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ORIGINAL ARTICLE

Loop-mediated isothermal amplification for detection of the tomato and potato late blight pathogen, *Phytophthora infestans*

Z.R. Hansen[1], B.J. Knaus[2], J.F. Tabima[3], C.M. Press[2], H.S. Judelson[4], N.J. Grünwald[2,3] and C.D. Smart[1]

1 Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, Geneva, NY, USA
2 Horticultural Crops Research Laboratory, USDA Agricultural Research Service, Corvallis, OR, USA
3 Botany and Plant Pathology, Oregon State University, Corvallis, OR, USA
4 Department of Plant Pathology and Microbiology, University of California, Riverside, CA, USA

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agriculture, diagnostics, late blight, loop-mediated isothermal amplification, *Phytophthora infestans*, plant pathology, potato, tomato.

Correspondence
Christine D. Smart, Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, Geneva, NY 14456, USA.
E-mail: .cds14@cornell.edu

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Abstract

Aims: To design and validate a colorimetric loop-mediated isothermal amplification assay for rapid detection of *Phytophthora infestans* DNA.
Methods and Results: Two sets of loop-mediated isothermal amplification (LAMP) primers were designed and evaluated for their sensitivity and specificity for *P. infestans*. ITSII primers targeted a portion of the internal transcribed spacer region of ribosomal DNA. These primers had a limit of detection of 2 pg *P. infestans* DNA and cross-reacted with the closely related species *Phytophthora nicotianae*. Rgn86_2 primers, designed to improve assay specificity, targeted a portion of a conserved hypothetical protein. These primers had a limit of detection of 200 pg *P. infestans* DNA and did not cross-react with *P. nicotianae*. The specificity of the Rgn86_2 assay was tested further using the closely related species *P. andina*, *P. ipomoeae*, *P. mirabilis* and *P. phaseoli*. Cross-reactions occurred with *P. andina* and *P. mirabilis*, but neither species occurs on tomato or potato. Both primer sets were able to detect *P. infestans* DNA extracted from tomato late blight leaf lesions.
Conclusions: Two colorimetric LAMP assays detected *P. infestans* DNA from pure cultures as well as infected leaf tissue. The ITSII primers had higher sensitivity, and the Rgn86_2 primers had higher specificity.
Significance and Impact of the Study: This is the first report of a LAMP assay for the detection of *P. infestans*, the causal organism of potato and tomato late blight. These assays have potential for immediate utility in plant disease research and diagnostic laboratories.

Introduction

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, continues to cause significant economic losses in both potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) production (Haverkort *et al.* 2008; Fry *et al.* 2015). The ability to sensitively detect and accurately diagnose the cause of a disease is a crucial step towards effectively managing it. To this end, many DNA-based diagnostic tools have been developed for *P. infestans*. Potential applications of such diagnostic tools have previously been discussed, and include potato seed lot and tomato seedling testing (Tooley *et al.* 1997; Keil *et al.* 2010), potato and tomato late blight disease diagnostics (Trout *et al.* 1997) and various experimental applications (Llorente *et al.* 2010; Lees *et al.* 2012). The numerous *P. infestans* diagnostic tools developed to date are not redundant; rather, each offers a unique capability relative to previously described methods. For example, a DNA-based diagnostic method for *P. infestans* was first published by Niepold and Schöber-Butin in 1995. This method utilized a standard PCR protocol to amplify a

repetitive sequence of *P. infestans* DNA. The internal transcribed spacer (ITS) regions of ribosomal DNA are commonly used in diagnostics due to their relatively high copy number and species specificity (Cassidy *et al.* 1984; Russell *et al.* 1984; Tooley *et al.* 1997; Trout *et al.* 1997; Liew *et al.* 1998; Ristaino *et al.* 1998; Lees *et al.* 2012; Hussain *et al.* 2013). Following the work of Niepold and Schöber-Butin, an ITS-targeting PCR assay was developed and tested for specificity. This assay was found to be specific for *P. infestans* and the closely related species *P. mirabilis* and *P. cactorum* (Trout *et al.* 1997). Another assay targeting the ITS region was developed for the specific purpose of differentiating three *Phytophthora* potato pathogens (*P. infestans*, *P. erythroseptica* and *Phytophthora nicotianae*) (Tooley *et al.* 1997). Highly repetitive DNA sequences have also been targeted to improve detection sensitivity over ITS-based assays (Judelson and Tooley 2000; Llorente *et al.* 2010). The detection of *P. infestans* inoculum in soil samples was demonstrated using conventional PCR (Hussain *et al.* 2005), and later improved upon using quantitative PCR (Lees *et al.* 2012; Hussain *et al.* 2014).

The genus *Phytophthora* is currently divided into eight clades based on phylogenetic analyses of both mitochondrial and genomic sequence data (Kroon *et al.* 2004; Blair *et al.* 2008). *Phytophthora infestans* belongs to clade 1, along with several other species including *P. cactorum*, *P. nicotianae* and *P. mirabilis*. Clade 1 is further divided into subclades, with clade 1c containing *P. infestans* and four closely related species (*P. andina*, *P. ipomoeae*, *P. mirabilis*, *P. phaseoli*) (Blair *et al.* 2008). The high genetic similarity between clade 1 species, and especially clade 1c species, has posed a challenge for the development of *P. infestans*-specific diagnostic assays (Trout *et al.* 1997; Lees *et al.* 2012).

Currently existing methods for *P. infestans* detection require, at minimum, thermal cycling and gel electrophoresis equipment (Trout *et al.* 1997; Judelson and Tooley 2000). In some cases, more expensive quantitative fluorescence detection equipment is required (Llorente *et al.* 2010; Lees *et al.* 2012). Loop-mediated isothermal amplification is an attractive alternative to conventional DNA-based diagnostic techniques because of its minimal equipment requirements, sensitivity, specificity and ability to produce rapid results (Notomi *et al.* 2000; Francois *et al.* 2011). Loop-mediated isothermal amplification (LAMP) is a method of DNA amplification that utilizes *Bst* DNA polymerase, which exhibits strand displacement activity, with four to six primers to amplify target DNA under isothermal conditions (Notomi *et al.* 2000). Positive reactions can be detected several different ways including by fluorescence (Njiru *et al.* 2008; Goto *et al.* 2009; Yasuhara-Bell *et al.* 2013), turbidity (Mori *et al.* 2001) and colorimetric analysis (Goto *et al.* 2009). Intercalating dyes can be added to the reaction mix after the LAMP assay is complete, resulting in a colour change which can be visualized by the naked eye. However, the postreaction addition of dyes increases the risk of contamination, making this method more prone to false positives. The addition of hydroxynaphthol blue (HNB) to the reaction mix prior to running the assay avoids this issue and has been shown to be a reliable indicator of DNA amplification (Goto *et al.* 2009). Hydroxynaphthol blue is a metal ion indicator. As pyrophosphate ions are generated during the LAMP reaction, they react with Mg^{2+} ions in the reaction mix resulting in the formation of magnesium pyrophosphate. This decreases the Mg^{2+} ion concentration, which is indicated by a colour change from violet to blue in the presence of HNB (Goto *et al.* 2009).

LAMP detection assays have been developed for several pathosystems, including oomycetes, fungi, bacteria and viruses (Harper *et al.* 2010; Dai *et al.* 2012; Bühlmann *et al.* 2013; Chen *et al.* 2013; Moradi *et al.* 2013; Duan *et al.* 2014; Kil *et al.* 2015; Thiessen *et al.* 2015). A LAMP assay for the detection of *P. infestans*, an organism which significantly impacts potato and tomato production globally, has not yet been developed. The purpose of this study was to design and validate a colorimetric LAMP assay for rapid detection of *P. infestans* DNA. Such an assay would have utility in any application where rapid diagnosis of *P. infestans* is necessary, but conventional or quantitative PCR instruments are unavailable.

Materials and methods

Isolates used in LAMP assays

Phytophthora infestans isolates were originally collected between 2008 and 2013 (Table 1). Isolates were maintained on pea agar (120 g frozen peas, 15 g agar, 1 l DI H_2O) (Jaime-Garcia *et al.* 2000) with 0·1 g l^{-1} ampicillin, 0·0125 g l^{-1} rifampicin and 0·025 g l^{-1} pentachloronitrobenzene (Sigma-Aldrich, St. Louis, MO). Isolates were grown in pea broth (120 g frozen peas, 1 l DI H2O) (Goodwin *et al.* 1992) at ambient temperature (approx. 20°C) for 5–10 days prior to extracting DNA. Mycelia were harvested using vacuum filtration and qualitative P8 grade filter paper (Thermo Fisher Scientific, Waltham, MA), collected and stored at −20°C until DNA extractions were performed using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Isolates of additional *Phytophthora* spp. obtained from the Cornell University Culture Collection were originally collected from New York State between 2007 and 2010 (Table 1). Isolates were maintained on V8 agar, and

Table 1 Isolates used to test Loop-mediated isothermal amplification (LAMP) sensitivity and specificity, and assay results

Species*	Isolate code	Reference†	LAMP ITSII assay results‡	LAMP Rgn86_2 assay results‡
Alternaria alternata	09107	CUCC	–	–
Doratomyces purpureofuscus	92289	CUCC	–	–
Fusarium equiseti	0594-1	CUCC	–	–
Fusarium oxysporum f.sp. *phaseoli*	814109	CUCC	–	–
Fusarium oxysporum f.sp. *lycopersici*	84045	CUCC	–	–
Fusarium sambucinum	ATCC 44651 (1)	CUCC	–	–
Fusarium sambucinum	ATCC 44651 (2)	CUCC	–	–
Fusarium sambucinum	ATCC 44651 (3)	CUCC	–	–
Trichocladium asperum	93195	CUCC	–	–
Agrobacterium tumefaciens	90-087	CUCC	–	–
Clavibacter michiganensis ssp. *michiganensis*	0580	CUCC	–	–
Pseudomonas syringae pv. *tomato*	A9	CUCC	–	–
Erwinia carotovora ss. *carotovora*	93-066	CUCC	–	–
Streptomyces acidoscabies	90-034	CUCC	–	–
Streptomyces scabies	90-035	CUCC	–	–
Xanthomonas perforans	13091	CUCC	–	–
Pythium irregulare	SQ1	Guarnaccia *et al.* (2015)	–	–
Pythium irregulare	SQ2	Guarnaccia *et al.* (2015)	–	–
Phytophthora arecae	CC1	Guarnaccia *et al.* (2015)	–	–
Phytophthora arecae	CS2	Guarnaccia *et al.* (2015)	–	–
Phytophthora capsici	6·1C	CUCC	–	–
Phytophthora capsici	8·1	CUCC	–	–
Phytophthora capsici	7·2E	CUCC	–	–
Phytophthora capsici	E1·13	CUCC	–	–
Phytophthora capsici	1-1H	CUCC	–	–
Phytophthora nicotianae	ATCC 52638 (2)	CUCC	+	–
Phytophthora nicotianae	ATCC 52638 (1)	CUCC	+	–
Phytophthora nicoatinae	AA1	Guarnaccia *et al.* (2015)	+	–
Phytophthora nicoatinae	PMH1	Guarnaccia *et al.* (2015)	+	–
Phytophthora nicoatinae	PMH6	Guarnaccia *et al.* (2015)	+	–
Phytophthora nicoatinae	CS3	Guarnaccia *et al.* (2015)	+	–
Phytophthora nicoatinae	AA4	Guarnaccia *et al.* (2015)	+	–
Phytophthora niederhauserii	WIN1	Guarnaccia *et al.* (2015)	–	–
Phytophthora niederhauserii	GRR2	Guarnaccia *et al.* (2015)	–	–
Phytophthora andina	PI-08_044	USDA ARS HCRL	nt	+
Phytophthora andina	PI-08-047	USDA ARS HCRL	nt	+
Phytophthora andina	PI-08-053	USDA ARS HCRL	nt	+
Phytophthora ipomoeae	PIP-07-001	USDA ARS HCRL	nt	–
Phytophthora ipomoeae	PIP-07-097	USDA ARS HCRL	nt	–
Phytophthora ipomoeae	PIP-12-003	USDA ARS HCRL	nt	–
Phytophthora mirabilis	PM-07-001	USDA ARS HCRL	nt	+
Phytophthora mirabilis	PM-07-006	USDA ARS HCRL	nt	+
Phytophthora mirabilis	PM-07-099	USDA ARS HCRL	nt	+
Phytophthora phaseoli	PP-12-001	USDA ARS HCRL	nt	–
Phytophthora infestans (US8)	0882	CUCC	+	+
Phytophthora infestans (US8)	0982	CUCC	+	+
Phytophthora infestans (US8)	1083	CUCC	+	+
Phytophthora infestans (US8)	1084	CUCC	+	+
Phytophthora infestans (US8)	1087	CUCC	+	+
Phytophthora infestans (US8)	ENY08	CUCC	+	+
Phytophthora infestans (US8)	1281	CUCC	+	+
Phytophthora infestans (US11)	11114	CUCC	+	+
Phytophthora infestans (US11)	11115	CUCC	+	+
Phytophthora infestans (US11)	11116	CUCC	+	+

Table 1 (*Continued*)

Species*	Isolate code	Reference†	LAMP ITSII assay results‡	LAMP Rgn86_2 assay results‡
Phytophthora infestans (US11)	11117	CUCC	+	+
Phytophthora infestans (US11)	11118	CUCC	+	+
Phytophthora infestans (US11)	12112	CUCC	+	+
Phytophthora infestans (US23)	112322	CUCC	+	+
Phytophthora infestans (US23)	122312	CUCC	+	+
Phytophthora infestans (US23)	122322	CUCC	+	+
Phytophthora infestans (US23)	122329	CUCC	+	+
Phytophthora infestans (US23)	12237	CUCC	+	+
Phytophthora infestans (US23)	14112129851	CUCC	+	+
Phytophthora infestans (US23)	12239	CUCC	+	+
Phytophthora infestans (US23)	13816140	CUCC	+	+
Phytophthora infestans (US23)	12232	CUCC	+	+
Phytophthora infestans (US24)	1249	CUCC	+	+
Phytophthora infestans (US24)	1273	CUCC	+	+
Phytophthora infestans (US24)	1250	CUCC	+	+
Phytophthora infestans (US24)	1339	CUCC	+	+
Phytophthora infestans (US24)	1345	CUCC	+	+

**Phytophthora infestans* isolates are followed by clonal lineages in parentheses.
†CUCC = Cornell University Culture Collection. USDA ARS HCRL = Grunwald lab collection, USDA ARS, Horticultural Crops Research Laboratory.
‡Negative (–) and positive (+) LAMP assay results (nt = not tested). All LAMP assays were done at least twice, with the same results observed in each replicate.

grown on potato dextrose or V8 broth for 5–10 days before collecting mycelia for DNA extractions (Dunn *et al.* 2010).

Fungal and bacterial species known to be associated with tomato and/or potato were also included in specificity assays. Fungal isolates (Table 1) were maintained on potato dextrose agar and grown on potato dextrose broth before collecting mycelia for DNA extractions, which were performed using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Bacterial isolates (Table 1) were grown LB medium (Miller 1972), and DNA was obtained by transferring bacteria using a sterilized loop into 100 μl of sterile type I H_2O (*Agrobacterium* and *Erwinia*) or 100 μl 50 mmol l^{-1} NaOH (*Streptomyces*) and boiling for 5 min (Smith *et al.* 2001).

Genome mining to identify non-ITS LAMP target sequence

Whole *Phytophthora* genome assemblies were created using sequence reads derived from second generation sequencing technologies. Reads from published articles and online sources were obtained from publicly available databases (*Phytophthora infestans* Sequencing Project; Raffaele *et al.* 2010; Cooke *et al.* 2012; Yoshida *et al.* 2013; Martin *et al.* 2013; Table 2). We also resequenced genomes of US clonal lineages using paired-end 100 bp Illumina sequencing. This involved preparing libraries using the Paired End DNA Sample Prep Kit from Illumina (Illumina, San Diego, CA) following the manufacturer's instructions, followed by sequencing to an average depth of 27-fold using an Illumina HiSeq 2000. The CASAVA 1·8·2 pipeline (Illumina) was used for base calling and quality filtering. Reads were mapped to the T30-4 reference (Haas *et al.* 2009) using bowtie2 (Langmead and Salzberg 2012). A conserved sequence within the highly polymorphic Region 86, which we hypothesized to be specific to *P. infestans*, was chosen as a target for the design of LAMP primers.

Primer design

Two sets of LAMP primers were designed to target two separate regions of *P. infestans* genomic DNA using the LAMP primer design software PRIMEREXPLORER V4 (Eiken Chemical Co., Tokyo, Japan). The first set, called LAMP ITSII, target a portion of the end of the 5·8S subunit and the ITS2 region of ribosomal DNA (Table 3). Cross-reactivity was observed with the LAMP ITSII primers and *P. nicotianae*, a pathogen that is periodically observed on potato and tomato (Erwin and Ribeiro 1996; Everts 2013). Due to this cross-reactivity, a second set of LAMP primers were designed to improve the specificity of the LAMP assay compared to the LAMP ITSII primers. These primers, called Rgn86_2, target a

Table 2 Source of *Phytophthora* genomes mined for polymorphic loci

Sample	Taxon	Reference
T30-4	*P. infestans*	Haas *et al.* (2009)
PIC99189, 90128	*P. infestans*	Raffaele *et al.* (2010)
13_a2	*P. infestans*	Cooke *et al.* (2012)
DDR7602, LBUS, NL0743, P136, P6096, P1012, P1065, P1163, P1220, P1352, P1362, P1777	*P. infestans*	Yoshida *et al.* (2013)
RS2009P1_us8, IN2009T1_us2, BL2009P4_us2	*P. infestans*	Martin *et al.* (2013)
1306, Pi-11-007 (US8), 110145 (US11), Pi-11-016 (US22), Pi-11-017 (US23), Pi-11-019 (US24)	*P. infestans*	This study
P7722	*P. mirabilis*	Yoshida *et al.* (2013)

conserved region of the *P. infestans* genome obtained in the genomic mining procedure described above. These primers target Region 86, a region from Supercontig 21 that includes a conserved hypothetical protein gene (PITG 11903; GenBank accession XM_002901331) (Table 3, Fig. 1). Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

LAMP reactions

LAMP reactions were held at 65°C for 1 h using a C1000 Touch Thermal Cycler (BioRad, Hercules, CA). Each 25 μl reaction contained 2 ng DNA template (or 2 μl of a plant or bacterial DNA extraction), 0·8 mol l^{-1} betaine (MP Biomedicals, Santa Ana, CA), 120 μmol l^{-1} hydroxynaphthol blue (Sigma-Aldrich), 1·4 mmol l^{-1} of a mixture of each dNTP (Thermo Fisher Scientific), 6 mmol l^{-1} $MgSO_4$ (Sigma-Alrich), 2·5 μl 10X isothermal DNA buffer (New England Biolabs, Ipswich, MA), 2 μmol l^{-1} forward inner primer (FIP), 2 μmol l^{-1} backward inner primer (BIP), 0·2 μmol l^{-1} primer F3, 0·2 μmol l^{-1} primer B3, 0·8 μmol l^{-1} loop primer and eight U *Bst* DNA polymerase (New England Biolabs) (Njiru *et al.* 2008; Wastling *et al.* 2010; Yasuhara-Bell *et al.* 2013). All LAMP assays were done at least twice and included a no DNA template reaction (2 μl sterile type I H_2O as template) as a negative control and 2 ng *P. infestans* DNA template (isolate 12239) as a positive control.

LAMP sensitivity tests

LAMP sensitivity was tested using 10-fold serial dilutions of pure *P. infestans* DNA extracted from three separate isolates (*P. infestans* 12232, 12112 and 1281, Table 1). Dilution series were prepared in sterile DI H_2O. The limit of detection is defined here as the smallest amount of DNA detected in every test replicate, with each concentration of each isolate tested two times with each primer set (ITSII and Rgn86_2). DNA was quantified using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific), then diluted in 10-fold serial dilutions to concentrations from 1 ng μl^{-1} to 0·01 fg μl^{-1}. LAMP assays were run as described above with positive and negative controls. The addition of hydroxynaphthol blue and $MgSO_4$ to the reaction mix results in a colour change from violet to blue following DNA amplification (Goto *et al.* 2009) (Fig. 2). An assay was considered positive if the reaction mix changed from violet to blue following the 1 h incubation.

DNA was also extracted from the edge of three separate tomato (variety Mountain Fresh) late blight leaf lesions, and three healthy tomato leaves as negative controls. The sporangial suspension used to inoculate leaves was generated by rinsing sporulating tomato leaves in sterile DI H_2O, quantifying sporangia concentrations using a haemocytometer, and adjusting the final concentration to 4000 sporangia ml^{-1}. Leaf lesions were generated by inoculating the abaxial side of the main vein of each leaf collected from greenhouse-grown tomatoes with 20 μl of sporangial suspension and incubating in a humid chamber at 16°C for 5 days. DNA extractions were done using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. LAMP assays were run as described above including positive and negative controls. Each assay (ITSII and Rgn86_2) was run twice on each sample for a total of six LAMP assays using infected tissues and six with healthy tissue. The presence of tomato DNA was checked by amplifying the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene by PCR and checking for amplification products by gel electrophoresis.

LAMP-specificity tests

Forty four nontarget isolates representing 22 diverse species of oomycetes, fungi and bacteria were used to test the specificity of the LAMP assays (Table 1). The presence of DNA in each of the 44 samples was verified prior to LAMP testing by gel electrophoresis following a PCR reaction using the primers ITS4 and ITS5 (fungi and oomycetes) and L1 and G1 (bacteria) (White *et al.* 1990; Jensen *et al.* 1993). DNA was then quantified using a NanoDrop spectrophotometer ND-1000 and diluted to 1 ng μl^{-1}. Additionally, five to nine *P. infestans* isolates from each of four clonal lineages (US-8, US-11, US-23, US-24) were tested at a concentration of 1 ng μl^{-1} (Table 1). Each of the DNA samples was tested twice

Table 3 Loop-mediated isothermal amplification primer sequences

Primer name	Primer Sequence (5′ – 3′)
ITSII_F3	GCCTGTATCAGTGTCCGTAC
ITSII_B3	CATTAACGCCGCAGCAGA
ITSII_FIP	ACCAACCGCAAGACACTTCACAT**TTTT**TGGCTTTCTTCCTTCCGTG
ITSII_BIP	GCTCCAAAAGTGGTGGCATTGC**TTTT**CAAACCGGTCGCCAACTC
ITSII_LOOP	GGCATCTCCTCCACCGACTA
Rgn86_2F3	TCGACACGGGTGTCTACG
Rgn86_3B3	ACGCAGACGTTACCCG
Rgn86_2FIP	TCTCCTGGTGGTGGTGGTGG**TTTT**GGTGTGTGGCTAGCGCTAAG
Rgn86_2BIP	ACCAGAAGGTCGCGTGCCC**TTTT**CCCGACGCAACGCATC
Rgn86_2LOOP	CAGTGGACAACAAAGCATTGGT

TTTT linkers are indicated in bold.

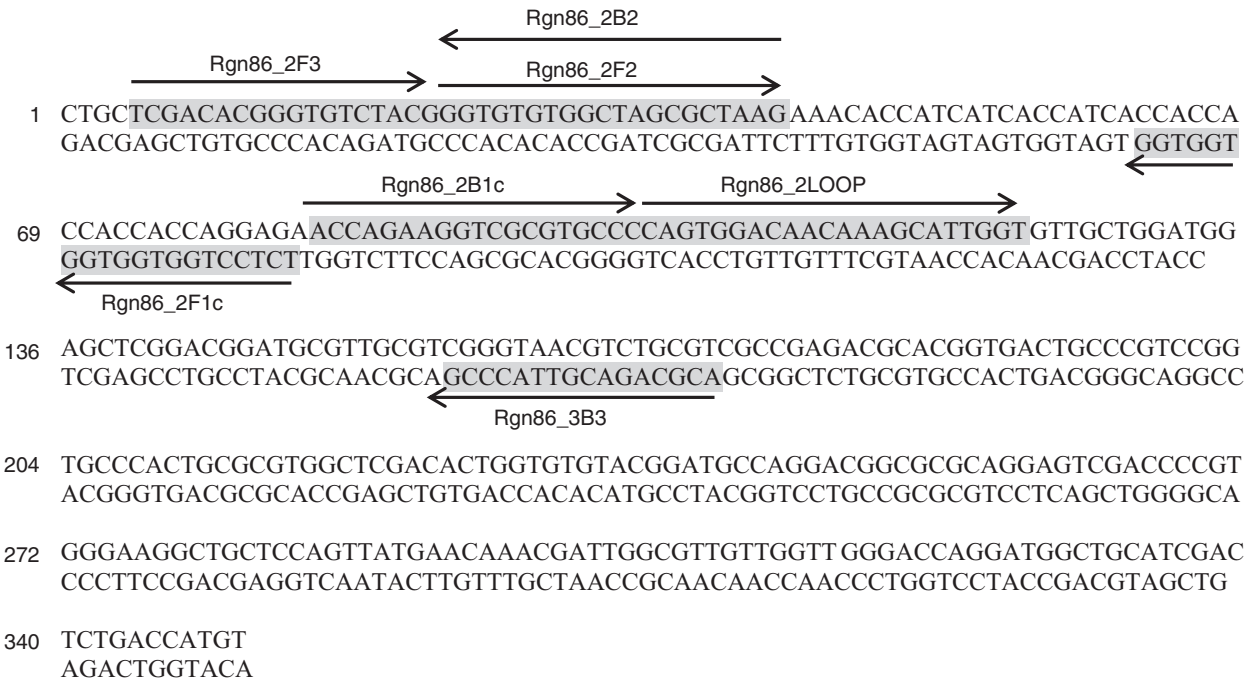

Figure 1 Rgn86_2 Loop-mediated isothermal amplification (LAMP) primers located within the forward and reverse DNA sequences of a conserved portion of Region 86. LAMP primer sequences are highlighted in grey.

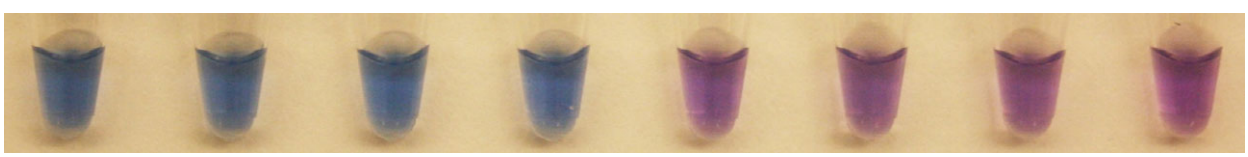

Figure 2 Loop-mediated isothermal amplification (LAMP) ITSII primers tested with a 10-fold *P. infestans* DNA dilution series. Positive LAMP reactions resulted in a colour change from violet to blue. *P. infestans* DNA quantities from left to right: 2 ng; 200 pg; 20 pg; 2 pg; 0·2 pg; 0·02 pg; 2 fg; no template (negative control).

with each primer set, with positive (2 ng *P. infestans* DNA, isolate 12239) and negative (2 μl sterile type I H_2O) controls included in each test. Only the Rgn86_2 primers were tested with 10 isolates representing the four *Phytophthora* species most closely related to *P. infestans* (Kroon *et al.* 2004; Blair *et al.* 2008). *Phytophthora andina*, *P. mirabilis*, *P. ipomoeae* and *P. phaseoli* isolates were sampled from the USDA ARS collection, Corvallis, OR

(Table 1). DNA was quantified using a Qubit 3·0 Fluorometer (Thermo Fisher Scientific). Reactions were considered positive when the colour of the reaction mixture changed from violet to blue following a 1-h incubation.

Results

LAMP ITSII primer sensitivity and specificity tests

LAMP ITSII primer sensitivity was tested with three separate 10-fold *P. infestans* serial DNA dilutions, with each test done twice. DNA template quantities ranged from 2 pg to 2 fg per reaction. The limit of detection (LOD) for the ITSII primers was 2 pg *P. infestans* template DNA (Fig. 2). This was the lowest concentration of DNA that was detected in every test replicate. In one replicate each of two different extracts 0·2 pg DNA was detected (twice total). LAMP ITSII primers resulted in positive reactions when tested with five to nine *P. infestans* isolates from four clonal lineages ($n = 27$). LAMP ITSII primers failed to produce positive reactions when tested against 27 nontarget isolates representing 18 oomycete, fungal and bacterial species. All seven isolates of *P. nicotianae* tested positive in each replicated test, indicated by a colour change from violet to blue. LAMP ITSII primers were not tested with the 10 isolates of *Phytophthora andina*, *P. mirabilis*, *P. ipomoeae* and *P. phaseoli* due to the cross-reactivity with the more distantly related *P. nicotianae*. Once it was discovered that the LAMP ITSII primers cross-reacted with *P. nicotianae*, the LOD was determined using 10-fold serial dilutions of *P. nicotianae* DNA extracted from three separate isolates (*P. nicotianae* AA4, CS3, and PMH6, Table 1). The LOD for *P. nicotianae* using the LAMP ITSII primers was 200 pg template DNA. The LAMP ITSII primers also detected *P. infestans* DNA extracted from each of three tomato late blight leaf lesions, while DNA extracted from healthy tomato leaves failed to produce a positive reaction. The leaf lesion assay was done twice on each of the three symptomatic and three healthy leaves, with identical results observed in each test. Tomato DNA was verified in all six tomato leaf DNA extracts by amplification of the GAPDH housekeeping gene by PCR and visualization of amplification products by gel electrophoresis.

LAMP Rgn86_2 primer sensitivity and specificity tests

LAMP Rgn86_2 primer sensitivity was also tested with three separate 10-fold *P. infestans* serial DNA dilutions, with each test done twice. DNA template quantities ranged from 2 pg to 2 fg per reaction. The LOD for the Rgn86_2 primers was 200 pg *P. infestans* template DNA. In one of six test replicates 20 pg DNA was detected. Rgn86_2 primers resulted in positive reactions when tested with five to nine *P. infestans* isolates from four clonal lineages ($n = 27$). Rgn86_2 primers showed high specificity for *P. infestans* when tested against 44 nontarget isolates. These primers produced negative reactions for closely related clade 1 species *P. nicotianae*, *P. ipomoeae*, and *P. phaseoli* (Table 1). Rgn86_2 primers produced positive reactions for the clade 1 species *P. andina* and *P. mirabilis*.

Due to the cross-reactivity observed with *P. andina* and *P. mirabilis*, the Region 86 sequence alignments were re-evaluated to determine if another portion of that region would be suitable for the design of primers capable of differentiating these two species from *P. infestans*. A total of five single-nucleotide polymorphisms (SNPs) were found with the potential to differentiate *P. mirabilis* from *P. infestans*. The distances separating these SNPs ranged from 53 bp to 650 bp within Region 86. Previous work performed in our laboratory indicated that two SNPs located within four base pairs of each other, and within the same B3 LAMP primer sequence, were not sufficient to separate two otherwise-identical *P. infestans* sequences following a LAMP reaction (Z.R. Hansen, unpublished data). The five SNPs differentiating *P. infestans* and *P. mirabilis* were not sufficiently close together to incorporate more than two into a new set of LAMP primers. Additionally, two of the five SNPs were flanked by sequences which were polymorphic within *P. infestans*, making them poor targets for a *P. infestans*-specific diagnostic assay. Therefore, the design of new LAMP primers without cross-reactivity with *P. andina* and *P. mirabilis* was not pursued.

In addition to amplifying *P. infestans* DNA collected from pure cultures, the Rgn86_2 primers also detected *P. infestans* DNA extracted from tomato late blight leaf lesions. DNA extracted from healthy tomato leaves failed to produce a positive reaction. The leaf lesion assay was done twice, with identical results observed in each test. Rgn86_2 primers had a LOD of 200 pg *P. infestans* template DNA. Each LAMP test included a negative control consisting of sterile DI H_2O in place of template, and a positive control consisting of 2 ng *P. infestans* DNA template (isolate 12239). Each LAMP test was done twice, and in each test the negative control remained violet while the positive control turned blue, indicating a positive LAMP reaction.

Discussion

In this study, a LAMP method capable of detecting *P. infestans* DNA isolated from pure cultures as well as plant tissue samples was developed. A portion of the *P. infestans* 5·8S subunit and ITS2 region of rDNA was chosen

as the target for the first set of LAMP primers (ITSII primers). This region was chosen because of the sensitivity and specificity achieved with other previously developed PCR and qPCR assays (Tooley *et al.* 1997; Trout *et al.* 1997; Ristaino *et al.* 1998; Lees *et al.* 2012). The LAMP ITSII primers had sensitivity comparable to several other published LAMP assays (limit of detection of 2 pg *P. infestans* DNA). Tomlinson *et al.* (2007) reported a LAMP assay targeting the *P. ramorum* ITS region as having a limit of detection between 10 pg and 50 pg of *P. ramorum* DNA. This assay relied on the intercalating dye PicoGreen for detection of positive reactions. A second LAMP assay developed by Tomlinson *et al.* (2010) amplified a portion of the *P. kernoviae* ITS region. This assay had a limit of detection of 17 pg DNA, and relied on a lateral flow device or gel electrophoresis for detection of positive reactions. Another LAMP assay developed by Storari *et al.* (2013) was designed to detect two strains of *Aspergillus*. Two LAMP primer sets were designed to target the polyketide synthase gene. These assays had limits of detection between 10 and 100 pg *A. carbonarius* or *A. niger* DNA. This assay utilized hydroxynaphthol blue for the detection of positive reactions.

A drawback to the highly sensitive ITSII primers is their cross-reactivity with the closely related *P. nicotianae*. *Phytophthora infestans* and *P. nicotianae* are both members of clade 1, with *P. infestans* belonging to the subclade 1c (Kroon *et al.* 2004; Blair *et al.* 2008). The positioning of *P. nicotianae* within clade 1 remains ambiguous, although it may be basal to subclade 1c (Blair *et al.* 2008). Given the close phylogenetic relationship between these two clade 1 species, it is not surprising that a diagnostic method targeting the ITS region could lack the specificity necessary to differentiate them. Lees *et al.* (2012) developed an ITS-based qPCR assay that failed to differentiate four clade 1 species, although it was able to differentiate *P. infestans* and *P. nicotianae*. Similarly, the ITS-based PCR assay developed by Trout *et al.* (1997) failed to differentiate two clade 1 species, although it could differentiate *P. infestans* and *P. nicotianae*. *Phytophthora nicotianae* has a wide host range and geographic distribution, and notably causes buckeye rot and root rot of tomato and tuber rot, pink rot and leaf and stem blight of potato (*Solanum tuberosum* L.) (Erwin and Ribeiro 1996; Tooley *et al.* 1997). Although *P. nicotianae* is less common than late blight as a foliar disease of either tomato or potato, it is occasionally observed as a foliar disease resembling late blight (Everts 2013).

Due to the cross-reactivity observed with the ITSII primers and *P. nicotianae*, a second set of LAMP primers were developed in an effort to improve the specificity of the LAMP assay. These primers (Rgn86_2) were designed to target a conserved portion of the PITG 11903 gene (a conserved hypothetical protein). The Rgn86_2 primers did not cross-react with *P. nicotianae*, *P. ipomoeae*, *P. phaseoli*, all of which are clade 1 species. However, these primers did produce positive reactions for two clade 1c species, *P. mirabilis* and *P. andina* (Kroon *et al.* 2004; Blair *et al.* 2008), the latter of which is thought to be a hybrid between *P. infestans* and an unknown *Phytophthora* clade 1c species (Goss *et al.* 2011). The ability to differentiate *P. infestans* from other clade 1 *Phytophthora* species using DNA-based diagnostics is challenging due to the high sequence similarity between closely related species (Trout *et al.* 1997; Flier *et al.* 2002; Blair *et al.* 2008; Lees *et al.* 2012). The enhanced specificity offered by the Rgn86_2 LAMP assay could make it particularly useful for *P. infestans* diagnostic applications given that no clade 1c species other than *P. infestans* infects tomato or potato.

Despite being less sensitive than the ITSII primers (limit of detection of 200 pg DNA), the Rgn86_2 primers still had sensitivity similar to that of other LAMP assays (Tomlinson *et al.* 2007, 2010; Storari *et al.* 2013). The lower sensitivity of the Rgn86_2 primers compared to the ITSII primers is probably due to the relatively higher copy number of the ribosomal RNA gene (Cassidy *et al.* 1984; Russell *et al.* 1984; Liew *et al.* 1998), although the copy number of the Rgn86 conserved hypothetical protein gene is currently unknown. Both primer sets were capable of detecting *P. infestans* DNA isolated from pure cultures, as well as *P. infestans* DNA extracted from tomato late blight lesions. This indicates the potential utility of the LAMP assays for diagnostics, especially where access to thermal cyclers, fluorescence detection or gel electrophoresis equipment is not available.

The LAMP assays described here offer an alternative to conventional PCR diagnostics. *Phytophthora infestans* DNA can be rapidly identified with the naked eye following a 1-h isothermal sample incubation. With the persistence of late blight as a global threat to potato and tomato production (Fry *et al.* 2015), rapid, reliable and accessible diagnostic methods must be made available to aid in studying and managing the disease. These LAMP assays provide another tool for diagnostic and research laboratories, and expand upon the options available for understanding, monitoring and combatting this important disease.

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Conflict of Interest

No conflict of interest declared.

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