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One-step reverse transcription loop-mediated isothermal amplification for the rapid detection of *cucumber green mottle mosaic virus*

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A B S T R A C T

Cucumber green mottle mosaic virus (CGMMV) has caused serious damage to *Cucurbitaceae* crops worldwide. The virus is considered one of the most serious *Cucurbitaceae* quarantine causes in many countries. In this study, a highly efficient and practical one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) was developed for the detection of CGMMV. The total RNA or crude RNA extracted from watermelon plants or seeds could be detected easily by this RT-LAMP assay. The RT-LAMP assay was conducted in isothermal (63 °C) conditions within 1 h. The amplified products of CGMMV could be detected as ladder-like bands using agarose gel electrophoresis or visualized in-tube under UV light with the addition of a fluorescent dye. The RT-LAMP amplification was specific to CGMMV, as no cross-reaction was observed with other viruses. The RT-LAMP assay was 100-fold more sensitive than that of reverse-transcription polymerase chain reaction (RT-PCR). This is the first report of the application of the RT-LAMP assay to detect CGMMV. The sensitive, specific and rapid RT-LAMP assay developed in this study can be applied widely in laboratories, the field and quarantine surveillance of CGMMV.

1. Introduction

Cucumber green mottle mosaic virus (CGMMV) is a member of the genus *Tobamovirus* in the family *Tombusviridae* (Hull and Matthews, 2002). CGMMV harm of cucumbers was first reported by Ainsworth (1935). Later, the UK, Germany, Denmark, Holland, Russia, India, Japan and Korea suffered huge socio-economic losses from this disease (Hseu et al., 1987; Rahimian and Izadpanah, 1977; Raychaudhuri and Varma, 1978; Zhou et al., 2008). The virus poses a serious threat to *Cucurbitaceae* crops production, and it is considered a serious *Cucurbitaceae* quarantine reason in many countries and regions in the world (Choi, 2001; Lee et al., 1990, 2011; Luo et al., 2010).

In 2003, in a Guangxi agricultural exhibition hall, CGMMV was observed for the first time in China in a greenhouse ornamental pumpkin whose seeds were imported from abroad (Qin et al., 2005). In 2006, there was a report published about CGMMV on watermelons in Gaizhou city in Liaoning province. This was the first report of CGMMV infecting watermelons in China (Chen et al., 2006). In December of the same year, the China Ministry of Agriculture issued announcement No. 788, which established national plant quarantine against this virus (Wu et al., 2009). Previous work proved that the virus contaminates rootstock seeds, and grafting tools were the critical factors influencing the disease epidemic in Liaoning province, where bottle gourd seeds imported from Korea were used as rootstocks for watermelon production (Chen et al., 2006). The occurrence of CGMMV in Beijing suburbs, along with Hebei and Gansu provinces, was also caused by importation of infected seeds. The introduction risk of CGMMV is very high due to the increased international exchange of cucurbit seeds (Chen et al., 2008). A sensitive, reliable and specific method for rapid surveillance is needed urgently to prevent the further spread of CGMMV.

The current methods of detecting CGMMV are enzyme-linked immunosorbent assay (ELISA), nucleic acid hybridization and reverse transcription polymerase chain reaction (RT-PCR) (Maroon and Zavriev, 2000; Roberts et al., 2000; Shang et al., 2010; Varveri

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et al., 2002). However, all of these techniques have some intrinsic disadvantages, such as being time-consuming or requiring expensive materials and equipment. Recently, a rapid nucleic acid detection method, loop-mediated isothermal amplification (LAMP), was developed (Notomi et al., 2000). For the method, only a water bath is needed to amplify large amounts of nucleic acids in 60 ~ 75 min. The LAMP and reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays have been proven to be rapid, simple, sensitive, specific and inexpensive for the detection of different DNA and RNA viruses. RT-LAMP has been used to detect various viruses in diseased plants such as *Tomato spotted wilt virus*, *Bean pod mottle virus, Peach latent mosaic viroid* and viruses in rice (Boubourakas et al., 2009; Fukuta et al., 2004; Le et al., 2010; Wei et al., 2012; Zhou et al., 2012).

In this study, a rapid and feasible one-step RT-LAMP method was developed for the detection of CGMMV in watermelon plants or seeds. The total RNA or crude RNA extracted from leaf or seed samples could be detected easily by one-step RT-LAMP. The amplification was very specific to CGMMV as no cross-reaction was observed with other viruses. The sensitivity of the RT-LAMP assay was hundreds-fold higher than conventional RT-PCR. This newly established RT-LAMP should be a very valuable and applicable tool for the detection of CGMMV.

2. Materials and methods

2.1. Source of materials

CGMMV was isolated from infected watermelon plants in Liaoning province in 2010. *Cucumber mosaic virus* (CMV) and *Tobacco mosaic virus* (TMV) were maintained in this laboratory. Watermelon seeds containing CGMMV were harvested from diseased plants provided by the Jiangsu Academy of Agricultural Sciences. The presence of CGMMV, TMV and CMV in infected or uninfected watermelon plants was confirmed by RT-PCR.

2.2. RT-LAMP primer design

The coat protein (CP) gene sequences of CGMMV were downloaded from the NCBI GenBank (NC_001801.1, EF611826.1, JN605349.1) and aligned for analysis of the most conserved sequence of the CP gene. RT-LAMP primers for CGMMV were designed using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html) and based on a conserved CP sequence. A set of four primers composed of two outer primers and two inner primers was designed. The details of the primers are given in Table 1.

2.3. Extraction of total RNA

Total RNA from the watermelon plants was extracted using the RNA simple Total RNA Purification Kit (TIANGEN Biotech, China) according to the manufacturer's instructions. The total RNA was eluted in RNase-free ddH_2O and stored at −80 °C until used for the RT-LAMP or RT-PCR.

2.4. Initial parameters of one-step RT-LAMP

For the initial test of one-step RT-LAMP, the reaction was conducted by mixing 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.8 mM dNTP, 0.8 μM each of the FIP and BIP primers, 0.2 μM each of the F3 and B3 primers, 0.8 M Betaine (Sigma), 3.2 U of Bst DNA Polymerase (New England Biolabs), 0.5 U of AMV Reverse Transcriptase (Promega) and 0.8 μL of total RNA in a 10 μL total reaction mixture. The RT-LAMP reaction was conducted at 63 °C for 60 min, followed by heating at 80 °C for 10 min to terminate the reaction (Wei et al., 2012). Negative controls were included in each experiment to check contamination. The RT-LAMP products were electrophoresed using 1% agarose gels.

2.5. Optimization of RT-LAMP parameters

The optimization of the RT-LAMP reaction was performed by evaluating different concentrations of $MgCl_2$ (2 ~ 6 mM), dNTP (0.2 ~ 1 mM), F3, B3 (0.05 ~ 0.25 μM), FIP, BIP (0.4 ~ 1.2 μM) and Betaine (0 ~ 1.6 M). The reaction temperatures (61–65 °C) and times (15, 30, 45, 60 and 75 min) were also optimized. The RT-LAMP products were electrophoresed using a 1% agarose gel to determine the optimal parameters.

2.6. In-tube visualization of RT-LAMP

To visualize the RT-LAMP products in-tube, 1 μL of SYBR Green I (Invitrogen) was added into the 10 μL reaction. The best way to add SYBR Green I is to drop it on the lid of the PCR tube, when the reaction is completed, mixing the drop with the RT-LAMP reagents to observe the color change under UV light.

2.7. Rapid extraction of crude RNA for RT-LAMP detection

The crude RNA was extracted rapidly as follows: 100 mg of leaves were ground in 400 μL of 0.5 M NaOH and kept shaking for 1 ~ 2 min. Then, 10 μL of the resulting solution was diluted quickly with 490 μL of 100 mM Tris–HCl buffer (pH 8.0), and 0.8 μL of the final solution was used as template in a 10 μL RT-LAMP reaction. The RT-LAMP reaction was conducted using the optimized reaction system and conditions (2.5). The RT-LAMP products were analyzed using 1% agarose gels.

2.8. Specificity analysis of the RT-LAMP

Crude RNA was extracted as above from watermelon plants infected by CGMMV, CMV or TMV. The RT-LAMP reaction was conducted using the optimized reaction parameters (Table 2). Three microliters of reaction products were electrophoresed using 1% agarose gels, or 0.7 μL of SYBR Green I was added into 7 μL of reaction products, and the tube was examined under UV light.

2.9. RT-PCR

The following procedure was used for the RT-PCR: first step, 10 μL of total RNA (RNA concentration as indicated in 2.10.) was

Table 1
RT-LAMP primers designed for the detection of CGMMV.

Primer name	Type	Length	Genome position[a]	Sequence(5′ → 3′)
F3	Forward outer	18	5821–5838	CCGTCAGGACTTTACTTA
B3	Backward outer	19	6004–6022	GAGCTGAGAAGCGAAACGA
FIP	Forward inner (F1c–F2)	44	5883–5904; 5842–5863	ACTCGCGGAAAGAATCTCTTCC-TTCTAGTTGCTTCACAAGGTAC
BIP	Backward inner (B1c-B2)	47	5926–5947; 5973–5993	CTGTCGTAGATATTAATTCTAG-ATTC-CAACACAGGACCGTTGAGGAA

[a] CGMMV SH strain (NC_001801.1) was used as the reference sequence. The CP gene sequence was used for designing the RT-LAMP primer.