Establishment and Application of Detection Techniques for Major Quarantine Diseases Affecting Citrus in Zhaoqing

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Abstract [**Objectives**] The paper was to develop a detection system applicable to major quarantine diseases affecting citrus in Zhaoqing. [**Methods**] qPCR was utilized for the detection of citrus Huanglongbing (HLB) and citrus bacterial canker disease (CBCD). Additionally, RT-PCR was employed for both single and dual RT-PCR assay of citrus HLB, citrus tristeza virus (CTV), and citrus yellow vein clearing virus (CYVCV). The dual RT-PCR assay system was optimized regarding reagent concentration and annealing temperature to establish a robust dual RT-PCR assay system suitable for the identification of citrus HLB, CTV and CYVCV in Zhaoqing. [**Results**] The established qPCR techniques for the detection of CBCD and HLB exhibited a high degree of specificity. The specific fragment sizes associated with the established single or dual RT-PCR assay techniques were as follows: HLB at 1 160 bp, CTV at 273 bp, and CYVCV at 614 bp, respectively. The amplification products generated by these techniques were characterized by their clarity, specificity, and sensitivity. [**Conclusions**] The established method for the detection of citrus diseases is systematic and capable of addressing the diverse needs of various users.

Key words Zhaoqing; Citrus disease; Detection; RT-PCR; Dual RT-PCR

1 Introduction

Citrus refers to a plant belonging to the genus *Citrus* L., which is part of the Citrus subfamily within the family Rutaceae. Notably, China holds the leading position globally in both the area dedicated to citrus cultivation and the annual production of citrus fruits[1]. Citrus huanglongbing (HLB), citrus bacterial canker disease (CBCD), citrus tristeza virus (CTV), and citrus yellow vein clearing virus (CYVCV) represent the primary quarantine diseases affecting citrus crops. These diseases are significant impediments to the advancement of the citrus industry. The pathogen responsible for citrus HLB is classified within the genus *Candidatus* Liberibacter spp. The disease is characterized by symptoms including yellowing of the lateral and midvein areas, yellowing of the leaf blades, softening of the leaf tissue, and a diminished luster on the leaf surface[2]. CBCD is a quarantine bacterial disease caused by *Xanthomonas axonopodis* pv. *citri*. Following the infection of plant leaves with canker disease, the reverse side of the leaf exhibits yellow to dark yellow oil-stained spots. Subsequently, the foliage may develop bulges that reveal beige spongy material. The fruits of infected plants display symptoms that are similar to those observed on the leaves[2]. CTV is a globally prevalent disease resulting from an infestation by a filamentous virus. The symptoms associated with CTV include decline, stem pitting, and yellowing of seedlings. This virus can induce various symptoms, including yellowing in seedlings, decline in tree health, and stem pitting in both young and mature trees, ultimately leading to a reduction in fruit yield[2]. Citrus yellow vein clearing virus (CYVCV) is a novel viral disease caused by the globally distributed virus. This disease adversely impacts the growth of the entire plant, particularly in lemon and lime species, and is characterized by symptoms such as vein clearing and leaf deformities[3]. The aforementioned diseases are prevalent in citrus production regions globally and can result in significant economic losses. In recent years, our research team has investigated citrus diseases within the citrus industrial zone of the Xijiang River Basin in Guangdong, specifically in Zhaoqing and Yunfu areas, as well as in various nurseries across other regions of Guangdong Province and neighboring provinces. Our findings indicate that different citrus varieties exhibit varying degrees of susceptibility to these diseases, with the majority of citrus plants showing the presence of two or more diseases simultaneously. Effective detection serves as a fundamental prerequisite for mitigating the prevalence of quarantine diseases, such as HLB. Presently, there exist academic reports detailing various detection methods pertinent to the aforementioned diseases, including serological techniques[4], reverse transcription polymerase chain reaction (RT-PCR)[5], and loop-mediated isothermal nucleic acid amplification assay[6]. However, there remains a notable deficiency in systematic citrus disease detection techniques within Zhaoqing production area. This study developed a single or dual RT-PCR assay system by optimizing both primer and template concentrations. This system offers a set of straightforward, rapid, and effective detection methods tailored to meet the diverse needs of fruit farmers. Furthermore, it addresses the gap in citrus quarantine disease detection technology within Zhaoqing region and provides a scientific foundation for the early detection, prevention, and control of citrus diseases.

2 Materials and methods

2.1 Materials The test materials utilized in this study included *Citrus flamea* Hort. ex Tseng. 'Shatangju' infected with HLB and CBCD, sourced from the citrus base of the Institute of Pomology at

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Zhaoqing University. Additionally, *C. sinensis* (L.) Osbeck. 'Tiancheng' infected with CTV were obtained from the China Citrus Research Institute. The test materials infected with CYVCV were collected from Anyue, Sichuan. Leaves from all aforementioned varieties served as positive controls, while healthy leaves of 'Shatangju' were employed as negative controls.

2.2 DNA extraction for HLB and CBCD in citrus plants and establishment of fluorescence quantitative PCR amplification assay DNA extraction for HLB and CBCD was performed in accordance with the protocols provided by the Safe Plant DNA Kit (Magen Biotechnology). The primers utilized in this study are presented in Table 1.

Table 1 Primers for HLB, CBCD, CTV, and CYVCV

Pathogen	Sequence of primer pairs (5'→3')	Fragment size//bp	Reference
HLB	F: GCGCGTATGCAATACGAGCGGCA	1 160	Ding Fang *et al.* [7]
	R: GCGTCGCGACTTCGCAACCCAT		
HLB	F: GCCGTTTTAACACAAAAGATGAATATC		Ren Suli[8]
	R: ATAAATCAATTTGTTCTAGTTTACGAC		
CBCD	F: GAGTCGCCTACCGAGAAA TCC	82	Zhao Yun[9]
	R: ACCACGGCAGGGTGAAGAC		
	TaqMan probe: AGTGTCTCGGAAATTCGACCTCTCCGAAC		
CTV	F: AACGCCCTTCGAGTCTGGGGTAGGA	273	Ding Fang *et al.* [7]
	R: TCAACGTGTGTTGAATTTCCCAAGC		
CYVCV	F: TACCGCAGCTATCCATTTCC	614	Chen Hongming *et al.* [10]
	R: GCAGAAATCCCGAACCACTA		

SYBR Green quantitative PCR reaction system for HLB consisted of 5 μL of SYBR Green PCR Master Mix, 0.2 μL of each homologous and complementary primer, 1 μL of template DNA, and ddH_2O to achieve a total reaction volume of 10 μL. The reaction protocol was as follows: pre-denaturation at 95 ℃ for 3 min, followed by denaturation at 95 ℃ for 10 sec, annealing at 60 ℃ for 30 sec, and extension at 72 ℃ for 30 sec, repeated for a total of 39 cycles.

TaqMan probe quantitative PCR reaction system for CBCD consisted of 5 μL of TaqMan PCR Master Mix, 0.2 μL of each homologous and complementary primer, 1 μL of template DNA, and ddH_2O to achieve a total reaction volume of 10 μL. The amplification procedure involved an initial denaturation step at 95 ℃ for 4 min, followed by 40 cycles of denaturation at 94 ℃ for 15 sec, and annealing/extension at 60 ℃ for 30 sec to collect fluorescence.

2.3 RNA extraction for HLB, CTV and CYVCV in citrus plants and establishment of RT-PCR amplification assay RNA extraction for HLB, CTV, and CYVCV was conducted in accordance with the protocols provided by the RNA Easy Fast Plant Tissue RNA Extraction Kit (Tiangen). The primers utilized in this study are presented in Table 1. The RT reaction system consisted of 4 μL of 5xFastKing-RT SuperMix, 2 μL of RNA, and RNase-free ddH_2O added to a final volume of 20 μL. The reaction procedure involved incubating at 42 ℃ for 15 min, followed by enzyme inactivation at 95 ℃ for 3 min. Subsequently, the reaction mixture was immediately placed on ice and stored at 4 ℃ for future use.

The single RT-PCR amplification protocol for CTV was conducted as follows: initial denaturation at 95 ℃ for 3 min, followed by 30 cycles of denaturation at 95 ℃ for 30 sec, annealing at 57 ℃ for 30 sec, and extension at 72 ℃ for 30 sec. The reaction concluded with a final extension at 72 ℃ for 10 min. For HLB, the amplification procedure involved an initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 30 sec, annealing at 63 ℃ for 30 sec, and extension at 72 ℃ for 30 sec, concluding with a final extension at 72 ℃. The protocol for CYVCV included a pre-denaturation step at 95 ℃ for 5 min, followed by denaturation at 95 ℃ for 30 sec, annealing at 56 ℃ for 30 sec, and extension at 72 ℃.

2.4 Dual RT-PCR reverse transcription technique The RT reaction system comprised 4 μL of 5xFastKing-RT SuperMix, 1 μL of total RNA, and RNase-free ddH_2O added to a final volume of 20 μL.

The reaction procedure involved incubating at 42 ℃ for 15 min, followed by enzyme inactivation at 95 ℃ for 3 min. Subsequently, the reaction mixture was immediately placed on ice and stored at 4 ℃ for future use.

2.5 Optimization of optimal primer concentration and annealing temperature for dual RT-PCR system The template concentrations were established at 10^0, 10^{-1}, and 10^{-2} mol/L of RNA. The annealing temperatures were set at 61 ℃, 62 ℃, and 63 ℃, and subsequently at 62 ℃, 63 ℃, and 64 ℃, respectively.

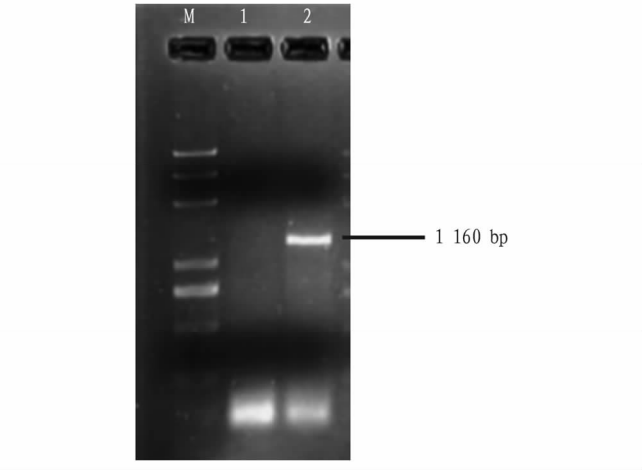
2.6 Agarose gel electrophoresis assay A mixture comprising 1.5 μL of PCR product and 0.5 μL of stain was spotted, and an agarose gel with a concentration of 1.8% was subsequently prepared. The amplification products were then detected by electrophoresis.

3 Results and analysis

3.1 Establishment of detection techniques for individual diseases (HLB, CBCD, CTV and CYVCV)

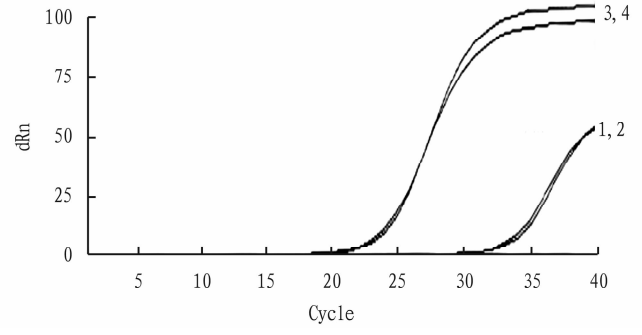
3.1.1 Establishment of detection techniques for two types of HLB in Zhaoqing region. Fig. 1 presents the results of the citrus HLB assay, demonstrating the successful amplification of the target fragment measuring 1 160 bp in the positive sample. In contrast, no corresponding target band was observed in the negative control. The results of qPCR indicated that a cycle threshold (*Ct*) value of 34 or higher was classified as negative, while a *Ct* value below 34 was classified as positive. As illustrated in Fig. 2, all

positive samples exhibited *Ct* values below 34, whereas all negative samples demonstrated *Ct* values exceeding 34.



NOTE M. 5 000 bp DNA Marker; 1. Negative sample; 2. Positive sample.

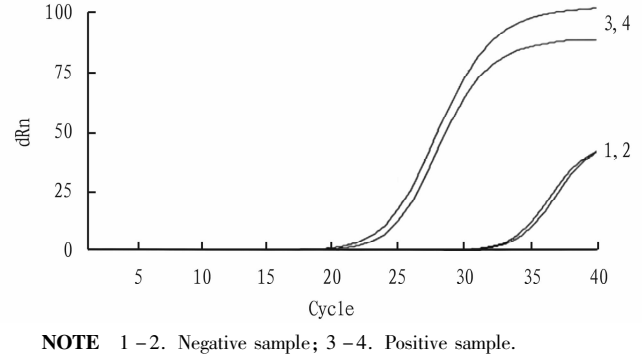
Fig. 1 Amplification effect of HLB by RT-PCR



NOTE 1 -2. Negative sample; 3 -4. Positive sample.

Fig. 2 Amplification effect of HLB by qPCR

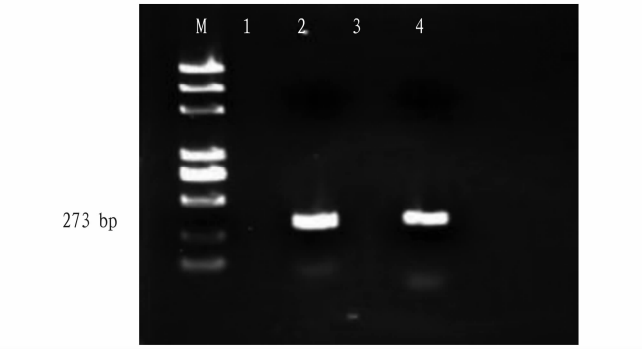
3.1.2 Establishment of detection techniques for CBCD in Zhaoqing region. Fig. 3 presents the results of qPCR analysis for CBCD. The positive samples of CBCD (3 and 4) exhibited distinct amplification curves, with *Ct* values of 24.41 and 25.12, respectively. In contrast, the negative samples (1 and 2) did not display clear amplification curves, yielding *Ct* values of 35.52 and 35.19, respectively. These findings indicate a strong specificity of the established qPCR assay.



NOTE 1 -2. Negative sample; 3 -4. Positive sample.

Fig. 3 Amplification effect of CBCD by qPCR

3.1.3 Establishment of detection techniques for two types of CTV in Zhaoqing region. The application of the RT-PCR assay for the detection of CTV materials, as illustrated in Fig. 4, resulted in the successful amplification of the target fragment measuring 273 bp in all positive samples. In contrast, no corresponding target band was observed in the negative control. Fig. 5 illustrates the application of a direct tissue dot immunoassay for the detection of CTV. The results indicated that when nitrocellulose membrane was observed under consistent external conditions, numerous small black-brown protuberances were observed around the periphery of the phloem in the positive control. In contrast, both the periphery and the inner circumference of the negative control exhibited sample imprints devoid of brown dots.



NOTE M. 2 000 bp DNA Marker; 1,3. Negative sample; 2,4. Positive sample.

Fig. 4 Amplification effect of CTV by RT-PCR

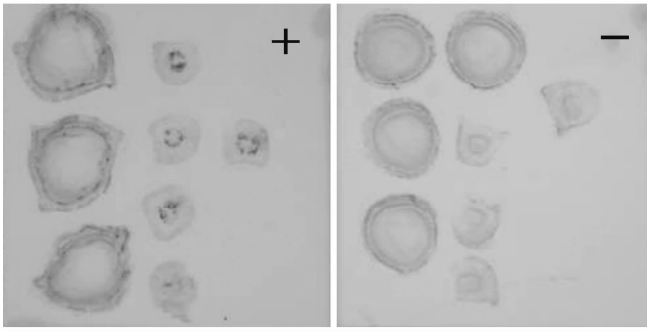
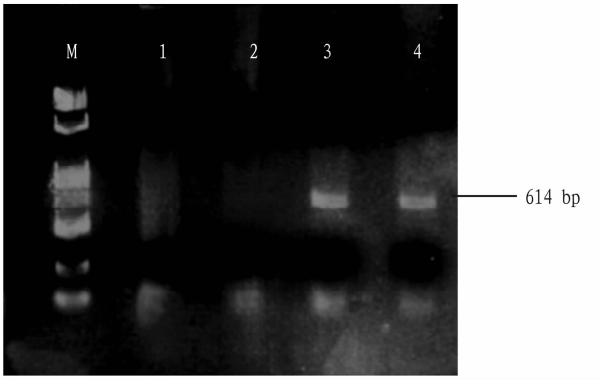


Fig. 5 Results of CTV direct tissue dot immunoassay

3.1.4 Establishment of detection techniques for CYVCV in Zhaoqing region. Fig. 6 illustrates the assay results for the CYVCV material, demonstrating that the target fragment of 614 bp was successfully obtained in all positive samples, while no corresponding target band was observed in the negative control.

3.2 Establishment of dual RT-PCR assay techniques

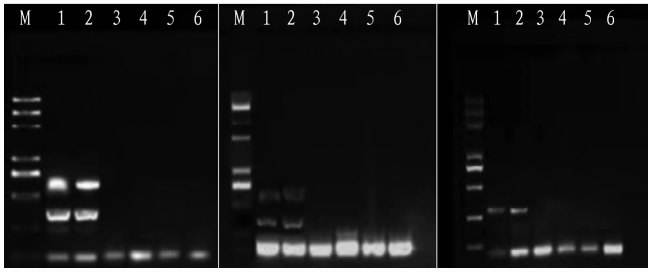
3.2.1 Establishment of dual RT-PCR assay techniques for CYVCV and CTV. (i) Optimization of RNA template concentration. The initial concentration of CYVCV RNA was measured at 70.5 μmol/L, while the initial concentration of CTV RNA was recorded at 83.2 μmol/L. A dilution series of the template concentration was subsequently performed, spanning a range from 10^0 to 10^{-2} fold. The results indicated that at a template concentration of 10^0 μmol/L, the electrophoretic amplification bands for CTV and CYVCV were most pronounced. In contrast, no electrophoretic



NOTE M. 2 000 bp DNA Marker; 1 – 2. Negative sample; 3 – 4. Positive sample.

Fig. 6 Amplification effect of CYVCV by RT-PCR

bands were observed in the negative control and blank groups. When the template concentration was diluted to 10^{-1} μmol/L, the intensity of the electrophoretic bands for CTV and CYVCV diminished. Further dilution to 10^{-2} μmol/L resulted in a significant reduction in band intensity, rendering them either faint or completely undetectable (Fig. 7).

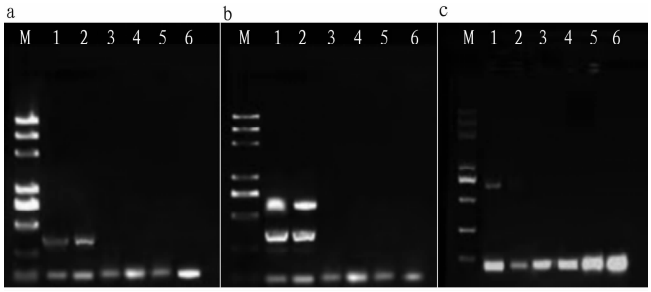


NOTE M. 2 000 bp DNA Marker; 1 – 2. CTV and CYVCV positive group; 3 – 4. CTV and CYVCV negative group; 5 – 6. Blank control group.

Fig. 7 Amplification results of RT-PCR at different RNA concentrations

(ii) Optimization of annealing temperature. Three distinct annealing temperatures of 62 ℃, 63 ℃, and 64 ℃ were evaluated (Fig. 8). At an annealing temperature of 62 ℃, the amplified bands of CTV were observed, albeit with a darker intensity. At 63 ℃, the amplified electrophoretic bands corresponding to both viruses exhibited increased brightness, while no bands were detected in the negative control group or the blank control group. Conversely, at an annealing temperature of 64 ℃, the bands were either weak or completely absent. In summary, the optimal annealing temperature was determined to be 63 ℃.

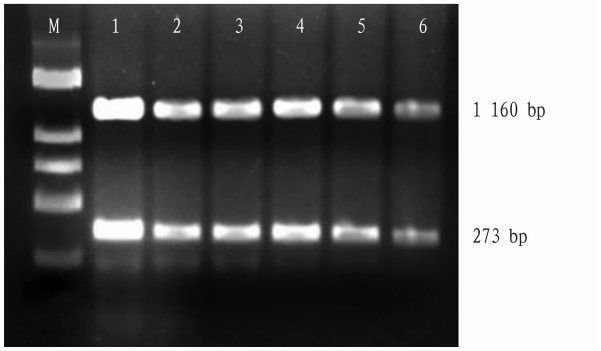
Based on the results of the aforementioned experiments, the dual RT-PCR reaction system for the two citrus viruses was established with a total volume of 20 μL. The composition of the reaction mixture included 10 μL of 2 × *Taq* PCR MasterMix II, 0.5 μmol/L of each homologous and complementary primer, 1 μL of template cDNA, and ddH_2O to achieve the final volume of 20 μL. The optimized reaction conditions were established as follows: reverse transcription was conducted at 42 ℃ for 15 min, followed by pre-denaturation at 95 ℃ for 3 min. This was succeeded by denaturation at 94 ℃ for 30 sec, annealing at 63 ℃ for 50 sec, and extension at 72 ℃ for 1 min, repeated for a total of 35 cycles. Finally, an extension step was performed at 72 ℃ for 10 min, after which the reaction was terminated at 10 ℃.



NOTE a. 62 ℃; b. 63 ℃; c. 64 ℃; M. 2 000 bp DNA Marker; 1 – 2. Positive control; 3 – 4. Negative control; 5 – 6. Blank control.

Fig. 8 Amplification effect of dual RT-PCR at different annealing temperatures

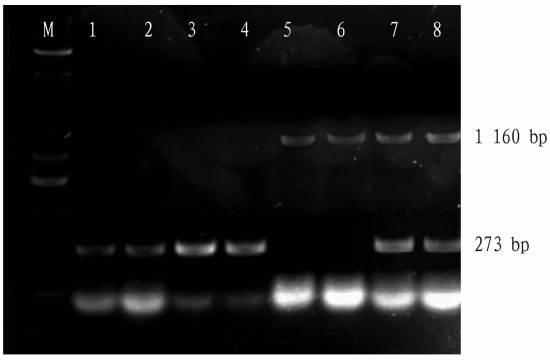
3.2.2 Establishment of dual RT-PCR for detection of HLB and CTV. (i) Optimization of RNA template concentration. To enhance the validation of the reliability of the dual RT-PCR assay technique, this study conducted a gradient experiment utilizing varying concentrations of RNA templates (Fig. 9). A 10-fold dilution series of the total RNA concentration was established, ranging from 10^{0} to 10^{-2}. The results indicated that the technique successfully detected pathogen RNA at significantly low concentrations (10^{-2}) with a high degree of sensitivity.



NOTE M. 2 000 bp DNA Marker; 1 – 2. 10^{-0} mol/L RNA; 3 – 4. 10^{-1} mol/L RNA; 5 – 6. 10^{-2} mol/L RNA.

Fig. 9 Amplification effect of dual RT-PCR at different RNA concentrations

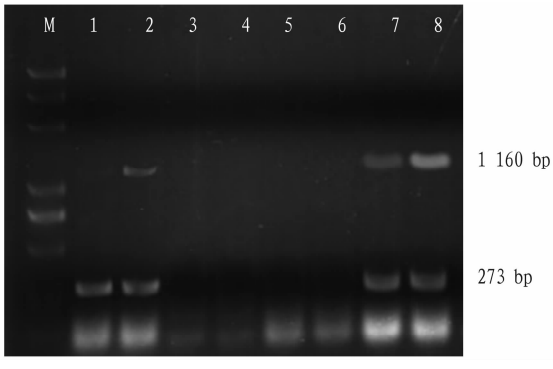
(ii) Optimization of annealing temperature. Three distinct annealing temperatures of 61 ℃, 62 ℃, and 63 ℃ were evaluated. The results indicated that at annealing temperatures of 61 ℃ and 62 ℃, the amplified bands corresponding to CTV were distinctly visible; however, the amplification of the bands associated with HLB was not pronounced (Fig. 10). In contrast, at an annealing temperature of 63 ℃, the amplified bands for both HLB and CTV were clearly observable. Consequently, 63 ℃ was determined to be the optimal annealing temperature for the RT-PCR targeting CTV and HLB.



NOTE M. 5 000 bp DNA Marker; 1 – 4. 61 ℃; 5 – 6. 62 ℃; 7 – 8. 63 ℃.

Fig. 10 Amplification effect of dual RT-PCR at different annealing temperatures

In this experiment, a dual RT-PCR amplification system for HLB and CTV was successfully developed. This system demonstrated the capability to efficiently amplify specific fragments of both HLB and CTV simultaneously. The amplification products exhibited distinct bands, and no non-specific amplification or cross-reactivity was detected (Fig. 11). This finding unequivocally demonstrated that the developed dual RT-PCR assay performed exceptionally well in terms of both sensitivity and specificity. The results of single RT-PCR amplification were compared with those of dual RT-PCR. The sizes of the target bands amplified by both methods corresponded, successfully yielding the target fragments of HLB and CTV in positive citrus samples, measuring 273 and 1 160 bp, respectively. In contrast, no target bands were observed in the negative control, which aligned with the anticipated sizes of the designed amplified fragments. This finding offers substantial evidence for the prompt and precise identification of citrus diseases. According to the results of the aforementioned experiments, the dual RT-PCR reaction system was established with a total volume of 20 μL, comprising 10 μL of 2 × *Taq* PCR Master-Mix II, 0.5 μmol/L of each homologous and complementary primer, 1 μL of template cDNA, and the remaining volume filled with ddH_2O. The optimized reaction conditions were as follows: reverse transcription at 42 ℃ for 15 min, followed by pre-denaturation at 95 ℃ for 3 min. This was succeeded by denaturation at 94 ℃ for 30 sec, annealing at 63 ℃ for 50 sec, and extension at 72 ℃ for 1 min, repeated for a total of 35 cycles. This was succeeded by a final extension at 72 ℃ for 10 min, with the reaction subsequently halted at 10 ℃.



NOTE M. 5 000 bp DNA Marker; 1, 3 – 6. Negative sample; 2, 7 – 8. Positive sample.

Fig. 11 Amplification effect of dual RT-PCR for HLB and CTV

4 Discussion

This study developed a detection technique for individual citrus diseases, including HLB, CBCD, CTV, and CYVCV. Additionally, a dual RT-PCR detection system was established for the simultaneous identification of citrus HLB and CTV, as well as CTV and CYVCV, through the optimization of template concentration and annealing temperature. The efficacy of the detection system was validated by comparing the results of single and dual detection methods.

Molecular detection techniques for citrus diseases encompass conventional PCR, quantitative fluorescence PCR, RT-PCR, *etc.* The outcomes of these techniques can differ significantly depending on the selected detection method. Various detection methods for citrus HLB are accessible to demanders. While quantitative fluorescence PCR demonstrates high sensitivity, it is also relatively costly. In our laboratory, branches infected with HLB were grafted, and the disease was not detectable until three months post-grafting. Consequently, quantitative fluorescence PCR should be prioritized for nursery testing. In the identification of adult trees within orchards, it is advisable to consider factors such as cost control, and conventional PCR may be favored. In instances where two diseases are detected simultaneously, dual RT-PCR can be employed.

Various citrus varieties exhibit differing susceptibilities to a range of diseases. Research indicates that the incidence of citrus ulcer disease is notably elevated in *C. nobilis* Lour. 'Wogan', 'Shatangju', *C. reticulata* cv. 'Jinqiushatangju', and *C. flamea* Hort. ex Tseng. 'Jinkuimiju'. Furthermore, CTV was detected in up to 100% of samples from *C. nobilis* Lour. 'Gonggan', *C. sinensis* Osbeck. 'Hongjiangcheng', *C. haniana* Hort. ex Tsen 'Chazhigan', and *C. sinensis* Osbeck. 'Qicheng'. Additionally, the presence of CYVCV was found to be more pronounced in varieties such as 'Gonggan' and 'Wogan'[11]. Consequently, in response to the diverse needs of users, this study presents several application models. In the detection of citrus complex diseases, various combinations can be formulated based on specific requirements. For instance, lemon and bergamot exhibit susceptibility to CYVCV, which can be detected individually. Additionally, 'Shatangju' and 'Gonggan' are susceptible to HLB and CTV, allowing for the selection of a dual RT-PCR method for the simultaneous detection of HLB and CTV. The citrus disease detection technology developed in this study is systematic and addresses the technological gap in Zhaoqing. In agricultural practice, fruit farmers can make informed decisions based on specific citrus varieties and associated diseases. Consequently, the combination of single and dual modes is more advantageous for application in citrus production.

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