Annals of Applied Biology ISSN 0003-4746

RESEARCH ARTICLE

Development and application of a simple, rapid and sensitive method for detecting moderately carbendazim-resistant isolates in *Botrytis cinerea*

Y.B. Duan[1], Y. Yang[1], M.X. Li[1], T. Li[1], B.A. Fraaije[2] & M.G. Zhou[1]

1 College of Plant Protection, State & Local Joint Engineering Research Center of Green Pesticide Invention and Application, Nanjing Agricultural University, Nanjing, China
2 Biointeractions & Crop Protection Department, Rothamsted Research, Harpenden, UK

Keywords
Botrytis cinerea; carbendazim; fungicide resistance; loop-mediated isothermal amplification; β-tubulin.

Correspondence
Prof. Y.B. Duan, College of Plant Protection, State & Local Joint Engineering Research Center of Green Pesticide Invention and Application, Nanjing Agricultural University, Nanjing 210095, China. Email: dyb@njau.edu.cn; and Prof. M.G. Zhou, College of Plant Protection, State & Local Joint Engineering Research Center of Green Pesticide Invention and Application, Nanjing Agricultural University, Nanjing 210095, China. Email: mgzhou@njau.edu.cn

Received: 18 November 2017; revised version accepted: 13 February 2018; published online: 13 March 2018.

doi:10.1111/aab.12426

Abstract

Sustainable disease management depends on the ability to monitor the development of fungicide resistance in pathogen populations. A point mutation resulting in an alteration (F200Y) at codon 200 of the target protein β-tubulin leads to a moderate level of resistance to carbendazim in *Botrytis cinerea*. Although traditional methods remain a cornerstone in detection of fungicide resistance, molecular methods that do not require the isolation of pathogens, can detect the presence of resistance alleles at low frequencies, and require less time and labour than traditional methods. In this study, we present an efficient, rapid, and highly specific method for detecting the moderately carbendazim-resistant isolates in *B. cinerea* based on loop-mediated isothermal amplification (LAMP). By using specific LAMP primers, we detected the resistance-conferring mutation underlying β-tubulin F200Y. The concentrations of LAMP components and LAMP parameters were optimised, resulting in reaction temperatures and times of 61–65°C and 45 min, respectively. The feasibility of the LAMP assay was verified by assaying the diseased samples with artificial inoculation in the different hosts. The LAMP assay developed in the current study was specific, stable, repeatable and sensitive, and was successfully applied for detection of moderately carbendazim-resistant isolates of *B. cinerea* in plant samples.

Introduction

Monitoring of fungicide resistance is becoming more important because of increased number of resistance cases in key agricultural pathogens. *Botrytis cinerea*, the causal agent of grey mould in many plant species, is regarded as a high-risk plant pathogenic fungus. At present, this pathogen has widely developed resistance to a variety of fungicides in many countries where disease management has relied upon the use of site-specific fungicides (Guido *et al.,* 2015; Liu *et al.,* 2016). The benzimidazole fungicides, mainly carbendazim, have been extensively used to control this disease for many years. Unfortunately, carbendazim-resistant populations of *B. cinerea* have evolved in recent years (Liu *et al.,* 2016). In order to design optimal resistance management strategies of grey mould, it is necessary to monitor carbendazim resistance in populations from different hosts.

Fungicide resistance has often been detected based on measurement of mycelial growth inhibition under fungicide exposure (Ma *et al.,* 2009; Schnabel *et al.,* 2015). However, this method is time consuming and tedious. With the rapid development of molecular biology, DNA-based molecular techniques have been introduced to detect fungicide resistance in plant pathogens. These methods can only be developed when the mechanism of fungicide resistance has been elucidated and suitable DNA markers can be used. According to previous studies (Leroux *et al.,* 2002; Ma & Michailides, 2005; Liu *et al.,* 2013; Hawkins & Fraaije, 2016), carbendazim resistance in *B. cinerea* is mediated by specific point mutations of the *β-tubulin* gene (U27198.1), leading to protein codon alterations E198A, E198G, E198K, E198L, E198V and F200Y (TTC → TAC). Mutations at codon 198 lead to a high level of resistance (minimum inhibitory concentration, MIC values >100 μg mL^{-1}, isolates growing in plates

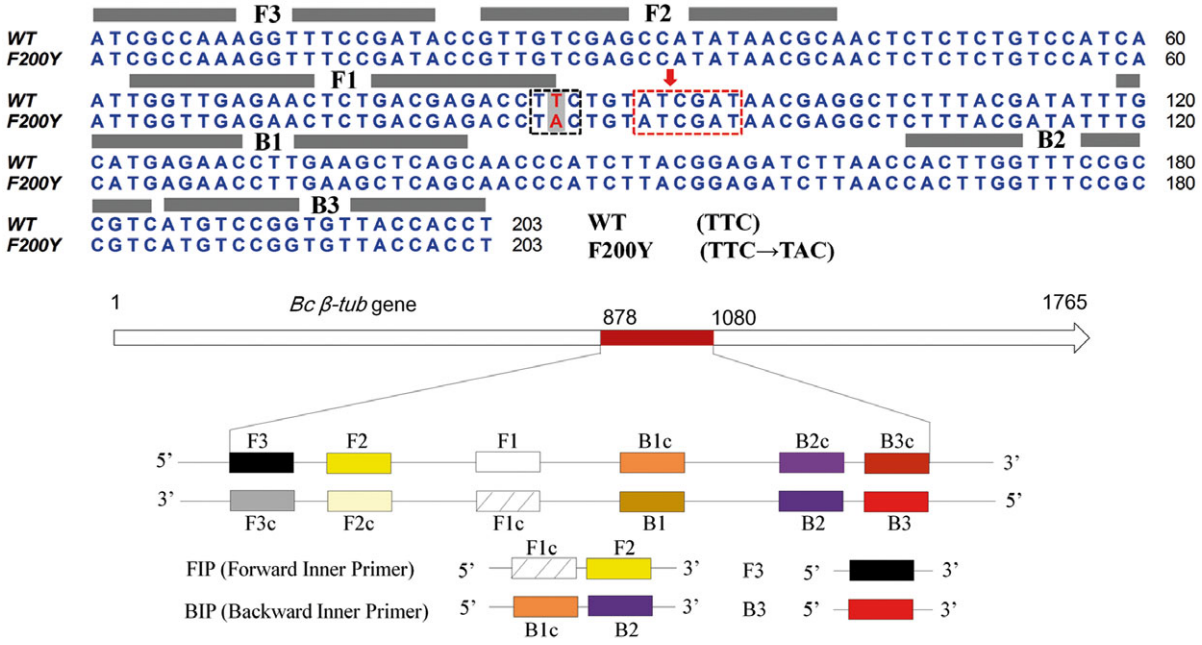

Figure 1 Schematic diagram of the loop-mediated isothermal amplification (LAMP) primers for detecting the moderately carbendazim-resistant *Botrytis cinerea* isolates carrying *β*-tubulin F200Y alleles. (A) Nucleotide sequence alignment of the *β*-tubulin target region of a wild-type isolate Bt4-1 (WT) and moderately carbendazim-resistant mutant B20 (F200Y). The sequences in the red frame indicate the target sequences of restriction enzyme *Cla*I. The red arrow indicates the cutting site of restriction enzyme *Cla*I. (B) Schematic representation of the LAMP primers.

containing 100 μg mL^{-1} carbendazim are considered as high level resistance to carbendazim), whereas the mutation at codon 200 results in a moderate level of carbendazim resistance (MIC values between 10 and 100 μg mL^{-1}, isolates growing in plates containing >10 μg mL^{-1} carbendazim and not growing in plates >100 μg mL^{-1} carbendazim are considered as moderate level resistance to carbendazim) (Ma & Michailides, 2005; Liu *et al.*, 2013). 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.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification technique, in which the reaction can be processed under isothermal conditions by a DNA polymerase with strand displacement activity (Notomi *et al.*, 2000). This technique involves the use of 4–6 different primers specifically designed to recognise 6–8 distinct regions on the target gene (Fig. 1); the reaction process proceeds at a constant temperature (60–65°C) and is completed within 60 min using the strand displacement reaction (Notomi *et al.*, 2000; Nagamine *et al.*, 2002). Furthermore, all steps from DNA amplification to detection in a LAMP assay are performed under isothermal conditions within one reaction tube. In our previous studies, the LAMP assay for detecting carbendazim-resistant populations has been established in *Fusarium asiaticum* and *Sclerotinia sclerotiorum*, respectively (Duan *et al.*, 2014c, 2015). However, the LAMP method for detection of moderately carbendazim-resistant populations in *B. cinerea* has not been developed and reported.

In this study, we developed a LAMP assay for detecting the moderately carbendazim-resistant mutants of *B. cinerea* carrying *β*-tubulin F200Y. This new assay can be used for early warning of the risk of carbendazim resistance in *B. cinerea* in the field and can provide important references to rationalise optimal disease management strategies for grey mould control.

Materials and methods

Fungal isolates and culture conditions

B. cinerea isolates B20, B05.10, SD2, SD4 and Bt4-1 were used in this study, all of which were isolated by a single-spore method (Choi *et al.*, 1999). The detailed information of these isolates was listed in Table 1 and maintained at 4°C on potato dextrose agar (PDA) at Laboratory of Fungicide Biology, Nanjing Agricultural University (China).

PDA was used for routine carbendazim sensitivity assays as described previously (Duan *et al.*, 2014c).

Table 1 Fungal isolates used for evaluating the specificity of the loop-mediated isothermal amplification (LAMP) assay for detecting moderately carbendazim-resistant isolates in *Botrytis cinerea*

Isolate	Fungal species	Host	Origin	Genotype description[a]	Phenotype[b]	LAMP
Bt4-1	*B. cinerea*	Strawberry	Jiangsu, China	Wild-type	MBC^{S}	–
B05.10	*B. cinerea*	Unknown	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	–	–	–
PD98	*Penicillium digitatum*	Citrus	Jiangsu, China	–	–	–
AA04	*Alternaria alternata*	Tomato	Jiangsu, China	–	–	–
CC15	*Corynespora cassiicola*	Cucumber	Henan, China	–	–	–
FV13	*Fusicladium virescens*	Pear	Anhui, China	–	–	–
FO11	*Fusarium oxysporum*	Cucumber	Jiangsu, China	–	–	–
SS07	*Sclerotinia sclerotiorum*	Lettuce	Jiangsu, China	–	–	–
CC13	*Colletotrichum capsici*	Pepper	Jiangsu, China	–	–	–

[a]Genotype represents the point mutation in the target gene *β-tubulin* of *B. cinerea*.
[b]MBC^{S}, MBC^{MR} and MBC^{HR} represent carbendazim sensitive (MIC value <10 μg mL^{-1}), moderately carbendazim resistant (MIC values between 10 and 100 μg mL^{-1}) and highly carbendazim resistant (MIC value >100 μg mL^{-1}), respectively.

According to the MIC (Ma & Michailides, 2005; Liu *et al.,* 2013), *B. cinerea* was assigned to three phenotypes as follows: sensitive to carbendazim, MIC <10 μg mL^{-1}; moderate carbendazim resistance, MIC values between 10 and 100 μg mL^{-1} and high carbendazim resistance, MIC >100 μg mL^{-1}.

DNA extraction

B. cinerea isolates were incubated for 3 days at 25°C on PDA. Mycelia were harvested to extract genomic DNA using the cetyltrimethyl ammonium bromide (CTAB) method with minor modification (Moller *et al.,* 1992; Duan *et al.,* 2014c). Hundred milligram fresh weight of mycelia and a steel ball (5 mm diameter) were placed into a 2-mL microtube containing 700 μL CTAB buffer. The samples were disrupted using mixer mill with speed set at 28 cycles s^{-1} for 30 s and spun at 10 000 ×g for 5 min at room temperature; 500 μL of the supernatant was transferred into a new 1.5-mL tube and mixed with 500 μL of isopropanol (stored at −20°C). The mixture was spun at 10 000 ×g for 5 min at room temperature. The supernatant was discarded. The pellet was washed with 500 μL of 70% ethanol (stored at −20°C) and spun at 10 000 ×g for 1 min. The ethanol was discarded, the pellet was dried and resuspended with 100 μL of sterile deionised water. DNA extracts were quantified using a spectrophotometer and stored at −20°C.

Primer design

LAMP primers were designed using the Primer Explorer V4 software program (Eiken, Japan) (http://primerexplorer.jp/e/) according the manufacturer's instructions. The structure of the LAMP primers and their complementarity to target DNA used in this study are shown in Fig. 1. A forward inner primer (FIP) consisted of F1c and F2, and a backward inner primer (BIP) consisted of B1c and B2. The outer primers F3 and B3 were required for initiation of the LAMP reaction. PCR primers were designed using Primer Premier 5.0 (Premier, Canada). Information of the primers is provided in Fig. 1 and Table 2.

Specificity of LAMP primers

A set of LAMP primers was obtained based on the point mutation linked with *β*-tubulin F200Y (Fig. 1A). According to a previous study (Duan *et al.,* 2015), we introduced a base mismatch into 5′ end of FIP to improve the specificity of the LAMP assay (Table 2, Fig. 1B). Thus, seven sets of LAMP primers were designed and used to specially distinguish the *β*-tubulin F200Y allele from the wild-type. Final LAMP assays were conducted as previously described (Duan *et al.,* 2015). Positive LAMP reactions could be visually determined using a colour change from violet to sky blue based on addition of hydroxynaphthol blue (HNB) (Goto *et al.,* 2009). In addition, positive LAMP reactions could produce a typical ladder-like pattern in 3.0% agarose gel electrophoresis stained with ethidium bromide under a UV transilluminator.

Optimisation of LAMP reaction components

With the optimised LAMP primers, optimisation of LAMP reaction components was performed using genomic DNA of *B. cinerea* isolate B20 as a template in accordance with Duan *et al.* (2015). The outcome of LAMP assays was

Table 2 Sequence and description of the loop-mediated isothermal amplification (LAMP) and PCR primers used to evaluate the specificity and sensitivity of the LAMP assay

Primer	Sequence (5′–3′)[a]	Description
F3	ATCGCCAAAGGTTTCCGATA	Forward outer primer
B3	AGGTGGTAACACCGGACAT	Backward outer primer
BIP	TGCATGAGAACCTTGAAGCTCAGC-GACGGCGGAAACCAAGTG	Backward inner primer
FIP1	TAGGTCTCGTCAGAGTTCTCAACCAGTTGTCGAGCCATATAACGCA	Forward inner primers to distinguish the mutation causing the F200Y variant of *β*-tubulin in *B. cinerea* for LAMP
FIP2	T**T**GGTCTCGTCAGAGTTCTCAACCAGTTGTCGAGCCATATAACGCA	
FIP3	TA**C**GTCTCGTCAGAGTTCTCAACCAGTTGTCGAGCCATATAACGCA	
FIP4	**C**TAGGTCTCGTCAGAGTTCTCAACCAGTTGTCGAGCCATATAACGCA	
FIP5	**C**TA**C**GTCTCGTCAGAGTTCTCAACCAGTTGTCGAGCCATATAACGCA	
FIP6	**TC**TAGGTCTCGTCAGAGTTCTCAACCAGTTGTCGAGCCATATAACGCA	
FIP7	**TC**TA**C**GTCTCGTCAGAGTTCTCAACCAGTTGTCGAGCCATATAACGCA	
Bcbeta442F	GGTAACAACTGGGCTAAGGG	To amplify a partial *β*-tubulin fragment (442 bp) covering codon 200
Bcbeta442R	GACCAGGGAAACGGAGACA	

[a]Nucleotides in frames represent the mutation site at codon 200 of the *β-tubulin* gene in *B. cinerea*. Nucleotides in bold represent the base pair mismatches manually introduced into the 5′ end of FIP.

assessed with HNB-visualised colour change and analysed with 3% gel electrophoresis.

Optimisation of LAMP parameters

With the optimal LAMP primers and reaction components, LAMP reaction temperature (58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C or 65°C) and time (15, 30, 45, 60, 75 or 90 min) were optimised using genomic DNA of B20 (F200Y) as template. The LAMP results were both visually assessed using HNB and gel electrophoresis.

Confirmation of the LAMP products

Restriction enzyme digestion was conducted in order to confirm that the designed LAMP assays amplified the intended 203-bp *β*-tubulin fragment target (Fig. 1A). Briefly, the restriction enzyme *Cla*I (Fig. 1A) was used to digest LAMP products and the resulting DNA fragments were analysed using gel electrophoresis (Niu *et al.*, 2012). Meanwhile, PCR was used to amplify the 203-bp fragment with the primer pair F3/B3, and the PCR products were cloned into the plasmid pEASY-T1 (Transgen, Beijing, China) for sequencing (Ge *et al.*, 2013; Li *et al.*, 2013).

Specificity of LAMP

The specificity of LAMP was tested with genomic DNA of B20 (F200Y), SD4 (E198V), B05.10 (E198A), SD2 (E198K), Bt4-1 (wild-type *β*-tubulin) and eight common plant-pathogenic fungi (Table 1). The LAMP results were assessed as described above.

Repeatability of LAMP

The reproducibility and specificity of the LAMP assay was tested using genomic DNA of eight *B. cinerea* isolates known to carry *β*-tubulin F200Y (Table 3) and the results were assessed as described above. DNA from wild-type isolate NJ84 were included as negative controls in these tests.

Sensitivity of LAMP

With the primer pair Bcbeta442F/Bcbeta442R, a 442-bp fragment containing the F200Y mutation was amplified by polymerase chain reaction (PCR) (Table 2). The PCR products were cloned to the vector pEASY-T1 to create plasmid pET442. The recombinant plasmid pET442 was transferred into *Escherichia coli* according to the previous studies (Ge *et al.*, 2013; Li *et al.*, 2013). The plasmid pET442 was extracted from the selected positive clones and 10-fold serially diluted to obtain from 6×10^9 to 6×10^0 copies (Zhang *et al.*, 2011). These dilutions were used as templates in the LAMP and PCR assays. The primer pair Bcbeta442F/Bcbeta442R was used for the PCR assay and the assay sensitivity was compared with the final LAMP assay. For LAMP, the optimised reaction components and conditions were used. The reaction results were analysed as described previously (Duan *et al.*, 2014c, 2015).

Application of LAMP on monitoring moderately carbendazim-resistant field isolates of *B. cinerea*

To verify the potential application of the LAMP assay for carbendazim-resistant monitoring of *B. cinerea* in the field, the fruits of cucumber, strawberry and tomato were inoculated with spore suspensions (10^4 spores mL^{-1}) of selected field isolates according to the previous studies (Liu *et al.*, 2007; Yang *et al.*, 2012; Duan *et al.*, 2013). In total, 16 strains were used of which three carried F200Y, four E198A, three E198K, four E198V and two

Table 3 *Botrytis cinerea* isolates used to evaluate the repeatability of the loop-mediated isothermal amplification (LAMP) assay for detecting moderately carbendazim-resistant isolates in *Botrytis cinerea*

Isolate	Host	Origin	Genotype description[a]	Phenotype[b]	LAMP
FJ03	Strawberry	Jiangsu, China	TTC → TAC, F200Y	MBC^{MR}	+
SG17	Cucumber	Shandong, China	TTC → TAC, F200Y	MBC^{MR}	+
DJ22	Tomato	Jiangsu, China	TTC → TAC, F200Y	MBC^{MR}	+
HA31	Strawberry	Jiangsu, China	TTC → TAC, F200Y	MBC^{MR}	+
HA52	Strawberry	Jiangsu, China	TTC → TAC, F200Y	MBC^{MR}	+
FJ04	Strawberry	Jiangsu, China	TTC → TAC, F200Y	MBC^{MR}	+
DJ38	Tomato	Jiangsu, China	TTC → TAC, F200Y	MBC^{MR}	+
NJ517	Strawberry	Jiangsu, China	TTC → TAC, F200Y	MBC^{MR}	+
NJ84	Strawberry	Jiangsu, China	No mutation	MBC^{S}	–

[a]Genotype represents the point mutation in the target gene *β-tubulin* of *B. cinerea*.
[b]MBC^{S} and MBC^{MR} represent carbendazim sensitive (MIC value <10 μg mL^{-1}) and moderately carbendazim resistant (MIC values between 10 and 100 μg mL^{-1}), respectively.

Table 4 Comparison of the loop-mediated isothermal amplification (LAMP) assay and fungicide sensitivity testing (determination of MIC values) for detecting the moderately carbendazim-resistant isolates of *B. cinerea* by the experiment of artificial inoculation

Isolate	Mutation[a]	Inoculated host	Total samples	Positive samples by LAMP	Positive samples by MIC
NJ78	No mutation	Tomato	16	0	0
YC14	No mutation	Cucumber			
B324	TTC → TAC, F200Y	Strawberry	24	24	24
HA21	TTC → TAC, F200Y	Cucumber			
SH38	TTC → TAC, F200Y	Tomato			
SD1	GAG → GTG, E198V	Strawberry	32	0	0
HY5	GAG → GTG, E198V	Cucumber			
YCS47	GAG → GTG, E198V	Tomato			
F71	GAG → GTG, E198V	Cucumber			
GCY004	GAG → GCG, E198A	Strawberry	32	0	0
B29	GAG → GCG, E198A	Tomato			
A24	GAG → GCG, E198A	Strawberry			
B11	GAG → GCG, E198A	Cucumber			
SD5	GAG → AAG, E198K	Cucumber	24	0	0
SD6	GAG → AAG, E198K	Tomato			
SH18	GAG → AAG, E198K	Strawberry			

[a]Genotype represents the point mutation in the target gene *β-tubulin* of *B. cinerea*.

wild-type *β*-tubulin alleles. There were eight replicates for each isolate (Table 4). After culturing for 3 days, a margin (5 mm × 5 mm) of a diseased region of the fruit samples was cut and used for extraction of genomic DNA in accordance with Duan *et al.* (2015). With genomic DNA of the tested isolates as templates, LAMP was performed to detect moderately carbendazim-resistant field isolates of *B. cinerea*. Meanwhile, the isolates obtained from diseased samples were isolated by tissue isolation method and tested by MIC method to further confirm the accuracy of the LAMP assay.

Results

Specificity of LAMP primers

A point mutation (F200Y) of the target protein *β*-tubulin confers moderate levels of carbendazim-resistance (MIC values between 10 and 100 μg mL^{-1}) in *B. cinerea* isolates. Based on this point mutation, seven sets of LAMP primers (Table 2) were designed by introducing base mismatches into the 5′ of FIP. Genomic DNA of the carbendazim-sensitive isolate Bt4-1, carrying wild-type *β*-tubulin, and a moderately resistant isolate B20, carrying *β*-tubulin F200Y, were used to perform the specificity test of the seven different LAMP primer sets. Based on HNB-visualised colour change and the ladder-like pattern of DNA amplification products in gel electrophoresis, LAMP primer set 1 detected both the F200Y mutant and wild-type *β*-tubulin alleles. Primer sets 5, 6 and 7 did not detect the two *β*-tubulin alleles, but specific detection of F200Y *β*-tubulin alleles was achieved with primer sets 2, 3 and 4 (Fig. 2A). Positive LAMP samples were indicated by a sky blue from HNB; while the negative samples remained violet blue (Fig. 2A). The changes in

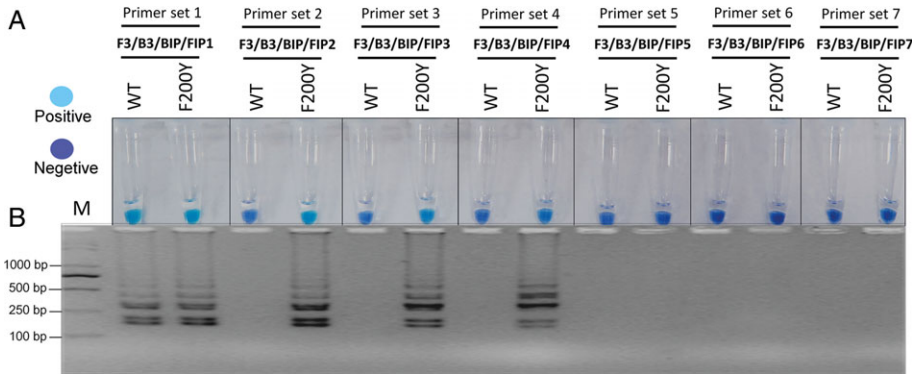

Figure 2 Specificity of loop-mediated isothermal amplification (LAMP) assays using different primer sets. (A) Assessment by hydroxynaphthol blue (HNB)-visualised colour change. (B) Detection of DNA amplification products from LAMP assays using gel electrophoresis. M, DNA marker (TransGen, Beijing, China).

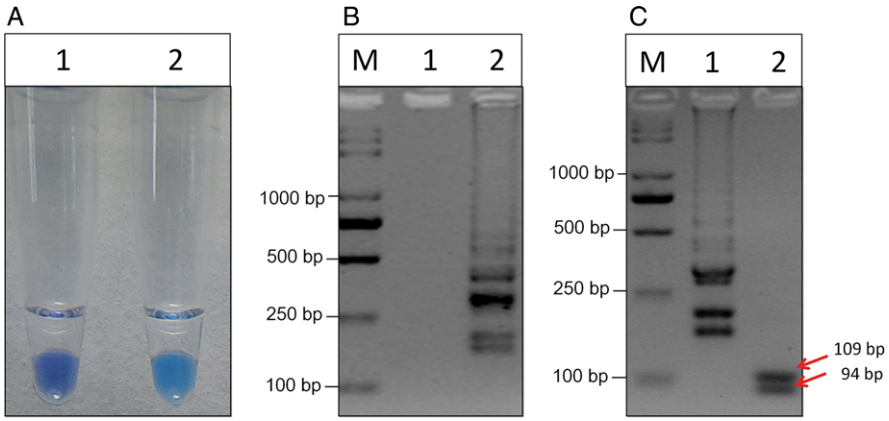

Figure 3 Loop-mediated isothermal amplification (LAMP) detection of *Botrytis cinerea* isolates carrying β-tubulin F200Y alleles and restriction enzyme digestion analysis of the LAMP products. (A) Identification of the positive LAMP assays based on hydroxynaphthol blue (HNB)-visualised colour change. 1, isolate Bt4-1 (WT); 2, isolate B20 (F200Y); (B) Assessment by gel electrophoresis analysis. M, DNA marker (TransGen); 1, isolate Bt4-1 (WT); 2, isolate B20 (F200Y); (C) LAMP products were digested with *Cla* I, and two bands of 94 and 109 bp were observed on agarose gel. M, DNA marker (TransGen); 1, without digestion; 2, digestion by *Cla I*.

colour were confirmed by gel electrophoresis, i.e. 3.0% gel electrophoresis produced a typical ladder-like pattern for LAMP positive samples but not for negative samples (Fig. 2B). The results indicated that primer sets 2, 3 and 4 (Fig. 2, Table 2) could be used to detect moderately carbendazim-resistant isolates carrying F200Y β-tubulin alleles. Primer set 3 was randomly selected for further optimisation of the assay.

LAMP reaction assay

With DNA of isolates carrying wild-type (Bt1-4) and F200Y β-tubulin alleles (B20) as templates, the LAMP assay was performed to optimise concentration of LAMP components. A positive LAMP reaction was only obtained with DNA from isolate B20 (Fig. 3A and Fig. 3B). The most efficient LAMP reaction (10 µL volume) consist of 2.4 U *Bst* DNA polymerase, 1.0 mM dNTPs, 3.5 mM $MgCl_2$, 0.64 M betaine, 1.0 µL 10× ThermoPol buffer, 200 µM HNB, 1.2 µM FIP, 1.2 µM BIP, 0.2 µM F3, 0.2 µM B3 and 10 ng template DNA.

Validation of the LAMP products

The positive LAMP products were digested with the restriction enzyme *Cla*I (Fig. 1A) to verify the specificity of LAMP for amplifying the target fragments. The results showed that the two DNA bands (94 and 109 bp) were observed on agarose gel (Fig. 3C). The results were in accord with those predicted based on the expected structures. The sequence of LAMP amplification was the same as that of the target gene *β-tubulin* for designing LAMP primers.

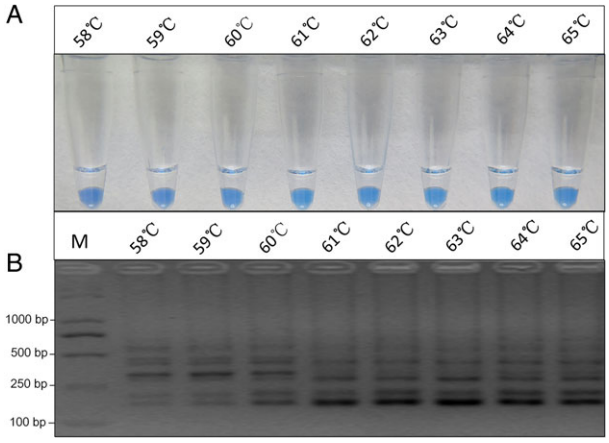

Figure 4 Optimisation of the loop-mediated isothermal amplification (LAMP) reaction temperature. (A) Assessment by hydroxynaphthol blue (HNB)-visualised colour change. (B) Assessment by gel electrophoresis analysis. M, DNA marker (TransGen).

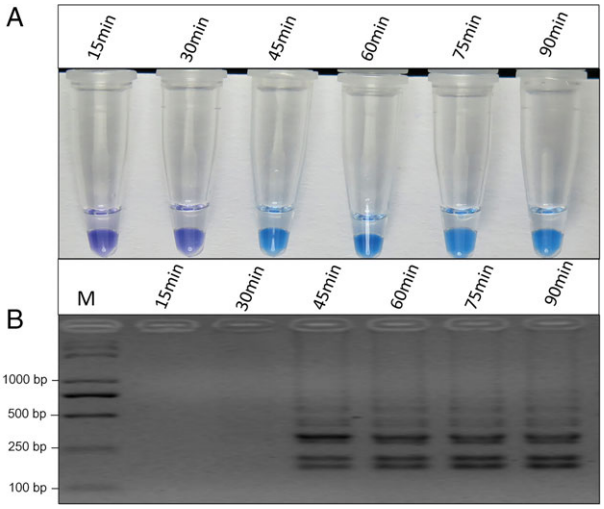

Figure 5 Optimization of the loop-mediated isothermal amplification (LAMP) reaction time. (A) Assessment by hydroxynaphthol blue (HNB)-visualised colour change. (B) Assessment by gel electrophoresis analysis. M, DNA marker (TransGen).

LAMP reaction conditions

Based on the above described LAMP components, the reaction temperature and time were optimised with the genomic DNA of isolate B20 carrying β-tubulin F200Y alleles as template. The LAMP products were evident at 58–65°C based on the HNB-visualised colour change (Fig. 4A) and the ladder-like pattern in agarose gel (Fig. 4B) and the DNA intensity was highest at 63°C (Fig. 4B). Therefore, 63°C was considered as the appropriate temperature. The results of the reaction time indicated that the positive reaction was not observed at a reaction time shorter than 45 min (Fig. 5A and Fig. 5B). Therefore, the optimised reaction temperature was set at 61–65°C and the optimised reaction time was identified at 45 min.

Specificity of LAMP

Based on the colour change and the ladder-like pattern in agarose gel, LAMP was only positive for the isolate (B20) that carried β-tubulin F200Y alleles (Fig. 6A and Fig. 6B). The results showed that LAMP established in this study had a high specificity for detecting the moderately carbendazim-resistant isolates of *B. cinerea*.

Sensitivity of LAMP

With the different dilutions of the plasmid pET442 as templates, the PCR and LAMP assays were tested to determine LAMP detection limits. The results indicated that the detection limits for the PCR and LAMP assays were 6×10^4 (Fig. 7C) and 6×10^2 copies (Fig. 7A and Fig. 7B). Thus, the sensitivity of the LAMP assay was 100-fold higher than the PCR assay.

Repeatability of LAMP

Carbendazim sensitivity of nine *B. cinerea* isolates (Table 3) was confirmed by the MIC method and sequencing before the repeatability was tested. Among the tested mutants or isolates, all the known F200Y isolates were positive based on colour change (Fig. 8A) or agarose gel analysis (Fig. 8B). This indicated that the LAMP assay was repeatable and robust.

Application of LAMP for monitoring moderately carbendazim-resistant mutants of *B. cinerea*

The results for LAMP and MIC for monitoring 221 samples were identical, i.e. both LAMP and MIC produced positive results for samples from the different hosts inoculated with isolates carrying β-tubulin F200Y alleles and negative results for samples from the different hosts inoculated with other isolates carrying different β-tubulin alleles (Table 4). This showed that LAMP could be used for detecting the moderately carbendazim-resistant isolates of *B. cinerea*.

Discussion

LAMP, a novel DNA amplification technique, is an auto-cycling and strand displacement DNA synthesis method involving the use of the large fragment of

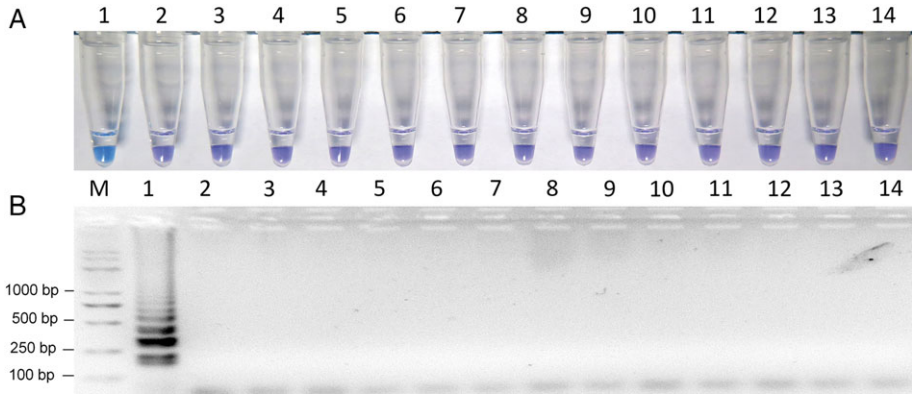

Figure 6 Specificity of loop-mediated isothermal amplification (LAMP) detection of *β*-tubulin F200Y alleles from moderately carbendazim-resistant *Botrytis cinerea* isolates. (A) Assessment by hydroxynaphthol blue (HNB)-visualised colour change. (B) Assessment by gel electrophoresis analysis. M, DNA marker (TransGen). M, DNA marker (TransGen); 1, isolate B20 (F200Y); 2, isolate Bt4-1 (WT); 3, isolate B05.10 (E198A); 4, isolate SD2 (E198K); 5, isolate SD4 (E198V); 6, *Penicillium italicum*; 7, *Penicillium digitatum*; 8, *Alternaria alternata*; 9, *Corynespora cassiicola*; 10, *Fusicladium virescens*; 11, *Fusarium oxysporum*; 12, *Sclerotinia sclerotiorum*; 13, *Colletotrichum capsici*; 14, ddH_2O.

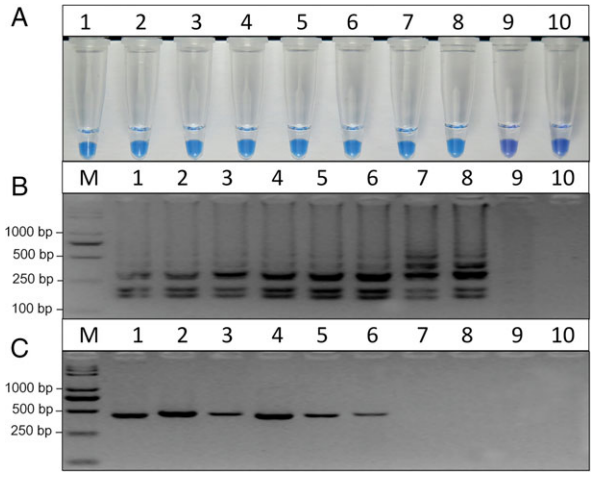

Figure 7 Sensitivity of loop-mediated isothermal amplification (LAMP) and PCR. (A) Detection by LAMP and hydroxynaphthol blue (HNB)-visualised colour change. (B) Detection by LAMP and gel electrophoresis. (C) Detection by PCR and gel electrophoresis. M, DNA marker (TransGen); 1, 6×10^9; 2, 6×10^8; 3, 6×10^7; 4, 6×10^6; 5, 6×10^5; 6, 6×10^4; 7, 6×10^3; 8, 6×10^2; 9, 6×10^1 and 10, 6×10^0.

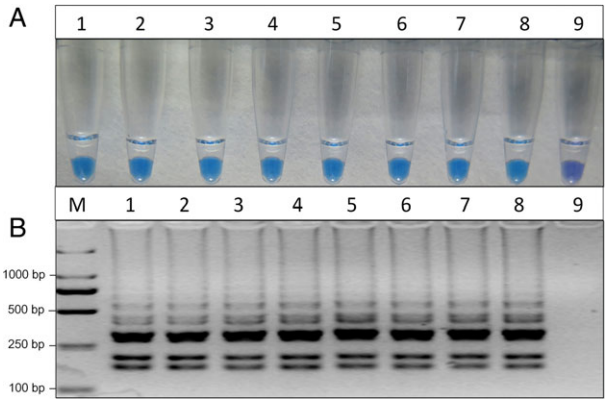

Figure 8 Repeatability of loop-mediated isothermal amplification (LAMP) detection of moderately carbendazim-resistant mutants of *Botrytis cinerea*. (A) Assessment by hydroxynaphthol blue (HNB)-visualised colour change. (B) Assessment by gel electrophoresis analysis. M, DNA marker (TransGen); 1–8, DNA samples of different isolates carrying *β*-tubulin F200Y alleles listed in Table 3; 9, DNA of wild-type isolate Bt1-4.

Bst DNA polymerase and a set of 4–6 specific primers (Notomi *et al.,* 2000; Mori *et al.,* 2001; Nagamine *et al.,* 2002; Curtis *et al.,* 2008; Goto *et al.,* 2009). Since this technique was developed in 2000, LAMP has been widely used for detection of pathogens of both plants and animals (Duan *et al.,* 2014a,b; Kogovšek *et al.,* 2015; Lee *et al.,* 2015; Niu *et al.,* 2015; Okuda *et al.,* 2015; Wang *et al.,* 2015; Zhuo *et al.,* 2015). However, it has been rarely used for detection of mutations conferring drug resistance in pathogenic microorganisms until recently. In our previous studies, we have reported several LAMP assays for detecting the carbendazim-resistant mutants in plant pathogens such as *F. asiaticum* and *S. sclerotiorum* (Duan *et al.,* 2014c, 2015).

Molecular mechanisms of resistance to carbendazim in *B. cinerea* has been demonstrated to be involved with the point mutation of the target gene *β-tubulin* (BC1G_00122), resulting in protein codon alterations at positions 198 (E198A, E198G, E198K, E198L and E198V) and 200 (F200Y) of the protein (Ma & Michailides, 2005; Liu *et al.,* 2013; Hawkins & Fraaije, 2016). Mutations resulting in *β*-tubulin alterations at codon 198 cause high level of resistance ($MIC > 100\,\mu g\,mL^{-1}$), while isolates carrying *β*-tubulin F200Y alleles are moderately resistant (MIC values between 10 and

100 μg mL^{-1}). Up to now, *B. cinerea* isolates with single point mutation were widely documented, but the isolates with more than one mutation have not been reported. In our recent studies, the LAMP assays for detecting the highly carbendazim-resistant *B. cinerea* isolates (*β*-tubulin E198A/K/V) have been developed. However, a LAMP detection method for moderately carbendazim-resistant *B. cinerea* isolates has not been developed. Thus, we developed the LAMP assay for detecting moderately carbendazim-resistant *B. cinerea* isolates carrying *β*-tubulin F200Y alleles.

One of the advantages of the LAMP assay is that the positive reaction products of LAMP can be visualised by adding DNA-intercalating dyes such as PicoGreen, SYBR Green and ethidium bromide, or metal-ion indicators such as $CuSO_4$, calcein and HNB (Parida *et al.*, 2005; Curtis *et al.*, 2008; Tomita *et al.*, 2008; Goto *et al.*, 2009; Zoheir & Allam, 2011). DNA intercalating dyes are added to reaction tubes after the reaction is completed. Exposed operation will increase the rates of contamination. To avoid such contamination, a visualisation indicator HNB is added to reaction tubes prior to amplification. The positive reaction is indicated by a colour change from violet to sky blue, and the negative reaction retains violet (Goto *et al.*, 2009). Meanwhile, the positive reaction was further verified with gel electrophoresis. In this study, we developed a LAMP assay with HNB for detection of the moderately carbendazim-resistant mutants of *B. cinerea*. The developed LAMP has a higher sensitivity and simplicity than conventional PCR and is very suitable for detection of fungicide resistance.

The parameters of LAMP primers can affect the concentration of reaction components and reaction conditions. With the primer set 3, the concentration of reaction components was optimised and the appropriate result was obtained. With selecting primer set 3 and reaction components, the LAMP reaction conditions were determined and the results showed that the optimal reaction temperature was 61–65°C and the optimal reaction time was 45 min.

In specificity tests, the wild-type isolate, *B. cinerea* isolates carrying E198A/K/V *β*-tubulin alleles and other commonly encountered plant-pathogenic fungi associated with fruits and vegetables were regarded as the negative control for LAMP. LAMP could specifically detect *B. cinerea* isolates carrying *β*-tubulin F200Y alleles. The repeatability tests showed that all F200Y isolates tested were positive. The sensitivity of the LAMP assay was also compared with conventional PCR and the detection limit of the LAMP assay was 100-fold more sensitive than PCR (Fig. 7). All these results showed the LAMP developed in this study had good specificity, sensitivity and repeatability.

To assess the applicability of LAMP for detecting the moderately carbendazim-resistant mutants in *B. cinerea*, fruits of tomato, cucumber and strawberry were artificially inoculated with wild-type isolates and carbendazim-resistant isolates and diseased samples were assessed by LAMP and carbendazim sensitivity testing (determination of MIC values). The results were similar for both LAMP and MIC but LAMP was easier to perform than MIC. Using a mobile device, the LAMP assay will be highly suitable for in-field detection of moderately carbendazim-resistant isolates of *B. cinerea*.

Acknowledgements

This study was funded by the National Key Research and Development Program of China (2016YFD0200501-04), Henan Science and Technology Cooperation Project (172106000006), and a grant from Innovation Team Program for Jiangsu Universities (2013).

References

Choi Y.W., Hyde K.D., Ho W.W.H. (1999) Single spore isolation of fungi. *Fungal Diversity*, **3**, 29–38.

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

Duan Y.B., Ge C.Y., Liu S.M., Wang J.X., Zhou M.G. (2013) A two-component histidine kinase *Shk1* controls stress response, sclerotial formation and fungicide resistance in *Sclerotinia sclerotiorum*. *Molecular Plant Pathology*, **14**, 708–718.

Duan Y.B., Ge C.Y., Zhang X.K., Wang J.X., Zhou M.G. (2014a) A rapid detection method for the plant pathogen *Sclerotinia sclerotiorum* based on loop-mediated isothermal amplification (LAMP). *Australasian Plant Pathology*, **43**, 61–66.

Duan Y.B., Ge C.Y., Zhang X.K., Wang J.X., Zhou M.G. (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.B., Zhang X.K., Ge C.Y., Wang Y., Cao J.H., Jia X.J., Wang J.X., Zhou M.G. (2014c) Development and application of loop-mediated isothermal amplification for detection of the F167Y mutation of carbendazim-resistant isolates in *Fusarium graminearum*. *Scientific Reports*, **4**, 7094.

Duan Y.B., Yang Y., Wang J.X., Liu C.C., He L.L., Zhou M.G. (2015) Development and application of loop-mediated isothermal amplification for detecting the highly benzimidazole-resistant isolates in *Sclerotinia sclerotiorum*. *Scientific Reports*, **12**, 17278.

Ge C.Y., Duan Y.B., Zhou M.G., Chen C.J. (2013) A protoplast transformation system for gene deletion and

complementation in *Sclerotinia sclerotiorum*. *Journal of Phytopathology*, **161**, 800–806.

Goto M., Honda E., Ogura A., Nomoto A., Hanaki K. (2009) Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxynaphthol blue. *BioTechniques*, **46**, 167–172.

Hawkins N.J., Fraaije B.A. (2016) Predicting resistance by mutagenesis: lessons from 45 years of MBC resistance. *Frontiers in Microbiology*, **7**, 1814.

Kogovšek P., Hodgetts J., Hall J., Prezelj N., Nikolić P., Mehle N., Lenarčič R., Rotter A., Dickinson M., Boonham N., Dermastia M., Ravnikar M. (2015) LAMP assay and rapid sample preparation method for on-site detection of flavescence dorée phytoplasma in grapevine. *Plant Pathology*, **64**, 286–296.

Lee D., Kim E.J., Kilgore P.E., Kim S.A., Takahashi H., Ohnishi M., Anh D.D., Dong B.Q., Kim J.S., Tomono J., Miyamoto S., Notomi T., Kim D.W., Seki M. (2015) Clinical evaluation of a loop-mediated isothermal amplification (LAMP) assay for rapid detection of *Neisseria meningitidis* in cerebrospinal fluid. *PLoS One*, **10**, e0130506.

Leroux P., Fritz R., Debieu D., Albertini C., Lanen C., Bach J., Gredt M., Chapeland F. (2002) Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Management Science*, **58**, 876–888.

Li B., Du J., Lan C., Liu P., Weng Q., Chen Q. (2013) Development of a loop-mediated isothermal amplification assay for rapid and sensitive detection of *Fusarium oxysporum* f. sp. *cubense* race 4. *European Journal of Plant Pathology*, **135**, 903–911.

Liu X.P., Wang J.Y., Gou P., Mao C.G., Zhu Z.R., Li H. (2007) *In vitro* inhibition of postharvest pathogens of fruit and control of gray mold of strawberry and green mold of citrus by aureobasidin A. *International Journal of Food Microbiology*, **119**, 223–229.

Liu S.M., Duan Y.B., Ge C.Y., Chen C.J., 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 Management Science*, **69**, 582–588.

Liu S.M., Che Z.P., Chen G.Q. (2016) Multiple-fungicide resistance to carbendazim, diethofencarb, procymidone, and pyrimethanil in field isolates of *Botrytis cinerea* from tomato in Henan Province, China. *Crop Protection*, **84**, 56–61.

Ma Z., Michailides T.J. (2005) Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Protection*, **24**, 853–863.

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

Möller E.M., Bahnweg G., Sandermann H., 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 Research*, **20**, 6115–6116.

Mori Y., Nagamine K., Tomita N., Notomi T. (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications*, **289**, 150–154.

Nagamine K., Kuzuhara Y., Notomi T. (2002) Isolation of single stranded DNA from loop-mediated isothermal amplification products. *Biochemical and Biophysical Research Communications*, **290**, 1195–1198.

Niu J.H., Jian H., Guo Q.X., Chen C.L., Wang X.Y., Liu Q., Guo Y.D. (2012) Evaluation of loop-mediated isothermal amplification (LAMP) assays based on 5S rDNA-IGS2 regions for detecting *Meloidogyne enterolobii*. *Plant Pathology*, **61**, 809–819.

Niu J.H., Gao Y.R., Yin J.M., Leng Q.Y., Yang G.S., Wang C., Ren Y. (2015) Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of bacterial blight pathogen (*Xanthomonas axonopodis* pv. *dieffenbachiae*) in anthurium. *European Journal of Plant Pathology*, **142**, 801–813.

Notomi T., Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N., Hase T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, **28**, e63.

Okuda M., Okuda S., Iwai H. (2015) Detection of *Cucurbit chlorotic yellows virus* from *Bemisia tabaci* captured on sticky traps using reverse transcription loop-mediated isothermal amplification (RT-LAMP) and simple template preparation. *Journal of Virological Methods*, **221**, 9–14.

Parida M., Horioke K., Ishida H., Dash P.K., Saxena P., Jana A.M., Islam M.A., Inoue S., Hosaka N., Morita K. (2005) Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay. *Journal of Clinical Microbiology*, **43**, 2895–2903.

Schnabel G., Hu M.J., Fernandez-Ortuno D. (2015) Monitoring resistance by bioassay: relating results to field use using culturing methods. In *Fungicide Resistance in Plant Pathogens*, pp. 281–293. Eds H. Ishii and D. Hollomon. Tokyo, Japan: Springer.

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

Wang X., Li X., Hu S., Qu H., Zhang Y., Ni H., Wang X. (2015) Rapid detection of active human cytomegalovirus infection in pregnancy using loop-mediated isothermal amplification. *Molecular Medicine Reports*, **12**, 2269–2274.

Yang Q.Q., Yan L.Y., Gu Q., Ma Z.H. (2012) The mitogen-activated protein kinase kinase kinase BcOs4 is required for vegetative differentiation and pathogenicity

in *Botrytis cinerea*. *Applied Microbiology and Biotechnology*, **96**, 481–492.

Zhang Y., Fu P., Li J., Jiang F., Li J., Wu W. (2011) Development of loop-mediated isothermal amplification method for visualization detection of the highly virulent strains of porcine reproductive and respiratory syndrome virus (PRRSV) in China. *African Journal of Biotechnology*, **10**, 13278–13283.

Zhuo X., Huang B., Luo J., Yu H., Yan B., Yang Y., Du A. (2015) Development and application of loop-mediated isothermal amplification assays based on ITS-1 for rapid detection of *Toxoplasma gondii* in pork. *Veterinary Parasitology*, **208**, 246–249.

Zoheir K.M., Allam A.A. (2011) A rapid improved method for sexing embryo of water buffalo. *Theriogenology*, **76**, 83–87.