

A rapid detection method for the plant pathogen *Sclerotinia sclerotiorum* based on loop-mediated isothermal amplification (LAMP)

Yabing Duan · Changyan Ge · Xiaoke Zhang ·
Jianxin Wang · Mingguo Zhou

Received: 17 May 2013 / Accepted: 11 June 2013 / Published online: 3 July 2013
© Australasian Plant Pathology Society Inc. 2013

Abstract The ascomycete *Sclerotinia sclerotiorum* is a devastating plant pathogen with a very broad host range. In this study, we developed a loop-mediated isothermal amplification (LAMP) assay targeting the *Ssos5* sequence for visual detection of *S. sclerotiorum*. The LAMP reaction was optimal at 63 °C for 45 min. When hydroxynaphthol blue (HNB) was added before amplification, samples with *S. sclerotiorum* DNA developed a characteristic sky blue colour but those without DNA or with the DNA of nine other plant-pathogenic fungi did not. Results obtained with LAMP and HNB were confirmed when LAMP products were subjected to gel electrophoresis. The detection limit of this LAMP assay for *S. sclerotiorum* was 0.1 fg μL^{-1} of genomic DNA per reaction, while that of conventional PCR was 100 fg μL^{-1} . Detection results were identical when 13 samples of rapeseed tissue infected with *S. sclerotiorum* were subjected to LAMP, conventional PCR, and conventional isolation. Because the LAMP assay reported here is simple, rapid, sensitive, and specific, it should be valuable for the detection of *S. sclerotiorum* in quarantine efforts and field surveys.

Keywords Hydroxynaphthol blue (HNB) · Loop-mediated isothermal amplification (LAMP) · Rapid detection · *Sclerotinia sclerotiorum*

Introduction

Sclerotinia sclerotiorum is a necrotrophic, ascomycetous, phytopathogenic fungus with worldwide distribution. This fungus can infect 450 plant species and subspecies from 278 genera and 75 families including many agriculturally important and economically valuable crops; its wide host range enables *S. sclerotiorum* to persist and spread (Boland and Hall 1994; Duan et al. 2013a, b). Stem rot caused by *S. sclerotiorum* is a sporadic but serious threat to production of rapeseed. With the increase in the trade of rapeseed among countries, rapid detection of *S. sclerotiorum* in soil and plant tissues is important not only for maintaining rapeseed yield and quality but also for controlling the pathogen's spread. Detection methods that are rapid, cost-effective, and efficient are required. In particular, rapid detection methods that can be performed in the field could provide new reference data for the control of *S. sclerotiorum*.

The most common method currently used for the rapid detection of *S. sclerotiorum* is based on conventional polymerase chain reaction (PCR) (Freeman et al. 2002). Although PCR has become a standard method in biotechnology, PCR has several intrinsic disadvantages, including the requirement of rapid thermal cycling, insufficient specificity, and rather low amplification efficiency (Freeman et al. 2002). Taking such disadvantages into account, in this study we developed a new nucleic acid amplification method that can be used in the field to detect *S. sclerotiorum* in the absence of a thermal cycler.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method reported first in 2000 by Notomi et al. (2000). This method requires a *Bst* DNA

Yabing Duan, Changyan Ge and Xiaoke Zhang authors contributed equally to this work.

Y. Duan · C. Ge · X. Zhang · J. Wang · M. Zhou (✉)
College of Plant Protection, Nanjing Agricultural University, Key
Laboratory of Pesticide, Jiangsu Province, Nanjing 210095, China
e-mail: mgzhou@njau.edu.cn

Y. Duan
e-mail: yabing0324@hotmail.com

C. Ge
e-mail: changyan8713@126.com

X. Zhang
e-mail: xiaokezxc@163.com

J. Wang
e-mail: jlz489@aliyun.com

polymerase with strand-displacement activity and a set of four to six specially designed primers based on six or eight distinct regions of the target DNA. LAMP products can be visualized with the unaided eye by adding DNA-intercalating dyes such as ethidium bromide, SYBR Green I, propidium iodide, or Quant-iT PicoGreen; by adding metal-ion indicators such as hydroxynaphthol blue (HNB) (Goto et al. 2009), CuSO_4 (Zoheir and Allam 2011), or calcein (Tomita et al. 2008) or by measuring the increase in turbidity derived from magnesium pyrophosphate formation (to infer increases in amplified DNA concentration). LAMP products can also be detected by real-time detection methods (Bekele et al. 2011). The simplicity of the LAMP method, which does not require a thermal cycler, makes it suitable for field testing. Although this method has been applied in the field of microbiology for detection and identification of bacteria (Pan et al. 2011), viruses (Parida et al. 2004), and fungi (Niessen and Vogel 2010), the LAMP method has not been applied for detection of *S. sclerotiorum*.

In the current study, we developed a LAMP assay for detection of *S. sclerotiorum* based on the *Ssos5* gene and demonstrated that the assay is specific and efficient. The new LAMP assay will provide important reference data for monitoring and controlling stem rot caused by *S. sclerotiorum*.

Materials and methods

Fungi and reagents

S. sclerotiorum isolates were collected from infected rapeseed plants in Jiangsu Province, China. All tested *S. sclerotiorum* isolates were obtained from single sclerotium. Other plant pathogens used in this study are maintained in a collection in the Laboratory of Plant Disease Control and Phytopharmacy, Nanjing Agricultural University, China.

Bst DNA polymerase was purchased from NEB. Betaine and hydroxynaphthol blue (HNB) were purchased from Sigma, and MgCl_2 and dNTPs were purchased from Takara. Double-distilled water was used in all experiments. All other reagents were analytical grade.

Culture conditions and DNA extraction

Fungi were maintained on potato sucrose agar medium (PSA) (200 g of potato, 20 g of sucrose, and 15 g of agar per liter of distilled water). Mycelia were grown in potato sucrose broth (PSB) at 25 °C for 2 days and harvested by filtration and frozen at -20 °C. Genomic DNA was extracted from the frozen mycelia using the Plant Genomic DNA Kit (TIANGEN) according to the manufacturer's protocol. DNA concentrations were determined spectrophotometrically, and extracted DNA was stored at -20 °C.

Primer design

Four specific LAMP primers were designed based on the *S. sclerotiorum Ssos5* gene (SS1G_14143). Briefly, a BLASTN search indicated that the sequence of *Ssos5* of *S. sclerotiorum* has 82 % similarity with that of *os5* (BC1G_07633) of *B. cinerea*. Then, we obtained the *os5* sequences in the genome databases for *S. sclerotiorum* and *B. cinerea* (<http://www.broadinstitute.org/>). In this way, specific primers based on the *os5* sequence alignment were designed for LAMP detection of *S. sclerotiorum* (Fig. 1a) using the Primerexplorer V4 software program (<http://primerexplorer.jp/e/>). The structure of the LAMP primers and their complementarity to target DNA used in this study are shown in Fig. 1b. A forward inner primer (FIP) consisted of the complementary sequence of F1 (F1c) and F2, and a backward inner primer (BIP) consisted of B1c and B2. The outer primers F3 and B3 are required for initiation of the LAMP reaction. Primer pair P1 and P2 was used for conventional PCR of the *Ssos5* gene. Information regarding the primer names and sequences is provided in Table 1.

Optimization of LAMP reaction

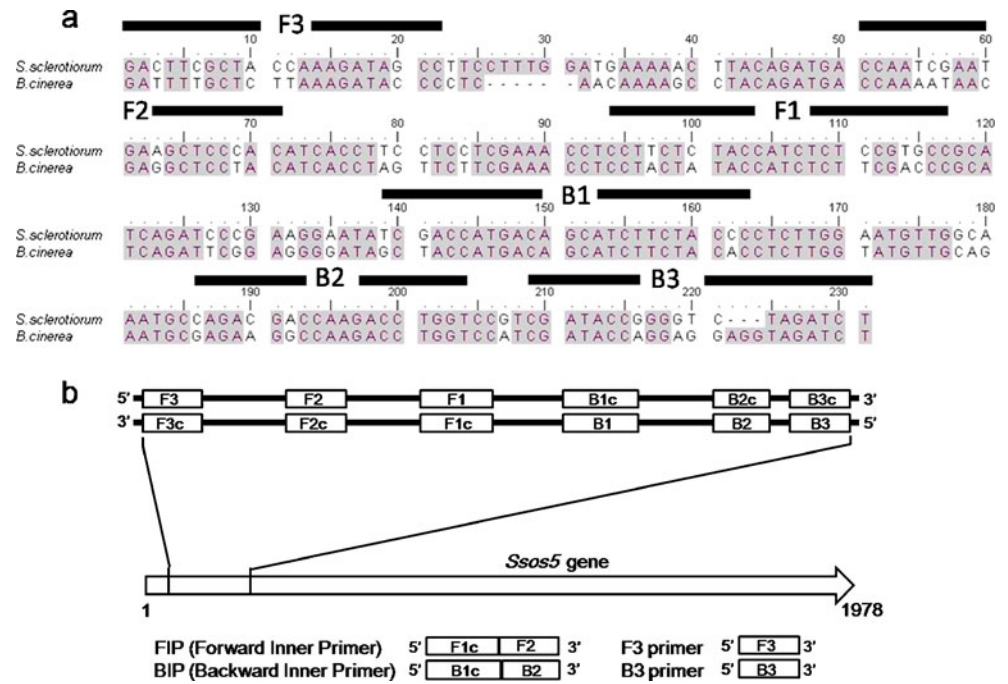
The visualization indicator HNB (Sigma-Aldrich) was added to the reaction mixture before amplification. As explained in the Discussion, HNB produces a distinctive colour if the reaction is positive; as a consequence, LAMP products can be detected without gel electrophoresis. The LAMP reaction used a total volume of 25 μL . For optimization of reagents, a range of concentrations of Mg^{2+} (2–8 mM), dNTPs (0.2–2 mM), primers (0.2–2 μM), betaine (0.8–1.6 M) (Sigma-Aldrich), HNB (100–200 μM), and *Bst* DNA polymerase large fragments (0.16–0.64 $\text{U } \mu\text{L}^{-1}$) (NEB) were evaluated. The reaction was performed in 0.2-mL microcentrifuge tubes that were placed in a water bath for 45 min at 63 °C, and the reaction was terminated by treatment at 80 °C for 10 min. A positive control (a sample known to contain *S. sclerotiorum* DNA) and a negative control (a sample to which no template was added) were included in each run. When the LAMP reaction was completed, the reaction mixtures in the microcentrifuge tubes were visually assessed (see Results). For confirmation of the LAMP assessment based on HNB-visualized colour change, 3 μL of each LAMP mixture was then subjected to 3.0 % agarose gel electrophoresis, and the gels were stained with ethidium bromide.

Optimization of LAMP reaction conditions

The LAMP reaction mixtures (with HNB and with or without *S. sclerotiorum* DNA as template) were incubated for 45 min at 61, 62, 63, 64, or 65 °C to determine the optimal reaction temperature. Then, the LAMP was performed at the optimal reaction temperature (63 °C, see Results) for 15, 30, 45, 60, and 90 min to determine the optimal reaction time

Fig. 1 Design of LAMP primers for detection of *S. sclerotiorum*.

(a) Nucleotide sequence alignment of the target region *Ssos5* in *S. sclerotiorum* and *B. cinerea*. The sequences used for LAMP primers are indicated by bold lines. (b) Schematic representation of the LAMP primers used in this study. Construction of the inner primers FIP and BIP are shown. F1c and B1c are complementary to F1 and B1, respectively



(45 min, see Results). The reactions were terminated by heat inactivation at 80 °C for 10 min. The assays were assessed based on HNB-visualized colour change and then on gel electrophoresis as described in the previous section.

Specificity of the LAMP

LAMP specificity was determined by performing the assay with DNA of *S. sclerotiorum* and of nine other plant-pathogenic fungi, including *Botrytis cinerea*, *Fusarium graminearum*, *Rhizoctonia cerealis*, *Rhizoctonia solani*, *Verticillium dahlia*, *Alternaria solani*, *Colletotrichum gloeosporioides*, *Pyricularia oryzae*, and *Fusarium moniliforme*. The LAMP assay was done as described earlier at 63 °C for 45 min. As before, the assays were assessed based on HNB-visualized colour change and then by gel electrophoresis. Each fungal sample was represented by three replications, and the experiment was performed three times.

Table 1 Information on the primers used in this study

Primer name	Sequence (5'-3')	Length (bp)
F3	GACTTCGCTACCAAAGATAGCC	22
B3	AGATCTAGACCCCGGTATCG	20
FIP	GGCACGGAGAGATGGTAGAGAAGG- CCAATCGAATGAAGCTCCAC	45
BIP	TCGACCATGACAGCATCTTCTACCC- ACCAGGTCTTGGTCGTCTG	44
LF	GGTTTCGAGGAGGAAGGTGAT	21
P1	AGCATCTTCTACCCCTCTTG	20
P2	CCAGGTGTTGAAACTGTAGC	20

LAMP sensitivity

Template DNA from *S. sclerotiorum* was prepared as described earlier and was 10-fold serially diluted (from 10^5 to 10^{-6} fg μL^{-1}). The samples were then subjected to LAMP (with HNB) and PCR assays. When the reactions were complete, the LAMP products were visualized as described earlier, while the PCR products were observed by gel electrophoresis. There were three replications for each sample, and the experiment was performed three times.

Evaluation of the LAMP assay using rapeseed tissue

Rapeseed stems with symptoms of stem rot were collected from 13 fields in Jiangsu province. The 13 samples were subjected to LAMP, conventional PCR, and tissue isolation. When LAMP reactions were finished, they were assessed based on colour change and based on gel electrophoresis as described earlier.

Results

Optimization of LAMP reaction

When the LAMP assay was performed with *S. sclerotiorum* DNA as the template, the best results were obtained in a 25 μL volume containing 1.2 μM each of FIP and BIP, 0.2 μM of F3 and B3, 1.28 M betaine, 1 mM dNTPs, 4 mM MgCl_2 , 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 0.1 % Triton X-100, 8 U of *Bst* DNA polymerase,

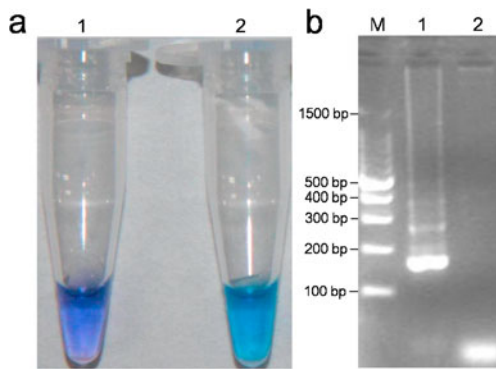
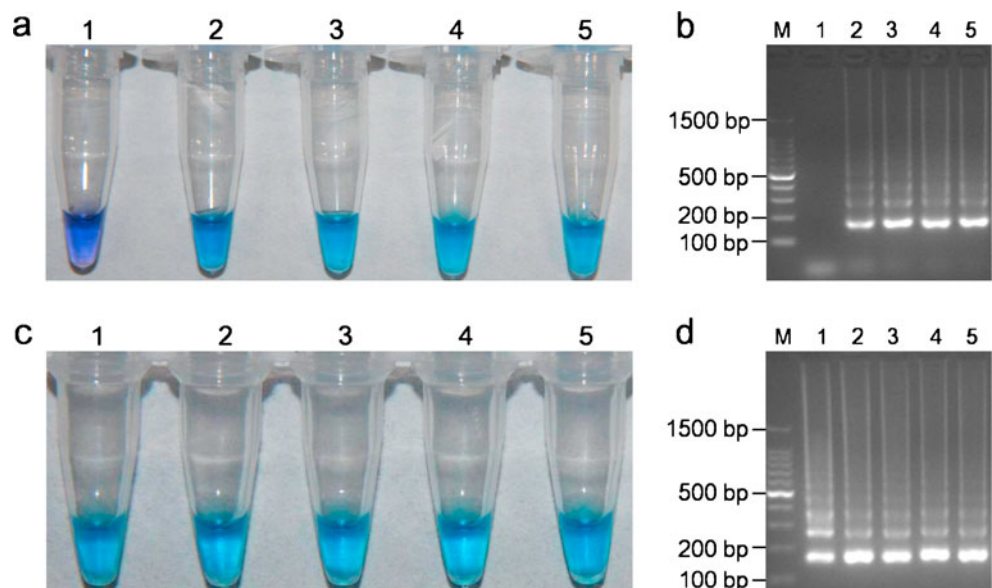


Fig. 2 LAMP detection of the *Ssos5* gene in *S. sclerotiorum*. **(a)** LAMP for detection of *S. sclerotiorum* using HNB as a visual indicator. The reaction becomes sky blue if the *Ssos5* gene is present but remains violet if the gene is absent; **(b)** Agarose gel electrophoresis of LAMP products. The positive reaction is manifested as a ladder-like pattern on the 3.0 % agarose gel. M=100-bp ladder. In **(a)** and **(b)**, the positive reaction (with target DNA) is labeled “1”, and the negative reaction (without target DNA) is labeled “2”

150 μ M HNB, and 2 μ L of target DNA. As noted in the Methods, the reactions were performed in a 0.2-mL microcentrifuge in a water bath for temperature control. When the tubes were examined before gel electrophoresis, a positive LAMP reaction was indicated by a sky blue colour; the color remained violet for negative reactions (Fig. 2a). After the tubes were visually assessed for colour change, the samples were subjected to agarose gel electrophoresis; characteristic bands were evident in the gel if the product was present but not if the product was absent (Fig. 2b). The results showed that the primers were effective, and that the same result was obtained with HNB-visualization and gel electrophoresis.

Fig. 3 Optimization of LAMP reaction time **(a, b)** and reaction temperature **(c, d)**. Assessment was based on HNB visualization of colour change in **(a)** and **(c)** and on gel electrophoresis in **(b)** and **(d)**. In **(a)** and **(b)**, 1=15 min, 2=30 min, 3=45 min, 4=60 min, and 5=90 min. In **(c)** and **(d)**, 1=61, 2=62, 3=63, 4=64, and 5=65 °C. M indicates a 100-bp ladder



Optimization of LAMP reaction conditions

With *S. sclerotiorum* DNA as the template and the reagents optimized as indicated in the previous section, the optimal LAMP reaction time and temperature were determined. When LAMP was conducted at 63 °C, positive results were obtained with times of 30 to 90 min whether assessment was based on HNB-visualization (Fig. 3a) or gel electrophoresis (Fig. 3b) but the ladder-like pattern produced by gel electrophoresis was strongest at 45 min. When LAMP was conducted for 45 min with a range of test temperatures, all temperatures produced a positive reaction whether assessment was based on HNB-visualization (Fig. 3c) or gel electrophoresis (Fig. 3d) but the bands obtained with gel electrophoresis were most intense at 63 °C (Fig. 3d). In summary, LAMP of the *Ssos5* gene was optimal when conducted at 63 °C for 45 min.

Specificity of the LAMP assay

The LAMP assay was positive only for *S. sclerotiorum*, i.e., no positive DNA products were observed when other plant pathogenic fungi (*B. cinerea*, *F. graminearum*, *R. cerealis*, *R. solani*, *V. dahlia*, *A. solani*, *C. gloeosporioides*, *P. oryzae*, and *F. moniliforme*) were used as templates. This was true whether assessment was based on HNB-visualization (Fig. 4a) or gel electrophoresis (Fig. 4b).

Sensitivity of the LAMP assay

The limit for LAMP detection of genomic DNA of *S. sclerotiorum* was 0.1 fg μ L⁻¹ whether detection involved HNB (Fig. 5a) or gel electrophoresis (Fig. 5b). In contrast, the detection limit for conventional PCR was 100 fg μ L⁻¹

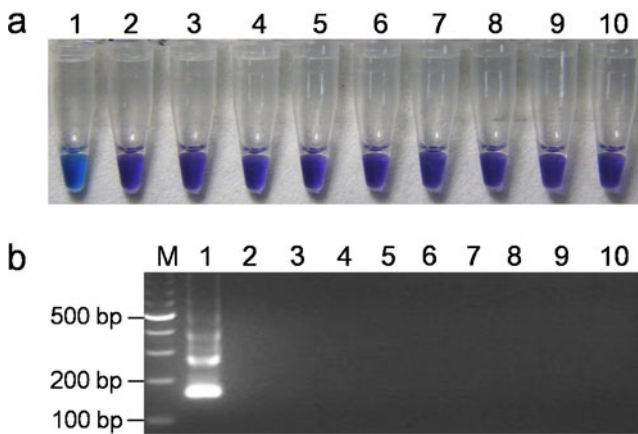


Fig. 4 Specificity of LAMP detection of *S. sclerotiorum*. Assessment based on (a) HNB visualization of colour change or (b) agarose gel electrophoresis analysis of the LAMP products. M indicates a 100-bp ladder; 1, *S. sclerotiorum*; 2, *B. cinerea*; 3, *F. graminearum*; 4, *R. cerealis*; 5, *R. solani*; 6, *V. dahlia*; 7, *A. solani*; 8, *C. gloeosporioides*; 9, *P. oryzae*; 10, *F. moniliforme*

(Fig. 5c). Similar detection limits for LAMP (with HNB or gel electrophoresis) and for conventional PCR were obtained with ten other *S. sclerotiorum* isolates (data not shown).

Evaluation of the LAMP assay using rapeseed tissues

To evaluate the LAMP assay for detection of *S. sclerotiorum* in the field, 13 diseased rapeseed tissues collected from different areas of Jiangsu province in 2012 were tested by the LAMP assay and conventional PCR as described above.

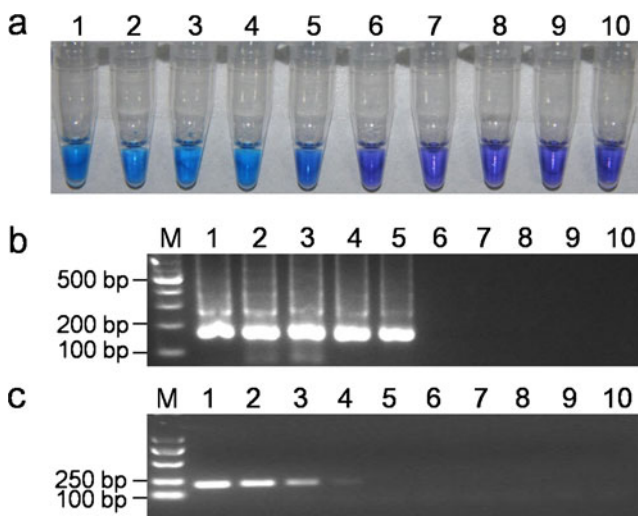


Fig. 5 Sensitivity of LAMP vs. conventional PCR for detection of *S. sclerotiorum* genomic DNA. Detection by (a) LAMP and HNB visualization, (b) LAMP and gel electrophoresis, and (c) conventional PCR. Concentrations of template DNA ($\text{fg } \mu\text{L}^{-1}$) per reaction in (a) and (b) were: 1= 10^3 , 2= 10^2 , 3=10, 4=1, 5= 10^{-1} , 6= 10^{-2} , 7= 10^{-3} , 8= 10^{-4} , 9= 10^{-5} , and 10= 10^{-6} . Concentrations of template DNA ($\text{fg } \mu\text{L}^{-1}$) per reaction in (c) were: 1= 10^5 , 2= 10^4 , 3= 10^3 , 4= 10^2 , 5=10, 6=1, 7= 10^{-1} , 8= 10^{-2} , 9= 10^{-3} , and 10= 10^{-4} . In (b) and (c), M indicates a 100-bp ladder

Isolation of *S. sclerotiorum* from these samples was performed using standard tissue isolation methods. Surprisingly, the results tested by LAMP, conventional PCR, and the baiting method were consistent (data not shown).

Discussion

To the best of our knowledge, this is the first report on the application of the LAMP assay for detection of *S. sclerotiorum*. Relative to conventional PCR, the LAMP assay reported here is easier to perform and more rapid, and the results are easier to evaluate. LAMP operates under isothermal conditions; the optimal temperature for detection of *S. sclerotiorum* was determined to be 63 °C in this study. LAMP is also rapid; for detection of *S. sclerotiorum*, 45 min was determined to be optimum. Because LAMP is conducted at one temperature, no time is lost as a result of changes in temperature, as is the case with thermal cycling with PCR. Moreover, LAMP requires only a regular laboratory bath or heat block that can provide a constant temperature of 63 °C. Another very important advantage of LAMP is that the amplified products can be visually detected by adding the dye HNB, i.e., electrophoresis is not required. Because the LAMP assay is simple, it should be useful even for those laboratories and research institutes that are unfamiliar with PCR or other methods of molecular analysis.

The LAMP assay developed here for detection of *S. sclerotiorum* uses four primers: F3, B3, FIP, and BIP. To confirm the efficiency and specificity of the four primers, we used DNA extracted from *S. sclerotiorum* and from nine other important plant-pathogenic fungi as templates for LAMP assay. The LAMP assay correctly distinguished between *S. sclerotiorum* and the other pathogens, i.e., the LAMP assay and the primers designed here are specific for *S. sclerotiorum*.

As the LAMP reaction progresses, pyrophosphate ions are produced; these bind to Mg^{2+} ions and form a white precipitate of magnesium pyrophosphate. Therefore, the results of the LAMP can be judged by the unaided eye. This characteristic feature of the LAMP reaction means that the reaction end point can be detected simply by gauging the presence of a precipitate.

The addition of HNB before the LAMP reaction facilitates the determination of a positive result. HNB is a colorimetric indicator of calcium and alkaline earth metal ions. In a LAMP reaction mixture, dNTPs can influence the colour of HNB by the chelating with the Mg^{2+} ions. In the presence of HNB, the colour gradually changes from violet to sky blue as the dNTPs decrease during amplification (Goto et al. 2009). In this study, 150 μM HNB successfully distinguished between positive and negative samples. Compared to other methods used to visually detect endpoints, such as those based on the visualization of turbidity (Nowotny et al. 1994), the addition of DNA intercalating dyes (Curtis et al.

2008; Hill et al. 2008; Parida et al. 2005), or the use of calcein (Tomita et al. 2008), the use of HNB is simpler (Goto et al. 2009; Wastling et al. 2010). HNB can be added before incubation so that amplification is completed in a closed tube system, and detection of the colour change requires no equipment. The positive and negative reactions obtained with LAMP and HNB were confirmed when the LAMP products were subjected to gel electrophoresis analysis in the current study.

The limit for detection of *S. sclerotiorum* DNA using the LAMP method was 0.1 fg μL^{-1} . This detection limit is lower (i.e., the sensitivity is greater) than that previously reported for LAMP methods used to detect *Phytophthora sojae* and *Phytophthora* spp. (Dai et al. 2012; Tomlinson et al. 2007; Tomlinson et al. 2010).

Researchers have reported that the LAMP reaction might be facilitated by the addition of loop-forward and loop-backward primers (Nagamine et al. 2002). In the present study, we could not identify a suitable loop-backward primer, and so we used only the loop-forward primer to accelerate the reaction (Table 1). This improved the reaction time and efficiency (data not shown).

The utility of the LAMP method for detection of *S. sclerotiorum* was confirmed by the collecting and processing of 13 field samples of diseased rapeseed tissues. All samples were subjected to LAMP, conventional PCR, and isolation. Compared with the other methods, the newly developed LAMP assay significantly improved the detection efficiency. Therefore, the LAMP assay may be used for detection of *S. sclerotiorum* in plants.

In conclusion, we have demonstrated that a LAMP assay combined with HNB is simple, rapid, sensitive, and specific. Because this LAMP assay does not require specialized equipment, it can be used in the field for the rapid detection of *S. sclerotiorum*. It follows that the new LAMP assay will be useful for monitoring and controlling the spread of *S. sclerotiorum*.

Acknowledgments This research was supported by the Special Fund for Agro-scientific Research in the Public Interest (201103016 and 201303023).

References

- Bekele B, Hodgetts J, Tomlinson J, Boonham N, Nikolić P, Swarbrick P, Dickinson M (2011) Use of a real-time LAMP isothermal assay for detecting 16SrII and 16SrXII phytoplasmas in fruit and weeds of the Ethiopian Rift Valley. *Plant Pathol* 60:345–355
- Boland GJ, Hall R (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Can J Plant Pathol* 16:93–108
- Curtis KA, Rudolph DL, Owen SM (2008) Rapid detection of HIV-1 by reverse-transcription, loop-mediated isothermal amplification (RT-LAMP). *J Virol Methods* 151:264–270
- Dai TT, Lu CC, Lu J, Dong SM, Ye WW, Wang YC, Zheng XB (2012) Development of a loop-mediated isothermal amplification assay for detection of *Phytophthora sojae*. *FEMS Microbiol Lett* 334:27–34
- Duan YB, Ge CY, Liu SM, Chen CJ, Zhou MG (2013a) Effect of phenylpyrrole fungicide fludioxonil on morphological and physiological characteristics of *Sclerotinia sclerotiorum*. *Pestic Biochem Phys* 106:61–67
- Duan YB, Ge CY, Liu SM, Zhou MG (2013b) A two-component histidine kinase *Shk1* controls stress response, sclerotial formation and fungicide resistance in *Sclerotinia sclerotiorum*. *Mol Plant Pathol* doi:10.1111/mpp.12041
- Freeman J, Ward E, Calderon C, McCartney A (2002) A polymerase chain reaction (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum*. *Eur J Plant Pathol* 108:877–886
- Goto M, Honda E, Ogura A, Nomoto A, Hanaki K (2009) Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques* 46:167–172
- Hill J, Beriwal S, Chandra I, Paul VK, Kapil A, Singh T, Wadowsky RM, Singh V, Goyal A, Jahnukainen T, Johnson JR, Tarr PI, Vats A (2008) Loop-mediated isothermal amplification assay for rapid detection of common strains of *Escherichia coli*. *J Clin Microbiol* 46:2800–2804
- Nagamine K, Kuzuhara Y, Notomi T (2002) Isolation of single-stranded DNA from loop-mediated isothermal amplification products. *Biochem Biop Res Co* 290:1195–1198
- Niessen L, Vogel R (2010) Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal amplification (LAMP) assay. *Int J Food Microbiol* 140:183–191
- Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28:e63
- Nowotny N, Mostl K, Maderbacher R, Odorfer G, Schuh M (1994) Serological studies in Austrian fattening pigs with respiratory disorders. *Acta Vet Hung* 42:377–379
- Pan W, Wang JY, Shen HY, Zhao MQ, Ju CM, Dong XY, Chen JD (2011) Development and application of the novel visual loop-mediated isothermal amplification of *Omp25* sequence for rapid detection of *Brucella* sp. *J Anim Vet Adv* 10:2120–2126
- Parida M, Posadas G, Inoue S, Hasebe F, Morita K (2004) Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. *J Clin Microbiol* 42:257–263
- Parida M, Horioka K, Ishida H, Dash PK, Saxena P, Jana AM, Islam MA, Inoue S, Hosaka N, Morita K (2005) Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay. *J Clin Microbiol* 43:2895–2903
- Tomita N, Mori Y, Kanda H, Notomi T (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* 3:877–882
- Tomlinson J, Barker I, Boonham N (2007) Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. *Appl Environ Microb* 73:4040–4047
- Tomlinson J, Dickinson M, Boonham N (2010) Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathology* 100:143–149
- Wastling SL, Picozzi K, Kakembo AS, Welburn SC (2010) LAMP for human African trypanosomiasis: a comparative study of detection formats. *PLOS Neglect Trop D* 4:e865
- Zoheir KM, Allam AA (2011) A rapid improved method for sexing embryo of water buffalo. *Theriogenology* 76:83–87