Development and evaluation of specific PCR and LAMP assays for the rapid detection of *Phytophthora melonis*

Qinghe Chen · Benjin Li · Peiqing Liu · Chengzhong Lan · Zhixiong Zhan · Qiyong Weng

Abstract *Phytophthora melonis* is a widespread and devastating pathogen for the Cucurbitaceae family. Early and accurate detection of *P. melonis* is essential to control the disease in the field. To establish a simple, visual, and rapid detection system for *P. melonis*, we developed nested polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) systems based on the Ras-related protein (*Ypt1*) gene. All 36 isolates of *P. melonis*, from geographically distinct counties in China, yielded positive detection results on LAMP or nested PCR assays. No cross reaction was observed with other oomycetes or fungal pathogens. A sensitivity assay showed that both methods had a detection limit of 10 fg genomic DNA. We also detected *P. melonis* in diseased cucumber tissues and soils, and evaluated positive detection rates using LAMP, nested PCR, and conventional isolation methods. The results suggest that the LAMP assay has the greatest potential for active detection of *P. melonis* in regions that are at risk of contracting the disease, and for use in resource-poor settings.

Keywords Loop-mediated isothermal amplification (LAMP) · Molecular detection · *Phytophthora melonis* · Ras-related protein (*Ypt1*) gene

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Introduction

The oomycete *Phytophthora melonis* Katsura, which was first described by Katsura (1976), is conspecific with *P. sinesis* and has been reported in China, Japan, Egypt, Turkey, Korea, and India (Ho et al. 2007). *P. melonis* can cause severe damage to cucumbers (*Cucumis sativus*) in affected fields, and areas receiving heavy rain may suffer from plant mortality and yield losses of up to 80 % (Wang et al. 2007). *P. melonis* also infects zucchini (*Cucurbita pepo L.*), hami melon (*Cucumis melo L.*), wax gourd [*Benincasa hispida* (Thunb.) Cogn.], pointed gourd (*Trichosanthes dioica* Roxb.), and pistachio (*Pistacia vera* L.) (Chen et al. 2012), causing blight, dieback, and root, foot, and crown rots (Mirabolfathy et al. 2001). A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences shows that *P. melonis* is closely associated with *P. sinensis* and they are placed in Clade 7b containing a variety of species, including *P. cajani*, *P. vignae*, *P. pistaciae*, *P. sojae*, among others (Blair et al. 2008). To control the spread of *P. melonis*, early detection and diagnosis of the pathogen in the field is essential.

Traditional detection methods, such as baiting and culturing from plant tissue, are difficult, time consuming, and require extensive experience. With the development of DNA-based techniques, polymerase chain reaction (PCR) has become a primary method of plant pathogen identification and detection (Henson and French. 1993; Trout et al. 1997). *P. melonis*–specific primers based on the internal transcribed spacer (ITS) have been used for rapid identification of the pathogen in infected plant tissues, soil, and water (Wang et al. 2007). Previous studies have suggested that the ITS regions are highly conserved in different fungal species, evolve more quickly than other regions, and may vary among species within a genus (Pavón et al. 2008; Silvar et al. 2005). However, ITS regions are not always sufficiently diverse to allow the separation of closely related taxa (Kroon et al. 2004; Schena and Cooke. 2006; Schena et al. 2006). Therefore, more recent studies have also examined the Ras-related protein gene *Ypt1*, β-tubulin, elicitin, and the spacer region between the mitochondrially encoded *cox1* and *cox2* genes in *Phytophthora* species (Martin et al. 2012; Meng and Wang. 2010; Schena et al. 2008). The *Ypt1* gene appears to be a more promising target because its coding (exons) and non-coding (UTRs and introns) regions have polymorphisms which can be used as molecular markers (Haubruck et al. 1987). Based on the *Ypt1* gene, specific primers were designed for the detection of *Phytophthora* species, including *Phytophthora inundata*, *Phytophthora kernoviae*, and *Phytophthora lateralis* (Martin et al. 2012).

The PCR technique offers great promise for plant pathogen detection, most significantly for its advantages in speed, sensitivity, specificity, and flexibility (Trout et al. 1997). However, the associated diagnostic procedures still require long periods of time and expensive laboratory instrumentation and reagents. These drawbacks limit its routine use, especially in poorly resourced laboratories and in rural areas of developing countries. Thus, a rapid, sensitive, simple, and economical detection method for practical applications is preferred. Recently, the novel loop-mediated isothermal amplification (LAMP) technique has been developed (Nagamine et al. 2001; Notomi et al. 2000; Rigano et al. 2010). This assay uses the *Bst* DNA polymerase, which has displacement activity and a set of four specially designed primers that recognise a total of six distinct sequences of the target DNA (Notomi et al. 2000). This technique, which employs four primers, has been used to perform highly specific

and sensitive amplifications of DNA to detect pathogens, including viruses, bacteria, and fungi (Nagamine et al. 2002; Notomi et al. 2000). The LAMP assay can be performed under isothermal conditions ranging between 60 °C and 65 °C, and it produces large amounts of replicate DNA (Notomi et al. 2000). The reaction shows a high tolerance to biological contaminants (Kaneko et al. 2007), which can help to avoid false-negative results due to inhibition of the enzyme (a common problem with PCR). Although LAMP amplification products can be detected by gel electrophoresis, they also should be detected using simple visual inspection by SYBR Green I and calcein intercalating DNA dyes (Niu et al. 2012).

Recently, the LAMP assay has proven to be effective in the diagnosis of oomycetes such as *Phytophthora* spp. (Martin et al. 2012). Faster, simpler, and more-specific molecular detection of *P. ramorum* by LAMP in the field was first described by Tomlinson et al. (2007). However, no study has reported the detection of *P. melonis* using a LAMP assay. In this study, we developed specific PCR and LAMP assays for *P. melonis* detection based on the Ras-related protein gene *Ypt1*, and demonstrated that this method is specific and efficient.

Materials and methods

Sources of isolates

The origin, host, and number of isolates used in this study are listed in Table 1. We tested 36 *P. melonis* isolates sampled from a wide geographic distribution in China, as well as 34 isolates of 13 different oomycetes (e.g. *P. sojae, P. vignae, P. capsici, P. parasitica* and *P. infestans*) and 22 isolates of other fungi isolated (e.g. *Fusarium oxysporum, Colletotrichum capsici, Alternaria alternate* and *Sclerotinia sclerotiorum*) from cucumbers and other hosts.

Culture conditions and DNA extraction

Isolates of *Phytophthora* spp. were cultured in a tomato juice medium (20 % v/v tomato juice, 0.1 g l^{-1} CaCO$_3$, and 15 g l^{-1}agar). Mycelia of each *Phytophthora* and *Pythium* isolate were obtained by growing the isolates in tomato juice broth at 18–25 °C (temperature dependent on isolate) for at least 5 days. Mycelia of the fungi

Table 1 Oomycetes and fungi isolates used in the PCR assay

Species[a]	Isolation/Origin		No. of isolates	PCR product[b]	
	Host	Source		PCR	LAMP
Oomycetes					
Phytophthora melonis	Cucumis sativus	Fuzhou, CHINA	13	+	+
Phytophthora melonis	Cucumis sativus	Jianou, CHINA	8	+	+
Phytophthora melonis	Cucumis sativus	Zhangzhou, CHINA	3	+	+
Phytophthora melonis	Cucumis sativus	Longhai, CHINA	6	+	+
Phytophthora melonis	Cucumis sativus	Sanming, CHINA	5	+	+
Phytophthora melonis	Cucumis sativus	Guangdong, CHINA	1	+	+
Phytophthora capsici	Capsicum annuum	Fujian, CHINA	8	−	−
Phytophthora sojae	Glycine max	Fujian, CHINA	5	−	−
Phytophthora parasitica	Nicotiana tabacum	Fujian, CHINA	3	−	−
Phytophthora infestans	Solanum tuberosum	Fujian, CHINA	5	−	−
Phytophthora cactorum	Malus pumila	Fujian, CHINA	3	−	−
Phytophthora boehmeriae	Gossypium	Jiangsu,CHINA	3	−	−
Phytophthora fragariae	Fragaria ananassa	Fujian, CHINA	1	−	−
Phytophthora megasperma	Rosa chinensis	Fujian, CHINA	1	−	−
Phytophthora cambivora	Castanea sativa	CBS 248.60	1	−	−
Phytophthora palmivora	Scindapsus aureus	Fujian, CHINA	1	−	−
Phytophthora vignae	Vigna unguiculata	Fujian, CHINA	1	−	−
Pythium aphanidermatum	Cucumis sativus	Fujian, CHINA	1	−	−
Pythium ultimum	Glycine max	Fujian, CHINA	1	−	−
Pseudoperonospora cubensis	Cucumis sativus	Fujian, CHINA	1	−	−
Fungi					
Fusarium oxysporum f.sp. cucumerimum	Cucumis sativus	Fujian, CHINA	1	−	−
Fusarium oxysporum f.sp.batatas	Ipomoea batatas	Fujian, CHINA	1	−	−
Fusarium oxysporum f.sp.fabae	Vicia faba	Fujian, CHINA	1	−	−
Fusarium oxysporum f.sp.pisi	Pisum sativum	Fujian, CHINA	1	−	−
Fusarium oxysporum f.sp.niveum	Citrullus lanatus	Fujian, CHINA	1	−	−
Fusarium oxysporum	Glycine max	Fujian, CHINA	1	−	−
Fusarium lateritium	Morus alba Linn	CGMCC	1	−	−
Fusarium catenulatum	Gossypium spp.	CGMCC	1	−	−
Fusarium chlamydosporum	Soil	Fujian, CHINA	1	−	−
Rhizoctonia solani	Cucumis sativus	Fujian, CHINA	1	−	−
Colletotrichum lagenarium	Cucumis sativus	Fujian, CHINA	1		
Colletotrichum capsici	Capsicum annuum	Fujian, CHINA	1	−	−
Colletotrichum gloeosporioides	Capsicum annuum	Fujian, CHINA	1	−	−
Colletotrichum truncatum	Glycine max	Fujian, CHINA	1	−	−
Magnaporthe oryzae	Oryza glaberrima	Fujian, CHINA	1	−	−
Ustilagrnoidea virens	Oryza glaberrima	Fujian, CHINA	1	−	−
Sclero tium rolfsii Sacc	Capsicum annuum	Fujian, CHINA	1	−	−
Botrytis cinerea Pers	Capsicum annuum	Fujian, CHINA	1	−	−
Sclerotinia sclerotiorum	Capsicum annuum	Fujian, CHINA	1	−	−
Alternaria alternate	Solanum tuberosum	Fujian, CHINA	1	−	−
Alternaria solani	Solanum lycopersicum	Fujian, CHINA	1	−	−

Table 1 (continued)

Species[a]	Isolation/Origin		No. of isolates	PCR product[b]	
	Host	Source		PCR	LAMP
Botryosphaeria rhodina	*Psidium guajava*	Fujian, CHINA	1	−	−
Total			92		

CGMCC China general microbiological culture collection

[a] All isolates of *P. melonis* and other *oomycetes* species and fungi were maintained in the collection of Fujian Academy of Agricultural Sciences

[b] Note that presence (+) or absence (−) are based on the presence of a PCR or LAMP product of the expected size

were grown in potato dextrose broth. The mycelia were harvested by filtration and freeze dried for 36 h. DNA was extracted using the cetyltrimethylammonium bromide (CTAB) procedure, as described previously. Purified DNA was quantified using a spectrophotometer, and aliquots were diluted to 100 ng μl^{-1} in distilled water as stocks at −20 °C.

Primer design for *P. melonis*

Alignment of the *Ypt1* gene sequences present in National Center for Biotechnology Information databases and belonging to *Phytophthora* species closely related to *P. melonis* was used to identify conserved and differing regions, which include *P.cinnamomi* (DQ270317); *P. melonis* (EF649778); *P. capsici* (FJ535571); *P. sojae* (DQ162958); *P. parasitica* (DQ864604); *P. infestans* (JN678988); *P. cactorum* (HQ850001) (Blair et al. 2008). On the basis of *P.*

melonis (EF649778) regions of divergence, the specific primer pair PmY1F/PmY1R was designed to amplify the *P. melonis*–specific regions of the *Ypt1* gene, and the nested PCR primer pair PmY1F/PmY2R was also developed to increase the sensitivity (Table 2, Fig. 1). Based on the *P. melonis Ypt1* gene sequence, a set of LAMP primers comprising two outer (F3 and B3) and two inner (FIP and BIP) primers was designed using the LAMP primer software PrimerExplorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). The designed primer sequences for *P. melonis* and their relative position in the sequence are shown in Table 1 and Fig. 1.

Extraction of *P. melonis* DNA from cucumber and soil

DNA samples from *P. melonis*–infected cucumber were performed as previously described (Tooley et al.

Table 2 PCR and LAMP primers used in this study

Primer type	Primer	Primer sequences (5′ to 3′)
PCR	PmY1F	GGTCGAGCGGGCGTTTTCGGGCT
	PmY1R	ACAACTGTTAGCACCGATCCGCA
Nested-PCR	PmY1F	GGTCGAGCGGGCGTTTTCGGGCT
	PmY2R	GCATTGATAAGATCATGTACCG
Universal *Ypt1*[a]	Yph1F	CGACCATKGGTGTGGACTTT
	Yph2R	ACGTTCTCMCAGGCGTATCT
LAMP	F3	AAATTCGCACGATCGAGCT
	B3	CCGTGACGTCGTACACCA
	FIP	GCGCTAAGTCGCGAATGTACCGGAC GGCAAGACCATCAAGC
	BIP	CTATTGTAGTGGGACACGGCCGCGAT AATACCGTGGGCACC

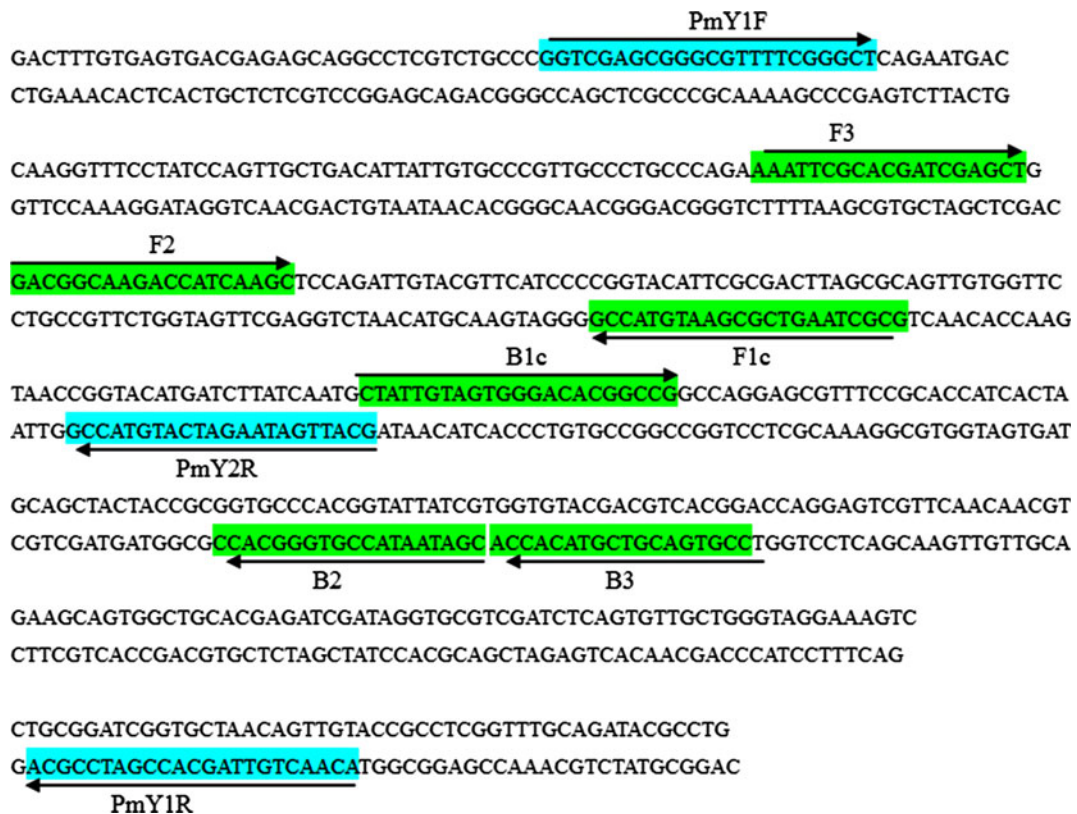[a] *Phytophthora*-genus-specific primers for *Ypt1* gene from Schena et al. (2008)

Fig. 1 Partial sequence of *Phytophthora melonis Ypt1* gene (EF649778) and the location and sequences of three PCR primers (PmY1F, PmY1R, and PmY2R) and four LAMP primers (F3, B3, F2, F1c, B2, and B1c) targeting the *P. melonis* isolate. FIP is a hybrid primer consisting of the F1c sequence and the F2 sequence, BIP is a hybrid primer consisting of the B1c sequence and the B2 sequence. *Arrows* indicate the direction of extension

1997). A sample (10 mg) of tissue (stem or leaf) was cut from each plant, placed into 10 μl freshly prepared 0.5 M NaOH, and macerated with a plastic pestle. The tubes were then centrifuged at 12,000×g for 5 min, and 5 μl supernatant was removed and immediately diluted with 195 μl 100 mM Tris (pH 8). DNA from soil and water were isolated as previously described (Wang et al. 2007). Soil DNA samples collected from infected and uninfected cucumbers in the field were extracted using a FastDNA® SPIN Kit for Soil from BIO 101® Systems (Q-Biogene Ltd, Irvine, CA). The samples were then used immediately for PCR or frozen at −20 °C for later use.

Detection of *P. melonis* by PCR

The Reaction mixtures (final volume, 25 μl) contained 1.4 mM MgCl₂, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 200 μM each dNTPs, 10 pmol of each primer, 1U Taq DNA polymerase, and 20 ng template. All reactions were performed in a PTC200 Thermo Cycler (MJ Research, Watertown, MA, USA) and programmed for an initial denaturation step at 95 °C (5 min) followed by 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 58 °C, extension for 1 min at 72 °C, and a final extension for 5 min at 72 °C. Nested PCR included two rounds of amplification using the universal primers Yph1F/Yph2R for the first round and the *P. melonis*–specific primers PmY1F/PmY2R for the second round (Table 2), and 1 μl amplified product from the first round as the template and a 203-bp product was amplified. The sensitivity of the primer pair PmY1F/PmY2R was tested by using different concentrations of the template, ranging from 1,000 ng to 1 fg of isolate purified DNA as a template. Negative controls lacking template DNA were performed in each experiment to test for contaminated

reagents. All reagents used for PCR amplification were purchased from TaKaRa (Dalian, China). All of the experiments were repeated at least three times.

LAMP reaction

The LAMP reaction was performed using a LoopAmp DNA amplification kit (Eiken Chemicals Co. Ltd, Tokyo, Japan) in a 25-μl volume. The reaction mixture contained 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3 primers, 2.0 μl template DNA (~10 ng), 1 μl *Bst* DNA polymerase (8 units), and 12.5 μl 2× reaction mix prepared in the kit. Negative controls containing nuclease-free water instead of DNA were included in each assay. To identify the optimal temperature and time for visual LAMP amplification, the reactions were performed in 60 °C, 62 °C, 63 °C, and 65 °C water baths for 15, 30, 45, 60, and 75 min. Finally, the reaction was terminated by heating the reaction mixture at 80 °C for 10 min. Amplification was monitored with SYBR Green I on UV lamp or the products (2 μl) were electrophoresed on 2.0 % agarose gels to determine the optimal conditions (Li et al. 2013), and the results of flourescence correlated well with gel electrophoresis. All of the experiments were repeated at least three times.

LAMP testing of diseased cucumbers

To develop field application of LAMP for *P. melonis* surveys and management, 15 cucumber wilt samples (stem and leaves) induced by *P. melonis* and 13 soil samples collected from a greenhouse and inoculated artificially with the pathogen were surveyed using the LAMP method. Diseased cucumbers were grown in a growth chamber. Zoospores for inoculation were performed as previously described (Ristaino 1990). *P. melonis* was grown on V8 agar petri plates for 1 week. The agar with fungal hyphae was then cut into 0.5-cm squares and placed in a sterile petri dish with sterile distilled water. The solution was replaced with fresh water every 3 days. After 1 week, the sporangia were given a cold shock (1 h at 4 to 6 °C), followed by a 1-h equilibration at room temperature. The zoospores were separated from the solution by filtering through 2 layers of cheesecloth. Zoospores (10^6 spores/ml) were applied to the cucumbers within 30 min of filtering. Non-infested cucumbers were used as controls. We also collected cucumbers and soil samples from

different natural fields in the Fujian province for LAMP detection. DNA extraction was performed and the LAMP react ions were performed at 63 °C for 60 min. Amplification was monitored with gel electrophoresis and SYBR Green I.

Results

Detection of *P. melonis* using a PCR assay

The specificity of PCR primers was assessed using a large collection of 36 *P. melonis* isolates, and 56 isolates of other oomycetes and fungi (Table 1). The primer pair PmY1F/PmY1R was able to amplify a unique DNA fragment approximately 410 bp in length (Fig. S1) in all *P. melonis* isolates from different regions and host plants in China. This primer pair did not yield amplification products when using isolates of any other oomycete or fungus tested. *Phytophthora* spp. *Ypt1* universal primers Yph1F/Yph2R (Table 2), designed to amplify a DNA fragment in *Phytophthora* spp. (Schena et al. 2008), produced positive PCR reactions in all *Phytophthora* isolates tested, although the amplification of fungal DNA was not achieved using this primer pair (data not shown).

Optimisation of LAMP assay

LAMP was performed using *P. melonis* DNA as a template to determine the optimal temperature and reaction time. Optimisation of LAMP reaction temperature and time revealed that the ideal conditions for the primer were 63 °C for 60 min. Therefore, the LAMP assays were performed under these conditions. As expected, the typical ladder-like pattern was observed on 2.0 % agarose gel electrophoresis in all positive samples, but not in the negative controls. Under visual fluorescence detection with SYBR Green I, positive and negative results were easily determined; positive reactions appeared green, whereas the negative control remained orange (Fig. 2).

Specificity of detection of *P. melonis* using LAMP

The specificity of the LAMP reaction was confirmed by electrophoresis in 2.0 % agarose gels stained with ethidium bromide and direct visual inspection of the LAMP products by adding SYBR Green I dye. As
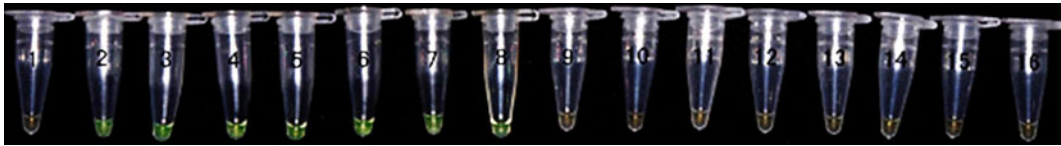
Fig. 2 Specificity of *Phytophthora melonis* DNA detection by LAMP. LAMP assay and visual inspection by adding SYBR Green I dye and observing under visible light. Positive reactions turned green after the addition of SYBR Green I. Lane 1, negative control; lane 2, positive *P. melonis* control; lanes 3–8, *P. melonis* isolates; lanes 9–16, other oomycete and fungal isolates (*P. capsici, P. sojae, P. parasitica, P. infestans, Pythium aphanidermatum, Fusarium oxysporum f.sp. cucumerimum, Rhizoctonia solani, Colletotrichum lagenarium*). The same results were obtained in all three replicates

expected, positive reactions were observed in all *P. melonis* isolates, but not in other *Phytophthora* spp. or isolates of true fungi (Fig. 2). The LAMP reaction yielded the same results as the PCR assay (Fig. S1). The results indicated that the LAMP technique developed in this study is highly specific for *P. melonis*.

Sensitivity detection of *P. melonis* using nested PCR and LAMP assays

The sensitivity of the primers PmY1F/PmY1R was tested by using different concentrations of DNA, ranging from 10 μg to 1 pg *P. melonis* isolate as a purified DNA template. The sensitivity of the primers was further tested by a nested PCR. All of the experiments were repeated at least three times and the amplicons were detected by agarose gel electrophoresis and visual inspection. As shown in Table 3, the minimum detection concentration required for the LAMP assay was 10 fg genomic DNA, i.e. the sensitivity of the LAMP assay was 1000-fold higher than that of conventional PCR with a detection limit of 10 pg for a 60-min reaction (Table 3). However, a second amplification conducted using 1.0 μl amplified product from the first round PCR as the template and the primers PmY1F/PmY2R increased the sensitivity of the assay by at least 1,000 times to 10 fg per 25.0 μl reaction volume (Table. 3). Our results suggest that the nested

PCR assay has the same sensitivity as the LAMP assay (Table 3).

Detection of *P. melonis* from inoculated samples

To demonstrate the applicability of the LAMP assay to field samples, this method was evaluated using plant tissue and soil infested with *P. melonis*. The plant tissue and soil from healthy cucumbers grown axenically in a greenhouse were used as negative controls, and purified DNA from *P. melonis* was used as a positive control. DNA extracted from infected tissue and soil samples was subjected to LAMP. A total of 28 samples of *P. melonis*–infected plant tissue and soil, and three healthy plant tissues were also analysed. All infected plant tissue and infested soil samples tested positive for *P. melonis* by LAMP, while no healthy sample showed any electrophoresis band or green stain (Fig. 3).

Evaluation of PCR and LAMP assays using diseased cucumbers in the field

To evaluate PCR and LAMP assays for the detection of *P. melonis*, 56 healthy-looking but pathogen-infected cucumbers and eight healthy cucumbers collected from different areas of Fujian, China, between 2009 and 2012 were tested using nested PCR and LAMP assays. The isolation of *P. melonis* from these samples was

Table 3 The sensitivity of LAMP, Nested PCR and PCR assays in detecting *Phytophthora melonis* using serial DNA dilutions

	1000 ng	100 ng	10 ng	1 ng	100 pg	10 pg	1 pg	100 fg	10 fg	1 fg
PCR	+	+	+	+	+	+	−	−	−	−
Nested PCR	+	+	+	+	+	+	+	+	+	−
LAMP	+	+	+	+	+	+	+	+	+	−

+ triplicate assay showed all positive; − triplicate assay showed all negative
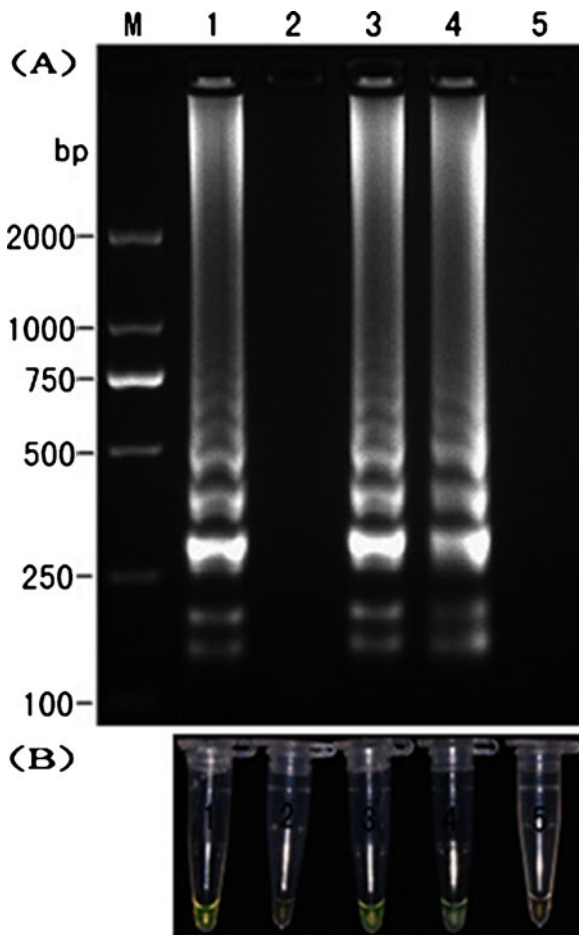
Fig. 3 LAMP detection of *Phytophthora melonis* from infected tissues and soil. **a** LAMP assay detected by agarose gel electrophoresis. **b** LAMP assay and visual inspection by adding SYBR Green I dye and observing under visible light. Three independent replicates gave the same results. Lane 1, purified DNA (*positive control*); lanes 2 and 5, DNA from healthy cucumbers and soil (*negative controls*); lanes 3 and 4, DNA from infected tissues and infested soil. Lane M, DL2000 DNA molecular marker. The same results were obtained in all three replicates

Table 4 Comparison of isolation, nested-PCR, LAMP for the detection of *Phytophthora melonis* in collected plant samples

Location	No. of sample	No. of positive sample		
		Isolation[a]	Nested-PCR	LAMP
Fuzhou	15	10	12	13
Jianou	6	4	6	6
Zhangzhou	8	5	7	7
Longhai	13	8	9	10
Sanming	4	4	4	4
Putian	10	6	8	8
Total	56	37	46	48
% positive		66.1	82.1	85.7

[a] Culture isolation positive samples were all confirmed to be *P. melonis* by sequencing the *Ypt1* gene

Ypt1 LAMP assay reported here, therefore, be used for the visual detection of *P. melonis* in plants and production fields.

Discussion

Traditional methods for the identification of *P. melonis* are time consuming and require considerable expertise to differentiate between species of *Phytophthora* based on morphologic characteristics. The LAMP reactions may be completed within 60 min by incubating at 63 °C and terminated by heating the reaction mixture at 80 °C for 10 min. Results can be visually inspected using SYBR Green I and confirmed by gel electrophoresis. The LAMP method has an extremely high amplification efficiency, due in part to its isothermal nature (Notomi et al. 2000); there is no time lost arising from changes in temperature for enzyme function, and the inhibition that occurs at later stages of amplification, a typical problem with PCR, is less likely to take place (Osawa et al. 2007). In addition, LAMP amplifies DNA at higher concentrations than PCR, making it convenient for visualisation of amplification products without gel electrophoresis in resource-poor settings. Moreover, the sensitivity of the Nested PCR assay was 1000-fold higher than that of conventional PCR with a detection limit of 10 fg, the detection limit of the Nested PCR assay is equivalent to the LAMP assay (Table 3) and Real-time PCR (Poppert et al. 2005; Skottman et al. 2007). Our results suggest that

performed using conventional isolation methods. The positive-sample ratios were 46/56 (82.1 %) for nested PCR, 48/56 (85.7 %) for the LAMP assay, and 37/56 (66.1 %) for the traditional isolation method (Table 4). Samples obtained from the control group were inspected using *Phytophthora* isolation, nested PCR, and LAMP assays, and all samples were negative for *P. melonis*. The isolation of *P. melonis* from these samples was performed using conventional methods for detecting *P. melonis* involving the visual examination and culturing of plant tissue (Wang et al. 2007). The

nested PCR assay has the same sensitivity as the LAMP assay in the *P. melonis* detection.

Historically, the ITS region has been used because it is possible to design general primers and the regions shows relatively high polymorphism. More recently, several alternative nuclear loci such as 60S ribosomal protein L10, β-tubulin, enolase, HS protein 90, large subunit rRNA, TigA gene fusion, and translation elongation factor 1α, and mitochondrial loci such as cox1, cox2, nad1, nad9, rps10, and secY, have been sequenced for phylogenetic resolution within *Phytophthora*, and these loci are also useful for molecular identification purposes (Martin et al. 2012). Molecular tools based on the restriction fragment length polymorphism (RFLP) of ITS region and ITS sequencing in Indian *P. melonis* isolate has suggested that they are 100 % similar with the five isolates of GenBank including a *P. sinensis* (Guharoy et al. 2006). Meanwhile, the *Ypt1* gene has been applied for the rapid detection of *P. nicotianae* (Meng and Wang. 2010). To the best of our knowledge, this is the first report of the application of the *Ypt1* for the detection of *P. melonis*. The greatest advantage of this gene is that its alternating conserved and variable regions allow the development of nested PCR with a first round using universal primers Yph1F/Yph2R, and a second round using species-specific primers. This allows the use of a common amplified product from the first amplification as a template for all specific primers, significantly reducing time and cost (Schena et al. 2006). LAMP is a novel, sensitive and rapid detection technique and can be applied for disease diagnosis in the field. The LAMP primers were selected from the *Ypt1* gene, which is highly specific to *P. melonis*. The four primers (F3, B3, forward inner [FIP], and backward inner [BIP]) targeted six regions of *P. melonis* (Fig. 1) and provided additional levels of specificity when compared to PCR primers (i.e. by targeting two regions). Using this detection system, target DNA was amplified within 60 min under isothermal condition at 63 °C plus 10 min at 80 °C and visualised on agarose gel. Also, LAMP was observed directly in the reaction tube by addition of SYBR Green I for a naked-eye inspection. The sensitivity assay showed that this method also had a detection limit of 10 fg DNA template. Of the 36 *P. melonis* and other non–*P. melonis* strains tested, the LAMP assay had 100 % inclusivity and 100 % exclusivity. This level of specificity was the same as that of nested PCR–based assays evaluated simultaneously in this study. Overall, these data revealed that the LAMP method was equivalent to the nested PCR method in specificity and sensitivity for *P. melonis* detection. Considering that the LAMP method has an advantage in its performance and low cost, this technique is more suitable for the detection of *P. melonis* in the field.

Several studies have reported the use of the LAMP method to detect various pathogens using an expensive real-time turbidimeter for reaction confirmation (Mori and Notomi. 2009; Nakao et al. 2010; Yamazaki et al. 2010). The use of expensive equipment decreases the versatility of LAMP and limits the use of this procedure, especially in developing countries. Thus, rapid and unambiguous visual inspection of LAMP results is essential for diagnostics, and several fluorescent intercalating dyes (such as SYBR Green I or calcein) have been developed to enable visual discrimination of positive samples. Here, the addition of SYBR Green I after incubation resulted in a colour change from orange to green in positive amplifications, which could be judged under natural or ultraviolet light, and agreed with the results of gel electrophoresis. However, all fluorescent intercalating dyes should be systematically compared and analysed for their rapidity, simplicity, sensitivity, and cost effectiveness in the LAMP assay (Dai et al. 2012; Mao et al. 2012). Then, the most effective and practical indicator should be employed for LAMP detection.

The LAMP protocol described in this study represents a very sensitive, specific, and rapid diagnostic protocol for *P. melonis* detection. To the best of our knowledge, this is the first study to use the LAMP technique to detect *P. melonis*. This protocol is useful for detecting low levels of *P. melonis* in plant tissues and it can be used to confirm the early stages of *P. melonis* infection at a relatively low level. Thus, *P. melonis*–carrying cucumbers can be identified during the early stages of infection, and management can be devised before the infection becomes epidemic.

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