



## Article

# Detection of a Point Mutation (G143A) in Cyt b of *Corynespora cassiicola* That Confers Pyraclostrobin Resistance

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**Abstract:** Point mutation G143A in the cytochrome b (Cyt b) protein commonly confers resistance to quinone outside inhibitor (QoI) fungicides in phytopathogenic fungi, including *Corynespora cassiicola*, which causes cucumber target spot disease. However, the effect of G143A on the binding between the QoI fungicide and the Cyt b protein, and the use of LAMP (loop-mediated isothermal amplification) to detect this point mutation had not been reported previously in *C. cassiicola*. In this study, the sensitivity of 131 *C. cassiicola* isolates—collected from Shandong province, China in 2019 and 2020—to pyraclostrobin was determined. The EC<sub>50</sub> values ranged from 1.67 to 8.82 µg/mL, and sequencing results showed that all *C. cassiicola* isolates contained the G143A mutation. Molecular docking results suggested that G143A significantly alters the affinity of pyraclostrobin to the Cyt b protein. Following development of three LAMP primer pairs, the best reaction condition for LAMP analysis was 65 °C for 60 min, and the detection limit was 0.01 ng/µL of DNA containing the point mutation. In conclusion, the G143A mutation conferring pyraclostrobin resistance is widespread in *C. cassiicola* from Shandong province, and the LAMP method can be used to monitor QoI resistance in *C. cassiicola* caused by the G143A mutation in the field.

**Keywords:** fungicide resistance; molecular docking; cucumber target spot; QoI



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## 1. Introduction

Cucumber target spot is a worldwide fungal disease in cucumber production [1,2]. It is caused by *Corynespora cassiicola*, which can infect a wide range of hosts, including nematode cysts and the human skin [3–6]. In addition to cucumber, it can also cause disease on leaves, stems, roots, flowers, and fruits in up to 530 plant species [7]. A saprophytic, endophytic lifestyle complicates *C. cassiicola* management [8].

Chemical control is the main method to limit cucumber target spot disease in China. At present, the domestically registered agrichemicals for the prevention and control of this disease include quinone outside inhibitors (QoI) (pyraclostrobin, trifloxystrobin, azoxystrobin), succinate dehydrogenase inhibitors (SDHI) (pydiflumetofen, fluopyram, fluxapyroxad), and sterol biosynthesis inhibitors (SBI) (difenoconazole, tebuconazole, prochloraz) (<http://www.chinapesticide.org.cn/> (accessed on 9 May 2021)). However, the long-term and frequent use of these chemical fungicides has caused *C. cassiicola* populations to gradually develop fungicide resistance [2,9–14]. Thus, timely detection is of particular importance for fungicide resistance management and guidance on the scientific use of fungicides in the field.

Traditional in vitro methods, such as mycelial growth rate and spore germination methods, are widely used for determining fungicide sensitivity (<https://www.frac.info/>

(accessed on 9 May 2021)). Often, highly resource-intensive methods limit the number of isolates that can be assayed, and some pathogens are difficult to culture in vitro. Thus, the isolation, culture, identification, and preservation of target pathogens is time-consuming and labor-intensive. Based on a clear resistance mechanism (point mutation in a target protein), many rapid and progressive molecular tools, such as AS-PCR (allele-specific PCR), PCR-RFLP (PCR-restriction fragment length polymorphism), qPCR, droplet digital PCR, and pyrosequencing, have been developed or used to detect fungicide-resistant populations in different plant pathogens [15–19]. However, sophisticated and expensive instruments and complicated test techniques limit the application of these methods in small laboratories.

LAMP (loop-mediated isothermal amplification) is a constant-temperature nucleic acid amplification technology, using *Bst* (*Bacillus stearothermophilus*) DNA polymerase developed by Japanese workers [20,21]. Compared with PCR methods, LAMP is more rapid and sensitive, and the results are more easily analyzed. The products of LAMP can be analyzed by agarose gel electrophoresis, turbidity, or fluorescence. This method has been extensively used to identify pathogenic microorganisms (viruses, fungi, bacteria, oomycetes) [22–25]. From 2014, LAMP assays were gradually developed to monitor SBI, QoI, SDHI, and MBC (methyl benzimidazole carbamate) fungicide resistance in different plant pathogens [26–30]. Although QoI resistance and the point mutation G143A in the Cyt b protein have been widely detected in *C. cassiicola* [12], the effect of this point mutation on the binding between pyraclostrobin and Cyt b, and an LAMP assay for this point mutation in *C. cassiicola* have not been reported. Thus, the aim of the current study was to (1) investigate the pyraclostrobin sensitivity of *C. cassiicola* isolated from infected cucumber leaves collected from Shandong province during 2019 and 2020; (2) confirm the relationship between point mutation G143A and pyraclostrobin resistance; and (3) develop an LAMP method for detecting G143A in *C. cassiicola* isolates.

## 2. Materials and Methods

### 2.1. Pathogens and Plants

*C. cassiicola* isolates, 131 in total, were obtained from cucumber leaves with typical symptoms from 8 cities in Shandong province during 2019 and 2020. Pyraclostrobin-sensitive isolates Cc4 and Cc10 were kindly provided by Associate Professor Yabing Duan of Nanjing Agricultural University.

Cucumber seeds (cv. Youliangwang) were sown in plastic pots (10 × 10 × 10 cm, 2 seeds per pot) filled with a peat/vermiculite mixture (2:1 v/v). All seedlings were grown under greenhouse conditions (27 ± 2 °C, 80% relative humidity, and 12/12 h photoperiod) until the four-leaf stage.

For mycelium collection, *C. cassiicola*, *Didymella bryoniae*, and *Botrytis cinerea* were maintained on PDA (potato dextrose agar), and *Phytophthora melonis* was inoculated on WKB (white kidney bean) agar [31]. For detached cucumber leaf inoculation, a mycelial plug of four of the above pathogens was placed on the upper surface of leaves for 4 days at 25 °C.

### 2.2. Determination of Pyraclostrobin Sensitivity

Pyraclostrobin (98%, active ingredient) was provided by Shandong United Pesticide Industry Co. Ltd. (Taian, China). A stock solution (10<sup>4</sup> µg/mL) was prepared with DMSO (dimethyl sulfoxide). Sensitivity of *C. cassiicola* to pyraclostrobin was investigated using the mycelial growth rate method. Agar disks (diameter 5 mm) containing *C. cassiicola* mycelium were inoculated onto AEA (alkyl ester agar medium) plates containing a series of concentrations (0.2, 0.5, 1, 5, 10, 20, 40, 80 µg/mL) of pyraclostrobin and 50 µg/mL of SHAM (salicylhydroxamic acid). *C. cassiicola* was incubated at 25 °C for 6 days in darkness, and then colony diameters were measured. Each treatment had three replicates, and the experiment was repeated twice. EC<sub>50</sub> values were calculated according to a previous study [12].

### 2.3. Genomic DNA Extraction and Analysis of the *Cyt b* Gene

Mycelium (100 mg) or the infected cucumber leaf sample (100 mg), which was frozen in liquid nitrogen, was ground in a ball mill for 2 min. (1) A total of 800 µL of 3% CTAB was added to each tube, followed by 1 h incubation at 60 °C in a water bath. (2) A total of 800 µL phenol–chloroform–isoamyl alcohol (25:24:1, *v:v*) was added. The sample was mixed gently by inversion and then spun at 12,000 rpm for 10 min. (3) The supernatant was transferred into a new centrifuge tube, and 650 µL of chloroform–isoamyl alcohol (24:1, *v:v*) was added. (4) The supernatant was transferred into a new centrifuge tube after centrifuging at 12,000 rpm for 10 min. (5) A 0.6-fold isoamyl alcohol was added and incubated for 30 min at –20 °C. (6) Tubes were centrifuged at 12,000 rpm for 10 min. (7) The liquid was decanted, and the precipitate was washed with ice-cold 70% ethanol. This step was done twice. (8) Tubes were placed into a Concentrator Plus (Eppendorf, Hamburg, Germany) with the caps open. The Concentrator Plus was run at 42 °C for approximately 10 min. (9) Dry precipitate was resuspended in 50 µL of ddH<sub>2</sub>O. The concentration of genomic DNA was tested with a NanoDrop ONE spectrophotometer (ThermoScientific, Waltham, MA, USA). The *Cyt b* gene was amplified using Primers CcCytb-F/CcCytb-R (Table 1) [12] and M5 Taq PCR Mix with Blue Dye (Mei5 Biotechnology, Co., Ltd., Beijing, China). PCR products were sequenced by Tsingke Biotechnology Co., Ltd. (Xian, China), and sequences were analyzed using DNAMAN 6.0 Software.

**Table 1.** Primers used for LAMP and partial *Cyt b* gene amplification from *Corynespora cassiicola*.

Primer Name	Sequence (5'-3') <sup>a</sup>	Use
F3	CCTGGGTATGTTTTACCATAC	Forward outer primer for LAMP
B3	AAGTGCATTAGTGCTAAAAGC	Backward outer primer for LAMP
BIP	TTGAGGAGGTTTCTCTGTAAACAATGCTAATACGAAAGGTAAAACGAAAT	Backward inner primer for LAMP
FIP1	ACTCAAGGGATGGCACTCATTAGGTCAAATGTCCTTATGACC	Forward inner primers to detect the G143A mutants of <i>Cyt b</i> gene in <i>C. cassiicola</i> for LAMP
FIP2	ACTCAAGGGATGGCACTCATTAGGTCAAATGTCCTTATGAAC	
FIP3	ACTCAAGGGATGGCACTCATTAGGTCAAATGTCCTTATGATC	
F	GTCAAATGTCCTTATGATC	To amplify partial fragments (163 bp) of <i>Cyt b</i> gene of <i>C. cassiicola</i>
R	GCTAATACGAAAGGTAAAACGAAAT	
CcCytb-F	GCGAATTCCTATTAGTTGATTC	PCR primers to amplify the partial <i>Cytb</i> from <i>C. cassiicola</i> [12]
CcCytb-R	GGTTACCTGATCCAGCTGTATC	

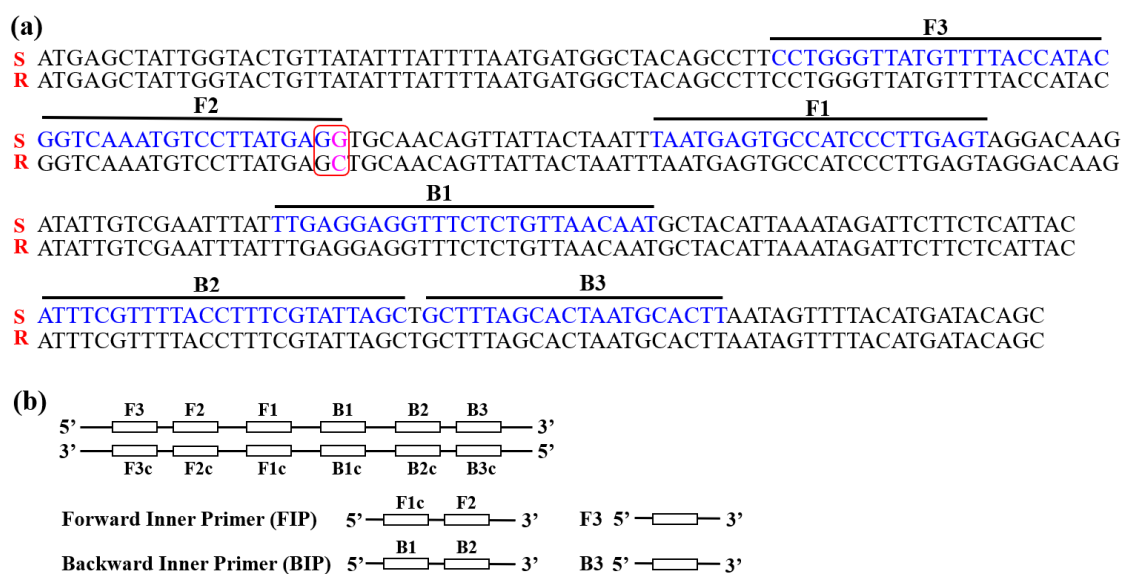
<sup>a</sup> Nucleotides with underline were mutant in the *Cyt b* gene sequence from pyraclostrobin-sensitive and -resistant *C. cassiicola* isolates. Nucleotides in boxes are mismatches manually introduced to detect G143A resistant genotypes of *C. cassiicola*.

### 2.4. Molecular Docking of Pyraclostrobin in *Cyt b* of *C. cassiicola*

An in silico model of the *C. cassiicola* Qo-site was built by comparative modelling using MODELLER v9.19, with the yeast structure (PDB 1KB9) [32] as a template, that exhibits 57.89% sequence identity with the sequence from *C. cassiicola*. To evaluate the stereochemical quality of the CcCyt b 3D model, a Ramachandran map was generated using PROCHECK. Verify3D was used to analyze the compatibility of the CcCyt b model with its own amino acid sequence. The structure of the substrate (pyraclostrobin) was constructed using AutoDock 4.2.6 and optimized using the MOPAC program. To exclude unreasonable spatial structures and make the binding model more stable, energy optimization was performed using an Amber14 force field. Autodock results were analyzed with AutoDock Tools 1.5.6., and illustrations of the 3D models were generated using Ligplot 1.4.5 and Pymol 1.8.6.

### 2.5. LAMP Primer Design

The LAMP primer was designed on the website Primer Explorer (v5) (<http://primerexplorer.jp/e/>) (accessed on 8 February 2021) according to the mutation GGT (Gly) → GCT (Ala) at the 143 amino acid codon of *Cyt b*, and the mutation site was located at the 3' end of the forward inner primer (FIP). Three pairs of primers with a mismatch at the penultimate position were designed to improve the specificity of the LAMP primers (Table 1, Figure 1).



**Figure 1.** LAMP primers for the G143A mutation of *Cyt b* in *Corynespora cassiicola*. (a) Sequence alignment of sensitive and resistant *Cyt b* alleles. The sequences used as LAMP primers are indicated by vertical lines. For each mutation, the F2 primer ends with its corresponding G143A mutant nucleotide. (b) Schematic diagram of LAMP primers used in this work. The internal primer FIP is composed of F1c and F2, whereas BIP is composed of B1 and B2c. The primers ending with “c” are complementary to the primers without “c”.

## 2.6. LAMP Reaction Mixtures

WarmStart Colorimetric LAMP 2× Master Mix (New England Biolabs, Beijing, China) was used in this study. The LAMP reaction mixture (25 µL) included a 12.5 µL WarmStart Colorimetric LAMP 2× Master Mix (New England Biolabs), 1.6 µM FIP and BIP, 0.2 µM F3 and B3, 0.4 µM loop F and loop B, and a 1 µL DNA template. The results were double confirmed through visual color changes (from pink to yellow) and 2% agarose gel electrophoresis.

## 2.7. Optimization of LAMP Reaction Time and Temperature

A series of constant temperatures (i.e., 61, 63, 65, 67, 69 °C), and a series of reaction times (i.e., 30, 45, 60, 75, and 90 min) were set to investigate the optimal reaction time and temperature.

## 2.8. LAMP Assay Specificity

To test the specificity of the LAMP assay, pyraclostrobin-sensitive *C. cassiicola* isolates (Cc4 and Cc10), pyraclostrobin-resistant *C. cassiicola* isolates (Cc21, Cc23, Cc31, Cc35, Cc42, Cc53, Cc71, Cc66, Cc96, Cc123), *D. bryoniae*, *B. cinerea*, and *P. melonis*, isolated from diseased cucumber plants, were selected. Genomic DNA of wild-type Cc4, Cc10, and ddH<sub>2</sub>O were used as negative controls and blank, respectively. The LAMP assay was performed at 65 °C for 60 min. Experiments were repeated three times.

## 2.9. LAMP Assay Detection Limit

A concentration series (100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng/µL) of DNA template was used to determine the detection limit of the LAMP assay for the G143A mutation in *C. cassiicola*.

## 3. Results

### 3.1. Pyraclostrobin Sensitivity of *C. cassiicola* Isolates and Sequence of *Cyt b* Gene

The EC<sub>50</sub> values of pyraclostrobin for the 131 *C. cassiicola* isolates ranged from 1.67 to 8.82 µg/mL, with a mean of 4.74 µg/mL (Table 2). There was no correlation between the

fungicide sensitivity and source of isolates (Table 2). Sequencing of the *Cyt b* gene showed that there was a G143A mutation in the *Cyt b* gene in all *C. cassiicola* isolates.

**Table 2.** Sensitivity of *Corynespora cassiicola* isolates collected from Shandong province to pyraclostrobin.

Source	Pyraclostrobin		
	N <sup>†</sup>	Range of EC <sub>50</sub> (µg/mL)	Mean ± SD <sup>**</sup> (µg/mL)
Lanling County, Linyi City	25	1.98–8.68	5.09 ± 2.25 <sup>ns</sup>
Xin County, Liaocheng City	16	1.67–6.94	4.74 ± 1.58
Daiyue District, Taian City	22	1.74–8.74	5.09 ± 1.79
Laicheng District, Laiwu City	4	1.95–4.69	3.71 ± 2.20
Pingyuan County, Dezhou City	12	1.68–8.82	3.92 ± 2.04
Shouguang City, Weifang City	26	1.99–8.00	5.32 ± 1.92
Jiyang County, Jinan City	12	1.70–6.21	3.68 ± 1.71
Zhoucun County, Zibo City	14	1.88–8.69	4.43 ± 1.95
Cumulative	131	1.67–8.82	4.74 *
Duan's lab <sup>§</sup>	2	0.006–0.01	0.008

<sup>†</sup> N = Number of isolates. <sup>\*\*</sup> SD = standard deviation. <sup>ns</sup> not significant data ( $p = 0.05$ ). \* The mean EC<sub>50</sub> value of pyraclostrobin to 131 *Corynespora cassiicola* isolates. <sup>§</sup> Two pyraclostrobin-sensitive isolates Cc4 and Cc10 were kindly provided by Associate Professor Yabing Duan of Nanjing Agricultural University.

### 3.2. Pyraclostrobin Docking in the *Cyt b* of *C. cassiicola*

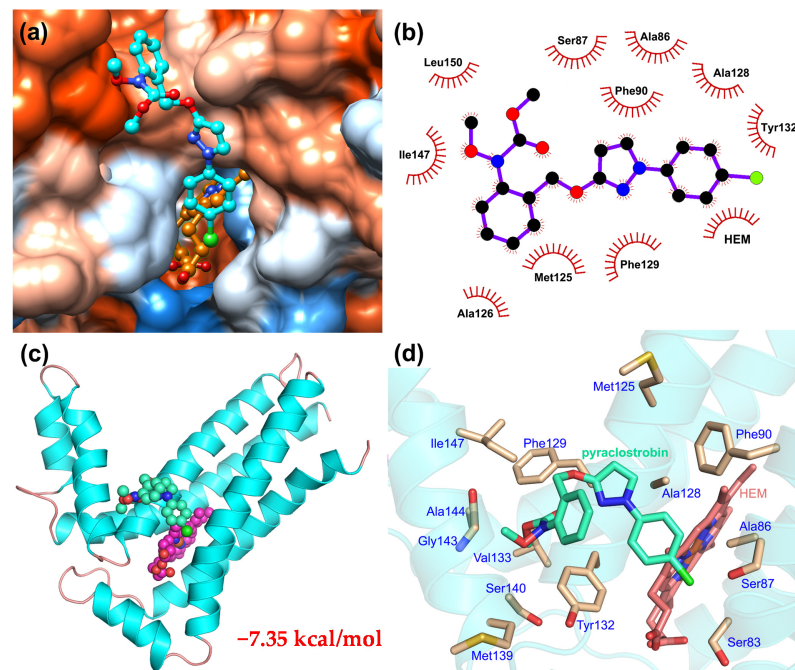
Docking results showed that pyraclostrobin can be bound to the vicinity of heme and form a more stable structure (Figures 2 and 3). For wild-type CcCyt b, pyraclostrobin binds in a hydrophobic pocket composed of 14 amino acid residues, including Ser83, Ser86, Ser87, Phe90, Met125, Ala128, Phe129, Tyr132, Val133, Met139, Ser140, Gly143, Ala144, and Ile147. Among them, Phe90, Ala128, Phe129, Ala144, and Ile147 have strong hydrophobicity, and hydrophobic effects can enhance the affinity between pyraclostrobin and CcCytb (−7.35 kcal/mol) (Figure 2). For mutated CcCyt b with G143A, pyraclostrobin is incorporated in a hydrophobic pocket consisting of 10 amino acid residues, including Ser86, Ser87, Phe90, Met125, Ala126, Ala128, Phe129, Tyr132, Ile147, and Leu150. The number of residues recognized by pyraclostrobin is reduced. Because of the G143A mutation, the side chain is changed to a methyl group. Due to the effect of the spatial position, the direction of pyraclostrobin is changed, resulting in a changed molecular conformation. Thus, the binding capacity of pyraclostrobin and CcCyt b with G143A was weakened (−6.59 kcal/mol) (Figure 3).

### 3.3. LAMP Reaction Optimization

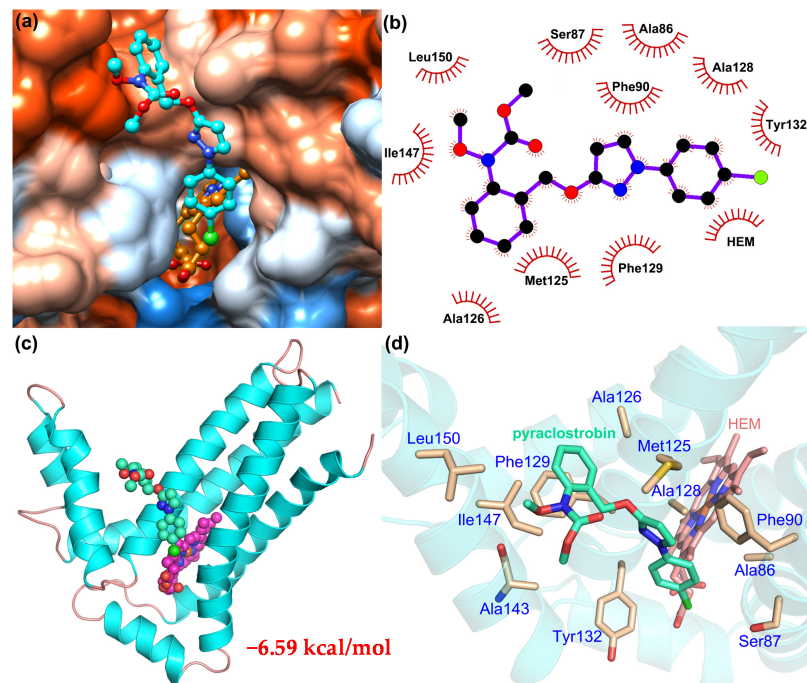
The two primer sets, containing FIP2 or FIP3, amplified the target *Cyt b* gene with the G143A mutation, according to a color change from pink to orange (Figure 4a). Thus, the primer set F3, B3, FIP3, and BIP, and corresponding genomic DNA were used for further optimization of the LAMP reaction. When the LAMP reaction was performed at 65 °C, the color change (pink to yellow) and intensity of the ladder-like band were significant (Figure 4b). Thus, the optimal reaction temperature was 65 °C, the same as the recommended temperature. In addition, the reaction time was also optimized at 65 °C. The target gene could be amplified within 45–75 min, and the color change (pink to yellow) and ladder-like band were most distinct after 60 and 75 min, respectively (Figure 4c). Thus, the LAMP reaction condition for the tested primer set was incubation at 65 °C for 60 to 75 min.

### 3.4. Detection Limit of LAMP Assay and Conventional PCR

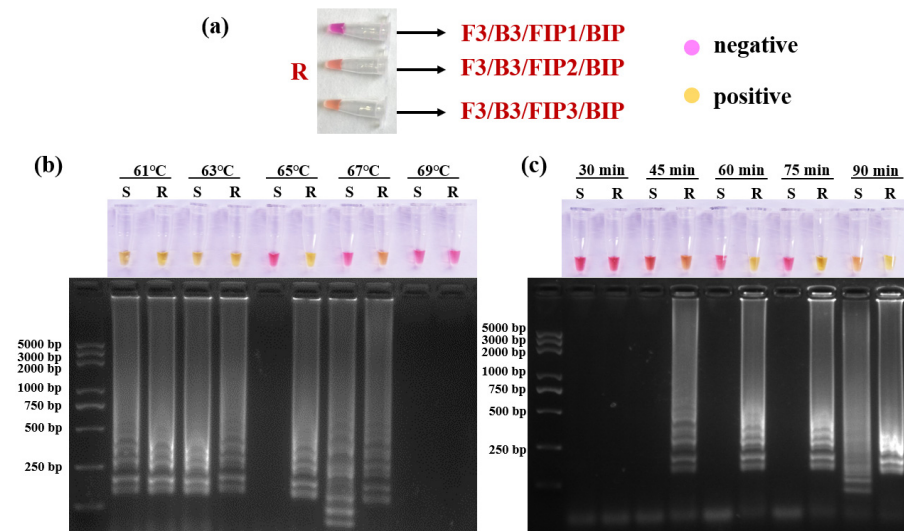
The lowest detection limit of the LAMP reaction was determined using the primer set (F3, B3, FIP3, and BIP) and tenfold serial dilutions of genomic DNA containing the G143A mutation from 100 ng/µL. The detection limit of LAMP is 0.01 ng/µL, and the detection limit of conventional PCR is 1 ng/µL (Figure 5).



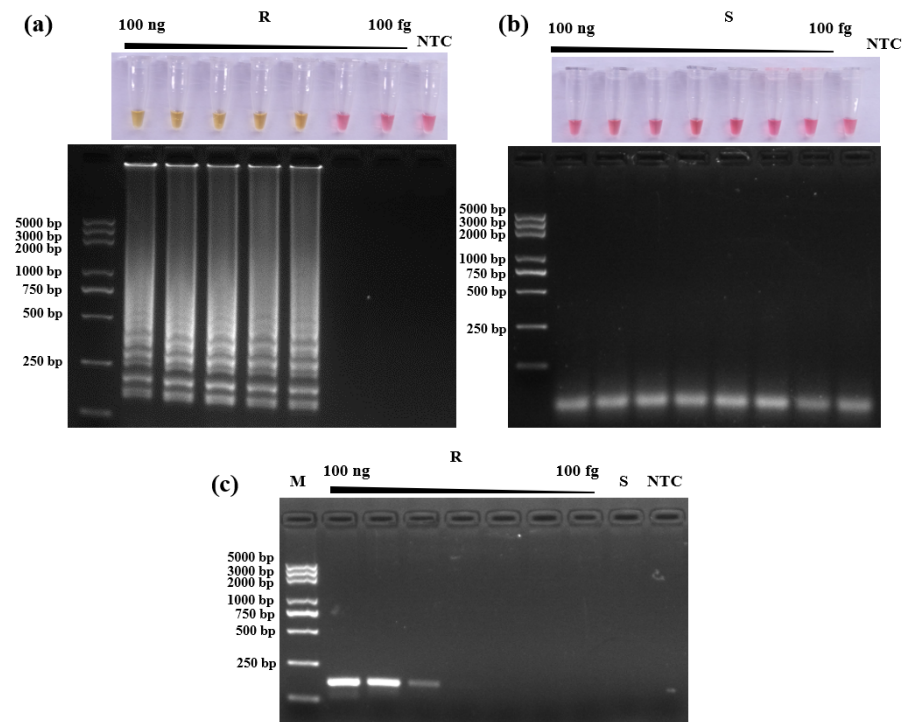
**Figure 2.** Binding mode of pyraclostrobin and CcCyt b protein. (a) Binding mode of pyraclostrobin, and hydrophobic and hydrophilic surfaces of CcCyt b protein. Blue and orange represent hydrophilic and hydrophobic regions, respectively. (b) Two-dimensional interaction of pyraclostrobin and CcCyt b, whereby red zigzag represents hydrophobic effect. (c) Binding position of pyraclostrobin and CcCyt b protein. (d) Interaction of pyraclostrobin and the amino acids of CcCyt b.



**Figure 3.** Binding mode of pyraclostrobin and CcCyt b protein with G134A. (a) Binding mode of pyraclostrobin and hydrophobic and hydrophilic surfaces of CcCyt b-G134A protein. Blue and orange represent hydrophilic and hydrophobic regions, respectively. (b) Two-dimensional interaction of pyraclostrobin and CcCyt b-G134A, whereby red zigzag represents hydrophobic effect. (c) Binding position of pyraclostrobin and CcCyt b-G134A protein. (d) Interaction of pyraclostrobin and the amino acids of CcCyt b-G134A.



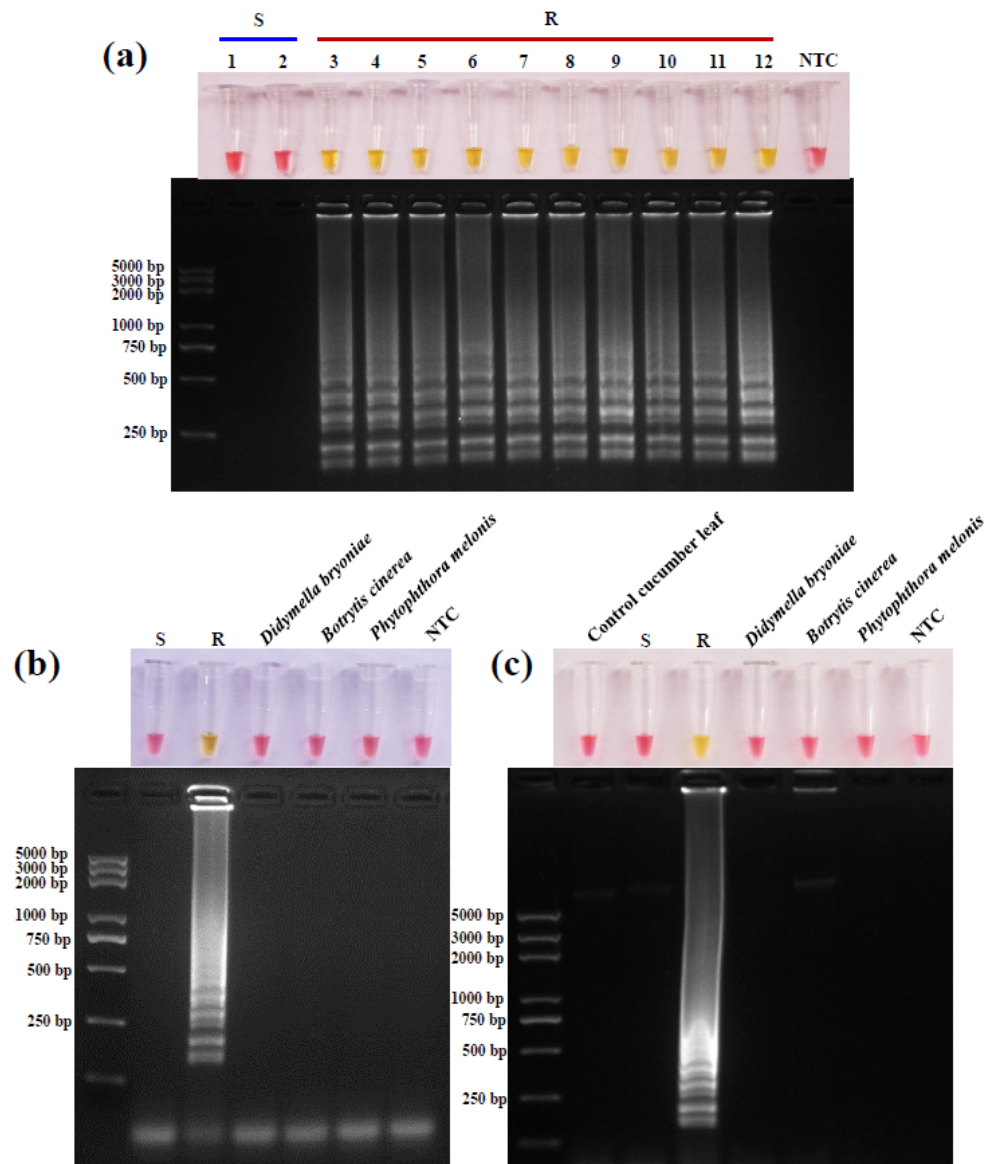
**Figure 4.** Optimization of LAMP reaction conditions for G143A in *Corynespora cassiicola*. (a) Selection of FIP primer of LAMP reaction. Optimization of the LAMP reaction temperature (b) and time (c) on the basis of color change (upper) and corresponding gel electrophoresis detection (lower). Reaction temperature gradient was set to 61, 63, 65, 67, and 69 °C, and the reaction time gradient was 30, 45, 60, 75, and 90 min. “S”, pyraclostrobin-sensitive isolate Cc4; “R”, pyraclostrobin-resistant isolate Cc66.



**Figure 5.** Sensitivity test of the LAMP reaction (a,b) and conventional PCR (c) for G143A in *Corynespora cassiicola*. LAMP reaction products of pyraclostrobin-resistant isolate (a) and -sensitive isolate (b) were evaluated on the basis of color change (upper) and corresponding gel electrophoresis detection (lower); conventional PCR products were assessed by 2% gel electrophoresis (c). The template genomic DNA concentrations used in reaction tubes from left to right were 100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng, and 100 fg. “S”, pyraclostrobin-sensitive *C. cassiicola* isolate Cc4; “R”, pyraclostrobin-resistant *C. cassiicola* isolate Cc66. “NTC”, negative treatment control with dH<sub>2</sub>O as reaction template.

### 3.5. LAMP Method Specificity

Genomic DNA was extracted from mycelia or infected cucumber leaves of several common pathogens of cucumber. The LAMP reaction results were positive only for *C. cassiicola* isolates containing the G143A mutation, and negative for pyraclostrobin-sensitive *C. cassiicola* isolates and *D. bryoniae*, *B. cinerea*, and *P. melonis*, irrespective of the DNA sampled from mycelia (Figure 6a,b) or infected leaves (Figure 6c).



**Figure 6.** Specificity of LAMP assay for the G143A mutation in the *Cyt b* gene of *Corynespora cassiicola* or other causal agents (*Didymella bryoniae*, *Botrytis cinerea*, and *Phytophthora melonis*) that cause common cucumber diseases. Genomic DNA of mycelia (a,b) or infected cucumber leaves (c) of four important pathogens. “S”, pyraclostrobin-sensitive *C. cassiicola* isolates (Cc4, Cc10); “R”, pyraclostrobin-resistant *C. cassiicola* isolates (Cc21, Cc23, Cc31, Cc35, Cc42, Cc53, Cc71, Cc66, Cc96, Cc123). “NTC”, negative control using dH<sub>2</sub>O as reaction template.

## 4. Discussion

Duan et al. reported that all 619 *C. cassiicola* isolates (obtained in 2017 and 2018) from greenhouse-cultivated cucumbers in China contained the G143A mutation in *Cyt b* [12]. Thus, QoI resistance is a very serious problem in *C. cassiicola* populations from cucumber in China. Continuous resistance monitoring is of high significance for rational



application of QoI in the protection and control of cucumber target spot disease. In the current study, all 131 *C. cassiicola* isolates obtained from Shandong province collected in 2019 and 2020 contained the G143A mutation. The EC<sub>50</sub> values of pyraclostrobin for field isolates collected in this study were 1.67–8.82 µg/mL, similar to the data (0.51–7.01) for eight G143A-containing *C. cassiicola* strains for pyraclostrobin reported by Duan et al. [12]. Compared with the two QoI-sensitive wild-type *C. cassiicola* isolates (Cc4 and Cc10), which had a low range of EC<sub>50</sub> values varying from 0.006 to 0.01 µg/mL (Table 2), all 131 *C. cassiicola* isolates had high levels of QoI resistance.

Apart from the relatively high inherent resistance risks of *C. cassiicola* and QoI, long-term continuous use is likely the main cause of current QoI resistance in *C. cassiicola*. The first QoI—trifloxystrobin—was registered to control cucumber target spot disease in 2014, and pyraclostrobin was registered in 2018 (<http://www.chinapesticide.org.cn/> (accessed on 9 May 2021)). However, QoIs have been registered in China to control cucumber downy mildew, powdery mildew, and anthracnose for approximately 15 years. The symptoms of cucumber target spot are similar to those of downy mildew, bacterial angular leaf spot, and anthracnose, and it is not easy to distinguish them. The use of fungicides for downy mildew, bacterial angular leaf spot, and anthracnose during production is often not appropriate. This might be an important reason for the development of QoI resistance in *C. cassiicola* in China, but the actual field control effect of pyraclostrobin to *C. cassiicola* should be evaluated further.

It has been reported that QoI resistance of *Podosphaera xanthii* and *Pseudoperonospora cubensis* was observed to decline under no QoI fungicide selection pressure [33,34]. Furthermore, heteroplasmy (the coexistence of wild-type and mutated genes) was also found in QoI-resistant isolates of *C. cassiicola*, *Mycovellosiella natrassii*, *Colletotrichum gloeosporioides*, and *Podosphaera leucotricha* [35,36]. Thus, it is recommended to suspend or postpone the use of QoI fungicides in the prevention and control of cucumber target spot disease.

G143A, F129L, and G137R are the three most widely reported main causes of QoI resistance. Many reports have shown that F129L and G137R mediate only low to moderate resistance levels and might have larger fitness penalties [37–40]. Interestingly, F129L and G137R were not found in any reported QoI-resistant *C. cassiicola* isolates obtained from cucumber, and F129L was found only in three moderately QoI-resistant *C. cassiicola* isolates obtained from tomato [12,13,36]. Thus, G143A is the most widespread mutation in QoI-resistant pathogens. Docking results showed that the G143A mutation causes a change in the binding pattern of pyraclostrobin and CcCyt b, and the number of amino acid residues that are recognized around the recognition is reduced. Hydrophobic interaction is reduced, leading to lowered binding ability of pyraclostrobin and the active center of CcCyt b–G143A, thereby conferring high resistance. Thus, rapid detection methods should be developed for G143A in Cyt b of *C. cassiicola*.

For traditional fungicide sensitivity determination, a mycelial growth inhibition assay, spore germination assay, and detached leaflet assay have been widely used (<https://www.frac.info/> (accessed on 9 May 2021)). These methods require large amounts of medium and space, and are time consuming. Recently, a simple, accurate, and fast microtiter method was used to monitor fungicide sensitivity in *C. cassiicola* [14]. However, this method also requires the isolation and culturing of pathogen isolates. More rapid molecular detection methods have been developed to detect fungicide resistance. For G143A in *C. cassiicola*, PCR-RFLP was developed by Ishii et al. [36], but more recent molecular methods based on PCR or sequencing require sophisticated equipment and technical experience. Thus, further development of LAMP offers the best option for on-site detection, because this method only requires a heated chamber and samples. In this study, the WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA) Kit was used. In this kit, an enhanced version of Bst DNA Polymerase (WarmStart® Bst 2.0 DNA Polymerase) and a visible pH indicator are mixed together to provide a rapid visual detection. To a certain extent, this approach can solve the problem of false positives. Our results showed that the LAMP system established for G143A in *C. cassiicola* based on this kit had high specificity and sensitivity (10 times

better than conventional PCR), and the reaction takes only 1 h. Thus, the LAMP system established in this study can be used for rapid detection of G143A in *C. cassiicola*.

## 5. Conclusions

All tested isolates of *C. cassiicola* from Shandong province during 2019 and 2020 were resistant to pyraclostrobin and contained the G143A mutation in Cyt b. Docking results demonstrated that G143A mutation results in a change in the binding pattern of pyraclostrobin and CcCyt b, and the binding ability of pyraclostrobin with the active center of CcCyt b–G143A is lowered. The LAMP assay with a WarmStart Colorimetric LAMP 2× Master Mix kit was specific, rapid, and sensitive for detecting the G143A mutation in *C. cassiicola*. It will provide rapid and valid information for fungicide resistance management.

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