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

Jin-yu Li [a], Qi-wei Wei [a], Yong Liu [b], Xin-qiu Tan [b], Wen-na Zhang [a], Jian-yan Wu [a], Miriam Karwitha Charimbu [a], Bai-shi Hu [a,c], Zhao-bang Cheng [d], Cui Yu [e], Xiao-rong Tao [a,*]

[a] *Key Laboratory of Integrated Management of Crop Diseases and Pests, Ministry of Education, Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, China*
[b] *Institute of Plant Protection, Hunan Academy of Agricultural Sciences, Changsha 410125, China*
[c] *National Engineering Research Center For Cucurbits, Changji 831100, China*
[d] *Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China*
[e] *Shanghai Entry Exit Inspection and Quarantine Bureau, Shanghai 200135, China*

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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

* Corresponding author. Tel.: +86 25 84399027; fax: +86 25 84395325.
E-mail address: taoxiaorong@njau.edu.cn (X.-r. Tao).

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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.

Table 2
Optimized parameters for the one-step RT-LAMP reaction.

Component (concentration)	Volume (μL) per 10 μL reaction
10× ThermoPol buffer	1
25 mM $MgCl_2$ (4 mM final)	1.6
10 mM dNTP (0.8 mM final)	0.8
20 μM FIP (1 μM final)	0.5
20 μM BIP (1 μM final)	0.5
10 μM F3 (0.2 μM final)	0.2
10 μM B3 (0.2 μM final)	0.2
5 M Betaine (0.8 M final)	1.6
Bst DNA polymerase 8 U/μL (3.2 U final)	0.4
AMV reverse transcriptase 10 U/μL (0.5 U final)	0.05
Total RNA	0.8
DEPC H_2O	2.35

denatured at 65 °C for 5 min in the presence of 2 μL of 10 μM reverse primer B3 and 1 μL of 10 mM dNTP, followed by 2 min on ice. Then, the RT mixture containing 4 μL of 5 × AMV RT buffer (Promega), 1 μL of 10 U/μL AMV reverse transcriptase (Promega), 1 μL of 40 U/μL recombinant ribonuclease inhibitor (RRI) (TaKaRa) and 1 μL of RNase-free ddH_2O was prepared. Reverse transcription (RT) was performed at 42 °C for 60 min, followed by 15 min at 70 °C. In the second step, 10 μL of a PCR mixture containing 10 × PCR buffer, 0.2 μL of dNTP (10 mM), 0.4 μL of F3 (10 μM), 0.4 μL of B3 (10 μM), 0.1 μL of Taq DNA Polymerase and 1.6 μL of cDNA from the RT reaction was prepared. PCR was conducted with denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 15 s and further extension at 72 °C for 10 min. The RT-PCR products were electrophoresed using a 1% agarose gel.

2.10. Sensitivity comparisons of RT-LAMP and RT-PCR

RT-LAMP and RT-PCR were conducted using various concentrations of total RNA extracted as template. The total RNA was quantified by NanoDrop 1000 (Thermo Scientific, USA) and was diluted serially 10-fold from 72 ng/μL to 7.2×10^{-6} ng/μL as template for RT-LAMP and RT-PCR. After the reaction was completed, the reaction products was electrophoresed using a 1% agarose gel.

2.11. Watermelon seeds for RT-LAMP detection

Fifty watermelon seeds were soaked in 3 mL of water for 1 h, and the total RNA was extracted from 100 μL of soaked solution using a RNA simple Total RNA Purification Kit (TIANGEN Biotech, China). For extraction of crude RNA, 5 μL of the soaked solution was mixed with 5 μL of NaOH and incubated for 5 min. Then, 10 μL of the resulting solution was neutralized in 490 μL of 100 mM Tris–HCl buffer (pH 8.0), and 0.8 μL of the final solution was used as template in 10 μL of the RT-LAMP reaction. The RT-LAMP products were examined using a 1% agarose gel.

3. Results

3.1. Design and testing of the RT-LAMP primer set for CGMMV

A set of RT-LAMP primers was designed using Primer Explorer V4 software based on highly conserved regions in the CP gene of CGMMV (Table 1). To determine whether the RT-LAMP primer set can be used for detection of CGMMV, the total RNA was isolated from a CGMMV-infected watermelon plant and assayed using a one-step RT-LAMP initial reaction. As shown in Fig. 1, the RT-LAMP products of the total RNA of CGMMV generated a ladder-like band in the 1% agarose gel. Whereas the negative control without the RNA template did not generate such a band, indicating that the selected RT-LAMP primer set was functional.

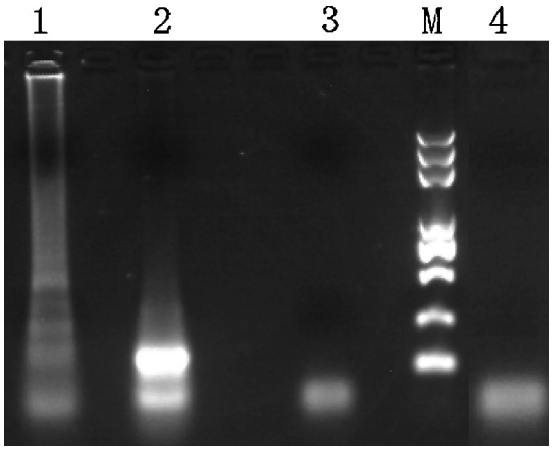

Fig. 1. Testing of the RT-LAMP primer set for the detection of CGMMV. Lane 1: The RT-LAMP amplification of the total RNA isolated from a CGMMV-infected watermelon plant using designed RT-LAMP primers based on CP; lane 2: RT-LAMP products of the CGMMV digested with *Xba* I; lane 3: negative control without RNA template for RT-LAMP reaction; lane 4: negative control with RNA template from a healthy watermelon plant.

To confirm that the RT-LAMP amplification products had sequences corresponding to the target DNA, the amplified products of the CGMMV CP gene were digested with the restriction enzyme *Xba* I and analyzed by electrophoresis. The sizes of the fragments were generated as predicted (123 bp) (Fig. 1). The band was purified, and the sequencing of this band confirmed that it was amplified from the CP gene of CGMMV, suggesting the CGMMV primer designed for RT-Lamp was suitable for the detection of CGMMV.

3.2. Optimization of the parameters for RT-LAMP

In the initial experiment involving the one-step RT-LAMP, the ladder-like band from the CGMMV-infected watermelon plant was not as adequate as expected (Fig. 1). To improve the efficiency of RT-LAMP amplification, a series of experiments to optimize the RT-LAMP parameters was conducted. The optimized RT-LAMP parameters were as follows: 4 mM Mg^{2+} (Fig. 2A), 0.8 mM dNTP (Fig. 2B), 0.2 μM outer primer (Fig. 2C), 1 μM inner primers (Fig. 2D), 0.8 M Betaine (Fig. 2E), incubation temperature at 63 °C (Fig. 2F) and incubation time of 1 h (Fig. 2G). With these parameters (Table 2), the ladder-like bands from the total RNA purified from the CGMMV-infected watermelon plant could be amplified adequately by RT-LAMP (Fig. 3A).

3.3. One-step RT-LAMP detection of CGMMV using crude RNA

To make the RT-LAMP assay a more cost-effective and rapid method, crude RNA was fast extracted from watermelon plant leaves ground in NaOH and neutralized immediately with Tris–HCl buffer. The crude RNA was assayed using optimized RT-LAMP (Table 2). As shown in Fig. 3A, the ladder-like bands could be amplified successfully from watermelon plants infected by CGMMV using a combination of RT-LAMP and crude RNA. No band was amplified from the crude RNA of healthy plants. The validity of RT-LAMP detection of CGMMV from crude RNA was confirmed with conventional RT-PCR, though the specific band amplified by RT-PCR using crude RNA was much weaker compared with the total RNA band (Fig. 3B).

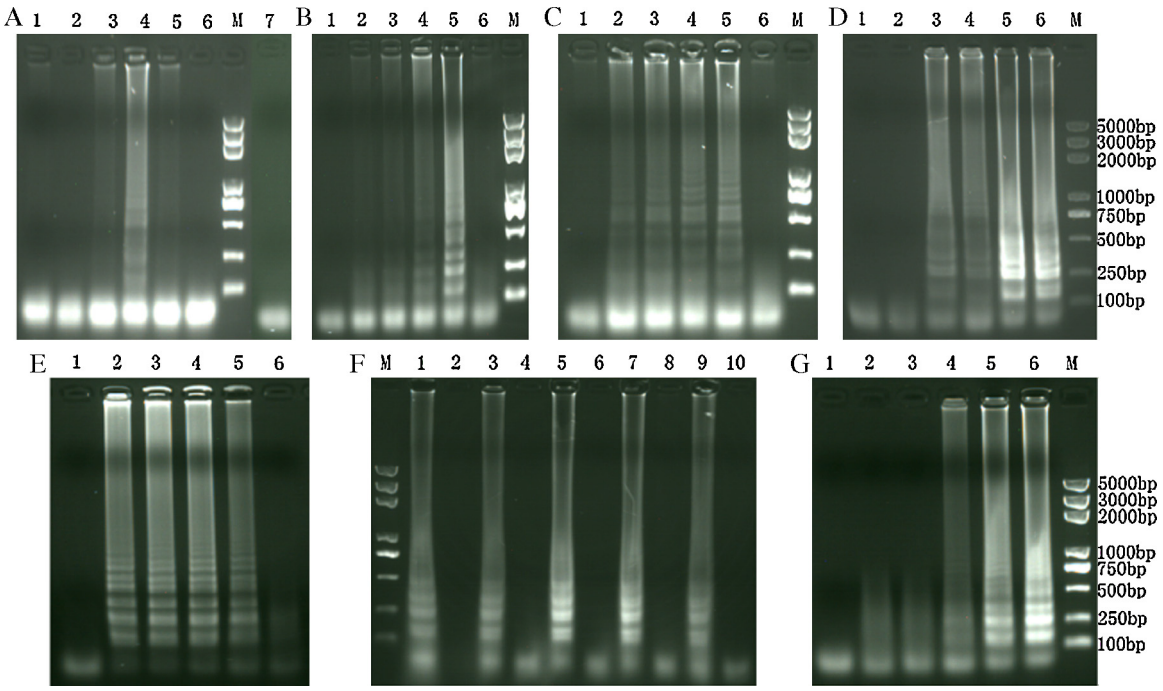

Fig. 2. Optimization of the RT-LAMP parameters for CGMMV detection. (A) Optimization of Mg^{2+} in the RT-LAMP detection of CGMMV. Lane 1: negative control without RNA template; lane 2–6: 2–6 mM final concentration of Mg^{2+}, respectively. Lane 7: Negative control with RNA template from healthy watermelon plant. M: 2-kb PLUS size marker. (B) Optimization of dNTP in the RT-LAMP. Lane 1: Negative control; lane 2–6: 0.2, 0.4, 0.6, 0.8 and 1 mM final concentration of dNTP; M: 2-kb PLUS size marker. (C) Optimization of outer primer. Lane 1: Negative control; lane 2–6: 0.05, 0.10, 0.15, 0.20 and 0.25 μM final concentration of outer primer, respectively; M: 2-kb PLUS size marker. (D) Optimization of inner primer. Lane 1: Negative control; lane 2–6: 0.4, 0.6, 0.8, 1.0 and 1.2 μM final concentration of inner primer; M: 2-kb PLUS size marker. (E) Optimization of Betaine. Lane 1: Negative control; lane 2–6: 0, 0.4, 0.8, 1.2 and 1.6 M final concentration of Betaine. (F) Optimization of reaction temperature for RT-LAMP. M: 2-kb PLUS size marker. Lane 1, 3, 5, 7 and 9: RT-LAMP reaction temperature at 61–65 °C, respectively; lane 2, 4, 6, 8 and 10: RT-LAMP reaction without RNA template incubated at 61–65 °C, respectively. (G) Optimization of RT-LAMP reaction time. Lane 1: Negative control; lane 2–6: RT-LAMP reaction incubated for 15, 30, 45, 60 and 75 min, respectively; M: 2-kb PLUS size marker.

3.4. Specificity of RT-LAMP for detection of CGMMV

To examine the specificity of RT-LAMP for CGMMV amplification, crude RNA isolated from watermelon plants infected by CMV or TMV was also tested by RT-LAMP. As shown in Fig. 4A (upper panel), the RT-LAMP amplification of the crude RNA from the watermelon plant infected by CGMMV could generate ladder-like bands in the agarose gel, whereas the crude RNA from watermelon plants infected with TMV or CMV, as well as a healthy plant, did not generate such a ladder-like band, suggesting that the RT-LAMP amplification was specific to CGMMV.

3.5. In-tube visualization of RT-LAMP

To visualize the RT-LAMP products in a naked-eye detectable manner, a drop of SYBR Green I was added into the tube after the reaction was completed. As shown in Fig. 4B (lower panel), the fluorescent dye of the RT-LAMP reaction containing the CGMMV-infected samples changed from an orange to green color that was visualized easily in-tube under UV light, whereas no color change was observed in the tubes containing TMV- or CMV-infected samples.

3.6. Sensitivity of RT-LAMP vs RT-PCR

To evaluate the sensitivity of RT-LAMP, it was compared with RT-PCR. Using a ten-fold dilution of total RNA extracted from a CGMMV-infected watermelon plant, RT-LAMP amplifications were observed at dilutions of 7.2×10^{-2} ng/μL (Fig. 5A), whereas RT-PCR produced positive results up to dilutions of 7.2 ng/μL (Fig. 5B). Comparisons between the RT-LAMP and RT-PCR amplification indicated that RT-LAMP is 100-fold more sensitive than RT-PCR (Fig. 5A and B).

3.7. RT-LAMP detection of CGMMV in watermelon seeds

CGMMV is one of the most economically important seed-borne viruses. To detect the viruses in seeds, watermelon seeds containing CGMMV were ground and their total RNA was extracted. However, the diagnostic ladder-like band could not be amplified from this total RNA by RT-LAMP. The watermelon seeds were then soaked in water, and the total RNA was extracted from the soaked solutions. Using the total RNA from the solutions, the ladder-like band was amplified successfully by RT-LAMP (Fig. 6). In addition, no band was amplified from the total RNA from the soaked solutions of healthy seeds. The crude RNA was also extracted rapidly by NaOH from the soaked solutions of infected watermelon seeds and neutralized by Tris–HCl buffer. The positive ladder-like bands were amplified successfully using rapid extracted RNA from soaked solutions (Fig. 6). Thus, RT-LAMP was also applied successfully to detect CGMMV in watermelon seeds.

4. Discussion

In this study, a highly efficient and practical method for the detection of CGMMV in infected watermelon plants and their seeds was developed. The designed RT-LAMP primers and optimized parameters were able to amplify adequately the ladder-like bands of the CP gene of CGMMV. The products were also visualized readily in-tube under the UV light with the addition of SYBR Green I. Using the total RNA or rapidly extracted crude RNA, RT-LAMP could detect CGMMV in infected watermelon plants or seeds. The

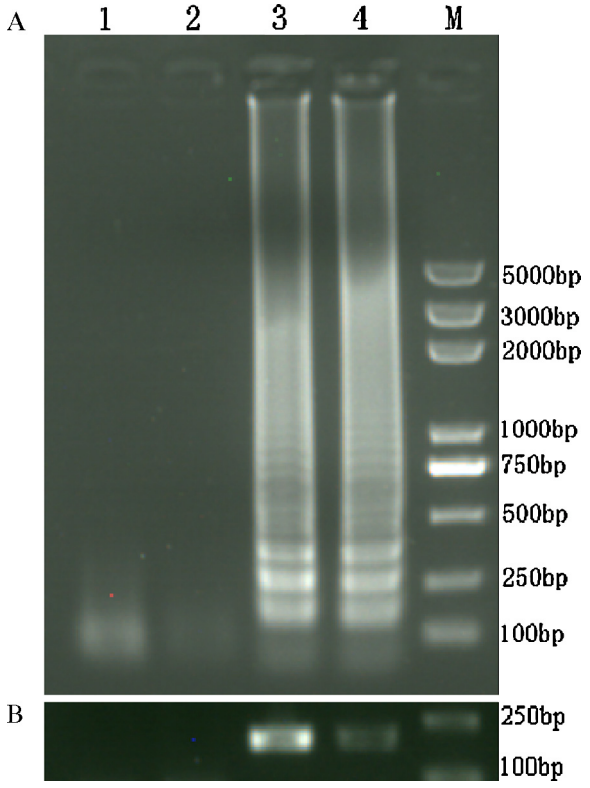

Fig. 3. RT-LAMP detection of CGMMV from total RNA or crude RNA using optimized parameters. (A) Detection of CGMMV by RT-LAMP. (B) Evaluation of RT-LAMP detection of CGMMV by RT-PCR. Lane 1: Total RNA from healthy watermelon plant; lane 2: crude RNA from healthy watermelon plant; lane 3: total RNA from CGMMV-infected watermelon plant; lane 4: rapidly extracted crude RNA from CGMMV-infected watermelon plant; M: 2-kb PLUS size marker.

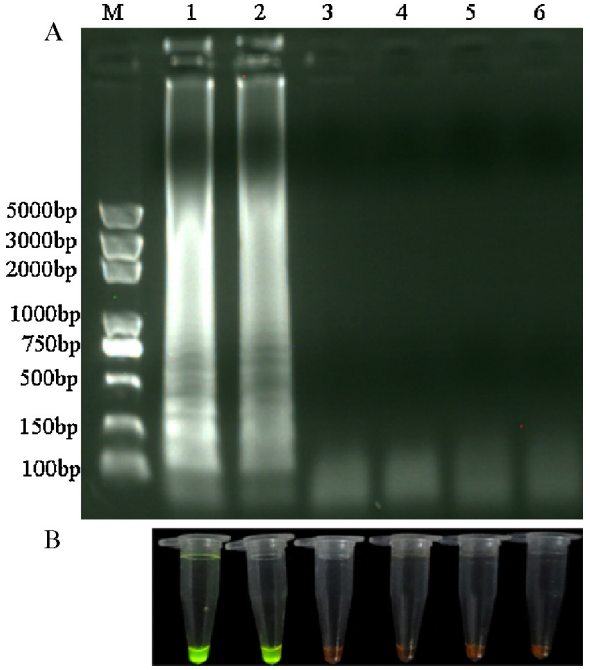

Fig. 4. Specificity of RT-LAMP detection of CGMMV. (A) Specificity analysis of CGMMV RT-LAMP assay by agarose gel electrophoresis. (B) Visualization of CGMMV RT-LAMP after adding SYBR Green I for color change under UV light. M: 2-kb PLUS size marker; Lane 1: RT-LAMP detection of crude RNA from CGMMV infected watermelon plant; lane 2: RT-LAMP detection of the rapidly extracted crude RNA from CGMMV-infected watermelon plant; lane 3: RT-LAMP detection of the rapidly extracted crude RNA from CMV-infected watermelon plant; lane 4: RT-LAMP with the crude RNA from TMV-infected watermelon plant; lane 5: RT-LAMP with the crude RNA from healthy watermelon plant; lane 6: RT-LAMP without RNA template.

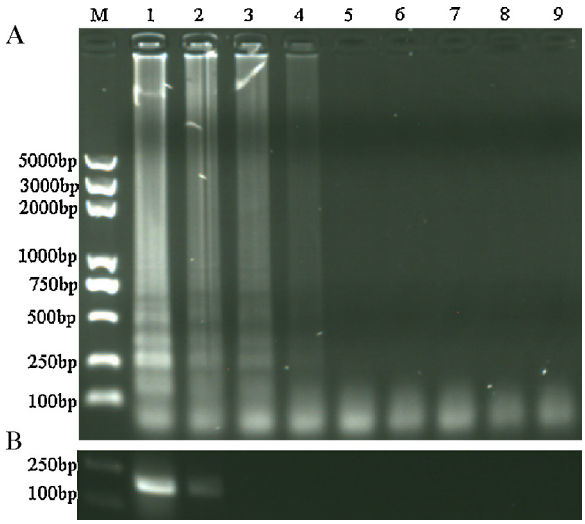

Fig. 5. Sensitivity comparison of RT-LAMP and RT-PCR for the detection of CGMMV. (A) Sensitivity of RT-LAMP for the detection of CGMMV as analyzed by agarose gel electrophoresis. Lane 1–8: Ten-fold dilution of the total RNA (57.6, 5.76, 5.76×10^{-1}, 5.76×10^{-2}, 5.76×10^{-3}, 5.76×10^{-4}, 5.76×10^{-5}, 5.76×10^{-6} ng, respectively) isolated from CGMMV-infected watermelon plant was included in each 10 μL total volume of RT-LAMP reaction. (B) Sensitivity of RT-PCR for the detection of CGMMV by agarose gel electrophoresis. Lane 1–8: Ten-fold dilution of total RNA (same order as panel A) was included in RT-PCR. M: 2-kb PLUS size marker; lane 9: negative control without RNA template.

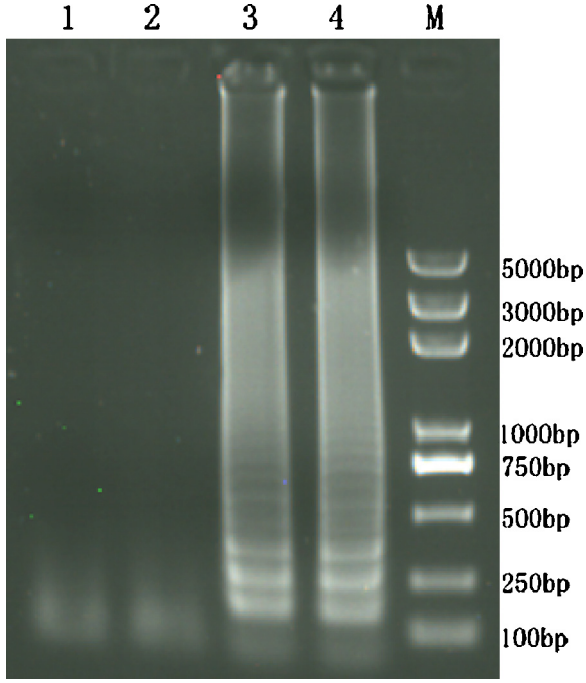

Fig. 6. RT-LAMP detection of CGMMV in watermelon seeds. Lane 1: RT-LAMP detection of the total RNA extracted from the soaked solution of healthy watermelon seeds; lane 2: RT-LAMP detection of the crude RNA from the soaked solution of healthy watermelon seeds; lane 3: RT-LAMP detection of the total RNA extracted from the soaked solution of watermelon seeds containing CGMMV; lane 4: RT-LAMP amplification of the crude RNA from the soaked solution of watermelon seeds containing CGMMV; M: 2-kb PLUS size marker.

detection of CGMMV from crude RNA makes this method quick and convenient. The RT-LAMP amplification was specific to CGMMV as no cross-reaction was observed with other viruses. The sensitivity of RT-LAMP detection for CGMMV was 100-fold greater than conventional RT-PCR. This sensitivity is similar to earlier reports on RT-LAMP for the detection of other viruses (Boubourakas et al., 2009; Fukuta et al., 2013; Wei et al., 2012; Zhang et al., 2013).

The diagnostic ladder-like band could not be amplified from the total RNA extracted directly from watermelon seeds by RT-LAMP; however, the positive bands could be amplified successfully from the total or crude RNA extracted from soaked solutions of CGMMV-infected seeds. The quality of the total RNA extracted from watermelon seeds was comparable with that from watermelon plants (data not shown). Thus, unknown components, such as the high content of fat in seeds, may interfere with the RT-LAMP reaction. The positive amplification of CGMMV from soaked solutions suggests that the CGMMV virus may attach to the surface of the watermelon seeds, as soaking the seeds without breaking the coat of the seeds was sufficient to elute the virus for RT-LAMP detection. The importation or trade of *Cucurbitaceae* seeds is the main risk factor for the introduction of CGMMV in many regions or countries (Chen et al., 2008; Wu et al., 2011). The one-step RT-LAMP method developed in this study enables the fast, specific and reproducible detection of CGMMV in seeds.

5. Conclusion

A one-step RT-LAMP assay was developed as an alternative method for the rapid detection of CGMMV with high sensitivity and specificity. This method enables the detection of the virus in both infected watermelon plants and seeds. To the best of our knowledge, this is the first study to demonstrate the application of a one-step RT-LAMP amplification technique for the diagnosis of CGMMV. This technique could be applied for health management programs and disease surveillance of watermelon plants and seeds in laboratories as well as to field detection of CGMMV to prevent disease outbreak.

Acknowledgments

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