Rapid on-site evaluation of the development of resistance to quinone outside inhibitors in Botrytis cinerea

X. R. Hu1, D. J. Dai1,2, H. D. Wang1,2 & C. Q. Zhang1

Botrytis cinerea, a typical “high-risk” pathogenic fungus that rapidly develops resistance to fungicides, affects more than 1,000 species of 586 plant genera native to most continents and causes great economic losses. Therefore, a rapid and sensitive assay of fungicide resistance development in B. cinerea populations is crucial for scientific management. In this study, we established a Loop-mediated isothermal amplification (LAMP) system for the monitoring and evaluation of the risk of development of B. cinerea resistance to QoI fungicides; the method uses two LAMP assays. The first assay detects G143A mutants of B. cinerea, which are highly resistance to QoI fungicides. Bcbi143/144 introns in B. cinerea are then detected by the second assay. HNB acts as a visual LAMP reaction indicator. The optimum reaction conditions of the LAMP assays were 61 °C for 50 min, and the detection limit of the LAMP assays was $100 \times 10^{-4}$ ng/μl. We directly pre-treated the field samples by using All-DNA-Fast-Out to extract DNA within ten minutes, then performed the LAMP assay to achieve one-step rapid detection. In conclusion, we established a rapid and sensitive LAMP assay system for resistance risk assessment and for monitoring QoI-resistance of B. cinerea in the field.

Gray mold is caused by Botrytis cinerea Pers:Fr., which is one of the most economically important pathogens of strawberry plants4,5. The infection may occur in the flower, maintain latency until fruit maturation, and develop abundantly into visible infection, causing fruit rot accompanied by profuse sporulation during harvest operations6. The management of gray mold disease is achieved by frequent application of fungicides. Quinone outside inhibitors (QoIs) were developed on the basis of the natural products of β-methoxyacrylate acid, including strobilurin A and oudemansin A4. QoIs commonly used in agricultural production include pyraclostrobin and azoxystrobin5. The activity of QoIs is derived from the ability to bind to the Qo site in cytochrome b in fungi, thereby inhibiting mitochondrial respiration6. Cytochrome b is part of the cytochrome bc1 complex located in the mitochondrial inner membrane. After an inhibitor is bound to cytochrome b, it prevents electron transfer between cytochrome b and c1. Because QoIs have a wide range of control efficiency for most important fungal diseases in agriculture, they have become crucial parts of plant disease management practices5. Unfortunately, strains highly resistant to QoIs have been reported for different target pathogens, such as Podosphaera fusca7, Venturia inaequalis8, Mycosphaerella graminicola9,10, Plasmopara viticola11,12, Erysiphe necator12, Colletotrichum graminicola13, and B. cinerea14. In general, a point mutation at codon 143 of cytb gene results in a substitution of glycine by alanine (G143 → A) in plant-pathogenic fungi, thus causing resistance to QoIs15,16. In addition, isolates of B. cinerea can be divided into two types: those with or without the Bcbi-143/144 intron in cytb14. QoI-resistant isolates have been found with the G143A mutation but without the Bcbi-143/144 intron. Isolates with Bcbi-143/144 introns are very low-risk for development of resistance to QoIs. This phenomenon has also been reported for other plant pathogens17–19.

The traditional method of detection or monitoring of fungicide resistance is the minimum inhibitory concentration (MIC) accompanied by long detection cycles, of a week or even longer20,21. In recent years, with the development of nucleic acid-related molecular detection, PCR-based detection techniques have been developed22,23. However, these techniques have inherent shortcomings, including the prolonged time and expensive

1Department of Plant Pathology, Zhejiang Agriculture and Forest University, Lin’an, 311300, China. 2Institute for the Control of Agrochemicals of Zhejiang Province, Hangzhou, 310020, China. Correspondence and requests for materials should be addressed to C.Q.Z. (email: cqzhang@zafu.edu.cn)
The optimum reaction condition of this LAMP was 61 °C for 50 min (Fig. 3c,d). Changes to determine the extent of reaction (Fig. 1c) and by gel electrophoresis (Fig. 1d). As expected, all positive samples showed significant color changes from violet to sky blue according to HNB, but the negative controls did not (Fig. 1).

Results
Visual detection of two LAMP reactions. For DNA samples extracted from the G143A genotype of B. cinerea strains, the specific G143A-LAMP mismatched primers S7 (Table 1) were screened, and the G143A mutants were specifically detected; the positive reaction was reflected in the color change from violet to sky blue according to HNB (Fig. 1a). The products of the G143A mutants showed a ladder-like pattern in the gel electrophoresis (Fig. 1b). Furthermore, positive samples with the BCbi143/144 intron were amplified by another specific primer set were determined by HNB visualization and gel electrophoresis analysis of amplification products. The results clearly indicated that the G143A-LAMP reaction could not be performed and that the color of the reaction tube did not change when the temperature was higher than 61 °C (Fig. 2a). However, the reaction mixture showed significant color changes, and clear LAMP bands were displayed in the gel electrophoresis (Fig. 2a,b). Therefore, to optimize the reaction time, G143A-LAMP was performed at 61 °C. The optimal running conditions for two LAMP assays. The optimal running conditions for LAMP reactions using a specific primer set were determined by HNB visualization and gel electrophoresis analysis of amplification products. The results clearly indicated that the G143A-LAMP reaction could not be performed and that the color of the reaction tube did not change when the temperature was higher than 61 °C (Fig. 2a). However, the reaction mixture showed significant color changes, and clear LAMP bands were displayed in the gel electrophoresis at 60 °C and 61 °C (Fig. 2a,b). Therefore, to optimize the reaction time, G143A-LAMP was performed at 61 °C. The results showed that suitable detection could be achieved at 61 °C for 50 min (Fig. 2c,d). Another LAMP assay to detect the BCbi143/144 intron in B. cinerea successfully proceeded at 59 °C to 63 °C, and was judged by color change or gel electrophoresis (Fig. 3a,b). Thus, 61 °C was selected as the reaction temperature to support both LAMP assays. The optimum reaction condition of this LAMP was 61 °C for 50 min (Fig. 3c,d).

Sensitivity of LAMP. For the sensitivity test, 10-fold diluted DNA samples were used as templates for LAMP sensitivity testing on the basis of the visible color change of HNB in the tube (Fig. 4a,c) and the results from gel electrophoresis (Fig. 4b,d). The detection limit of the G143A-LAMP assay and BCbi143/144-LAMP assay was 100 × 10⁻⁴ ng/µl.

On-site detection of strawberry samples within 1 h. All field samples were treated with All-DNA-Fast-Out at 80 °C for 5–10 minutes to extract genomic DNA. The supernatant obtained from the lysate was directly added to the LAMP mixture as DNA template. The LAMP results of the G143A assay showed 46 positive reactions from 78 samples. According to the MIC test, 46 isolates with high resistance to QoI fungicides were detected with an MIC > 100 μg ml⁻¹ (Fig. 5), and the frequency of resistant strains was 59% (a total of 78), as shown in Table 2. PCR sequencing analysis indicated that all isolates highly resistant to azoxystrobin had the G143A mutation in the cytb gene (Table 2). The G143A-LAMP assay specifically detected isolates with the G143A genotype with 100% accuracy (Fig. 5, Table 2). For the BCbi143/144-LAMP assay, three of the 78 field samples were positive. The PCR amplification results were consistent with those of the BCbi143/144-LAMP assay. In the gel electrophoresis (Fig. 5), there were two types of cytb in B. cinerea: type I was followed by an intron (1205 bp) at codon 143, and the products amplified by primers BC-cytb-F and BC-cytb-R were 1700 bp (3 samples); for

---

Table 1. Primers used in this study.

| Primer name | Primer set name | Sequence (5'-3') |
|-------------|----------------|------------------|
| G143A-F3    | Forward outer  | TGATGTTCTGGCCTACG |
| G143A-B3    | Reverse outer  | CCTTCTAGATGTTCTGG |
| G143A-FIP1  | S1 Forward inner| GTCCAAATCTATGGACAGCAGCAGGCAAATGTCACTGTAGAC |
| G143A-FIP2  | S2 Forward inner| GTCCAAATCTATGGACAGCAGCAGGCAAATGTCACTGTAGTC |
| G143A-FIP3  | S3 Forward inner| GTCCAAATCTATGGACAGCAGCAGGCAAATGTCACTGTAGCC |
| G143A-FIP4  | S4 Forward inner| GTCCAAATCTATGGACAGCAGCAGGCAAATGTCACTGTAGAC |
| G143A-FIP5  | S5 Forward inner| GTCCAAATCTATGGACAGCAGCAGGCAAATGTCACTGTAGTC |
| G143A-FIP6  | S6 Forward inner| GTCCAAATCTATGGACAGCAGCAGGCAAATGTCACTGTAGTC |
| G143A-FIP7  | S7 Forward inner| GTCCAAATCTATGGACAGCAGCAGGCAAATGTCACTGTAGTC |
| G143A-FIP8  | S8 Forward inner| GTCCAAATCTATGGACAGCAGCAGGCAAATGTCACTGTAGTC |
| BCh143/144-F3 | S9 Forward outer | CCTAAATCAATGTCAATGAT |
| BCh143/144-B3 | S10 Forward outer | CGTACAGTAACCATGTAAG |
| BCh143/144-FIP | S11 Forward outer | TGACAATCACCATAAGTCAATG |
| BCh143/144-BIP | S12 Reverse outer | GATATTGTTGAGTGACCAACCATCTCATTCCACCATGAC |

C. y. cytb gene (Table 2). The G143A-LAMP assay specifically detected isolates with the G143A genotype with 100% accuracy (Fig. 5, Table 2). For the BCbi143/144-LAMP assay, three of the 78 field samples were positive. The PCR amplification results were consistent with those of the BCbi143/144-LAMP assay. In the gel electrophoresis (Fig. 5), there were two types of cytb in B. cinerea: type I was followed by an intron (1205 bp) at codon 143, and the products amplified by primers BC-cytb-F and BC-cytb-R were 1700 bp (3 samples); for...
type II, the 143rd codon was not followed by an intron, and the products were approximately 550 bp with primers BC-cytb-F and BC-cytb-R.

Discussion

LAMP is an innovative technique for gene amplification and a simple diagnostic tool for the early detection and identification of diseases. LAMP provides a simpler method of diagnosing pathogenic fungi and can has the specificity sufficient to detect single-base differences in DNA fragments. In the current research, mismatched primers were designed and screened to detect QoI-resistance in B. cinerea. For this purpose, mismatched bases were introduced at the 3’ end of FIP to separate G143A mutant genotype strains from sensitive strains. Eight sets of primers were screened, and primers S7 with a change in the second and third nucleotide at the 3’ end of FIP (from A and G to T and T) specifically detected G143A B. cinerea. Additionally, another primer set for intron detection were designed according to the 1205 bp of the BCbi143/144 intron sequence and was used to monitor and evaluate the low-resistance risk of a B. cinerea population. In this study, a visual color change to sky blue from violet indicated HNB in positive samples through the LAMP system, whereas negative samples remained violet. This sealed reaction decreased the risk of false positives observed when the DNA-intercalating dye SYBR Green is added after amplification.

The detection method of B. cinerea resistance to Qols was designed to meet some of the main requirements for on-site rapid testing. To make LAMP suitable for field diagnostics, pretreatment of samples must be as simple as possible. In the current one-step LAMP, samples were pre-treated with All-DNA-Fast-Out to extract DNA, and the lysates, as DNA templates, were added to prepared LAMP reaction mixtures and incubated in a heated block. The procedure is sufficiently simple to potentially allow this on-site assay to be performed without precise equipment and experienced staff. Conventional nucleic acid-based methods have many equipment...
requirements, such as liquid nitrogen, centrifuges and expensive thermocyclers with a series of different temperatures to achieve amplification in techniques such as AS-PCR, PCR-RFLP, real-time PCR, and RAPD-PCR. Compared with these existing PCR-based detection methods, this assay is relatively simple and generates easily interpreted results in just over 1 hour, including the time required for DNA extraction. A variety of field samples were assessed by using All-DNA-Fast-Out in a single step (approximately 5–10 minutes). The supernatant of the lysate can be directly used for LAMP amplification without centrifugation, extraction or other operational steps for traditional DNA extraction, thereby minimizing sample pretreatment time and decreasing contamination between samples to achieve rapid detection.

Figure 3. Optimization of reaction conditions for BCbi143/144-LAMP. Reaction temperature gradient of BCbi143/144-LAMP were set to 66.0 °C, 65.0 °C, 64.0 °C, 63.0 °C, 62.0 °C, 61.0 °C, 60.0 °C, 59.0 °C. (a) Optimization of temperature gradient on the basis of HNB color change. (b) Optimization of temperature on the basis of gel electrophoresis detection. Reaction times of BCbi143/144-LAMP were set to 15 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min, 90 min. (c) Optimization of reaction time on the basis of HNB color change. (d) Optimization of reaction time on the basis of gel electrophoresis detection.

Figure 4. Sensitivity test of G143A-LAMP and BCbi143/144-LAMP. Reaction tubes 1–7 were 10-fold dilutions of the cleavage products, with DNA concentrations of 100, 100 × 10^{-1}, 100 × 10^{-2}, 100 × 10^{-3}, 100 × 10^{-4}, 100 × 10^{-5}, 100 × 10^{-6} ng/μl, and the tube DW was ddH₂O blank control. (a) Sensitivity detection on the basis of HNB color change. (b) Sensitivity detection on the basis of gel electrophoresis detection.

The B. cinerea population constitutes three sub-populations: resistant to QoIs (common of G143A), sensitive to QoIs with Bcbi-143/144 intron and sensitive to QoIs without Bcbi-143/144 intron. The Qol-sensitive isolates without the Bcbi-143/144 intron have a high risk of developing resistance to Qol through the G143A single mutation, which is the main resistance mechanism of resistance to QoIs in B. cinerea. In contrast, the Qol-sensitive isolates with the Bcbi-143/144 intron generally have very low risk of developing resistance to Qols. In this study, to evaluate the use of LAMP as a field diagnostic tool for the application of QoIs against gray mold disease, we established a LAMP system for monitoring and evaluation of the risk of Botrytis cinerea.
resistance to QoIs. G143A-LAMP detected isolates resistant to QoIs, and BCbi143/144-LAMP indicated the percentage of the sub-population with a very low risk of resistance development. In general, gray mold results in 20 to 30% yield loss, up to 50% when the environment is favorable for B. cinerea epidemics. The management of

Figure 5. Detection of field strawberry samples by LAMP assays in fields and MIC testing in laboratory. (a) Strawberry samples from field, label 1 healthy samples, label 2–6 strawberry fruits of gray mold in field. (b) G143A-LAMP detection based on the color change of HNB, label 1–8 G143A mutant phenotypes of B. cinerea, labels 9–11: no mutant strains of B. cinerea. (c) Detection of field samples by MIC method, S represents QoI-sensitive strain, R represents strain of QoI-resistance. (d) Bcbi 143/144-LAMP reaction causing change in color, labels 1–3: B.cinerea with Bcbi –143/144 intron, lables 4–16: B.cinerea without Bcbi –143/144 intron. (e) Detection of the intron of the strain using PCR amplification.

| Origin                | Number of samples | G143A-LAMP | BCbi143/144-LAMP | MIC Positive | Number of t mutation genotypes |
|-----------------------|-------------------|-------------|-------------------|--------------|--------------------------------|
| Jiashan, Zhejiang     | 14                | 3           | 0                 | 3            | 3                              |
| Jiaode, Zhejiang      | 11                | 4           | 0                 | 4            | 0                              |
| Xiahsa, Zhejiang      | 10                | 7           | 0                 | 7            | 0                              |
| Zhuji, Zhejiang       | 10                | 9           | 1                 | 9            | 1                              |
| Linan, Zhejiang       | 21                | 12          | 2                 | 12           | 2                              |
| Tongxiang, Zhejiang   | 12                | 11          | 0                 | 11           | 0                              |
| Total                 | 78                | 46          | 3                 | 46           | 3                              |

Table 2. Field sample testing with LAMP, MIC and PCR. MIC Positive indicates a MIC (minimum inhibitory concentration) >100μg ml⁻¹.
gray mold is somewhat reliant on frequent applications of fungicides. QoI should not be used anymore for the management of B. cinerea with serious resistance as the tested populations in this study. According to the results of these two assays, however, QoIs can be applied in the scientific and sustainable management of B. cinerea in context with low or without resistance has occurred.

**Materials and Methods**

Isolates for developing LAMP assays. Three isolates of B. cinerea were adopted to develop the LAMP assays. ANB13-07 is the type with the Bcbi-143/144 intron inserted between the 143rd and 144th codon in the cyt b gene and is sensitive to azoxystrobin. LAB12-06 is highly resistant to azoxystrobin and has a point mutation at codon 143 in the cyt b gene (G143A). TMB15-06 contains the cyt b gene without the Bcbi-143/144 intron and is sensitive to azoxystrobin. Mycelia (approximately 2 mg) of B. cinerea were collected from each purely cultured strain on PDA, placed in a 0.2 ml tube containing 50 μl of DNA-EZ Reagents V All-DNA-Fast-Out (Sangon, Shanghai) and heated at 80 °C for 5 min in a water bath. The lysed supernatant was used as a DNA template to develop the LAMP assay. The extracted DNA was quantified by spectrophotometry and diluted with distilled water.

LAMP primers design and screening. In the current study, two LAMP tests were developed. One LAMP assay, referred to as the G143A-LAMP assay, detected G143A mutants of B. cinerea, which is highly resistant to QoI fungicides. The G143A-LAMP primers were designed and mismatched on the basis of the point mutation at codon 143 (G143A). Several sets of mismatched LAMP primers were screened from specificity and sensitivity to select a set of optimal primers. In addition, to detect the presence of the BCb143/144 intron in B. cinerea, another LAMP primer set was designed according to the intron 1205 bp DNA sequence and a unique 200–300 bp sequence from this intron, referred to as BCb143/144-LAMP assay. The LAMP primer sequences used are shown in Fig. 6 and Table 1.

LAMP reaction mixtures. Each LAMP assay was performed in a 25-μl reaction mixture, including a final concentration of 8 μl Bst DNA polymerase (New England Biolabs, Beijing), 2.5 μl 10 × ThermoPol buffer, 1 mM dNTPs, 5 mM MgCl₂, 1.6 μM FIP and BIP, 0.2 μM F3 and B3, 0.6 M betaine, 150 μM hydroxynaphthol blue (HNB, metal ion indicator), and 1 μl DNA sample (a concentration of 100 ng/μl that extracted by All-DNA-Fast-Out), and the volume was adjusted to 25 μl with nucleic acid-free water. In the G143A-LAMP assay, DNA samples extracted from the G143A mutant genotype (LAB12-06) were used as positive samples, and TMB15-06 with no mutation at codon 143 was selected as a negative control, and nucleic acid-free water was used as a blank control. For the BCb143/144-LAMP assay, isolate ANB13-07 was used as a positive control, and negative samples of TMB15-06 were used. Both LAMP reactions were incubated at 61 °C for 60 min and 80 °C for 5 min. Each treatment was repeated at least three times, as below. The reaction results were examined via visual color changes of HNB (from violet to sky blue) after the reaction and/or further confirmed via 1% agarose gel electrophoresis.

Determination of running-conditions. After the LAMP reactions were complete, the HNB color change in the reaction mixture was observed by naked eye under sunlight. For positive results, sky blue with HNB was observed, and negative or blank treatments remained violet. If confirmation was required, the reaction products (5 μl) were further assayed by 1% agarose gel electrophoresis, and the positive results showed typical LAMP ladder-like bands; negative reactions lacked these bands. To determine the optimal running conditions for the reactions, we set a series of constant temperatures, e.g., 59.0, 60.0, 61.0, 62.0, 63.0, 64.0, 65.0, 66.0 °C to detect the optimal reaction temperature. The LAMP assay was performed for 15, 30, 40, 50, 60, 70, 80, and 90 min at the optimal reaction temperature to determine the shortest optimal time.

Detection limit of LAMP assay. From the PDA surface of a pure culture of B. cinerea, hyphae were picked to extract DNA with ALL-DNA-Fast-Out. DNA samples were 10-fold diluted in water and used as templates for LAMP sensitivity testing; the final concentration of DNA samples was 100, 100 × 10⁻¹, 100 × 10⁻², 100 × 10⁻³, 100 × 10⁻⁴, 100 × 10⁻⁵, and 100 × 10⁻⁶ ng/μl. The detection limit represented the lowest DNA concentration at which positive results were observed. Samples were observed for HNB color change and further analyzed by 1% agarose gel electrophoresis.

Application to on-site rapid detection. To assess this LAMP for on-site detection, a total of 78 diseased strawberry fruits from greenhouses in six different geographical regions in Zhejiang Province, China during 2017 were tested. For each fruit, approximately 2 mg mold was collected from the fruit surface and added to 0.2 ml PCR tubes containing 50 μl of ALL-DNA-Fast-Out (Sangon, Shanghai). After incubation at 80 °C for 5–10 min in a heated block, the supernatant obtained was directly used in the LAMP assay as described above. Furthermore, each tested strawberry fruit was taken to the laboratory to verify the results of the on-site LAMP detection. B. cinerea was isolated and purified from a single colony isolated from the 78 strawberry samples. The sensitivity to azoxystrobin was evaluated using the traditional method of minimum inhibitory concentration (MIC). Briefly, mycelial plugs (5 mm) of each isolate were placed on potato dextrose agar (PDA) plates with a series of 0, 5, 20, and 100 μg/ml azoxystrobin (Syngenta, China). Each fungicide concentration was treated three times. All plates were incubated at 23 °C for 3 d. If the isolate had an MIC > 100 μg/ml, it was designated as highly resistant to QoIs. The DNA fragment including the 143rd codon of the B. cinerea cyt b gene was amplified by conventional PCR using the designed primers BC-cytb-F (5'-TAAAGTGGTATACCGCAGG-3') and BC-cytb-R (5'-CCATCTCCATCCACCATACCT-3'). The reactions contained the following reagents: 25 μl 2 × PCR Master, 0.4 μM primers of BC-cytb-F and BC-cytb-R, 1 μl DNA template, and ddH₂O to adjust the volume to 50 μl. The
thermal cycling of the conventional PCR program was 95 °C for 5 min; 30 reaction cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s with an extension at 72 °C for 5 min. The PCR products were separated by 1% agarose gel electrophoresis, and genomic DNA was purified using a UNIQ-10 Column DNA Purification Kit (Sangon, Shanghai). The purified product was directly inserted into the T-Vector PMD-19 (TAKALA, Dalian), according to the manufacturer's instructions. All purified PCR products were sequenced by Sangon, Shanghai. The resulting sequences were aligned using Clustal W software.

References
1. Mertely, J. C., Mackenzie, S. J. & Legard, D. E. Timing of fungicide applications for Botrytis cinerea based on development stage of strawberry flowers and fruit. Plant Dis. 86, 1019–1024 (2002).
2. Romanazzi, G., Nigro, F., Ippolito, A. & Salerno, M. Effect of short hypobaric treatments on postharvest rots of sweet cherries, strawberries and table grapes. Postharvest Bio. Tec. 22, 1–6 (2001).
3. Kovach, J., Petzoldt, R. & Harman, G. E. Use of honey bees and bumble bees to disseminate Trichoderma harzianum 1295-22 to strawberries for Botrytis control. Biol. Control 18, 235–242 (2000).
4. Kraiczy, P. et al. The molecular basis for the natural resistance of the cytochrome bc1 complex from strobilurin-producing basidiomycetes to center Qp inhibitors. Eur. J. Biochem. 235, 54–63 (1996).
5. Bartlett, D. W. et al. The strobilurin fungicides. Pest Manag. Sci. 58, 649–662 (2002).
6. Von, J. G. & Link, T. A. Use of specific inhibitors on the mitochondrial bc1 complex. Methods Enzymol. 126, 253–271 (1986).
7. Fernández-Ortuño, D. et al. Occurrence and distribution of resistance to QoI fungicides in populations of Podosphaera fusca in south central Spain. Eur. J. Plant Pathol. 115, 215–222 (2006).
8. Köller, W., Parker, D. M., Turecek, W. W., Avila-Adame, C. & Cronshaw, K. A two-phase resistance response of Venturia inaequalis populations to QoI fungicides kresoxim-methyl and trifloxystrobin. Plant Dis. 88, 537–544 (2004).
9. Frawje, B. A. et al. Role of ascospores in further spread of QoI-resistant cytochrome b alleles (G143A) in field populations of Mycosphaerella graminicola. Phytopathology 95, 933–941 (2005).
10. Torrini, S. F., Goodwin, S. B., Kema, G. H., Pangilinan, J. L. & Mcdonald, B. A. Intraspecific comparison and annotation of two complete mitochondrial genome sequences from the plant pathogenic fungus Mycosphaerella graminicola. Fungal Genet. Biol. 45, 628–637 (2008).
11. Genet, J. L., Jaworska, G. & Deparis, F. Effect of dose rate and mixtures of fungicides on selection for QoI resistance in populations of Plasmopara viticola. Pest Manag. Sci. 62, 188–194 (2006).
12. Baudoin, A., Olaya, G., Delmotte, F., Colcol, J. F. & Sierotzki, H. QoI resistance of Plasmopara viticola and Erysiphe necator in the mid-Atlantic United States. Plant Health Progress 22, 22–24 (2008).
13. Aviladame, C., Olaya, G. & Köller, W. Characterization of Colletotrichum graminicola isolates resistant to strobilurin-related QoI fungicides. Plant Dis. 87, 1426–1432 (2004).
14. Zhang, C. B., Liu, Y. H., Ding, L. & Zhu, G. N. Shift of sensitivity of Botrytis cinerea to azoxyestrogen in greenhouse vegetables before and after exposure to the fungicide. Phytoparasitica 39, 293–302 (2011).
15. Markoglu, A. N., Malandrakis, A. A., Vitoratos, A. G. & Ziogas, B. N. Characterization of laboratory mutants of Botrytis cinerea resistant to QoI fungicides. Eur. J. Plant Pathol. 115, 149–162 (2006).
16. Yin, Y. N., Kim, Y. K. & Xiao, C. L. Molecular characterization of pyraclostrobin resistance and structural diversity of the cytochrome b gene in Botrytis cinerea from apple. Phytopathology 102, 315–322 (2012).
17. Vega, B., Libertti, D., Harmon, P. F. & Dewdney, M. A. A rapid resazurin-based microtiter assay to evaluate QoI sensitivity for Alternaria alternata isolates and their molecular characterization. Plant Dis. 96, 1262–1270 (2012).
18. Sierotzki, H. et al. Cytochrome b gene sequence and structure of Pyrenophora teres and P. triticivora reis and implications for QoI resistance. Pest Manag. Sci. 63, 225–233 (2007).
19. Grassro, V., Palermo, S., Sierotzki, H., Garibaldi, A. & Gisi, U. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. Pest Manag. Sci. 62, 465–472 (2006).
20. Myresiotis, C. K., Karaglanidis, G. S. & Travella-Klonari, K. Resistance of Botrytis cinerea isolates from vegetable crops to antinopyrimidine, phenylpyrrole, hydroxanilide, benzimidazole, and dicarboxamide fungicides. Plant Dis. 91, 407–413 (2007).
21. Liu, S., Che, Z. & Chen, G. Multiple-fungicide resistance to carbendazim, diethofencarb, procymidine, and pyrimethanil in field isolates of Botrytis cinerea from tomato in Henan Province, China. Crop Prot. 84, 56–61 (2016).
22. Ziogas, N. B., Nikou, D., Markoglu, N. A., Malandrakis, A. A. & Vontas, J. 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 (2009).
23. Malandrakis, A. A., Markoglu, N. A. & Ziogas, B. N. PCR-RFLP detection of the E198A mutation conferring resistance to benzimidazoles in field isolates of Monilinia laxa from Greece. Crop Prot. 39, 11–17 (2012).
24. Notomi, T., Mori, Y., Tomita, N. & Kanda, H. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. J. Microbiol. 53, 1–5 (2015).
25. Iosi, A., Kadhim, M. J. & Olszewski, K. LAMP - a method of isothermal DNA amplification. Med. Weter. 72, 22–27 (2016).
26. Tomita, N., Mori, Y. & Kanda, H. & Notomi, T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. 3, 877–882 (2008).
27. Tomlinson, J. A., Dickinson, M. J. & Boonham, N. Detection of Botrytis cinerea by loop-mediated isothermal amplification. Lett. Appl. Microbiol. 51, 650–657 (2010).
28. Shen, W., Xu, G., Sun, L., Zhang, L., & Liang, Z. Development of a loop-mediated isothermal amplification assay for rapid and sensitive detection of Sporisorium scitaninum in sugarcane. Ann. Appl. Biol. 168, 321–327 (2016).
29. Huang, W. et al. Loop-mediated isothermal amplification method for the rapid detection of Ralstonia solanacearum phytopath type I nullberry strains in China. Front Plant Sci. 8, 76, https://doi.org/10.3389/fpls.2017.00076 (2017).
30. Duan, Y. et al. Development of a rapid and high-throughput molecular method for detecting the F200Y mutant genotype in benimidazole-resistant isolates of Fusarium asiaticum. Pest Manag. Sci. 72, 2128–2135 (2016).
31. Duan, Y. B. et al. Development and application of loop-mediated isothermal amplification for detecting the highly benzimidazole-resistant isolates in Sclerotinia sclerotiorum. Sci Rep 5, 17278, https://doi.org/10.1038/srep17278 (2015).
32. Pan, L., Li, J., Zhang, W. N. & Dong, L. Detection of the 11781L mutation in fenoxyacop-p-ethyl-resistant American sloughgrass (Beckmannia syzigachne Steud.), based on the loop-mediated isothermal amplification method. Pest Manag. Sci. 71, 123–130 (2015).
33. Goto, M., Honda, E., Ogura, A., Nomoto, A. & Hanaki, K. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue dye. Biotechniques 46, 167–172 (2009).
34. Luo, J. et al. Visual detection of norovirus genogroup II by reverse transcription loop-mediated isothermal amplification with hydroxy napthol blue dye. Food Environ. Virol. 6, 196–201 (2014).
35. Baptista, F. J., Bailey, B. J. & Menses, J. F. Effect of nocturnal ventilation on the occurrence of Botrytis cinerea in Mediterranean unheated tomato greenhouses. Crop Prot. 32, 144–14 (2012).
36. Williamson, B., Tudzynski, B., Tudzynski, P. & Van Kan, J. A. Botrytis cinerea: the cause of grey mold disease. Mol. Plant Pathol. 8, 561–580 (2007).
37. Roslenbroich, H. J. & Stuebler, D. Botrytis cinerea - history of chemical control and novel fungicides for its management. Crop Prot. 19, 557–561 (2000).

Acknowledgements
This work was supported by a grant from the Key Research and Development Project of Zhejiang Province, China (No. 2015C02G1320008).

Author Contributions
Zhang C.Q. and Wang H.D. designed the study, Hu X.R. and Dai D.J. were responsible for conducting experiments, analyzing and interpreting results and initial drafting of the manuscript. Zhang edited the manuscript. All authors read and approved the manuscript.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
