Plant Disease • 2018 • 102:2016-2024 • https://doi.org/10.1094/PDIS-03-18-0542-RE

e-Xtra*

Simultaneous Detection of Multiple Benzimidazole-Resistant β-Tubulin Variants of *Botrytis cinerea* using Loop-Mediated Isothermal Amplification

Ya Bing Duan, Ying Yang, Jian Xin Wang, and **Chang Jun Chen,** College of Plant Protection, State & Local Joint Engineering Research Center of Green Pesticide Invention and Application, Nanjing Agricultural University, Nanjing, Jiangsu Province, 210095, China; **Gero Steinberg,** Biosciences, University of Exeter, Stocker Road, Exeter, EX4 4QD, UK; **Bart A Fraaije,** Biointeractions & Crop Protection Department, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK; and **Ming Guo Zhou,**† College of Plant Protection, State & Local Joint Engineering Research Center of Green Pesticide Invention and Application, Nanjing Agricultural University

Abstract

Optimal disease management depends on the ability to monitor the development of fungicide resistance in plant pathogen populations. Benzimidazole resistance is caused by the point mutations of the β-tubulin gene in *Botrytis cinerea*, and three mutations (E198A, E198K, and E198V) at codon 198 account for more than 98% of all resistant strains. Although traditional methods remain a cornerstone in monitoring fungicide resistance, molecular methods that do not require the isolation of pathogens can detect resistance alleles present at low frequencies, and require less time and labor than traditional methods. In this study, we present an efficient, rapid, and highly specific method for detecting highly benzimidazole-resistant *B. cinerea* isolates based on loop-mediated isothermal amplification (LAMP). By using specific primers, we could simultaneously detect all three resistance-conferring mutations at codon 198. The LAMP reaction components and conditions were optimized, and the best reaction temperatures and times were 60 to 62°C and 45 min, respectively. When *B. cinerea* field isolates were assessed for benzimidazole resistance, similar results were obtained with LAMP, minimal inhibition concentration, and sequencing. The LAMP assay developed in the current study was highly suitable for detection of highly benzimidazole-resistant field isolates of *B. cinerea*.

Botrytis cinerea is a destructive phytopathogenic fungus with a broad host range and a worldwide distribution. This species can attack more than 200 plants, including many important agricultural and horticultural crops (Williamson et al. 2007). Although phytopathogenic fungi can often be controlled by resistant host cultivars, resistance is difficult to develop against *B. cinerea*, because this pathogen attacks a wide range of hosts and can adapt rapidly. Therefore, application of fungicides is the primary tool for controlling gray mold on most crops caused by *B. cinerea* (Janisiewicz et al. 2016; Vitale et al. 2016). The benzimidazole fungicides, particularly carbendazim, have been extensively used for controlling *B. cinerea* since the early 1970s (Richmond and Phillips 1975). Unfortunately, benzimidazole-resistant populations of *B. cinerea* have evolved and spread rapidly. Benzimidazoles are still used to control the fungus but this requires the close monitoring of benzimidazole resistance over large regions to ensure a high level of efficacy can still be achieved.

According to previous studies (Geeson 1978; Hawkins and Fraaije 2016; Liu et al. 2013; Ma and Michailides 2005; Ziogas et al. 2009), benzimidazole resistance of *B. cinerea* is mediated by the point mutations of β-*tubulin*, leading to target site alterations at codons 198 (E198V, GAG→GTG; E198A, GAG→GCG; E198K, GAG→AAG; E198G, GAG→GGG; and E198L, GAG→CTG) and 200 (F200Y, TTC→TAC). Mutations at codon 198 result in a high level of resistance, whereas the mutation at codon 200 is associated with a moderate level resistance in *B. cinerea*. The three mutations at codon 198 account for more than 98% of the resistant subpopulations of *B. cinerea* in the field in China (unpublished data from our laboratory). In recent years, we monitored carbendazim-resistant populations of *B. cinerea* from the different hosts from geographical regions in China. Among these carbendazim resistant isolates, the mutations E198L and E198G were not detected.

Fungicide resistance has often been detected based on mycelial inhibition concentration (Duan et al. 2014c, 2015; Ma et al. 2009). However, this laboratory-based procedure is time consuming and tedious, and the efficiency of resistance detection is low. In recent years, polymerase chain reaction (PCR)-based techniques have been developed, and these enable the rapid detection of resistance-conferring mutations in fungicide target genes (Chen et al. 2009; Hou et al. 2011; Luo et al. 2009; Zhang et al. 2015). PCR-based techniques also have disadvantages (i.e., they require expensive instruments and lab space, are often insufficiently specific, are prone to contamination, and need to be conducted by trained personnel).

Loop-mediated isothermal amplification (LAMP) is a novel gene amplification method that can rapidly amplify nucleic acids with high efficiency, specificity, and sensitivity under isothermal conditions (Notomi et al. 2000). This method is characterized by the use of a set of specially designed LAMP primers and a DNA polymerase with strand displacement activity (Mori et al. 2001; Nagamine et al. 2002). Because of the isothermal nature of LAMP, no time is lost during thermal changes, and the amplification efficiency is much higher in LAMP than in PCR.

Although LAMP has been successfully used to detect phytopathogens (Dai et al. 2012; Duan et al. 2014a; Niessen and Vogel 2010), it has been rarely used to detect mutations conferring target-site-mediated fungicide resistance in phytopathogens till recently. Our research group successfully developed LAMP assays for detecting fungicide resistance in phytopathogens (Duan et al. 2014c, 2015, 2016a, b) but these assays can only detect a single mutation in each reaction. This is inadequate for *B. cinerea* because all three β-tubulin target alterations at codon 198 (E198A, E198K, or E198V) cause high levels of benzimidazole resistance in *B. cinerea*.

In the current study, we developed a LAMP assay for simultaneously detecting three mutation genotypes of highly benzimidazole-resistant populations in *B. cinerea*, and we demonstrated the

†Corresponding author: M. G. Zhou; E-mail: mgzhou@njau.edu.cn

Funding: This work was supported by the Fundamental Research Funds for the Central Universities (KYTZ201604), the National Natural Science Foundation of China (31572025), and Henan Science and Technology Cooperation Project (172106000006).

*The *e*-Xtra logo stands for "electronic extra" and indicates that one supplementary table is published online.

Accepted for publication 4 April 2018.

usefulness of the assay by comparing it with traditional assays in tests involving large numbers of field isolates. This new assay is simple and rapid and allows early detection of resistance development in plant samples.

Materials and Methods

Fungal isolates and culture conditions. *B. cinerea* isolates B05.10, SD2, and SD4 carrying β-tubulin E198A, E198K, and E198V, respectively, are highly resistant to carbendazim. Isolate B20 carrying β-tubulin F200Y is moderately resistant to carbendazim, and isolate Bt4-1 carrying wild-type β-tubulin is sensitive to carbendazim. Background information on these isolates is provided in Table 1.

Potato dextrose agar (PDA) medium was used in routine assays to assess the carbendazim sensitivity in vitro. According to the minimum inhibitory concentration (MIC), *B. cinerea* isolates were divided into three phenotypes as follows: sensitive to carbendazim, MIC values <10 μg ml^{-1}; moderately resistant to carbendazim, MIC values between 10 and 100 μg ml^{-1}; and highly resistant to carbendazim, MIC values >100 μg ml^{-1}.

DNA extraction. *B. cinerea* isolates was incubated for 3 days at 25°C on PDA. Mycelia were harvested to extract genomic DNA

Table 1. Fungal isolates used in this study

Isolate	Fungal species	Host	Origin	β Tubulin variant	Type[a]	LAMP[b]
Bt4-1	*Botrytis cinerea*	Strawberry	Jiangsu, China	Wild type	MBC^{S}	–
B05.10	*B. cinerea*	Strawberry	Germany	GAG→GCG, E198A	MBC^{HR}	+
SD2	*B. cinerea*	Tomato	Shandong, China	GAG→AAG, E198K	MBC^{HR}	+
SD4	*B. cinerea*	Tomato	Shandong, China	GAG→GTG, E198V	MBC^{HR}	+
B20	*B. cinerea*	Strawberry	Jiangsu, China	TTC→TAC, F200Y	MBC^{MR}	–
PI01	*Penicillium italicum*	Citrus	Jiangsu, China	TTC→TAC, F200Y	MBC^{HR}	–
PD98	*P. digitatum*	Citrus	Jiangsu, China	Wild type	MBC^{S}	–
AA04	*Alternaria alternate*	Tomato	Jiangsu, China	Wild type	MBC^{S}	–
CC15	*Corynespora cassiicola*	Cucumber	Henan, China	GAG→GCG, E198A	MBC^{HR}	–
FV13	*Fusicladium virecens*	Pear	Anhui, China	Wild type	MBC^{S}	–
FO11	*Fusarium oxysporum*	Cucumber	Jiangsu, China	Wild type	MBC^{S}	–
SS07	*Sclerotinia sclertiorum*	Lettuce	Jiangsu, China	Wild type	MBC^{S}	–
CC13	*Colletotrichum capsici*	Pepper	Jiangsu, China	Wild type	MBC^{S}	–

[a] Phenotypes MBC^{S}, MBC^{MR}, and MBC^{HR} indicate that the isolate is sensitive, moderately resistant, and highly resistant to benzimidazole (MBC), respectively.
[b] Symbols + and – indicate positive and negative results, respectively, for loop-mediated isothermal amplification (LAMP).

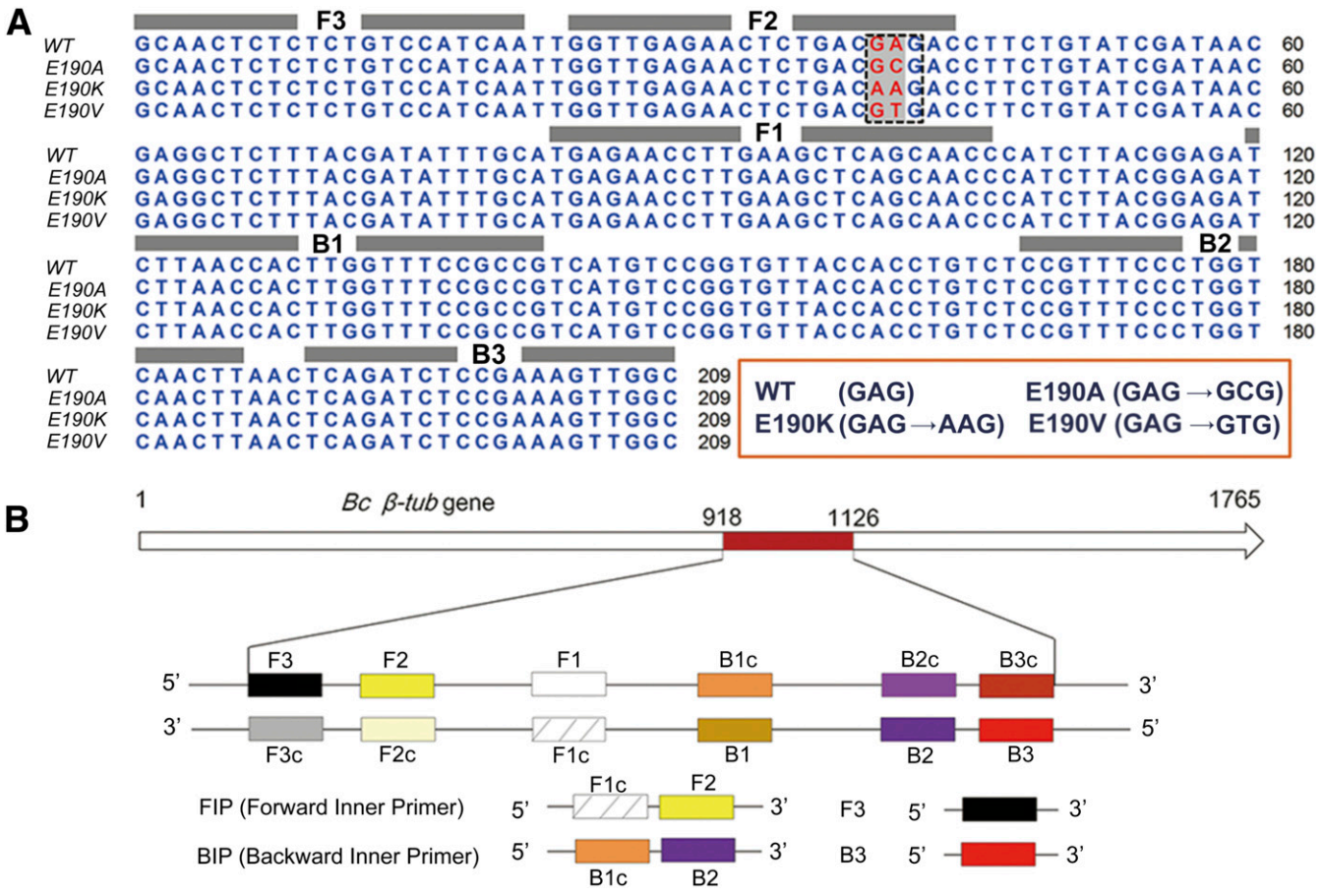

Fig. 1. Schematic diagram of loop-mediated isothermal amplification (LAMP) primers for detection of highly carbendazim-resistant β-tubulin variants of *Botrytis cinerea*. **A,** Nucleotide sequence alignment of the β-*tubulin* target fragments of the wild-type isolate Bt4-1 (WT) and the highly carbendazim-resistant mutants B05.10 (E198A), SD2 (E198K), and SD4 (E198V). Sequences used for LAMP primers are indicated by bold lines. **B,** Schematic representation of the LAMP primers.

using the cetyltrimethylammonium bromide method, according to a previous study (Duan et al. 2014b; Möller et al. 1992).

Specificity of LAMP primers. Based on three highly benzimidazole-resistant β-tubulin variants (E198V, E198A, and E198K) (Fig. 1A), a set of LAMP primers was designed with PrimerExplorer V4, as previously described (Table 2) (Duan et al. 2016a,b). To increase the specificity of LAMP amplification, we introduced an artificial base mismatch within three nucleotides of the point mutation locus (Table 2). In addition, a degenerate base was introduced in the point mutation site to simultaneously distinguish the three mutated β-tubulin variants from the wild type. Thus, five sets of LAMP primers (Table 2, Set1 to Set5) were obtained and the specificity of LAMP primers was assessed with the genomic DNA of Bt4-1, B05.10, SD4, and SD2 as templates. The LAMP products were analyzed by both hydroxynaphthol blue (HNB)-visualized color change and agarose gel electrophoresis.

Optimization of LAMP reaction components. To simultaneously distinguish the three mutated β-tubulin variant mutation genotypes from the wild type after primer evaluation, we determined the various concentrations of the LAMP components in a total volume of 10 μl, as described previously (Duan et al. 2016a, b). The LAMP assay was performed at 63°C for 60 min and the LAMP reaction products were once again evaluated by HNB-visualized color change and agarose gel electrophoresis, as described above.

Optimization of LAMP reaction conditions. Using the optimal LAMP primers and reaction components (see Results), we determined the optimal reaction temperature by incubating LAMP reaction mixtures at 58, 59, 60, 62, 63, 65, 66, or 67°C for 60 min. We then used the optimal reaction temperature to perform the optimization test of the reaction time by incubating the optimal LAMP reaction mixtures for 15, 30, 45, 60, 75, or 90 min. The test was performed twice, and each temperature was replicated twice in each test. After LAMP amplification was finished, the LAMP results were assessed as described before.

Verification of the LAMP products. To confirm that LAMP amplified the intended targets, the LAMP products were digested with restriction enzyme *Cla*I and then the digested LAMP products were analyzed by gel electrophoresis. Additionally, the primers F3 and B3 were used to amplify the target fragment for sequencing according to a previous method (Duan et al. 2016a, b).

Specificity of LAMP. Using the suitable LAMP primer Set5, we tested the specificity tests with gDNA of the wild-type isolate Bt4-1, the E198V mutant SD4, the E198A mutant B05.10, the E198K mutant SD2, the F200Y mutant B20, and eight common plant-pathogenic fungi (Table 1). The LAMP products were assessed as described above.

Sensitivity of LAMP and PCR. With the primers Bcbeta442F/Bcbeta442R (Table 2), PCR was used to amplify a 442-bp DNA fragment covering the positions of the point-mutations at codon 198 of β-*tubulin*. The PCR products were purified and cloned into the vector pEASY to create plasmid pET442. The plasmid pET442 was transferred into *Escherichia coli* cells and the positive clones was used to extract the plasmid for further testing. DNA copies were calculated by the formula number of copies (molecules) = [amount (ng) × 6.022×10^{23} (molecules/mole)]/[length(numbers of base pairs) × 660 (g/mole) × 1×10^{9} (ng/g)]. Then, the extracted plasmid was 10-fold serially diluted to obtain 2×10^{7} to $2 \times 10^{\circ}$ copies, and the dilutions were used as templates in LAMP and PCR. For LAMP, the optimized reaction components and conditions were used. PCR was performed with the primers Bcbeta442F and Bcbeta442R. The LAMP results were assessed as described above and the PCR results was analyzed in gel electrophoresis.

Table 2. Information on the primers used in this study

Primer	Sequence (5′–3′)[a]	Use[b]
F3	GCAACTCTCTCTGTCCATCAA	Forward outer primer for LAMP
B3	GCCAACTTTCGGAGATCTGA	Backward outer primer for LAMP
BIP	TCTTAACCACTTGGTTTCCGCCG-AAGTTGACCAGGGAAACGG	Backward inner primer for LAMP
FIP1	GGTTGCTGAGCTTCAAGGTTCTCA-GGTTGAGAACTCTGAC[AT]GAC	Forward inner primers to simultaneously detect three β-tubulin variants (E198A, E198K, and E198V) of *Botrytis cinerea* using LAMP
FIP2	GGTTGCTGAGCTTCAAGGTTCTCA-GGTTGAGAACTCTGAC[AT]G**T**C	
FIP3	GGTTGCTGAGCTTCAAGGTTCTCA-GGTTGAGAACTCTGAC[AC]GAC	
FIP4	GGTTGCTGAGCTTCAAGGTTCTCA-GGTTGAGAACTCTGAC[AC]G**T**C	
FIP5	GGTTGCTGAGCTTCAAGGTTCTCA-GGTTGAGAACTCTGAC[AY]GAC	
Bcbeta442F	GGTAACAACTGGGCTAAGGG	To amplify the partial fragments (442-bp) of the β-tubulin gene containing the 198 position
Bcbeta442R	GACCAGGGAAACGGAGACA	

[a] Nucleotides in frames differ in the sensitive phenotype versus the resistant phenotype. Nucleotides in bold are mismatches manually added to distinguish three mutation genotypes (E198A, E198K, and E198V) from carbendazim-sensitive wild types in *B. cinerea*.
[b] LAMP = loop-mediated isothermal amplification.

Table 3. Comparison of loop-mediated isothermal amplification (LAMP), minimum inhibitory concentration (MIC), and sequencing for detecting the benzimidazole-resistant populations of *Botrytis cinerea* from fields in Jiangsu Province, China[a]

			LAMP		MIC (>10 μg ml^{-1})		Sequencing	
Geographical origin	Host	*N*[b]	Pos	Freq (%)	Pos	Freq (%)	Pos	Freq (%)
Baitu, Zhenjiang	Strawberry	28	19	67.9	20	71.4	20	71.4
Fujiabian, Nanjing	Strawberry	31	18	58.1	18	58.1	18	58.1
Taolin, Lianyungang	Cucumber	38	26	68.4	26	68.4	26	68.4
Dingji, Huaian	Cucumber	32	25	78.1	26	81.3	26	81.3
Suojin, Nanjing	Strawberry	54	47	87.0	48	88.9	48	88.9
Xuyi, Huaian	Tomato	42	30	71.4	30	71.4	30	71.4
Total	…	225	165	73.3	168	74.7	168	74.7

[a] Isolates were obtained in 2012. Pos = positive and Freq = resistance frequency.
[b] Number of samples.

Ability of LAMP to detect field isolates known to be highly resistant to benzimidazole. The ability of LAMP to detect field isolates known to be highly resistant to carbendazim was assessed with the genomic DNA of 96 *B. cinerea* field isolates originating from different geographical regions in China (Supplementary Table S1). Some of these isolates have a point mutation resulting in β-tubulin alterations E198K, E198V, or E198A based on sequence analysis and MIC values that were previously determined.

Table 4. Detection of the highly benzimidazole-resistant populations of *Botrytis cinerea* in fields in Jiangsu Province, China by loop-mediated isothermal amplification (LAMP), minimum inhibitory concentration (MIC)[a]

Geographical origin	Host	*N*[b]	Positive in MIC	Resistance frequency by MIC (%)	Positive in LAMP	Resistance frequency by LAMP (%)
Baitu, Zhenjiang	Strawberry	84	73	86.90	72	85.71
Fujiabian, Nanjing	Strawberry	78	71	91.03	68	87.18
Suojin, Nanjing	Strawberry	65	60	92.31	58	89.23
Jianhu, Yancheng	Strawberry	28	24	85.71	23	82.14
Xuyi, Huaian	Tomato	36	32	88.89	32	88.89
Xuyi, Huaian	Cucumber	94	87	92.55	87	92.55
Taolin, Lianyungang	Tomato	103	96	93.20	94	91.26
Taolin, Lianyungang	Cucumber	85	77	90.59	77	90.59
Dingji, Huaian	Strawberry	77	75	97.40	72	93.51
Dingji, Huaian	Tomato	65	59	90.77	59	90.77
Dingji, Huaian	Cucumber	89	79	88.76	77	86.52
Shouguang, Shandong	Cucumber	63	57	90.48	57	90.48
Total	…	867	790	91.12	776	89.50

[a] Isolates were obtained in 2013.
[b] Number of samples.

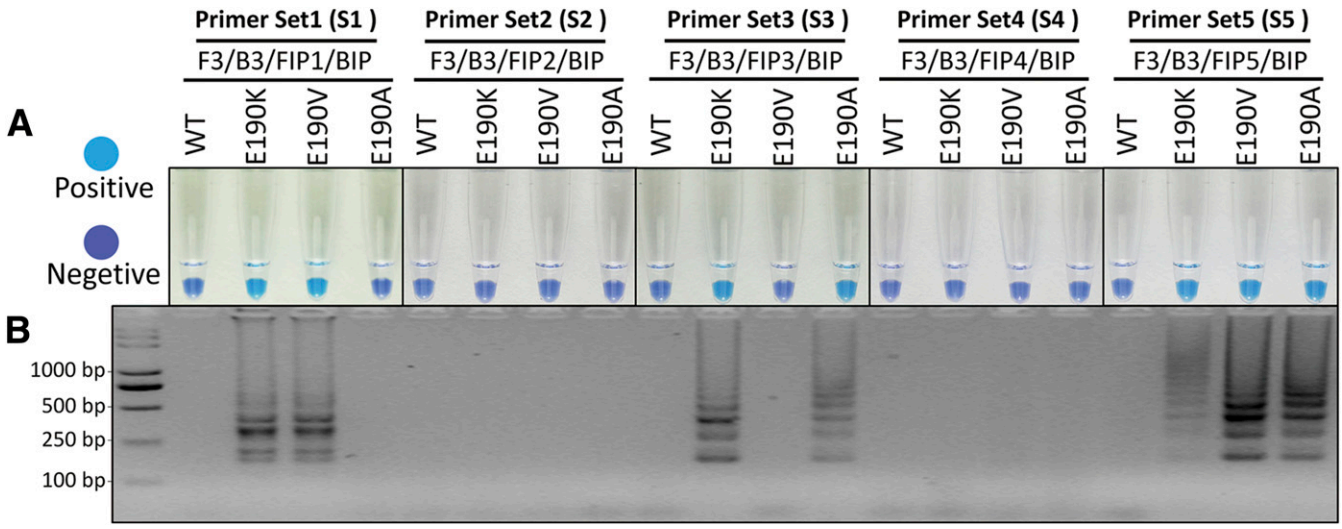

Fig. 2. Specificity of loop-mediated isothermal amplification (LAMP) assays using different primer sets. **A,** Hydroxynaphthol blue-visualized color change and **B,** gel electrophoresis analysis of the LAMP products. WT = wild type.

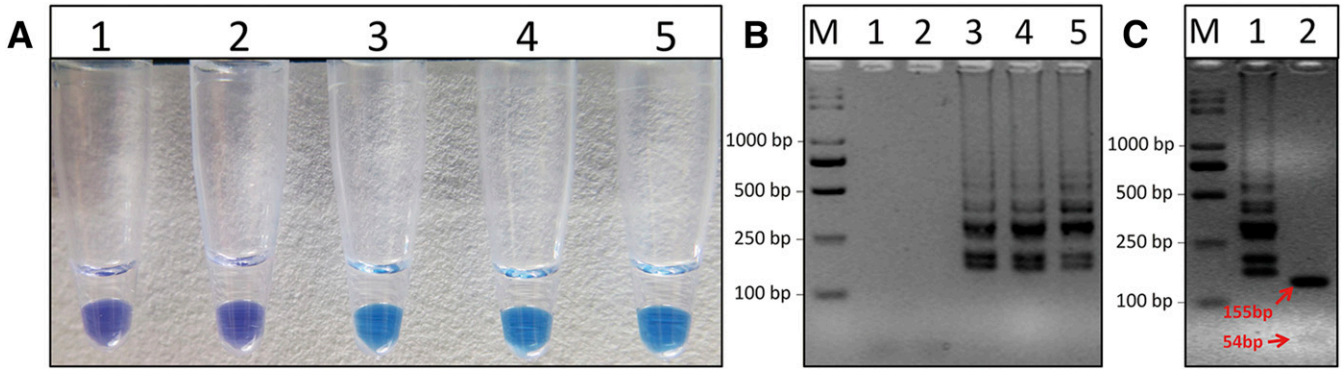

Fig. 3. Loop-mediated isothermal amplification (LAMP) detection of highly carbendazim-resistant *Botrytis cinerea* isolates and restriction enzyme digestion analysis of the LAMP products. **A,** LAMP detection of highly carbendazim-resistant isolates using hydroxynaphthol blue as a visual indicator. The reaction becomes sky blue if the β-tubulin gene has a point mutation resulting in E198A, E198K, or E198V at codon 198 but remains violet if the β-tubulin gene has no mutation at codon 198. Lane 1, double-distilled H_2O (ddH_2O); lane 2, Bt4-1 (wild type [WT]); lane 3, SD2 (E198K); lane 4, SD4 (E198V); and lane 5, B05.10 (E198A). **B,** Gel electrophoresis analysis of LAMP products. The positive reaction is indicated by a ladder-like pattern on the 3.0% agarose gel. Lane M, 2K plus (TransGen); lane 1, ddH_2O; lane 2, Bt4-1 (WT); lane 3, SD2 (E198K); lane 4, SD4 (E198V); and lane 5, B05.10 (E198A). **C,** LAMP products were digested with *Cla*I, and two fragments (54 and 155 bp) were observed by gel electrophoresis. Lane M, 2K plus (TransGen); lane 1, LAMP products without digestion; and lane 2, LAMP products digested by *Cla*I.

Comparison of LAMP, MIC, and sequencing. The optimized LAMP method was compared with MIC and sequencing results for detecting the three β-tubulin variants (E198V, E198A, and E198K) linked with high levels of carbendazim resistance in *B. cinerea*. LAMP and MIC (>10 μg ml^{-1}) were performed as described above using 225 isolates obtained from different hosts in China in 2012 (Table 3). Additionally, PCR was performed using the primers Bcbeta442F and Bcbeta442R (Table 2) and the PCR products were cloned into pEASY and sequenced.

Application of LAMP for monitoring *B. cinerea* with high levels of benzimidazole resistance in the field. To assess the usefulness of LAMP for monitoring the resistance of *B. cinerea* to carbendazim in agricultural production, 867 diseased samples were collected from the fruit of hosts strawberry, cucumber, or tomato from different fields in China in 2013. Infected lesions of these disease samples were divided into two parts. One part was used for extraction of genomic DNA according to the previous study (Duan et al. 2016a) and then subjected to LAMP. The other part was used for single-spore isolation and then subjected to MIC (Table 4).

Results

Specificity of LAMP primers. Based on color change (Fig. 2A) and the ladder-like pattern in gel electrophoresis (Fig. 2B), LAMP primer Set1 only detected the E198K and E198V β-tubulin variants and Set3 only detected E198K and E198A β-tubulin variants but neither Set2 nor Set4 detected any β-tubulin variants, including the wild type (Fig. 2A). To simultaneously detect all three mutated β-tubulin variants, a degenerate base was added to the 3′ end of the forward inner primer (FIP) to create the primer Set5. Set5 simultaneously distinguished all three mutated β-tubulin variants from the wild type (Fig. 2A). The positive samples were indicated by sky-blue color, while the negative samples remained violet (Fig. 2A). The changes in color were confirmed by gel electrophoresis (i.e., 3.0% gel electrophoresis produced a typical ladder-like pattern for positive samples but not for negative samples) (Fig. 2B). The results indicated that Set5 (Fig. 2; Table 2) could be used to detect field isolates with high levels of benzimidazole resistance resulting from the presence of one of the three known mutations in codon 198.

Optimization of LAMP reaction components. With genomic DNA of the wild type and three mutated β-tubulin variants (E198A, E198K, and E198V) as the templates, the optimization test of LAMP reaction components was performed. As expected, HNB-visualized color change (Fig. 3A) and a ladder-like pattern with 3% gel electrophoresis (Fig. 3B) were detected with three mutated β-tubulin variants but not with the wild type or double-distilled H_2O. The optimal 10-μl reaction mixture contained 2.4 U of Bst DNA polymerase, 1.0 μl of 10× ThermoPol Buffer, 1.2 mM dNTP, 4.0 mM $MgCl_2$, 0.64 M betaine, 200 μM HNB, 1.2 μM each FIP and backward inner primer, 0.3 μM each F3 and B3, and 1 μl of target DNA (20 ng μl^{-1}).

Optimization of LAMP reaction conditions. Based on the above optimized reaction components, LAMP reaction conditions (temperature and time) were optimized using genomic DNA of an E198A β-tubulin variant as template. Color change (Fig. 4A) and gel electrophoresis (Fig. 4B) of the LAMP results were evident at 58 to 65°C,

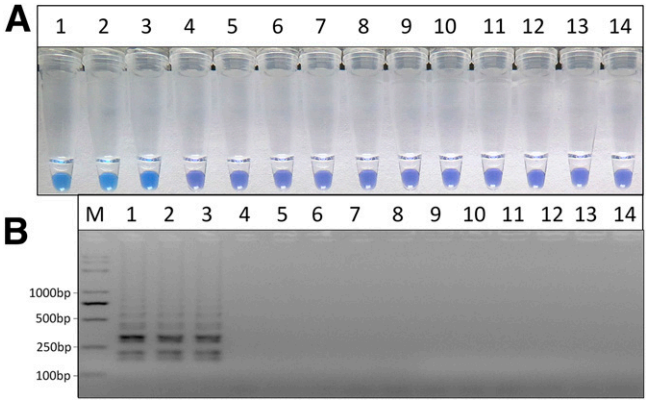

Fig. 5. Specificity of loop-mediated isothermal amplification (LAMP) detection of highly carbendazim-resistant *Botrytis cinerea* isolates. Assessment was based on **A,** hydroxynaphthol blue visualization of color change or **B,** agarose gel electrophoresis of the LAMP products. Lane M, DNA marker (TransGen); lane 1, isolate B05.10 (E198A); lane 2, isolate SD2 (E198K); lane 3, isolate SD4 (E198V); lane 4, isolate Bt4-1 (wild type); lane 5, isolate B20 (F200Y); lane 6, *Penicillium italicum*; lane 7, *P. digitatum*; lane 8, *Alternaria alternate*; lane 9, *Corynespora cassiicola*; lane 10, *Fusicladium virecens*; lane 11, *Fusarium oxysporum*; lane 12, *Sclerotinia sclertiorum*; lane 13, *Colletotrichum capsici*; and lane 14, double-distilled H_2O.

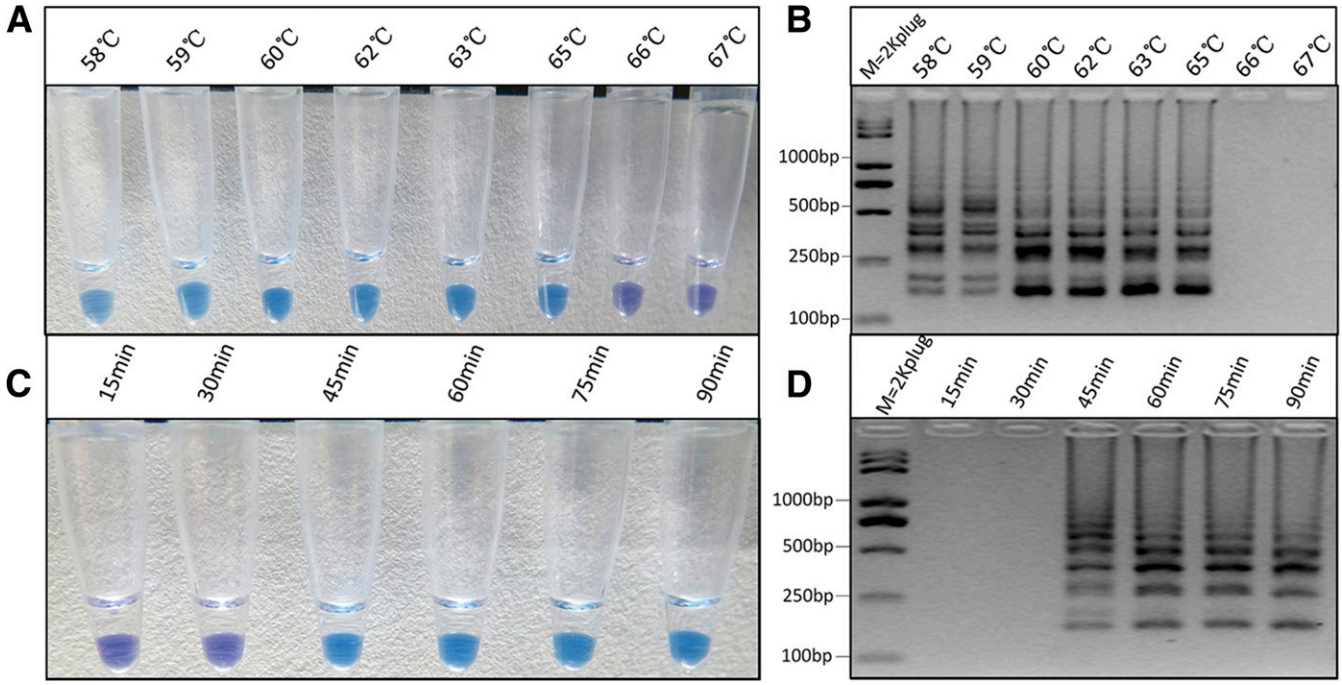

Fig. 4. Optimization of loop-mediated isothermal amplification (LAMP) reaction temperature and time. **A** and **C,** Assessment was based on hydroxynaphthol blue visualization of color change of the LAMP products. **B** and **D,** Assessment was based on gel electrophoresis of the LAMP products.

and DNA intensity was highest at 60 to 62°C (Fig. 4B). Based on the appropriate temperature (62°C), the optimization test of LAMP reaction time was further optimized. LAMP products were evident with reaction times ranging from 45 to 90 min based on color change (Fig. 4C) and gel electrophoresis (Fig. 4D). Therefore, the optimized reaction temperature was set at 60 to 62°C and the optimized reaction time was set at 45 min.

Verification of the LAMP products. The restriction enzyme analysis of LAMP products showed *Cla*I produced 54- and 155-bp bands in gel electrophoresis (Fig. 3C). This was in accord with those predicted based on the expected structures. Additionally, a 209-bp target fragment was obtained by PCR amplification using the primers F3 and B3 and transformed into *E. coli* for sequencing. The results indicated that the target fragment by PCR was consistent with β-*tubulin* used for primer design.

Specificity of LAMP. As indicated by HNB-visualization (Fig. 5A) and gel electrophoresis (Fig. 5B), LAMP assays using primer Set5 were only positive for the three mutated *B. cinerea* β-tubulin variants (E198V, E198A, and E198K). The results showed that LAMP established in this study had a high specificity for detecting the highly carbendazim-resistant isolates of *B. cinerea*.

Sensitivity of LAMP and PCR. With 10-fold serial dilutions of pET442 as templates, LAMP and PCR were performed to determine the detection limits. The limit of LAMP was 2×10^3 copies based on color change (Fig. 6A) and gel electrophoresis (Fig. 6B), whereas the limit of PCR was 2×10^4 copies based on gel electrophoresis (Fig. 6C). These results indicated that LAMP developed in this study was more sensitive than PCR.

Ability of LAMP to detect all field isolates known to be highly resistant to benzimidazoles. The 96 *B. cinerea* isolates used in the field test were originated from different geographical regions in China. They were previously tested (unpublished results) and 60 of them were found to be highly resistant to carbendazim and to carry E198K, E198V, or E198 β-tubulin variants. According to color change (Fig. 7A) and gel electrophoresis (Fig. 7B, C, D, and E), all of the highly carbendazim-resistant isolates carrying β-tubulin E198K, E198V, or E198 variants were positive in the LAMP assay (Fig. 7). All of the

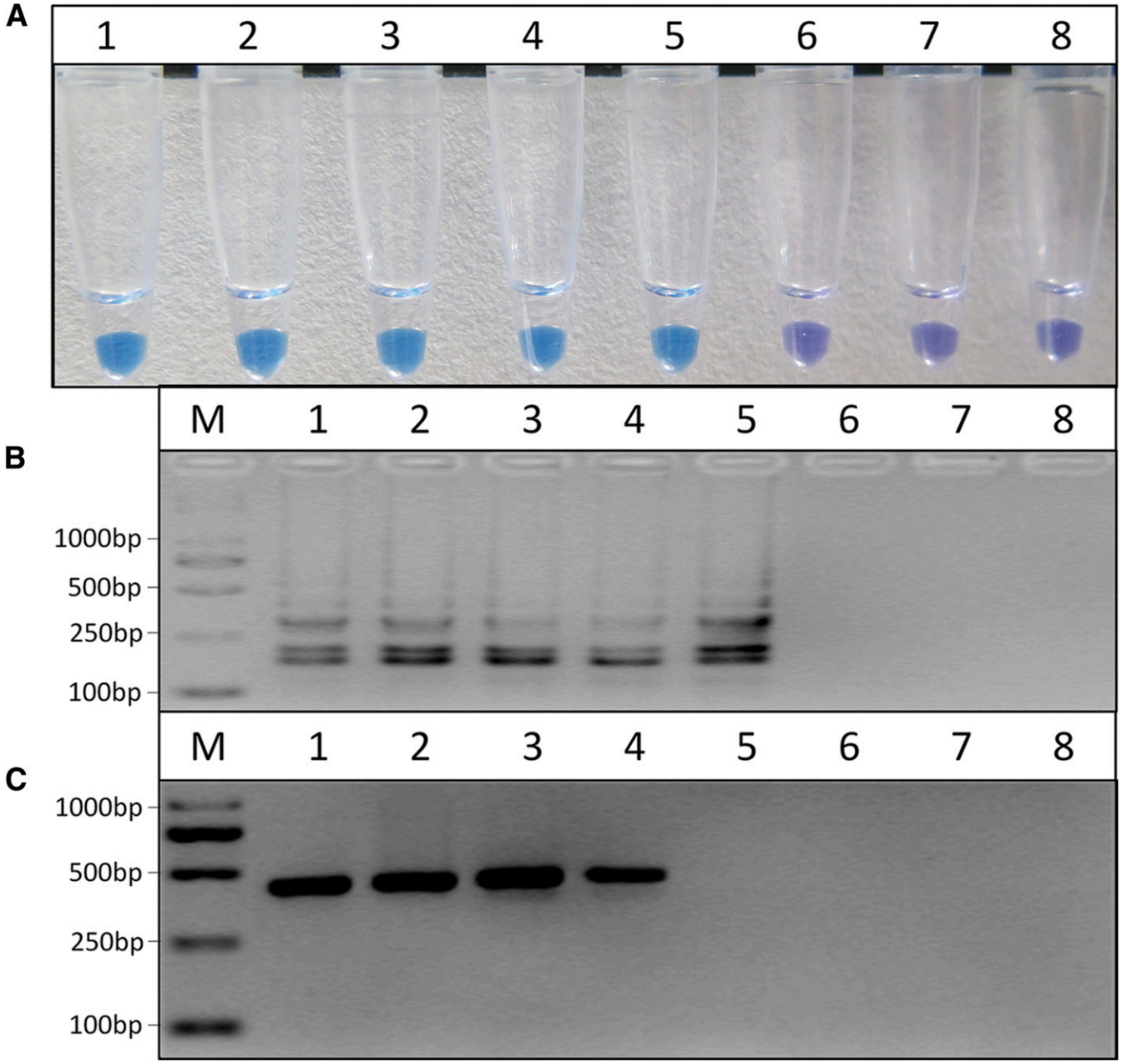

Fig. 6. Sensitivity of loop-mediated isothermal amplification (LAMP) versus conventional polymerase chain reaction (PCR) for detection of the plasmid DNA. Detection by **A,** LAMP and hydroxynaphthol blue visualization; **B,** LAMP and gel electrophoresis; and **C,** conventional PCR and gel electrophoresis. Concentrations of template DNA (copies) per reaction in were as follows: lane 1 = 2×10^7, lane 2 = 2×10^6, lane 3 = 2×10^5, lane 4 = 2×10^4, lane 5 = 2×10^3, lane 6 = 2×10^2, lane 7 = 2×10^1, and lane 8 = 2×10^0. B and C, M = 2K plus (TransGen).

benzimidazole-sensitive wild-type and moderately benzimidazole-resistant F200Y β-tubulin variants tested negative in the LAMP assay (Fig. 7).

Comparison of LAMP, MIC, and sequencing for detection of *B. cinerea* field isolates with high levels of benzimidazole resistance. In total, 238 samples of *B. cinerea*-diseased tissue were collected from strawberry, cucumber, and tomato hosts in China in 2012, and 225 single-spore isolates were obtained and tested by LAMP, MIC, and sequencing. The percentage of positive samples was 73.3, 74.7, and 74.7% for LAMP, MIC, and sequencing, respectively (Table 3). In other words, similar results were obtained with the three methods.

Application of LAMP for detecting *B. cinerea* isolates with high benzimidazole resistance in the field. In 2013, the infected lesions of 867 samples were directly used for extracting genomic DNA. When these templates were tested by LAMP in this study, 89.5% were found to be positive (Table 4). To further verify the

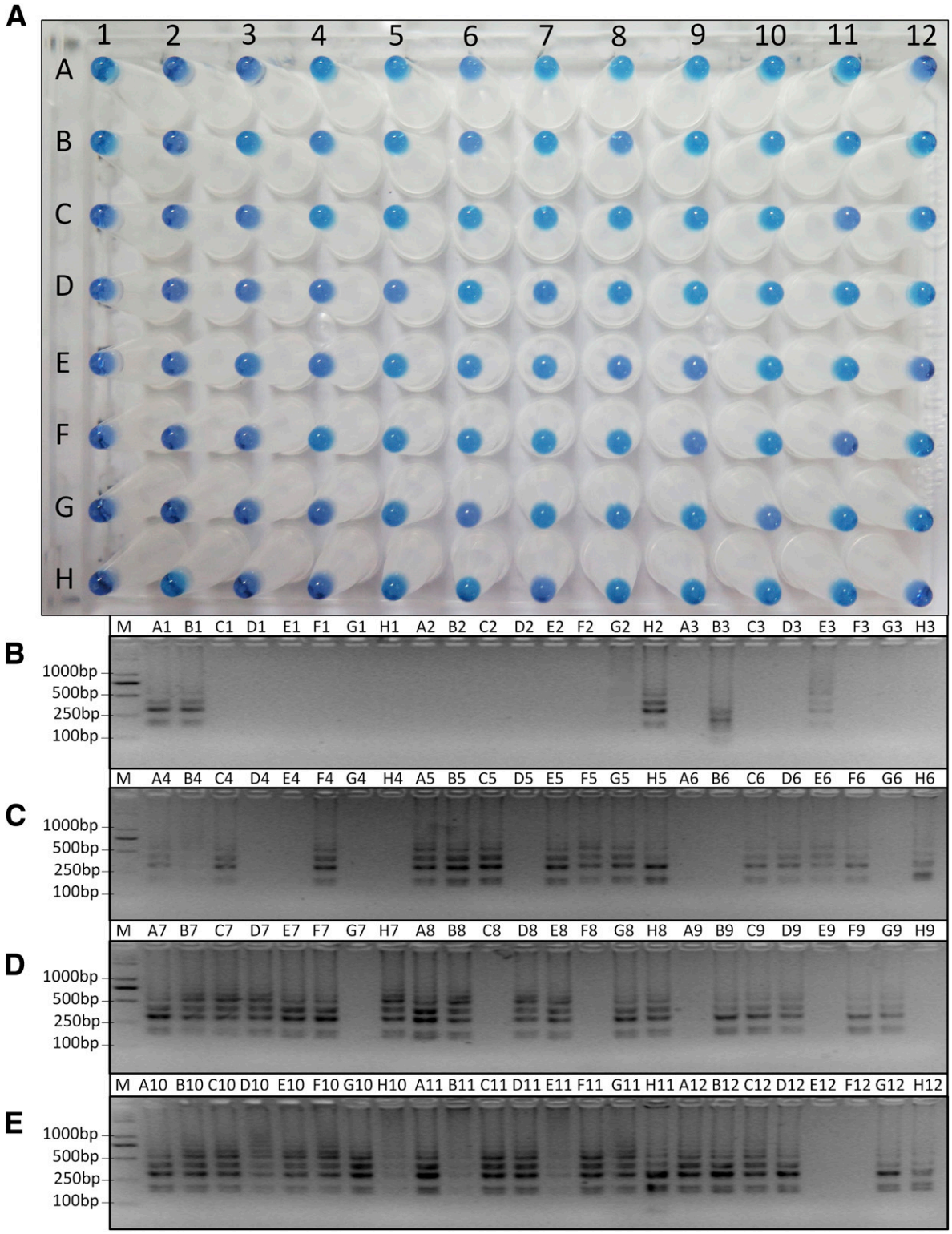

Fig. 7. Ability of loop-mediated isothermal amplification (LAMP) to detect *Botrytis cinerea* field isolates carrying β-tubulin variants E198A, E198K, or E198V. Assessment was based on **A,** hydroxynaphthol blue visualization of color change or **B, C, D,** and **E,** gel electrophoresis of the LAMP products. Each combination of letter and number indicates 1 of the 96 isolates.

application of LAMP for monitoring the resistance of *B. cinerea* to carbendazim in the field, MIC was performed using infected lesions of these samples and 91.12% were found to be positive (Table 4). In this test, we performed the LAMP assay without single-spore isolation, and the results from LAMP were similar to MIC. This further confirmed the simplicity, rapidity, and reliability of the LAMP assay compared with traditional detection methods.

Discussion

LAMP was previously used to detect single nucleotide mutations associated with fungicide resistance in fungal plant pathogens (Duan et al. 2014c, 2015, 2016a,b). In the current study, we described the development of LAMP to simultaneously detect multiple β-tubulin variants in one assay. In the fungal pathogen *B. cinerea*, a highly carbendazim-resistant level is caused by one of many point mutations at codon 198 of β-*tubulin*, resulting in the amino acid substitutions E198A, E198K, E198V, E198G, or E198L. During 2013 to 2014, our group monitored carbendazim-resistant populations of *B. cinerea* in China; resistance frequencies were 74.04 and 0.96% for highly resistant populations and moderately resistant populations, respectively. Among these highly resistant populations, resistance frequencies were 14.3, 3.9, and 81.8% for the mutation genotypes E198A, E198K, and E198V, respectively; neither E198G nor E198L were detected (unpublished data). We reported here the development of a LAMP assay for simultaneous detection of three mutations causing alterations at codon 198 of β-*tubulin* in *B. cinerea* and assessed its application for monitoring the frequency of highly carbendazim-resistant populations in the field. To our knowledge, this is the first report that describes the use of LAMP to detect multiple mutations in one assay.

An advantage of LAMP is that the results can be visually assessed. The LAMP products can be detected by adding metal-ion indicators or DNA intercalating dyes such as HNB (Goto et al. 2009), $CuSO_4$ (Zoheir and Allam 2011), SYBR green (Parida et al. 2005), Picogreen (Curtis et al. 2008), or calcein (Tomita et al. 2008). DNA intercalating dyes, however, are added after the reaction is completed, and exposure of the products increases the chance of contamination. To avoid such contamination in the current study, HNB was added before amplification to visualize the LAMP products. With HNB, a color change from violet to sky blue indicates a positive reaction and violet indicates a negative reaction (Goto et al. 2009). Results of HNB color changes in the current study were confirmed by 3% agarose gel electrophoresis and staining with ethidium bromide.

After Set5 was selected as the best LAMP primer set for distinguishing the mutated β-tubulin variants (E198A, E198K, and E198V) from the wild type, the concentrations of LAMP components, temperature, and time were optimized. The reaction temperature was set at 60 to 62°C, which is consistent with that reported in previous studies (Duan et al. 2016a, b). The reaction time of LAMP in this study was short in comparison with previous studies (Duan et al. 2016a, b), indicating that LAMP primers had a good specificity. In addition, Nagamine et al. (2002) loop forward (LF) and loop backward (LB) primers can accelerate the LAMP reaction. Although we designed LF and LB primers to accelerate the amplification reactions in this study, the addition of LF and LB primers did not distinguish the carbendazim-resistant mutants from the wild types of *B. cinerea*.

The LAMP assay was compared with more traditional methods in several tests in the current study. LAMP was 10-fold more sensitive than the PCR used in this study (Fig. 6). The ability of LAMP to reliably detect highly benzimidazole-resistant *B. cinerea* isolates carrying E198A, E198K, or E198V was assessed using 96 field isolates that were previously characterized using MIC measurements and β-tubulin gene sequence analysis. The LAMP assay found that all highly benzimidazole-resistant *B. cinerea* isolates were correctly identified based on color change and gel electrophoresis (Fig. 7). The simplicity, rapidity, and reliability of the LAMP assay was further demonstrated by assessment of 867 diseased samples collected from different hosts. In 2013, when these samples were obtained, the traditional MIC assay indicated that 91.1% were benzimidazole resistant. The same samples were tested by the LAMP assay in the current study, and 89.5% were found to be resistant. The LAMP assay was also compared with MIC and sequencing for detection of benzimidazole-resistant isolates among a collection of single-spore *B. cinerea* isolates that were sampled from the field in 2012. The results of LAMP, MIC, and sequencing were very similar, with overall positive detection frequencies of 73.3, 74.7, and 74.7%, respectively. Only three isolates produced a false-negative reaction in LAMP, and this was due to the mutation genotype (F200Y) at codon 200 of β-tubulin in the three isolates. Overall, these findings indicate that the LAMP assay is very reliable and can be used for detecting highly benzimidazole-resistant *B. cinerea* isolates in the field.

The results presented here demonstrate that LAMP, when combined with HNB, is more sensitive, specific, and practical for detecting highly benzimidazole-resistant mutants of *B. cinerea* than traditional methods. Thus, we can use this assay to monitor the development of benzimidazole resistance in *B. cinerea* under laboratory conditions. However, the assay developed in this study still requires isolation of isolates and time-consuming DNA extraction, which restrict the application of LAMP as an in-field detection assay. Therefore, we need to further simplify the procedures of genetic DNA extraction from the diseased tissues. Meanwhile, we still need to identify newly evolved benzimidazole-resistant β-tubulin variants in *B. cinerea*. This will provide essential references for early warning of benzimidazole resistance and management of control strategies of gray mold caused by *B. cinerea*.

Literature Cited

Chen, C., Zhao, W., Lu, Y., Wang, J., Chen, Y., Li, H., and Zhou, M. 2009. High-throughput detection of highly benzimidazole-resistant allele E198A with mismatch primers in allele-specific real-time polymerase chain reaction. Pest Manage. Sci. 65:413-419.

Curtis, K. A., Rudolph, D. L., and Owen, S. M. 2008. Rapid detection of HIV-1 by reverse transcription, loop-mediated isothermal amplification (RT-LAMP). J. Virol. Methods 151:264-270.

Dai, T. T., Lu, C. C., Lu, J., Dong, S., Ye, W., Wang, Y., and Zheng, X. 2012. Development of a loop-mediated isothermal amplification assay for detection of *Phytophthora sojae*. FEMS Microbiol. Lett. 334:27-34.

Duan, Y., Ge, C., Zhang, X., Wang, J., and Zhou, M. 2014a. A rapid detection method for the plant pathogen *Sclerotinia sclerotiorum* based on loop-mediated isothermal amplification (LAMP). Australas. Plant Pathol. 43:61-66.

Duan, Y., Ge, C., Zhang, X., Wang, J., and Zhou, M. 2014b. Development and evaluation of a novel and rapid detection assay for *Botrytis cinerea* based on loop-mediated isothermal amplification. PLoS ONE 9:e111094.

Duan, Y., Yang, Y., Li, T., Zhao, D., Cao, J., Shi, Y., Wang, J., and Zhou, M. 2016a. Development of a rapid and high-throughput molecular method for detecting the F200Y mutant genotype in benzimidazole-resistant isolates of *Fusarium asiaticum*. Pest Manage. Sci. 72:2128-2135.

Duan, Y., Yang, Y., Wang, J., Liu, C., He, L., and Zhou, M. 2015. Development and application of loop-mediated isothermal amplification for detecting the highly benzimidazole-resistant isolates in *Sclerotinia sclerotiorum*. Sci. Rep. 5: Article 17278.

Duan, Y., Yang, Y., Wang, Y., Pan, X., Wu, J., Cai, Y., Li, T., Zhao, D., Wang, J., and Zhou, M. 2016b. Loop-mediated isothermal amplification for the rapid detection of the F200Y mutant genotype of carbendazim-resistant isolates of *Sclerotinia sclerotiorum*. Plant Dis. 100:976-983.

Duan, Y., Zhang, X., Ge, C., Wang, Y., Cao, J., Jia, X., Wang, J., and Zhou, M. 2014c. Development and application of loop-mediated isothermal amplification for detection of the F167Y mutation of carbendazim-resistant isolates in *Fusarium graminearum*. Sci. Rep. 4: Article 7094.

Geeson, J. D. 1978. Mutational tolerance to carbendazim in *Botrytis cinerea*. Ann. Appl. Biol. 90:59-64.

Goto, M., Honda, E., Ogura, A., Nomoto, A., and Hanaki, K. 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxynaphthol blue. Biotechniques 46:167-172.

Hawkins, N. J., and Fraaije, B. A. 2016. Predicting resistance by mutagenesis: Lessons from 45 years of MBC resistance. Front. Microbiol. 7:1814.

Hou, Y., Luo, Q., Chen, C., and Zhou, M. 2011. Application of cycleave PCR to the detection of a point mutation (F167Y) in the β_2-tubulin gene of *Fusarium graminearum*. Pest Manage. Sci. 67:1124-1128.

Janisiewicz, W. J., Takeda, F., Glenn, D. M., Camp, M. J., and Jurick, W. M. 2016. Dark period following UV-C treatment enhances killing of *Botrytis cinerea* conidia and controls gray mold of strawberries. Phytopathology 106:386-394.

Liu, S. M., Duan, Y. B., Ge, C. Y., Chen, C. J., and Zhou, M. G. 2013. Functional analysis of the β_2-tubulin gene of *Fusarium graminearum* and the β-tubulin gene of *Botrytis cinerea* by homologous replacement. Pest Manage. Sci. 69:582-588.

Luo, Q. Q., Xu, J. Q., Hou, Y. P., Chen, C. J., Wang, J. X., and Zhou, M. G. 2009. PIRA-PCR for detection of *Fusarium graminearum* genotypes with moderate resistance to carbendazim. Plant Pathol. 58:882-887.

Ma, H. X., Feng, X. J., Chen, Y., Chen, C. J., and Zhou, M. G. 2009. Occurrence and characterization of dimethachlon insensitivity in *Sclerotinia sclerotiorum* in Jiangsu Province of China. Plant Dis. 93:36-42.

Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853-863.

Möller, E. M., Bahnweg, G., Sandermann, H., and Geiger, H. H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Res. 20:6115-6116.

Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289:150-154.

Nagamine, K., Kuzuhara, Y., and Notomi, T. 2002. Isolation of single-stranded DNA from loop-mediated isothermal amplification products. Biochem. Biophys. Res. Commun. 290:1195-1198.

Niessen, L., and Vogel, R. F. 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., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28:e63.

Parida, M., Horioke, K., Ishida, H., Dash, P. K., Saxena, P., Jana, A. M., Islam, M. A., Inoue, S., Hosaka, N., and 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.

Richmond, D. V., and Phillips, A. 1975. The effect of benomyl and carbendazim on mitosis in hyphae of *Botrytis cinerea* Pers. ex Fr. and roots of *Allium cepa* L. Pestic. Biochem. Physiol. 5:367-379.

Tomita, N., Mori, Y., Kanda, H., and Notomi, T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. 3:877-882.

Vitale, A., Panebianco, A., and Polizzi, G. 2016. Baseline sensitivity and efficacy of fluopyram against *Botrytis cinerea* from table grape in Italy. Ann. Appl. Biol. 169:36-45.

Williamson, B., Tudzynski, B., Tudzynski, P., and van Kan, J. A. 2007. *Botrytis cinerea*: The cause of grey mould disease. Mol. Plant Pathol. 8:561-580.

Zhang, Z., Chen, Z., Hou, Y., Duan, Y., Wang, J., Zhou, M., and Chen, C. 2015. PIRA-PCR for detection of *Fusarium fujikuroi* genotypes with carbendazim-resistance alleles. Plant Dis. 99:1241-1246.

Ziogas, B. N., Nikou, D., Markoglou, A. N., Malandrakis, A. A., and Vontas, J. 2009. Identification of a novel point mutation in the β-*tubulin* gene of *Botrytis cinerea* and detection of benzimidazole resistance by a diagnostic PCR-RFLP assay. Eur. J. Plant Pathol. 125:97-107.

Zoheir, K. M., and Allam, A. A. 2011. A rapid improved method for sexing embryo of water buffalo. Theriogenology 76:83-87.