Loop-Mediated Isothermal Amplification for the Rapid Detection of the F200Y Mutant Genotype of Carbendazim-Resistant Isolates of *Sclerotinia sclerotiorum*

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Abstract

Duan, Y., Yang, Y., Wang, Y., Pan, X., Wu, J., Cai, Y., Li, T., Zhao, D., Wang, J., and Zhou, M. 2016. 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.

The point mutation at codon 200 (TTC→TAC, F200Y) confers moderate resistance to carbendazim in *Sclerotinia sclerotiorum*. This mutant genotype (F200Y) has been detected mainly by determining the minimum inhibitory concentration (MIC), which requires 3 to 5 days. Here, we developed a loop-mediated isothermal amplification (LAMP) assay for the rapid detection of the F200Y mutant genotype of carbendazim-resistant isolates of *S. sclerotiorum*. Specific LAMP primers were designed and concentrations of LAMP components were optimized. The optimal reaction conditions were 62 to 63°C for 45 min. The new LAMP assay requires no special equipment and is highly sensitive and specific (the i.e., it generated positive results with

F200Y mutant genotype but generated negative results with other carbendazim-resistant mutants and with a variety of carbendazim-resistant mutants of *Botrytis cinerea* and *Fusarium graminearum*). Inclusion of the loop backward (LB) primer reduced the reaction time to 15 min. Results were identical with LAMP and MIC determinations. The advantages of the LB-accelerated LAMP assay for detection of the F200Y mutant genotype were demonstrated by assaying sclerotia produced on rape stems that were artificially inoculated in the field. The results indicated that the new LAMP assay represents an improved way to detect the F200Y mutant genotype of carbendazim-resistant isolates of *S. sclerotiorum*.

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is a destructive disease of rape and other crops in many countries, including the United States (Bolton et al. 2006; Purdy 1979), Canada (Bardin and Huang 2001), Australia (Letham et al. 1976), and China (Zhou and Luo 1994). Although this disease is mainly controlled by the application of fungicides (Bardin and Huang 2001; Duan et al. 2013a; Steadman 1979), the extensive use of a single fungicide selects for fungicide-resistance in the pathogen. Therefore, resistance detection is important for risk assessment and integrated control of *Sclerotinia* stem rot.

Since the early 1980s, the benzimidazole fungicide carbendazim was frequently used to control *Sclerotinia* stem rot (Ma et al. 2009b). Because carbendazim-resistant populations of *S. sclerotiorum* have appeared with increasing frequency in rape fields (unpublished data), it is necessary to monitor carbendazim resistance over large regions for effective control of this disease. Previous detection methods have been based on determination of the minimum inhibitory concentration (MIC) (Li et al. 2003; Ma et al. 2009a), which requires substantial time and effort. As an important alternative, molecular methods have been developed for detection of carbendazim-resistant genotypes, and these molecular methods are based on the detection of the mutants that result in resistance. The point mutation at codon 198 (GAG→GCG, E198A) or 200 (TTC→TAC, F200Y) of the β -tubulin gene leads to carbendazim resistance in *S. sclerotiorum*. Although polymerase chain reaction (PCR) has been developed for detecting the E198A mutant genotype (Chen et al. 2009; Li et al. 2002), molecular detection of the F200Y mutant genotype has yet to be reported. Given the importance of *Sclerotinia* stem rot and of carbendazim resistance, a rapid, economic, and simple technique is needed for detecting the F200Y mutant genotype of carbendazim-resistant isolates of *S. sclerotiorum*.

Loop-mediated isothermal amplification (LAMP) can rapidly amplify nucleic acids with high specificity, sensitivity, and efficiency

under isothermal conditions (Notomi et al. 2000). The current study describes a LAMP assay for detection of the F200Y mutant genotype of carbendazim-resistant isolates of *S. sclerotiorum*. The new LAMP assay will be useful for assessing carbendazim resistance risk in *S. sclerotiorum* and will also provide important reference data for control of *Sclerotinia* stem rot of rape or other crops.

Materials and Methods

Fungal isolates, culture conditions, and reagents. This study used three *S. sclerotiorum* isolates (JY4016, TZ25, and HA61), all of which were obtained from single sclerotia (Table 1). JY4016 is moderately resistant to carbendazim because of a point mutation at codon 200 (Phe to Tyr, F200Y) in the β -tubulin gene (SS1G_04652); TZ25 is highly resistant to carbendazim because of a point mutation at codon 198 (Glu to Ala, E198A) in the β -tubulin gene; and HA61 is sensitive to carbendazim. Single-spore isolates of *Fusarium graminearum* and *Botrytis cinerea* with moderate or high resistance to carbendazim were also used (Table 1).

The sensitivity of *S. sclerotiorum* to carbendazim was determined on potato dextrose agar medium. Based on their MIC, the tested isolates were divided into three phenotypes: highly resistant, MIC > 100 $\mu\text{g ml}^{-1}$; moderately resistant, 100 $\mu\text{g ml}^{-1}$ > MIC > 5 $\mu\text{g ml}^{-1}$; and sensitive, MIC < 5 $\mu\text{g ml}^{-1}$.

Carbendazim and other reagents used in this study were the same as in a previous study (Duan et al. 2014c).

DNA extraction. Genomic DNA was extracted from sclerotia of *S. sclerotiorum* or from mycelia of *F. graminearum* and *B. cinerea* using a mixer mill (MM400; Retsh) according to the cetyltrimethylammonium bromide procedure (Duan et al. 2013b; Möller et al. 1992).

Primer design. LAMP primers were designed using Primer explorer V4 software (<http://primerexplorer.jp/e/>) according to a previous study (Duan et al. 2014c). LAMP primers used in this study are indicated in Table 2 and Figure 1.

Specificity of LAMP primers. To improve the specificity of the LAMP primers, one or two artificial base-pair mismatches were introduced within three nucleotides of the 3' end of the forward inner primer (FIP) (Table 2). Thus, six sets of LAMP primers (Table 2) were obtained and tested to distinguish the F200Y mutant genotype from the wild type. With the genomic DNA of JY4016 or HA61 as template and with the addition of hydroxynaphthol blue (HNB), LAMP was

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Accepted for publication 9 December 2015.

<http://dx.doi.org/10.1094/PDIS-10-15-1185-RE>
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Table 1. Fungal isolates used in this study

Fungus	Isolate	Genotype description	Origin	Resistance phenotype ^a	LAMP ^b
<i>Sclerotinia sclerotiorum</i>	HA61	Wild type	Jiangsu Province, China	MBC ^S	–
<i>S. sclerotiorum</i>	JY4016	Mutation at codon 200 of β tubulin (F200Y)	Jiangsu Province, China	MBC ^{MR}	+
<i>S. sclerotiorum</i>	TZ25	Mutation at codon 198 of β tubulin (E198A)	Jiangsu Province, China	MBC ^{HR}	–
<i>Botrytis cinerea</i>	CGY004	Mutation at codon 198 of β tubulin (E198A)	Jiangsu Province, China	MBC ^{HR}	–
<i>B. cinerea</i>	SD2	Mutation at codon 198 of β tubulin (E198K)	Shandong Province, China	MBC ^{HR}	–
<i>B. cinerea</i>	SD4	Mutation at codon 198 of β tubulin (E198V)	Shandong Province, China	MBC ^{HR}	–
<i>B. cinerea</i>	B20	Mutation at codon 200 of β tubulin (F200Y)	Shandong Province, China	MBC ^{MR}	–
<i>Fusarium graminearum</i>	JT04	Mutation at codon 198 of β_2 tubulin (E198L)	Anhui Province, China	MBC ^{HR}	–
<i>F. graminearum</i>	J-2	Mutation at codon 198 of β_2 tubulin (E198K)	Anhui Province, China	MBC ^{HR}	–
<i>F. graminearum</i>	NT7	Mutation at codon 200 of β_2 tubulin (F200Y)	Anhui Province, China	MBC ^{MR}	–
<i>F. graminearum</i>	R9	Mutation at codon 167 of β_2 tubulin (F167Y)	Anhui Province, China	MBC ^{MR}	–

^a MBC^S, MBC^{MR}, and MBC^{HR} indicate that the isolate is sensitive, moderately resistant, and highly resistant to MBC, respectively.

^b Loop-mediated isothermal amplification: + and – represent positive and negative results, respectively.

Table 2. Primers used in this study

Primer	Sequence (5'–3') ^a	Use ^b
F3	CTCTCTCTGTTTCATCAATTGG	FOP for LAMP
B3	CAACTTTCGGAGATCTGAGT	BOP for LAMP
BIP	CTACGGAGATCTTAACCACTTGGTTAAGTTGACCAGGGAACCGGA	BIP for LAMP
LB	TCCGCTGTCATGTCCGG	LB primer for LAMP
FIP1	GGTTCTCATGCAAATGTCGTAGAGCGAGAACTCTGACGAGACCTA	FIP F200Y
FIP2	GGTTCTCATGCAAATGTCGTAGAGCGAGAACTCTGACGAGACCAA	FIP F200Y
FIP3	GGTTCTCATGCAAATGTCGTAGAGCGAGAACTCTGACGAGACGTA	FIP F200Y
FIP4	GGTTCTCATGCAAATGTCGTAGAGCGAGAACTCTGACGAGACCTAG	FIP F200Y
FIP5	GGTTCTCATGCAAATGTCGTAGAGCGAGAACTCTGACGAGACCTACA	FIP F200Y
FIP6	GGTTCTCATGCAAATGTCGTAGAGCGAGAACTCTGACGAGACGAA	FIP F200Y
Ssbeta383F	TGATGTCGTTCTGCTGTGAGGC	Partial fragment
Ssbeta383R	GACCAGGGAACCGAGACAGG	Partial fragment

^a Nucleotides in frames were modified from the sequence of the β -tubulin gene in the sensitive genotype and the F200Y mutant genotype. Nucleotides in bold are mismatches manually added to specifically detect the F200Y mutant genotype of *S. sclerotiorum*.

^b FOP = forward outer primer, LAMP = loop-mediated isothermal amplification, BOP = backward outer primer, BIP = backward inner primer, LB = loop backward, FIP = forward inner primer, FIP F200Y = FIP for LAMP detection the F200Y mutant genotype of *S. sclerotiorum*, and Partial fragment = to amplify the partial fragments (383 bp) of the β -tubulin gene containing the F200Y mutation position.

Fig. 1. Schematic illustration of loop-mediated isothermal amplification (LAMP) primers used for detection of F200Y mutant genotype of *Sclerotinia sclerotiorum*. **A**, Nucleotide sequence alignment of the target region of the β -tubulin gene in the wild-type (WT) isolate HA61 and in the moderately carbendazim-resistant isolate JY4016. Sequences used for LAMP primers are indicated by bold lines. The point mutation in the black frame (TTC→TAC) leads to a moderate level of carbendazim resistance. **B**, Schematic representation of LAMP primers used in this study. Construction of the inner primers FIP and BIP is shown. F1c and B1c are complementary to F1 and B1, respectively.

performed at 63°C for 60 min and then at 80°C for 10 min to terminate the reaction. Each product was analyzed by 3.0% agarose gel electrophoresis; the gels were stained with ethidium bromide and photographed under a UV transilluminator. In addition, the amplification product could also be visually assessed with the unaided eye because HNB caused the color of the mixture in the reaction tube to change from violet to sky blue if the product was present but to remain violet if the product was absent.

Optimization of LAMP reaction components and conditions. As previously described (Duan et al. 2014c), concentrations of LAMP reaction components were optimized in a total volume of 10 µl using the optimized LAMP primers and the genomic DNA of *S. sclerotiorum* JY4016 as template. Then, LAMP reaction temperature and time were also optimized as previously described (Duan et al. 2014c). As before, the results of the LAMP assay were assessed based on both HNB-visualized color change and gel electrophoresis.

Confirmation of the LAMP products. LAMP products were digested in a final volume of 20 µl that contained 1 µl of *Msp*I (Takara), 2 µl of 10× T Buffer, 2 µl of 0.1% bovine serum albumen, 8 µl of LAMP products, and 7 µl of double-distilled (dd)H₂O. The mixture was incubated at 37°C overnight, after which the DNA bands were analyzed by 3% agarose gel electrophoresis; the gels were stained with ethidium bromide and photographed as described earlier. To confirm the identities of the LAMP products, the PCR products were amplified using primers F3 and B3. The PCR mixtures contained 10 µM F3 and B3 (2 µl of each primer), 2 µl of 2.5 mM dNTP, 2.5 µl of 10× PCR buffer (Mg²⁺ free), 1.5 µl of 25 mM MgCl₂, 0.125 µl of rTaq at 5 U µl⁻¹, 1 µl of template DNA, and 13.875 µl of ddH₂O. PCR assays were performed as follows: 94°C for 2 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. The PCR products were purified by with a gel extraction kit and were cloned into pEASY-T1 vector for sequence analysis.

Specificity of LAMP. The specificity of LAMP was tested with genomic DNA of the fungal isolates or mutants listed in Table 1. The LAMP assay was performed and assessed as described in the previous section and with the optimal LAMP primers and reaction parameters.

Sensitivity of LAMP and PCR. A 383-bp DNA fragment containing the point mutation at codon 200 of the β-tubulin gene was amplified by PCR using the primers Ssbeta383F and Ssbeta383R. The PCR products were purified and cloned into pEASY-T1 vector to create pET383, as described above. The recombinant plasmid pET383 was extracted from positive clones and was 10-fold serially diluted to obtain templates that contained from 1 × 10⁹ to 1 × 10⁰ copies. The templates were subjected to LAMP and PCR. Primers F3 and B3 were used for PCR detection. LAMP was performed as described earlier and with the optimal LAMP primers and reaction parameters.

LAMP detection of *S. sclerotiorum* F200Y mutants from different geographical regions. LAMP was conducted with the genomic DNA of 11 known *S. sclerotiorum* F200Y mutants from different geographical regions in China (Table 3). Four wild-type isolates and ddH₂O were used as negative controls.

Acceleration of LAMP by loop primers. To reduce the time required for the LAMP assay, the loop backward (LB) primer was added to the LAMP reaction. LAMP was performed as described earlier with the genomic DNA of JY4016 or HA61 as template.

LAMP field test with artificial inoculation. To evaluate the feasibility of using LAMP to detect the F200Y mutant genotype of carbendazim-resistant mutants of *S. sclerotiorum*, rape stems ('Qinyou number 7') in a field in Jiangsu Province were inoculated with the following mutants or isolates, as previously described (Freeman et al. 2002): four F200Y mutants, three E198A mutants, and three carbendazim-sensitive isolates (Table 4). For each mutant or isolate, one stem on each of three rape plants was inoculated. Multiple sclerotia developed on all inoculated stems. The sclerotia from each stem were divided into two groups; one group was assessed by LAMP (with and without the LB primer) and the other was assessed by MIC determination.

Results

Specificity of LAMP primers. Based on the point mutation, a LAMP primer set was designed. To determine whether the specificity of this primer could be improved, base pair mismatches were added to the 3' end of FIP, resulting in six sets of LAMP (Table 2). Genomic

Table 4. Comparison of loop-mediated isothermal amplification (LAMP) and minimum inhibitory concentration (MIC) for detecting the F200Y mutation in carbendazim-resistant isolates of *Sclerotinia sclerotiorum* in a field test with artificial inoculation

Isolate	Mutation	N ^a	Number of positive samples by		
			LAMP	LB ^b	MIC
JH4003	No mutation	32	0	0	0
RD4084	No mutation	38	0	0	0
NT4070	No mutation	44	0	0	0
JY4016	TTC→TAC, Phe to Tyr, F200Y	36	36	36	36
MAS117	TTC→TAC, Phe to Tyr, F200Y	28	28	28	28
ZG1106	TTC→TAC, Phe to Tyr, F200Y	22	22	22	22
LA305	TTC→TAC, Phe to Tyr, F200Y	16	16	16	16
JZ2067	GAG→GCG, Glu to Ala, E198A	43	0	0	0
NT4082	GAG→GCG, Glu to Ala, E198A	35	0	0	0
QQ2351	GAG→GCG, Glu to Ala, E198A	19	0	0	0

^a Total number of sclerotia from three replicates.

^b Loop backward (LB) primer-accelerated LAMP.

Table 3. Loop-mediated isothermal amplification (LAMP) and minimum inhibitory concentration (MIC) detection of the F200Y mutation in *Sclerotinia sclerotiorum* isolates obtained from rape fields in different areas in China

Isolate	Origin	Mutation of β-tubulin	LAMP	MIC
JH4003	Jianhu, Jiangsu Province	No mutation	–	–
RD4084	Rudong, Jiangsu Province	No mutation	–	–
NT4070	Nantong, Jiangsu Province	No mutation	–	–
YZ4018	Yizheng, Jiangsu Province	No mutation	–	–
JY4016	Jiangyan, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+
JK53D5	Lianyungang, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+
JY4142	Jiangyan, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+
NT1023	Nantong, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+
MAS117	Maanshan, Anhui Province	TTC→TAC, Phe to Tyr, F200Y	+	+
ZG1106	Zhangjiagang, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+
LA305	Liuan, Anhui Province	TTC→TAC, Phe to Tyr, F200Y	+	+
SY103	Sheyang, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+
SZ44	Suzhou, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+
JR1309	Jurong, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+
XY203	Xuyi, Jiangsu Province	TTC→TAC, Phe to Tyr, F200Y	+	+

DNA of the F200Y mutant genotype and the wild type were used to assess LAMP based on HNB-visualized color change (Fig. 2B) and gel electrophoresis (Fig. 2C). The positive LAMP reaction was indicated by a sky-blue color in tubes with HNB (Fig. 2B) and by a typical ladder-like pattern in gels (Fig. 2C); for negative samples, the tubes remained violet (Fig. 2B) and the gels lacked DNA bands (Fig. 2C). The results indicated that four of the six primer sets (S2, S3, S4, and S5) could be used to detect the F200Y mutant genotype of carbendazim-resistant mutants of *S. sclerotiorum*. The primer set S2 was randomly selected for the further tests in this study.

Optimization of LAMP reaction components. LAMP reaction components were optimized for detection of the F200Y mutant genotype using genomic DNA of JY4016, TZ25, and HA61 as the templates. The best results in terms of HNB-visualized color change (Fig. 3A) and the ladder-like pattern with 3% gel electrophoresis (Fig. 3B) were obtained when the 10- μ l reaction mixture contained 2.4 U of Bst DNA polymerase, 20 mM Tris-HCl, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Triton X-100, 4.5 mM MgCl_2 , 0.8 mM dNTP, 0.64 M betaine, 0.2 mM HNB, 1.2 μ M each of FIP and BIP, 0.3 μ M each of F3 and B3, and 1 ng of target DNA.

Restriction enzyme digestion and sequence analysis of the LAMP products. To confirm that the LAMP products were the target fragments, the positive LAMP products were digested with the restriction enzyme *MspI* and subjected to gel electrophoresis. The resulting 146- and 57-bp bands (Fig. 3C) were consistent with the bands that were predicted based on the structure of the target fragment. The 203-bp fragment was also amplified by PCR and cloned into the pEASY-T1 vector to create pET203. The plasmid pET203 was transformed into competent cells, and the positive clones were sequenced. The results showed that the 203-bp target fragment was 100% homologous to the β -tubulin gene used for primer design.

Optimization of LAMP reaction conditions. Reaction temperature and time were optimized using optimized reaction components and genomic DNA of JY4016 as the template. Color change from violet to sky blue in tubes was observed at 59 to 66°C (Fig. 4A) and the ladder-like pattern was observed on gels at 57 to 66°C (Fig. 4B) but the bands on gels were dense at 62 to 63°C (Fig. 4B). Thus, 62°C was selected as the reaction temperature for determining the optimal reaction time. HNB-visualized color change in tubes (Fig. 5A) and the ladder-like pattern in gels (Fig. 5B) were not observed until the

Fig. 2. Optimization of the loop-mediated isothermal amplification (LAMP) primers. **A**, LAMP primer sets used for optimization. Template DNA was obtained from a *Sclerotinia sclerotiorum* strain with the F200Y mutation (F200Y) or from a carbendazim-sensitive wild-type strain (WT). Assessment was based on **B**, hydroxynaphthol blue-visualized color change of the LAMP products and **C**, gel electrophoresis of the LAMP products.

Fig. 3. Loop-mediated isothermal amplification (LAMP) detection of the F200Y mutant genotype in carbendazim-resistant mutants of *Sclerotinia sclerotiorum*, and digestion of the positive LAMP products. **A**, LAMP detection as indicated by hydroxynaphthol blue-visualized color change. The reaction becomes sky blue if the β -tubulin gene has the point mutation at codon 200 but remains violet if the β -tubulin gene has no mutation or a different mutation. Tube 1, HA61; tube 2, TZ25; tube 3, JY4016; tube 4, double-distilled (dd)H₂O. **B**, LAMP detection as indicated by gel electrophoresis. The positive reaction is manifested as a ladder-like pattern on the 3.0% agarose gel. Lane M, 2K plus; lane 1, HA61; lane 2, TZ25; lane 3, JY4016; lane 4, ddH₂O. **C**, LAMP products were digested with *MspI* and then subjected to gel electrophoresis. Lane M, 2K plus; lane 1, LAMP products without digestion; lane 2, LAMP products after digestion by *MspI*; two fragments (146 and 57 bp) were observed.

reaction time was increased to 45 min. Therefore, 62 to 63°C for 45 min were selected as the best reaction conditions for LAMP detection of the F200Y mutant genotype of *S. sclerotiorum*.

Specificity of LAMP. Among the tested fungal isolates or mutants (Table 1), only the F200Y mutant genotype of *S. sclerotiorum* produced positive results based on HNB-visualized color change (Fig. 6A) and

gel electrophoresis (Fig. 6B). This indicated that the established LAMP method had good specificity for detecting the F200Y mutant genotype of carbendazim-resistant mutants of *S. sclerotiorum*.

Sensitivity of LAMP and PCR. To determine the detection limit, PCR and LAMP were performed with 10-fold serial dilutions of the plasmid pET383 as template. Based on HNB-visualized color change

Fig. 4. Optimization of the loop-mediated isothermal amplification (LAMP) reaction temperature for detection of the F200Y mutant genotype in carbendazim-resistant mutants of *Sclerotinia sclerotiorum*. The experiment used the optimized concentrations of LAMP reaction components, the optimized LAMP primers, and the genomic DNA of *S. sclerotiorum* JY4016 as template. Assessment based on **A**, hydroxynaphthol blue-visualized color change and **B**, gel electrophoresis.

Fig. 5. Optimization of the loop-mediated isothermal amplification (LAMP) reaction time for detection of the F200Y mutant genotype in carbendazim-resistant mutants of *Sclerotinia sclerotiorum*. The experiment used the optimized concentrations of LAMP reaction components, the optimized LAMP primers and temperature, and the genomic DNA of *S. sclerotiorum* JY4016 as template. Assessment based on **A**, hydroxynaphthol blue-visualized color change and **B**, gel electrophoresis.

in tubes (Fig. 7A) and the ladder-like pattern in gels (Fig. 7B), the detection limit of the established LAMP assay was 1×10^2 copies. Based on gel electrophoresis, the detection limit of PCR was 1×10^6 copies (Fig. 7C). This indicated that LAMP was 10,000 times more sensitive than PCR.

LAMP detection of *S. sclerotiorum* F200Y mutants from different geographical regions. Before this experiment was conducted, the carbendazim sensitivity of the 15 isolates from different areas was determined (Table 3); based on MIC, 4 of the isolates were sensitive to carbendazim and 11 were resistant. For the resistant isolates, the point mutant genotype at codon 200 in the β -tubulin gene

was confirmed by sequence analysis; the absence of the point mutation was also confirmed for the sensitive isolates. LAMP results were negative for the 4 carbendazim-sensitive isolates and for ddH₂O but were positive for the 11 F200Y mutants, as indicated by HNB-visualized color change in tubes (Fig. 8A) and by the ladder-like pattern in gels (Fig. 8B). The results indicated that LAMP could detect *S. sclerotiorum* F200Y mutants obtained from different fields and areas.

Acceleration of LAMP by loop primers. The LAMP reaction can be accelerated by the addition of two loop primers, termed loop forward (LF) and LB primers (Nagamine et al. 2002). In this study, only the LB primer was designed to accelerate the LAMP reaction

Fig. 6. Specificity of loop-mediated isothermal amplification (LAMP) detection of the F200Y mutant genotype in carbendazim-resistant mutants of *Sclerotinia sclerotiorum*. Assessment was based on **A**, hydroxynaphthol blue-visualized color change or **B**, gel electrophoresis. Lane M, 2K plus; lane 1, wild-type isolate HA61 of *S. sclerotiorum*; lane 2, E198A mutant of *S. sclerotiorum*; lane 3, F200Y mutant of *S. sclerotiorum*; lane 4, E198A mutant of *Botrytis cinerea*; lane 5, E198K mutant of *B. cinerea*; lane 6, E198V mutant of *B. cinerea*; lane 7, F200Y mutant of *B. cinerea*; lane 8, E198K mutant of *Fusarium graminearum*; lane 9, E198 L mutant of *F. graminearum*; lane 10, F167Y mutant of *F. graminearum*; lane 11, F200Y mutant of *F. graminearum*.

Fig. 7. Sensitivity of loop-mediated isothermal amplification (LAMP) versus conventional polymerase chain reaction (PCR) for detection of the F200Y mutant genotype in carbendazim-resistant mutants of *Sclerotinia sclerotiorum*. Detection by **A**, LAMP and hydroxynaphthol blue visualization; **B**, LAMP and gel electrophoresis; and **C**, conventional PCR and gel electrophoresis. The template contained the indicated number of copies of the pET383 recombinant plasmid; the pET383 plasmid contained the 383-bp DNA fragment with the F200Y mutation.

and to distinguish the F200Y mutants from the wild-type isolates. The results indicated that the signal increased at 15 min with the LB primer (Fig. 9A and B) rather than at 45 min without the LB primer (Fig. 5A and B). Although addition of the LB primer can accelerate the LAMP reaction, the LAMP assay with the LB primer failed to distinguish between F200Y mutants and wild types when the reaction time was increased to 45 min (Fig. 9A and B). Thus, the reaction time of the LAMP assay should be 15 min when the LB primer is used.

LAMP field test with artificial inoculation. In total, 313 sclerotia of *S. sclerotiorum* were collected from the artificially inoculated rape stems. The sclerotia were divided into two groups. One group was subjected to the LAMP assay (with and without the LB primer), and the other was subjected to the MIC assay. The results for three assays (LAMP, LB-accelerated LAMP, and MIC) were identical (i.e., the assays yielded positive results for sclerotia produced by F200Y mutants and negative results for sclerotia produced by E198A mutants or wild-type isolates) (Table 4).

Discussion

LAMP is a novel nucleic acid-amplification assay with high specificity and sensitivity, and is also simple to perform. LAMP has been successfully used for the detection of bacterial pathogens (Lee et al. 2015; Niu et al. 2015), viral pathogens (Okuda et al. 2015; Wang et al. 2015), fungal pathogens (Dai et al. 2012; Duan et al. 2014a,b; Niessen and Vogel 2010), and parasites (Kogovšek et al. 2015; Zhuo et al. 2015) in both animals and plants. LAMP has not been used, however, for detecting drug-resistant mutants. In the current study, we developed a LAMP assay for detection of the F200Y mutant genotype of carbendazim-resistant mutants of *S. sclerotiorum*. To our knowledge, this is the first published study to use LAMP for the detection of F200Y mutants of *S. sclerotiorum*.

LAMP is performed isothermally without a thermal cycler (Notomi et al. 2000), and the results can be visually assessed based on turbidity (Patel et al. 2015) or by adding DNA-intercalating dyes or metal-ion indicators such as SYBR green (Parida et al. 2005), Picogreen (Curtis et al. 2008), HNB (Goto et al. 2009), CuSO₄ (Zoheir and Allam 2011), or calcein (Tomita et al. 2008). Turbidity detection requires expensive equipment, which is not suitable for small laboratories. DNA-intercalating dyes are usually added after the reaction is complete, and such addition can easily result in contamination. The risk of contamination is reduced by adding the metal-ion indicator HNB before amplification and by keeping the reaction tubes closed. In HNB-visualized detection, positive samples change from violet to sky blue while negative samples remain violet. LAMP results can also be assessed by gel electrophoresis; positive samples generate a ladder-like pattern in the agarose gel.

To guarantee the specificity of the LAMP primers developed in this study, we tested six sets of LAMP primers, and four of these primer sets distinguished between *S. sclerotiorum* strains with and without the F200Y mutation. Although any one of these four sets

of primers could probably be used, we randomly selected S2 as the primer set to be used for optimization of LAMP reaction components and conditions and for further study of assay performance. Once component concentrations were optimized, we found that the optimal LAMP conditions were 62 to 63°C for 45 min.

Nagamine et al. (2002) reported that the LAMP reaction could be accelerated by the addition of LF and LB primers. To accelerate the LAMP reaction for detection of the F200Y mutant genotype of *S. sclerotiorum*, we designed an LB primer. The addition of this primer reduced the LAMP reaction time from 45 to 15 min. However, if the assay was run in the presence of the LB primer for 45 min, it failed to distinguish between an F200Y mutant and a wild-type isolate. Thus, the reaction should keep at 15 min if the LB primer is used.

We tested the specificity of the LAMP assay developed here with DNA extracted from different carbendazim-resistant mutants of *S. sclerotiorum*, *B. cinerea*, and *F. graminearum* whose resistance was based on various point mutations in the β -tubulin gene. The assay was specific in that it generated positive results only for the F200Y mutants in *S. sclerotiorum*. The new LAMP assay also generated positive results for all 11 *S. sclerotiorum* F200Y mutants that were obtained from different fields in different areas of China. The detection limits of the new LAMP assay and conventional PCR were compared using serial 10-fold dilutions of plasmid pET383 as templates; the plasmid

Fig. 9. Acceleration of loop-mediated isothermal amplification (LAMP) by the loop backward primer for the detection of the F200Y mutant genotype in carbendazim-resistant mutants of *Sclerotinia sclerotiorum*. Template DNA was obtained from a *Sclerotinia sclerotiorum* strain with the F200Y mutation (F200Y) or from a carbendazim-sensitive wild-type strain (WT). Assessment based on **A**, hydroxynaphthol blue-visualized color change or **B**, gel electrophoresis.

Fig. 8. Loop-mediated isothermal amplification (LAMP) detection of *Sclerotinia sclerotiorum* F200Y mutants from different geographical regions. Assessment based on **A**, hydroxynaphthol blue visualized color change or **B**, gel electrophoresis. Lane 1, double-distilled H₂O; lanes 2 to 5, wild-type isolates; lanes 6 to 16, known F200Y mutants. The order of isolates and mutants (from left to right) is the same as in Table 3 (from top to bottom).

contained a DNA fragment with the F200Y mutation. The new LAMP assay was 10,000 times more sensitive than conventional PCR.

The detection of carbendazim-resistant mutants of *S. sclerotiorum* by MIC often requires 2 to 5 days. Although PCR requires only 4 to 5 h, it requires expensive equipment, including a PCR thermocycler, an electrophoresis set, and a gel-documentation system. In addition, the PCR assay exposes researchers to toxic materials or conditions, including ethidium bromide, gel-red, and ultraviolet light. The LAMP assay greatly reduces the detection time and requires only a heating block, which is available in most laboratories. The results of the LAMP assay can be visually assessed without special or expensive equipment.

To assess the application of LAMP for detecting the F200Y mutant genotype in carbendazim-resistant mutants of *S. sclerotiorum* in the field, we artificially inoculated rape stems in a field with a mutant and wild-type isolate and subjected the sclerotia that developed to the three assay methods: LAMP, LB-accelerated LAMP, and MIC. The results were identical for all three assay methods, while the LAMP assay was easier to perform than the MIC assay and the LB-accelerated LAMP was the most rapid.

Therefore, we conclude that the LB-accelerated LAMP assay described here will be useful for the detection of the F200Y mutant genotype of carbendazim-resistant mutants of *S. sclerotiorum*. Although the current study reported on *S. sclerotiorum* isolates exclusively from rape fields, our unpublished data indicate that the LB-accelerated LAMP assay will be useful for isolates obtained from any crop.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (31401764), the Fundamental Research Funds for the central Universities (KJQN201508), the Youth Foundation of Jiang'su Scientific Committee (BK20140679), the Special Fund for Agro-scientific Research in the Public Interest (201303023), and the Students' Research Training Plan Program (1512A01).

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