler (PCR Thermal Cycler Dice,

TP600, Takara, Japan) with initial denaturation at 96 $^{\circ}$ C for 2 min, followed by 40 cycles of 94 $^{\circ}$ C for 30 s, 65 $^{\circ}$ C for 45 s, 72 $^{\circ}$ C for 1 min, and a final extension at 72 $^{\circ}$ C for 10 min. Amplification products were confirmed by electrophoresis in 1% agarose gels, and stained with ethidium bromide for visualisation under UV light.

Detection of *P. ultimum* in infected plants

To evaluate the application of LAMP as a tool for the diagnosis of *P. ultimum*, wheat, soybean, cucumber, and tobacco plants were inoculated with *P. ultimum* mycelia. Each plant leaf was inoculated with a small piece of V8 agar cut from the edge of a 3-day-old *P. ultimum* colony. After 24 h, tissue surrounding the infected region was harvested for DNA extraction by the CTAB method. These DNA samples were evaluated using the developed LAMP system. A color change in the products was assessed with the naked eye. The experiment was repeated at least three times.

Results

LAMP specificity test

In total, 4 *P. ultimum* isolates, 17 isolates from other *Pythium* species, 4 *Phytophthora* isolates, and 3 fungal isolates (Table 1) were used to test the specificity of the LAMP reaction. Initially, LAMP primers based on the ITS sequence were tested, but these failed to distinguish *P. ultimum* from related *Pythium* species. Then, a comparative genomics approach was performed to identify a target sequence, and a gene encoding a spore wall protein (GenBank accession number: MF765461) was found to be specific for *P. ultimum*. LAMP primer SW-1 was generated based on this gene, and was used to test the specificity of the assay. Primer SW-1 resulted in positive reactions when tested with four *P. ultimum* isolates, and no positive reaction was observed for related *Pythium* spp. (Table 1).

Fig. 1 Design of the LAMP primers specific for *P. ultimum* based on the selected target gene encoding a spore wall protein. The LAMP primer sequences are highlighted in grey

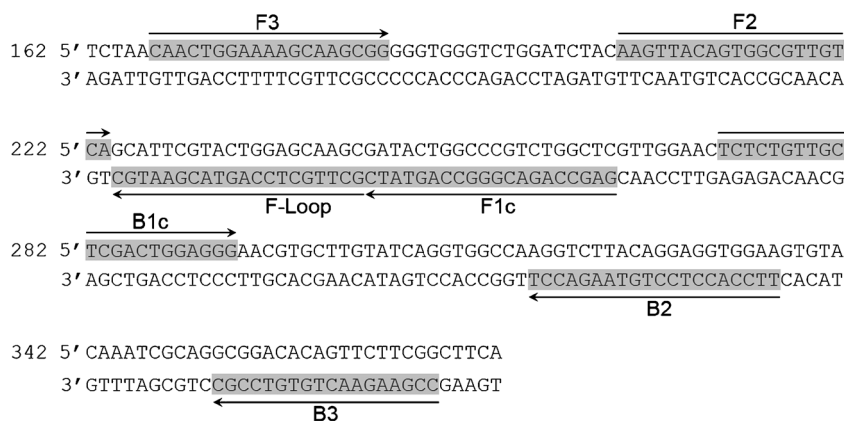
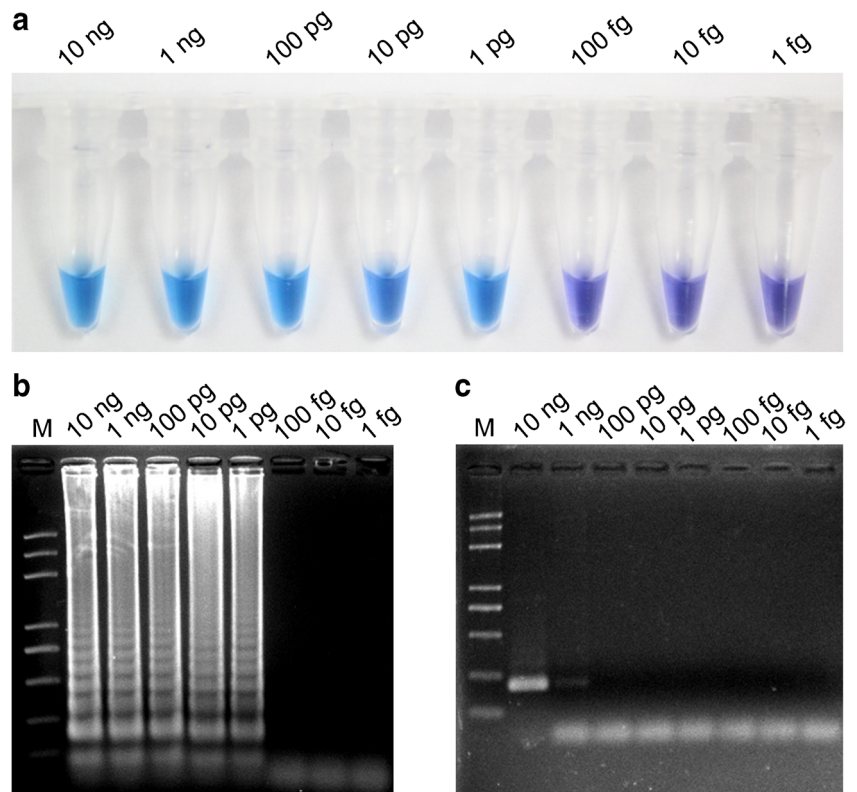


Fig. 2 Sensitivity of the LAMP and conventional PCR assays for detecting *P. ultimum*. **a** Detection of LAMP products using HNB as a visual indicator. **b** Detection of LAMP products by agarose gel electrophoresis. **c** Detection of conventional PCR products by agarose gel electrophoresis



Negative reactions were also seen for the *Phytophthora* spp. and fungal isolates. These results were evaluated with both the naked eye and gel electrophoresis, and three replicates were performed. The results indicate that primer SW-1 had good specificity for the LAMP –based detection of *P. ultimum*.

Comparison of the sensitivity of LAMP and conventional PCR assays

To determine the sensitivity of primer SW-1, ten-fold serial dilutions of *P. ultimum* genomic DNA were tested. The DNA template concentrations ranged from 10 ng μL^{-1} to 1 fg μL^{-1} . As indicated by a color change and DNA amplification of the reaction products, the lowest limit of *P. ultimum* detection was 1 pg μL^{-1} DNA for the LAMP assay (Fig. 2a,b). In comparison, the detection limit of the conventional PCR assay using the outer primers F3 and B3 was 1 ng μL^{-1} DNA (Fig. 2c). This demonstrates that the LAMP assay has a wider dynamic range, with a sensitivity nearly 1000 times greater than that of conventional PCR for the detection of *P. ultimum*.

Successful detection in infected plants

To assess the reliability of our LAMP method, wheat, soybean, cucumber and tobacco leaves were inoculated with *P. ultimum* mycelia. At 24 h after inoculation, genomic DNA was extracted from the infected region and subjected to the LAMP reaction. Positive reactions were observed in

all four infected plants, while negative reactions were seen in uninoculated plants (Fig. 3a).

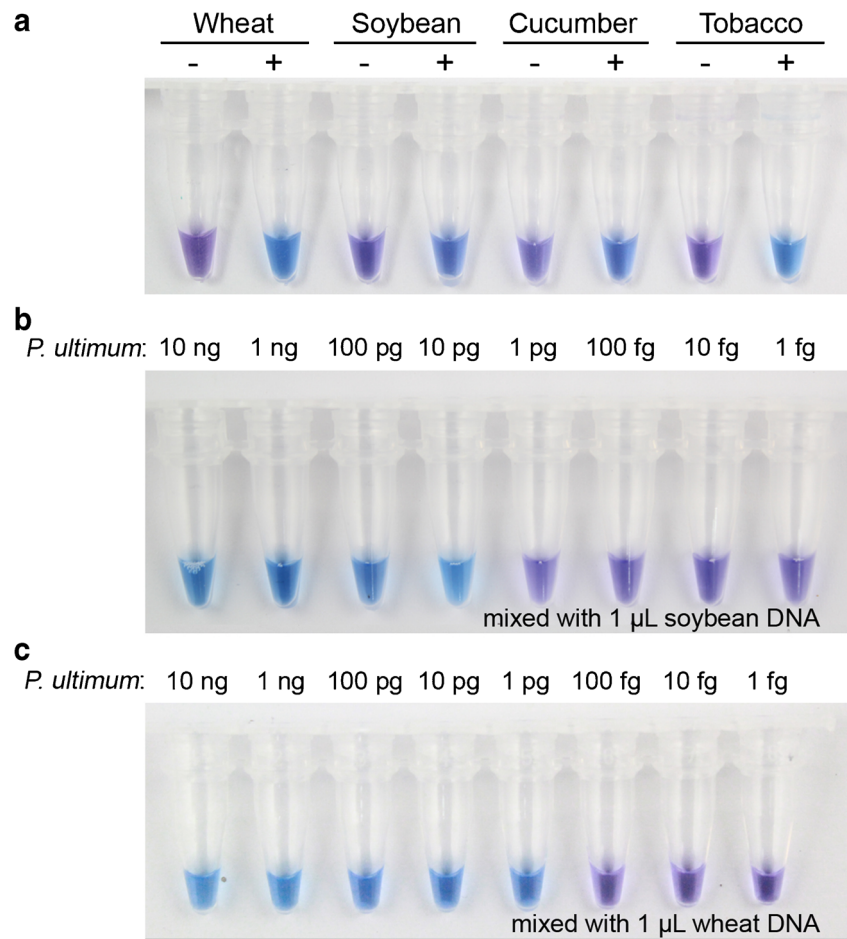
To investigate the detection limit of the LAMP method for the pathogen in plants, we established the detection limit of purified *P. ultimum* DNA when mixed with a fixed amount of purified plant DNA. The target DNA in the LAMP reaction was 2 μL , which included 1 μL of plant DNA (50 ng μL^{-1}) and 1 μL of *P. ultimum* DNA with concentrations ranging from 10 ng μL^{-1} to 1 fg μL^{-1} . As shown in Fig. 3b, the detection limit was 10 pg μL^{-1} DNA for *P. ultimum* when mixed with soybean DNA. The lowest limit was 1 pg μL^{-1} DNA for *P. ultimum* when mixed with wheat DNA (Fig. 3c). These results suggest that the detection limit for *P. ultimum* in plants was nearly the same as for in vitro samples.

Discussion

Pythium ultimum is a ubiquitous soil-borne pathogen with a broad host range that causes a variety of diseases, including damping-off and root rot. The process of disease control requires high specificity and sensitivity in the detection of *P. ultimum* infection. In this study, we developed a rapid, sensitive, and reliable LAMP detection method for *P. ultimum*.

A key factor influencing the LAMP method is target selection. Most previous targets were based on ITS or housekeeping gene sequences (Fukuta et al. 2013; Takahashi et al. 2014). We initially selected the ITS sequence as the target, but the LAMP

Fig. 3 Detection of *P. ultimum* in infected plants. Detection of LAMP products using HNB as a visual indicator. **a** For each plant, the left tube contained purified plant DNA and the right tube contained DNA from the infected region after inoculation with *P. ultimum* mycelia. **b** Investigation of the detection limit for *P. ultimum* DNA when mixed with 1 μL of soybean DNA. **c** Investigation of the detection limit for *P. ultimum* DNA when mixed with 1 μL of wheat DNA



primer sets failed to show specificity for *P. ultimum*. We believe that the ITS sequences of closely related pathogens are too similar or even identical, and are not appropriate for distinguishing some pathogens. As increasing numbers of pathogens have been sequenced, bioinformatics tools have been widely used to identify targets for molecular diagnostics. A unique target identified by a comparative genomics pipeline was successfully applied to the LAMP detection of *Xanthomonas arboricola* pv. *pruni* (Buhlmann et al. 2013). In this study, we used a similar target identification method and the selected target was suitable for the diagnosis of *P. ultimum*.

Compared with conventional and real-time PCR assays, the LAMP assay is simple, rapid, specific, and sensitive, and it should become a new reliable method for the diagnosis of *P. ultimum* infection. The LAMP assay requires only a heating block or water bath instead of a thermal cycler to incubate the reaction mixture in approximately 1 h under isothermal conditions, and the reaction products can be detected visually. Our results show that the SW-1 primer set designed in this study successfully distinguished *P. ultimum* from related species. In addition, the sensitivity evaluation showed that the detection limit of LAMP was 1 pg μL^{-1} , which is 1000 times more sensitive than conventional PCR. These results demonstrate the high specificity and sensitivity of the LAMP assay. We

also tested the reliability of the LAMP method in infected plants, and positive reactions were seen in all infected samples, indicating that this method is reliable and suitable for the diagnosis of *P. ultimum* in the field.

In conclusion, we developed a visual LAMP assay for the diagnosis of *P. ultimum*. This LAMP method had good specificity, sensitivity, and accuracy, and was useful for the identification and detection of *P. ultimum*. This method should facilitate the early and accurate diagnosis of *P. ultimum* in the field so that timely plant protection measures can be initiated.

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