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Rapid detection of benzimidazole resistance in *Botrytis cinerea* by loop-mediated isothermal amplification

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Abstract

Benzimidazole fungicides (MBCs) have been widely used in agriculture since 1970s, and resistance to this class of fungicides in *Botrytis cinerea* is reported worldwide. Resistance to MBCs in *B. cinerea* is related to mutations in the target β -tubulin gene (*TUB2*). Compared with the mutation at codon 200, the substitutions from glutamic acid to alanine (E198A), valine (E198V), or lysine (E198K) at codon 198 are currently the predominant mutations in MBC resistant populations of *B. cinerea*. In this study, a loop-mediated isothermal amplification (LAMP) method was established for rapid detection of benzimidazole resistance in *B. cinerea*. On the basis of the three mutations at *TUB2* codon 198, three sets of LAMP primers were designed, and each of these primer sets was able to specifically amplify the DNA containing its corresponding mutation, while no amplification was detected with other mutated or the wild type DNA. After optimization, the sensitivity and specificity tests illustrated that this LAMP assay had good sensitivity and specificity to detect specific resistant genotypes in *B. cinerea*. Result also showed that the LAMP assay had good reproducibility. Above all, boiled mycelia could be used as templates, which simplified the process and increased the efficiency of the assay. Considering its rapidity, simplicity, and high efficiency, the LAMP assay developed in this study is a promising tool for the diagnosis of benzimidazole resistance in *B. cinerea*, and will contribute to the monitoring of resistance development to MBCs in the future.

Keywords: *Botrytis cinerea*, Fungicide resistance, Benzimidazole fungicide (MBC), Loop-mediated isothermal amplification (LAMP), Mutation of *TUB2*

Background

Grey mold is a common disease on numerous crops worldwide, especially on vegetables and small fruits. The causal agent of this disease is the ascomycete fungus *Botrytis cinerea* (Williamson et al. 2007). To control this fungus, fungicides are widely used. At present, several classes of fungicides are available, and the group of benzimidazole fungicides (MBCs) is one of them. MBCs, including carbendazim, benomyl and the related agent thiophanate-methyl, are broad-spectrum fungicides and the first group of systemic single-site fungicide

introduced into agriculture in the early 1970s (Walker et al. 2013). MBCs bind to β -tubulin and prevent microtubule assembly, thereby inhibiting germ-tube elongation and mycelial growth (Leroux et al. 1999).

Soon after the introduction, resistance to this class of fungicides in *B. cinerea* was reported (Bolton 1976; Tripathi and Schlösser 1982). This resistance was related to several mutations in the β -tubulin gene (*TUB2*) at codon 198, which resulted in amino acid replacement of glutamic acid by alanine (E198A), valine (E198V), or lysine (E198K) (Yarden and Katan 1993; Banno et al. 2008). Moreover, a mutation at codon 200 substituting phenylalanine by tyrosine (F200Y) was also involved in the resistance to MBC fungicides (Yarden and Katan 1993). Interestingly, apart from benzimidazole resistance, mutations E198K and F200Y also conferred resistance to another anti-microtubule fungicide, diethofencarb, while strains with E198A and E198V mutations were sensitive

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to diethofencarb (Yarden and Katan 1993; Banno et al. 2008). In Japanese fields, most of benzimidazole-resistant *B. cinerea* isolates had the E198V or E198A mutation (Bardas et al. 2008). Our earlier research also showed that mutations at codon 198 were predominant in the isolates with resistance to carbendazim in Hubei, China (Fan et al. 2016; Fan et al. 2017).

To manage fungicide resistance, information on the occurrence and prevalence of resistance to registered fungicides is particularly significant for crop producers to adjust their management strategy promptly. Current methods for detection of MBC resistance in *B. cinerea* include the determination of EC_{50} values, visual analysis of growth on medium amended with a single discriminatory concentration of an MBC fungicide, and allele-specific polymerase chain reaction (Luck and Gillings 1995; Fernández-Ortuño et al. 2014). Additionally, real-time PCR, PCR-restriction-fragment length polymorphism (PCR-RFLP), and the two-step high-resolution melting (HRM) analysis were also applied in benzimidazole resistance identification (Banno et al. 2008; Ziogas et al. 2009; Chatzidimopoulos et al. 2014). Detection of resistance based on EC_{50} determination or discriminatory dose consumes time and labor, and cannot identify the point mutations, while modern molecular techniques mentioned require DNA extraction, thermal cycling instruments, and other sophisticated equipment. Therefore, these methods are not suitable for on-site detection.

A technique that has the potential to provide rapid, yet more feasible in-field detection of pathogens is loop-mediated isothermal amplification (LAMP). As a novel method of DNA amplification, LAMP combines high specificity, efficiency and rapidity (Notomi et al. 2000). Compared with Polymerase Chain Reaction (PCR), LAMP obviates the demand for a thermal cycler, due to isothermal conditions. Furthermore, the LAMP product can be visualized by several methods, such as staining with SYBR Green I, ethidium bromide (EB), hydroxynaphthol blue (HNB) and calcein or using generic lateral flow devices (Tomita et al. 2008; Goto et al. 2009; Tomlinson et al. 2010b). Observing turbidity increase derived from magnesium pyrophosphate formation is also a choice to analyze LAMP product (Njiru 2012). Because of the advantages of LAMP, it has been widely applied in field studies, especially for the diagnosis of bacteria, viruses, fungi, and transgenic plants (Niessen and Vogel 2010; Wang et al. 2012; Zhou et al. 2014; Kuan et al. 2016). Recently, LAMP has been applied successfully to detect fungicide and herbicide resistance, such as carbendazim resistance in *Fusarium graminearum* and *Sclerotinia sclerotiorum* (Duan et al. 2014a; Duan et al. 2015), and fenoxaprop-p-ethyl resistance in American slough grass (Pan et al. 2015). Even though this remarkable technique has been applied for

the detection of *B. cinerea* (Duan et al. 2014b), it has not been adopted to detect MBC resistance in *B. cinerea*.

Here, we established a LAMP assay for rapid detection of benzimidazole resistant isolates of *B. cinerea* based on point mutations at codon 198 of *TUB2*. Based on three mutations (E198A, E198V, and E198K), three sets of LAMP primers were designed. Our study showed that each of these three sets of primers was able to specifically amplify the DNA containing its corresponding mutation, while no amplification was detected with other mutations or the wild type DNA. It was demonstrated that the established LAMP assay had good sensitivity, specificity and reproducibility. Furthermore, the LAMP assay could be accomplished using boiled mycelia as templates, which made this assay simple and time-saving. We believe that this LAMP assay is a promising tool for the detection of benzimidazole resistance in *B. cinerea*, and will play a significant role in monitoring resistance against MBCs in the future.

Results

Design of LAMP primers

Using the sequence of *TUB2* with the E198V point mutation, eight sets of LAMP primers were designed. Only one set of LAMP primers named Tub-E198V (Fig. 1a and Table 1) was able to identify the E198V mutation. To increase the primer specificity, the position of F2 primer was changed to end with the mutation, and one nucleotide mismatch was introduced before the mutation (Fig. 1a and Table 1). After addition of SYBR Green I, the samples of E198A, E198K, sensitive isolate, and DNA-free control remained brown, but the sample of E198V changed to a fluorescent yellow color (Fig. 2a), which indicated the positive LAMP reaction. Agarose gel electrophoresis showed that this set of primers could only amplify DNA from the E198V isolate, but not from any other mutated isolates or the sensitive isolates (Fig. 2b). After the successful design of Tub-E198V, the F2 primer was modified to fit the other two mutations (E198A and E198K) following the same strategy (Fig. 1 and Table 1). Both dye treatment and electrophoresis demonstrated that primer sets Tub-E198A and Tub-E198K were capable of specifically recognizing their corresponding mutations respectively (Fig. 2).

Optimization of LAMP reaction

The LAMP reaction system was optimized using the primer set Tub-E198V and the corresponding genomic DNA. The optimized LAMP master mix contained 4 U of *Bst* DNA polymerase, 2.5 μ L 10 \times ThermoPol Buffer, 4 mM $MgSO_4$, 1 mM dNTPs, 1.2 mM each of primers FIP and BIP, 0.4 mM each of primers F3 and B3, 0.8 M betaine, and 1 μ L of target DNA in a final volume of 25 μ L. Best results were obtained when LAMP reaction was performed at 63 $^{\circ}C$ for 60 min.

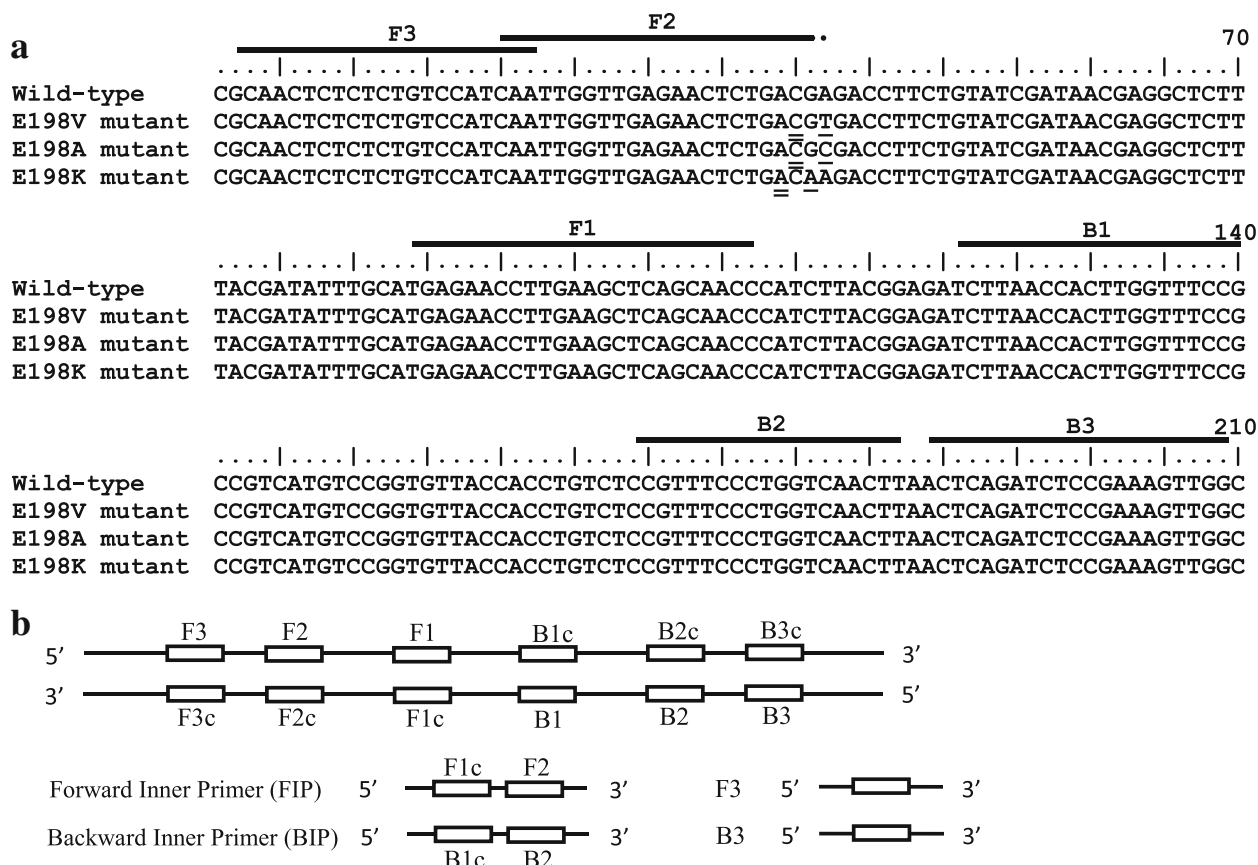


Fig. 1 Design of LAMP primers for detection of the E198K/A/V mutations of *TUB2* in *Botrytis cinerea*. **a** Nucleotide sequence alignment of sensitive and resistant genotypes. The sequences used as LAMP primers are indicated by bars. Point mutations are shown underlined with one line and mismatches are shown underlined with double lines. For each mutation, the F2 primer ends with its corresponding mutated nucleotide. **b** Schematic representation of the LAMP primers used in this work. The inner primer FIP consists of F1c and F2, and BIP comprised of B1c and B2. Primers ending with 'c' are complementary to their counterparts without 'c'.

Sensitivity of LAMP and conventional PCR

The sensitivity of LAMP and conventional PCR was investigated using Tub-E198V primer set and ten-fold serial dilutions of plasmid pMD18-TubA. When the concentration of plasmid reached 2×10^5 copies/ μ L, the ladder-like product was observed by electrophoresis, and the color change of SYBR Green I also occurred

(Fig. 3a, b), indicating that the LAMP detection limit for plasmid pMD18-TubA was approximately 2×10^5 copies/ μ L. For conventional PCR, a 936 bp band was amplified when the template plasmid was diluted to as low as 2×10^3 copies/ μ L (Fig. 3c), illustrating that the detection limit of PCR was about two orders of magnitude lower than that of LAMP.

Table 1 Design of LAMP primers for detection of the E198K/A/V mutations of *TUB2* in *Botrytis cinerea*

Primer	Type	Length (bp)	Sequence (5'-3')
F3	Forward outer	21	GCAACTCTCTCTGTCCATCAA
B3	Backward outer	21	CCAACTTTCGGAGATCTGAGT
BIP	Backward inner(B1c-B2)	42	TCTTAACCACTTGGTTTCCGCCGAAGTTGACCAGGGAACGG
Tub-E198V	Forward inner(F1c-F2)	47	GGTTGCTGAGCTTCAAGGTTCTCACAATTGGTTGAGAACTCTGAG ^b GT ^a
Tub-E198A	Forward inner(F1c-F2)	47	GGTTGCTGAGCTTCAAGGTTCTCACAATTGGTTGAGAACTCTGAT ^b GC ^a
Tub-E198K	Forward inner(F1c-F2)	46	GGTTGCTGAGCTTCAAGGTTCTCACAATTGGTTGAGAACTCTGT ^b CA ^a

^aNucleotides in bold are modified from the sequence of the *TUB2* gene in the sensitive and the resistant isolates

^bNucleotides underlined are mismatches introduced specifically to distinguish *B.cinerea* genotypes (E198K/A/V)

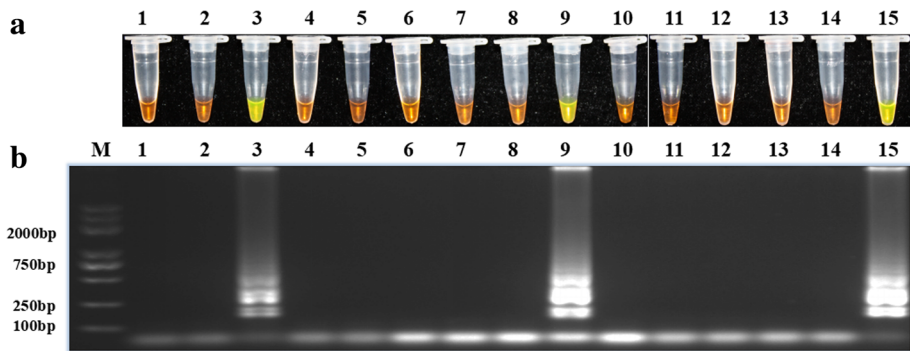


Fig. 2 LAMP primer set designed to amplify the E198K, E198A and E198V mutations in *Botrytis cinerea*, respectively. **a** LAMP products detected with 1000 × SYBR Green I. **b** Detection of LAMP products by agarose gel electrophoresis. Lane M: DNA 2 K plus Marker; tubes and lanes 1–5: primer set Tub-E198K; tubes and lanes 6–10: primer set Tub-E198A; tubes and lanes 11–15: primer set Tub-E198V; tubes and lanes 1, 6, 11: ddH₂O; tubes and lanes 2, 7, 12: HBStr-104; tubes and lanes 3, 8, 13: HBStr-155; tubes and lanes 4, 9, 14: HBStr-114; tubes and lanes 5, 10, 15: HBStr-496

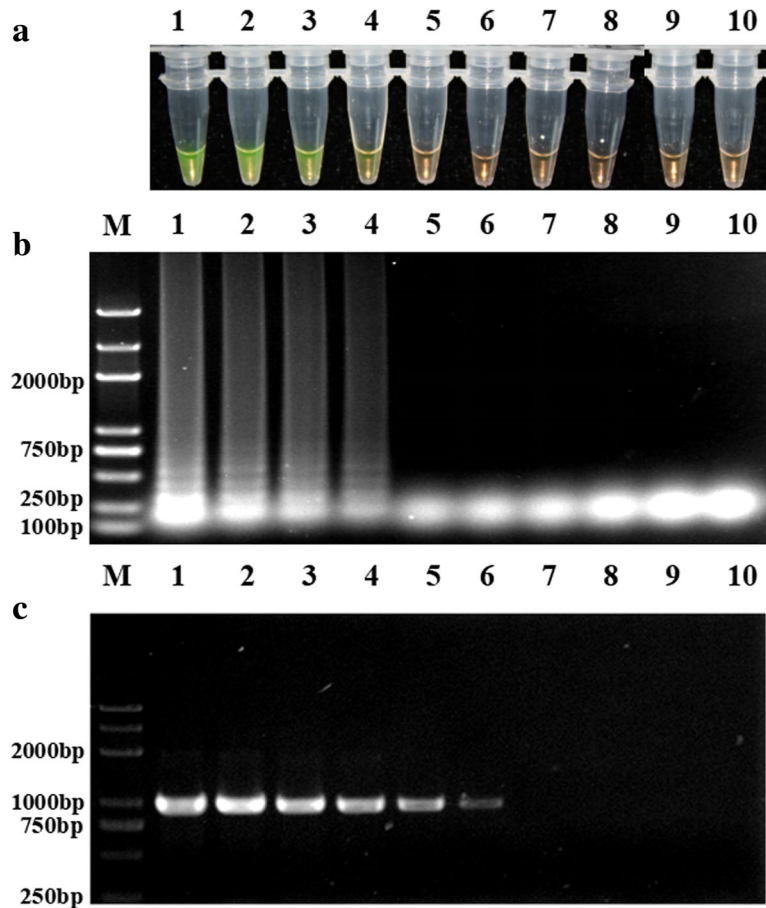


Fig. 3 Sensitivity comparison of LAMP and conventional PCR for the detection of the E198V mutation using plasmid pMD18-TubA. **a** LAMP products detected with 1000 × SYBR Green I. **b** LAMP assessment based on agarose gel electrophoresis. **c** Conventional PCR assessment based on agarose gel electrophoresis. Lane M: DNA 2 K plus Marker; tubes and lanes 1–9: concentration of plasmid pMD18-TubA at 2×10^8 , 2×10^7 , 2×10^6 , 2×10^5 , 2×10^4 , 2×10^3 , 2×10^2 , 2×10^1 , and 2×10^0 copies/μL, respectively; tube and lane 10: ddH₂O

Specificity and reproducibility test of LAMP

Genomic DNA was extracted from several relative species of *B. cinerea*, including *B. sinoviticola*, *B. sinoallii*, *B. porri*, *S. sclerotiorum*, *Monilinia fructicola*, *M. mumeicola*, *M. yunnanensis*, and *F. graminearum*, and the specificity of LAMP by using Tub-E198V primer set was subsequently tested. Both the color change in the tubes (Fig. 4a) and amplification bands (Fig. 4b) were only achieved with DNA of carbendazim-resistant *B. cinerea* isolates with E198V mutation, confirming the high specificity of LAMP.

To evaluate the reproducibility, all three LAMP primer sets were employed against genomic DNA from eight isolates for each of wild type, E198V, E198A and E198K mutant, respectively. Using primer set Tub-E198V, only the eight E198V resistant isolates showed positive reaction, whereas the reaction of the eight sensitive isolates, eight E198A resistant isolates, and eight E198K resistant isolates remained negative (Additional file 1: Figure S1). As expected, similar observations were made using primer sets Tub-E198A and Tub-E198K (Additional file 2: Figure S2 and Additional file 3: Figure S3).

LAMP detection using crude DNA from fungal mycelia

Outside the laboratory, DNA extraction may be a limiting step for the LAMP assay. To solve this problem and to increase the speed and economic efficiency, crude DNA extracted with 10 × TE buffer from mycelia was used as the template in LAMP detection. After LAMP amplification with primer set Tub-E198V, the fluorescent yellow of SYBR Green I occurred only with mycelia of four E198V resistant isolates, whereas SYBR Green I

stayed brown for sensitive isolates and the resistant isolates with the E198A or E198K mutation (Fig. 5a), which was also validated by agarose gel electrophoresis (Fig. 5b). The results showed that the LAMP detection could also be successful when crude mycelial extracts were used as template to detect the carbendazim resistance conferred by E198A/V/K mutations in *B. cinerea*.

Discussion

MBCs, such as carbendazim and thiophanate-methyl, have been used in agriculture since 1970s (Walker et al. 2013). They inhibit germ-tube elongation and mycelial growth by targeting β -tubulin, preventing microtubule assembly (Leroux et al. 1999). Due to the specific-site mode of action, the fungus can develop resistance based on single mutations. By now, four mutations in *TUB2* gene are known to be involved in resistance to MBCs in *B. cinerea*, namely E198A, E198V, E198K, and F200Y (Yarden and Katan 1993; Banno et al. 2008). Compared with the extremely rare occurrence of mutation at codon 200, mutations at codon 198 were found at a high frequency (Banno et al. 2008). In our previous studies, all resistant mutations occurred at codon 198 in sequenced isolates from Hubei Province, China (Fan et al. 2016; Fan et al. 2017). Interestingly, although four types of mutations conferred resistance to benzimidazoles, only mutations E198K and F200Y are capable of conferring resistance to both MBC fungicide and diethofencarb (Yarden and Katan 1993; Banno et al. 2008). Diethofencarb, belonging to *N*-phenylcarbamate, is another anti-microtubule fungicide but negative cross resistance was observed between diethofencarb and MBC

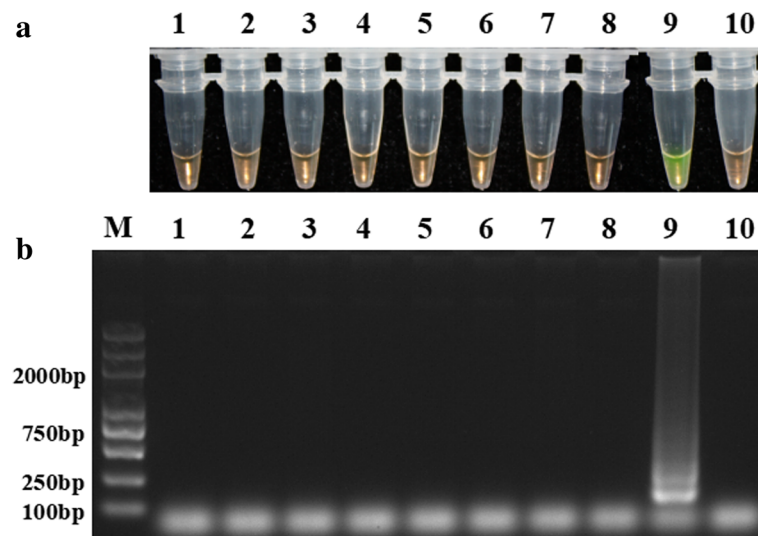


Fig. 4 Specificity of a LAMP primer set for detection of E198V mutation in *Botrytis cinerea*. **a** LAMP products detected with 1000 × SYBR Green I. **b** LAMP assessment based on agarose gel electrophoresis. Lane M: DNA 2 K plus Marker; tubes and lanes 1–9: *B. sinoviticola*, *B. sinoallii*, *B. porri*, *Monilinia fructicola*, *M. mumeicola*, *M. yunnanensis*, *Fusarium graminearum*, *Sclerotinia sclerotiorum*, and *B. cinerea* (E198V); tube and lane 10: ddH₂O

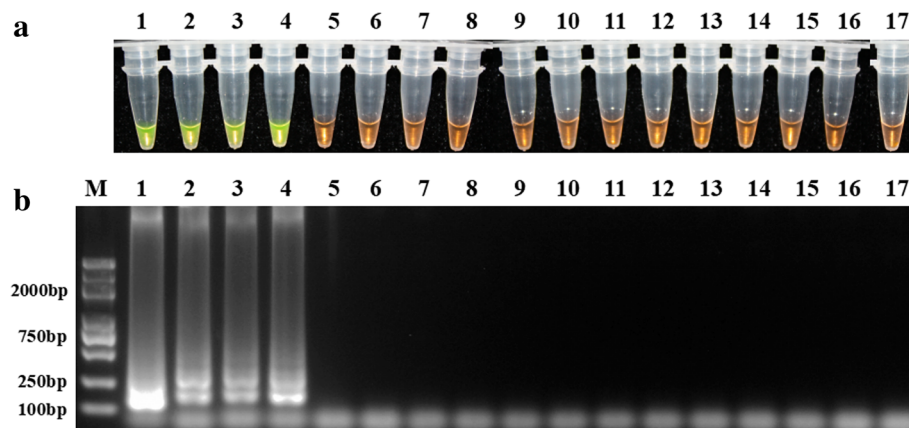


Fig. 5 Conduction of LAMP using mycelia. **a** LAMP products detected with 1000 × SYBR Green I. **b** Detection of LAMP products by agarose gel electrophoresis. Lane M: DNA 2K plus Marker; tubes and lanes 1–4: E198V strains; tubes and lanes 5–8: E198K strains; tubes and lanes 9–12: E198A strains; tubes and lanes 13–16: wild type strains; tube and lane 17: ddH₂O

fungicides in isolates with E198A or E198V mutation (Leroux et al. 1999).

Resistance monitoring, an indispensable step of resistance management, is valuable to guide the efficient use of fungicides. For resistance detection, growth assays on fungicide-amended media can be used, which consume time and labor, and do not allow identification of the resistance mutations. Modern molecular tools depending on PCR, such as conventional PCR, allele-specific PCR, real-time PCR, and PCR-RFLP, show some advantages over classic methods, but sophisticated equipment, reagents and skilled personnel are required. The technique based on loop-mediated isothermal amplification (LAMP) established in this paper for identification of MBCs resistance is specific, rapid, simple and efficient, and has the potential to be conducted in the field, which facilitates the efficient use of fungicides.

According to a previous study, LAMP is highly specific due to its six distinct primers (Notomi et al. 2000). In this study, LAMP primers designed using the *TUB2* gene sequence of *B. cinerea* could specifically amplify the DNA from resistant strains of *B. cinerea*. Such a high specificity of LAMP was also documented in other studies (Duan et al. 2014a; Duan et al. 2015). Moreover, LAMP was also found to be highly sensitive compared with conventional PCR (Notomi et al. 2000; Zhou et al. 2014; Pan et al. 2015). However, our experiments revealed that the detection limit of LAMP was approximately 100 times lower than that of conventional PCR. It is possible that the sensitivity of LAMP is influenced by the affinity of specific primers and target DNAs. Even though the LAMP assay developed in this study was not as sensitive as that in other studies, the sensitivity was still high enough for detection, even using the crude DNA obtained from boiled mycelia. This is an important simplification of LAMP detection, since this method

does not require sophisticated equipment or expensive reagent. Considering that there are large amounts of mycelia on diseased samples of grey mold, no isolation and cultivation of *B. cinerea* is required for the LAMP assessment.

The LAMP reaction can be accelerated by the addition of two loop primers, named loop forward (LF) primer and loop backward (LB) primer (Nagamine et al. 2002). By now, loop primers have been successfully applied in many studies (Tomlinson et al. 2010a; Wang et al. 2011; Moradi et al. 2012). However, with the loop primers, the LAMP assay failed to distinguish the resistant mutants from the wild type of *F. graminearum* in a previous study (Duan et al. 2014a). Similarly, LAMP assay with the loop primer could not differentiate the resistant mutation from wild type when the reaction time was increased to 45 min (Duan et al. 2016). It seems that the LAMP analysis with loop primers can lead to false positive reaction when conducting resistance monitoring. According to the manual for LAMP primer designing, the loop primers are not essential. Therefore, similar to other studies (Pan et al. 2015; Duan et al. 2016), the loop primers were not adopted in this study.

As LAMP is a promising method for diagnosis of pathogens, it continues to attract the attention of researchers in different fields (Mori et al. 2013). Still, LAMP technique has a major problem, the false positive results arising from product cross contamination (Njiru et al. 2008; Angamuthu et al. 2012; Li et al. 2014). Contamination was usually caused by aerosol of product, especially when analyzing the LAMP product (Zhou et al. 2014). To avoid contamination, various methods have been developed (Goto et al. 2009; Zoheir and Allam 2011; Karthik et al. 2014). In our study, LAMP reaction mixture was prepared in a laminar flow cabinet, and the detection area was separated from preparation and

amplification areas. In this way, false positive amplification was largely avoided. Since all the three sets of LAMP primers shared the same primer set, we were wondering if they could work together in a single reaction to facilitate the detection of MBCs resistance. Although multiplex PCR has been successfully applied in many studies (Côté et al. 2004; Hu et al. 2011), there has been no study on multiplex LAMP. In the current study, a lot of efforts were expended. Unfortunately, when using all the primers simultaneously, no DNA amplification was detected (data not shown). The complex structures formed among the primers might inhibit the reaction. Further attempts will be made to achieve the goal of multiplex LAMP.

The development of fungicide resistance in *B. cinerea* populations is one of the major limiting factors of successful control of gray mold (Hahn 2014). Resistance to MBCs in *B. cinerea* is reported worldwide (Zhang et al. 2010; Weber 2011; Fernández-Ortuño et al. 2014). In order to provide guidance on how to use MBCs efficiently, continuous monitoring of fungicide resistance is necessary. Since the mutations at codon 198 are predominant in benzimidazole-resistant isolates, the LAMP method established in the present study could be easily adopted as a simple, rapid and efficient method to identify resistant isolates of *B. cinerea*, especially by using templates directly from mycelia.

Conclusions

Resistance to MBCs in *B. cinerea* is related to the mutations in the β -tubulin gene, and the mutations E198A/V/K are predominant genotypes. For rapid detection of benzimidazole resistance caused by the E198A/V/K mutations, a LAMP method was established in this study. Results showed that this LAMP method had good sensitivity and specificity, as well as good reproducibility. Above all, boiled mycelia could be used as LAMP templates. Considering its rapidity, simplicity, and high efficiency, this LAMP assay is a promising tool for the detection of MBCs resistance in *B. cinerea*, and will significantly contribute to the monitoring of resistance development to MBCs in practice.

Methods

Fungal isolates and DNA extraction

Four isolates of *B. cinerea*, HBstr-104, HBstr-114, HBstr-155, and HBstr-496 were tested in this study. They were collected from greenhouse strawberries in 2012 in Hubei Province, China. DNA sequencing showed that HBstr-114, HBstr-155, and HBstr-496 contained the point mutation of E198A (GAG to GCG), E198K (GAG to AAG), and E198V (GAG to GTG) in *TUB2*, respectively. As a consequence, isolates HBstr-114, HBstr-155, and HBstr-496 were resistant to

benzimidazole fungicide carbendazim. Isolate HBstr-104 was used as a sensitive control. In the specificity test for the LAMP primers, several relative species of *B. cinerea* were tested, including *B. sinoviticola*, *B. sinoallii*, *B. porri*, *S. sclerotiorum*, *M. fructicola*, *M. mumecola*, *M. yunnanensis*, and *F. graminearum*. In reproducibility test, 32 *B. cinerea* isolates including eight for each of E198V, E198K, E198A and wild type strains were tested. All isolates were cultured on potato dextrose agar (PDA) medium at 22 °C in darkness.

To extract the genomic DNA, isolates were grown on PDA with cellophane for 3 days, and mycelia were collected using a toothpick. The genomic DNA was extracted and purified as described previously (Chi et al. 2009). The final concentration of extracted DNA was around 100 ng/ μ L.

Primer design

On the basis of the DNA sequence of the E198V mutation, a set of LAMP primers named Tub-E198V (Table 1) was designed using the Primer Explorer V4 software program (<http://primerexplorer.jp/elamp4.0.0/index.html>). Depending on this primer set, two new LAMP primer sets Tub-E198A and Tub-E198K were also designed to distinguish the other two mutations. The three primer sets shared all the same primers except primer F2, which was modified based on each mutation. Furthermore, a mismatch was introduced in primer F2 to avoid false positives.

Reaction mixture for LAMP

The initial LAMP reaction was performed according to a former study, with a little adjustment (Duan et al. 2014a). The LAMP reaction was carried out in a 25 μ L volume containing 6 U of *Bst* DNA polymerase large fragment (New England Biolabs, Massachusetts, USA), 2.5 μ L 10 \times ThermoPol Buffer, 4 mM MgSO₄, 1 mM dNTPs (Aidlab, Beijing, China), 1.6 mM each of FIP and BIP, 0.2 mM each of F3 and B3, 0.94 M betaine (Aladdin, Shanghai, China), and 1 μ L of target DNA. Sterilized water was used as negative control. The mixture was incubated at 63 °C for 60 min, followed by heating at 80 °C for 10 min to inactivate the enzyme and terminate the reaction. The LAMP products were analyzed by 1% agarose gel electrophoresis stained with GelRed (US Everbright Inc., Suzhou, China). Additionally, 0.2 μ L 10,000 \times SYBR Green I (Aidlab) was added (final concentration of SYBR Green I was 80 \times) into the product to facilitate the visualization of the reaction by naked eyes. The experiment was conducted twice.

Optimization of LAMP reaction

The primer set Tub-E198V and relevant genomic DNA were chosen to optimize the LAMP reaction. The

optimization was conducted in a total volume of 25 μ L. A series of concentrations of Mg^{2+} (2, 3, 4, 5 and 6 mM), dNTP (0.5, 1, 1.5 and 2 mM), betaine (0, 0.8 and 1.6 M), *Bst* DNA polymerase (2, 4, 6 and 8 U), inner primers (0.8, 1.2, 1.6 and 2.0 μ M) and outer primers (0.2, 0.4, 0.6 and 0.8 μ M) were evaluated. First, the concentration of Mg^{2+} was optimized while all the other components remained constant. Then, the concentration of dNTPs was optimized at the optimal concentration of Mg^{2+} . Accordingly, the concentrations of betaine, *Bst* DNA polymerase, inner primers and outer primers were determined, respectively. Moreover, using the optimized reaction components, the reaction was performed at 61 °C, 62 °C, 63 °C, 64 °C and 65 °C for 60 min, respectively, to examine the best temperature. Later, the reaction was carried out at the optimal temperature for 30, 45, 60, 75 and 90 min, respectively, to test the optimal time. The LAMP products were assessed based on agarose gel electrophoresis and color visualization with SYBR Green I as described above. The experiment was performed twice.

Sensitivity of LAMP and conventional PCR

The sensitivity of LAMP and PCR was analyzed using Tub-E198V primer set and ten-fold serial dilutions of plasmid pMD18-TubA, which contained the E198V fragment of *TUB2*. For construction of pMD18-TubA, the E198V fragment was obtained by PCR with primer pair TubA 1378F19/2314R20 (5'-GAT CTC CAA CTT GAG CGT A / TGG AAA CCT TAC CAC GGC TA-3'). The PCR was performed as described in the following sensitivity test and the product was purified using EasyPure PCR Purification Kit (TransGen, Beijing, China). The purified fragment was then cloned into pMD18-T vector using pMD18-T Vector Cloning kit (TaKaRa, Dalian, China). The recombinant plasmid pMD18-TubA was extracted using EasyPure Plasmid MiniPrep Kit (TransGen) and ten-fold serially diluted from 2×10^8 to 2×10^0 copies/ μ L prior to use.

For conventional PCR detection, primer pair TubA 1378F19/2314R20 was used to amplify a 936-bp DNA fragment. PCR amplification was performed in a total reaction volume of 25 μ L, containing 1.25 U of EasyTaq DNA Polymerase (TransGen), $1 \times$ EasyTaq buffer, 1.25 mM each dNTP, 0.4 μ M each primer, and 1 μ L corresponding amount of template DNA. Amplification was carried out in an iCycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA). The PCR program consisted of an initial preheating step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 60 s, with a final extension step at 72 °C for 10 min. For LAMP detection, LAMP reaction was performed at reaction conditions optimized as described. The PCR product was separated on 1% agarose gel stained with

ethidium bromide (EB) in $1 \times$ TAE buffer and visualized under UV light. The LAMP products were examined by both agarose gel electrophoresis and the color change of SYBR Green I. The analysis was conducted twice.

Specificity and reproducibility test of LAMP

Primer set Tub-E198V was also tested against other relative species of *B. cinerea*, including *B. sinoviticola*, *B. sinoallii*, *B. porri*, *S. sclerotiorum*, *M. fructicola*, *M. mumeicola*, *M. yunnanensis*, and *F. graminearum*, to determine the specificity of LAMP primers. Additionally, all three LAMP primer sets designed in this study were applied to evaluate the reproducibility using genomic DNA extracted from eight for each of wild type, E198V, E198A, and E198K isolates. Instead of DNA template, sterile water was used in negative control, while other reaction components remained identical. The LAMP was conducted at the optimal reaction parameters and the product was assessed as described above. All experiments were conducted twice.

LAMP application using mycelia

Four isolates for each of wild-type *B. cinerea* (sensitive to benzimidazole), and its benzimidazole-resistant phenotypes E198V, E198A, and E198K were analyzed in this test. Mycelia on approximately 0.5 cm² agar medium were prepared using toothpicks after culturing on PDA plates for a week. Then, the mycelia were suspended in 50 μ L $10 \times$ Tris-EDTA (TE) buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.0) in a 1.5 mL centrifuge tube. After boiling in water for 2 min, the suspension was chilled on ice for 2 min and then centrifuged at 12000 rpm for 1 min. The supernatants obtained were used as templates for LAMP. The LAMP assay was conducted and assessed with the primer set Tub-E198V using the optimal LAMP reaction parameters as described above. The experiment was performed twice.

Additional files

Additional file 1: Figure S1. Reproducibility of LAMP primer set Tub-E198V. **a** LAMP products detected with $1000 \times$ Sybr Green I. **b** Detection of LAMP products by agarose gel electrophoresis. Lane M: DNA 2 K plus Marker; tubes and lanes 1–8: E198V strains; tubes and lanes 9–16: E198K strains; tubes and lanes 17–24: E198A strains; tubes and lanes 25–32: wild type strains; tube and lane 33: ddH₂O. (DOCX 3108 kb)

Additional file 2: Figure S2. Reproducibility of LAMP primer set Tub-E198A. **a** LAMP products detected with $1000 \times$ Sybr Green I. **b** Detection of LAMP products by agarose gel electrophoresis. Lane M: DNA 2 K plus Marker; tubes and lanes 1–8: E198A strains; tubes and lanes 9–16: E198K strains; tubes and lanes 17–24: E198V strains; tubes and lanes 25–32: wild type strains; tube and lane 33: ddH₂O. (DOCX 3048 kb)

Additional file 3: Figure S3. Reproducibility of LAMP primer set Tub-E198K. **a** LAMP products detected with $1000 \times$ Sybr Green I. **b** Detection of LAMP products by agarose gel electrophoresis. Lane M: DNA 2 K plus Marker; tubes and lanes 1–8: E198K strains; tubes and lanes 9–16: E198A strains; tubes and lanes 17–24: E198V strains; tubes and lanes 25–32: wild type strains; tube and lane 33: ddH₂O. (DOCX 2935 kb)

Abbreviations

E198A, V, K: Substitutions from glutamic acid to alanine, valine, lysine at codon 198 of *TUB2*; EB: Ethidium bromide; F200Y: Mutation at codon 200 of *TUB2* substituting phenylalanine by tyrosine; HNB: Hydroxynaphthol blue; LAMP: Loop-mediated isothermal amplification; MBCs: Benzimidazole fungicides; PCR-RFLP: PCR-restriction-fragment length polymorphism; PDA: Potato dextrose agar; *TUB2*: β -tubulin gene

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FF and C-XL designed the research; FF performed research; FF, MH, G-QL, YL and C-XL wrote the manuscript, all authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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