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BRIEF REPORT

Detection of cucumber mosaic virus isolates from banana by one-step reverse transcription loop-mediated isothermal amplification

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Abstract Cucumber mosaic virus (CMV) is one of the most devastating threats to the banana industry. A single-tube, one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for the rapid detection of CMV-infected banana and plantain (*Musa* spp.). The reaction was performed in a single tube at 63 °C for 90 min using a real-time turbidimeter, with an improved closed-tube visual detection system in which fluorescent dye was added to the inside of the lid prior to amplification. This RT-LAMP assay is an alternative method for the rapid detection of CMV in banana plants and tissue culture materials.

Banana and plantain (*Musa* spp.) are among the most widely consumed fruits and a perennial fruit crop in many tropical and subtropical countries and regions. Banana mosaic disease caused by cucumber mosaic virus (CMV) has been a threat to the banana industry [1, 2]. CMV is a positive-sense, single-stranded RNA virus with a tripartite genome [3] and is the type member of the genus *Cucumovirus* in the family *Bromoviridae* [4]. No effective resistance to this virus in *Musa* is known; thus, control is still largely based on the use of virus-free propagative materials, roguing of infected plants and implementation of quarantine barriers.

Published methods for detecting CMV in infected banana include virus purification [5], enzyme-linked immunosorbent assay (ELISA) [6–9], dot-blot hybridization [7], reverse transcription-polymerase chain reaction (RT-PCR) [7, 10, 11], and immunocapture RT-PCR (IC-RT-PCR) [9, 12]. However, theses assays are relatively time-consuming, labour-intensive, and dependent on specialized equipment. Although ELISA and RT-PCR methods are widely applied, a method for nucleic acid amplification termed loop-mediated isothermal amplification (LAMP) is faster and simpler, using only a water bath or heating block [13], and has been successfully used to detect some plant pathogens [14–17]. The LAMP assay is carried out under isothermal conditions, and a large amount of by-product, pyrophosphate ions, is produced, yielding white precipitate of magnesium pyrophosphate in the reaction mixture. Judging the presence or absence of this white precipitate allows easy distinction of whether nucleic acid was amplified by the LAMP method. Since an increase in the turbidity of the reaction mixture due to the production of precipitate correlates with the number of DNA molecules synthesized, monitoring of the LAMP reaction can be done by real-time measurement of turbidity [18, 19].

In this study, we have developed a one-step RT-LAMP assay for rapid and sensitive detection of CMV in planting materials of banana to aid in the establishment of a vigorous, virus-free nuclear stock for a future supply of

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certified banana suckers. Additionally, an improved visual LAMP method in a closed-tube detection system was developed for high-throughput practical application.

CMV isolates were provided by the Environmental and Plant Protection Institute of CATAS in the form of virus-infected leaves. A total of 85 samples of plants showing CMV or virus-like symptoms were obtained from different banana plantations from Yunnan, Guangdong, Guangxi and Hainan provinces of China.

Total RNA was extracted from infected and healthy banana leaf tissues (approximately 0.5 g) using the CTAB method as reported previously [20]. The final RNA concentration was adjusted to 0.2 μg/μL.

Specific RT-LAMP primers for CMV were designed based on the coat protein gene sequence (accession no: EU926956.1) using PrimerExplorer V4 software (http://primerexplorer.jp/e/). A forward inner primer (FIP, 5′-CC GTGACTGAATCAGGAAGTAA-CTGAAACCGCCGAA GATA-3′, nt 271-292 / nt 223-240) consisted of F1c (the complementary sequence of F1) and F2, and a reverse inner primer (BIP, 5′-TCGAGTTAATCCTTTGCCGAA-AGGA ACTTTACGGACTGT-3′, nt 327-347 / nt 370-387) consisted of B1c (the complementary sequence of B1) and B2. The outer primers F3 (5′- GGTACACGTTCACATCTATC -3′, nt 200-219) and B3 (5′-GAACATAGCAGAGATGGC -3′, nt 412-429) were used for the initiation of the RT-LAMP reaction.

The RT-LAMP reaction was conducted as described previously with minor modifications and optimization [13, 21]. The RT-LAMP reaction contained 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 1.6 mM dNTPs, 1 M betaine, 4 mM $MgSO_4$, 10× ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1 % TritonX-100), 8 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 1 μL of template RNA, 0.2 μL (8 U) reverse transcriptase (Takara, Dalian, China), and double-distilled water to a final volume of 25 μL. Then, an equal volume of paraffin oil was added to the tube to prevent evaporation, followed by addition of 1 μL of 1:10-diluted SYBR Green I (Invitrogen, Carlsbad, CA) to the inside of the lid prior to amplification. The RT-LAMP reaction was carried out in a Loopamp real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan) at 63 °C for 90 min and terminated at 80 °C for 10 min. Real-time turbidity readings at 650 nm were obtained, and a turbidity threshold value of 0.1 was used. After the reaction, RT-LAMP products were detected directly by visual observation of the solution colour by mixing the pre-added 1 μl of SYBR Green I to the reaction solution through gentle centrifugation. Green fluorescence was clearly observed with the naked eye in the positive reaction, whereas the colour remained the original orange in the negative reaction. The LAMP products (5 μL) were analyzed by electrophoresis on a 2 % (w/v) agarose gel and subsequently stained with ethidium bromide.

For confirming the specificity of the RT-LAMP, the products were cloned and sequenced. The specificity of the assay was also tested by RT-LAMP reactions that used five banana-infecting pathogens: banana bunchy top virus (BBTV), banana streak OL virus (BSV-OL), *Xanthomonas campestris* pv. *musacearum*, *Fusarium oxysporum* f. sp. *cubense* race 1 (Foc1) and race 4 (Foc4).

To determine the sensitivity of the CMV RT-LAMP assay, a 547-bp specific cDNA fragment containing a target region from the CMV genome was amplified by RT-PCR using a pair of specific primers (sense, 5'-CACCCAACC TTTGTGGGTAG-3'; antisense, 5'-CAACACTGCCAAC TCAGCTC-3'). The RT was carried out by using the antisense primer according to the instructions of the AMV reverse transcription kit (Promega). The PCR was supplemented with 2 μL cDNA from the above-mentioned RT step and 0.2 μM corresponding primers in a reaction volume of 25 μL. The thermal cycling program consisted of the following steps: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 7 min. The RT-PCR products were cloned into the pMD18-T vector (Takara) according to the manufacturer's instructions. The recombinant plasmid, designated pMD18-T-CMV, was used to make dilutions as a reference for evaluating the detection limit of the LAMP protocol. The concentration of pMD18-T-CMV plasmid was adjusted to 100 ng/μL, and the sample was serially diluted tenfold (1×10^0 to 1×10^7 copies) before mixing with extracted RNA from healthy banana, which was used as a reference for sensitivity in the RT-LAMP reaction.

To evaluate the feasibility of the RT-LAMP method for the diagnosis of field samples, 85 samples that were collected from different places in South China were investigated by RT-PCR and RT-LAMP, respectively.

To design the primers for the RT-LAMP assay, sequences from various banana CMV isolates were examined to identify the conserved regions of the virus genome. A set of primers based on regions that are conserved among isolates was designed and used subsequently for the evaluation of the specificity of CMV RT-LAMP assay.

In the specificity test, only amplified products from CMV RNA showed a ladder-like pattern of bands, while no amplicons were detected for other banana pathogens or the control (Fig.1A). The colour of the RT-LAMP products changed from orange to green when CMV was detected with SYBR Green I, while the original orange colour remained when the healthy control and water were tested (Fig. 1B). The graph of turbidity over time obtained using real-time turbidimetry to monitor the DNA synthesis reaction indicated that the primer set was able to amplify

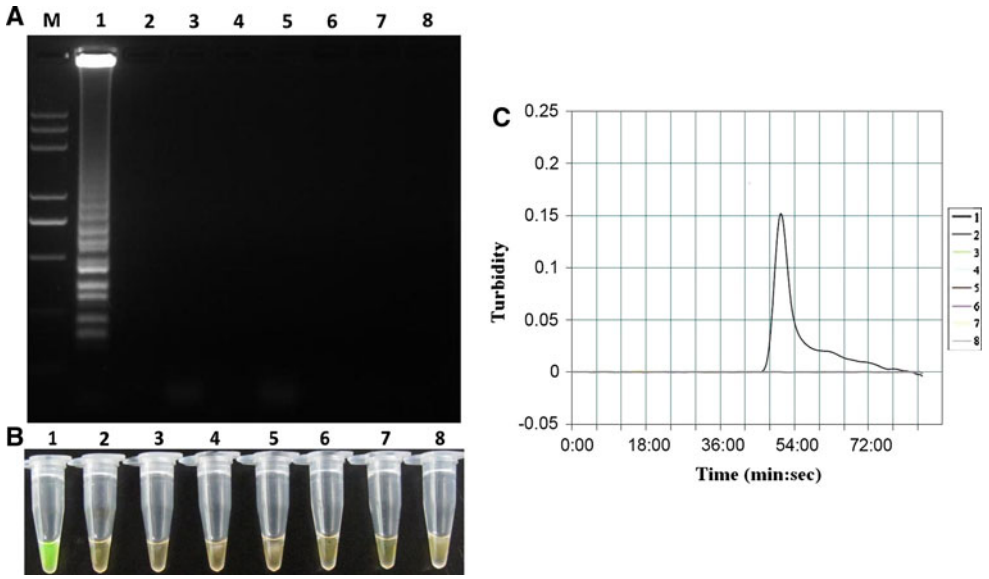

Fig. 1 Specificity of RT-LAMP assay for the detection of CMV. (**A**) Agarose gel electrophoresis analysis of the RT-LAMP amplicon, showing the specificity of the primers. (**B**) Visual inspection of the LAMP specificity assay corresponding to the agarose gel electrophoresis analysis. (**C**) Graph of turbidity over time for the CMV RT-LAMP reaction. Lane 1, cucumber mosaic virus (CMV); lanes 2-6, DNA from banana bunchy top virus (BBTV), banana streak OL virus (BSV-OL), *Ralstonia solanacearum*, *Fusarium oxysporum* f.sp. *cubense* race 1 (Foc1) and *Fusarium oxysporum* f.sp. *cubense* race 4 (Foc4), respectively; lane 7, healthy control; lane 8, water control; lane M, Trans2K *Plus* II DNA Marker

the target RNA sequence (Fig. 1C). The small fragment from the RT-LAMP product was cloned into the pMD18-T vector and subsequently sequenced. The sequencing results showed that the length of the fragment had over 95 % nucleotide sequence identity to known CMV strains (data not shown). These results indicated that this RT-LAMP method was highly specific for diagnosis of CMV infection.

It is difficult to quantify CMV genomic RNA in banana plants; thus, the plasmid DNA mixed with banana leaf RNA was used as both LAMP and PCR reference to evaluate the sensitivity of the CMV LAMP assay. While some inhibitory compounds are present in banana tissues, mixing plasmid DNA with extracted banana RNA is a convenient approach to evaluate the detection limit of either LAMP or PCR. However, it would be more useful as a reference if transcribed and quantitated CMV RNA could be obtained and employed to determine the detection limit of RT-LAMP and RT-PCR, respectively.

The sensitivity tests showed that this RT-LAMP method could detect as little as 1 pg/μL of DNA, while the detection limit of the RT-PCR is about 100 pg/μL plasmid DNA (Fig. 2). Essentially, the detection sensitivity of the RT-LAMP assay was 100 times higher than that of the RT-PCR. All of the experiments were performed independently three times, and nearly identical results were obtained.

To evaluate the usefulness of this one-step RT-LAMP assay for the detection of CMV in the field, a total of 85 samples were tested by RT-LAMP and RT-PCR, respectively. The RT-PCR detected 78 samples that were infected by CMV, with the remaining samples negative for the presence of CMV. Results from the RT-LAMP assay indicated that 80 samples were positive. Five samples that were negative by RT-LAMP were also negative by RT-PCR, but two RT-LAMP-positive samples were not detected by RT-PCR. The two samples were propagated by tissue culture, and when the plantlets were re-tested, they were positive by both RT-PCR and RT-LAMP. The detection rates of RT-PCR and one-step RT-LAMP were 78/85 (91.8 %) and 80/85 (94.1 %), respectively, for the field samples in this study (Fig. 3).

At present, banana plantlets from tissue culture are widely used in tropical agriculture, and CMV is recognized as one of the serious threats to banana production [2]. Therefore, it is necessary to develop highly sensitive and rapid detection protocols and establish virus-indexed banana plants, since virus-free indexed nuclear stocks are required for micropropagation. RT-PCR has been widely used for the detection of CMV [7, 10, 11]; however, RT-PCR requires a thermal cycler and post-reaction gel analysis. In this study, a one-step RT-LAMP method based on real-time turbidimetry was developed, which can detect CMV-infected banana faster than RT-PCR. Moreover, there is no need for any expensive equipment; a temperature-controlled water bath or a heating block is sufficient [13].

Fukuta et al. [22] developed an RT-LAMP assay for detection of a CMV isolate from chrysanthemum in Japan

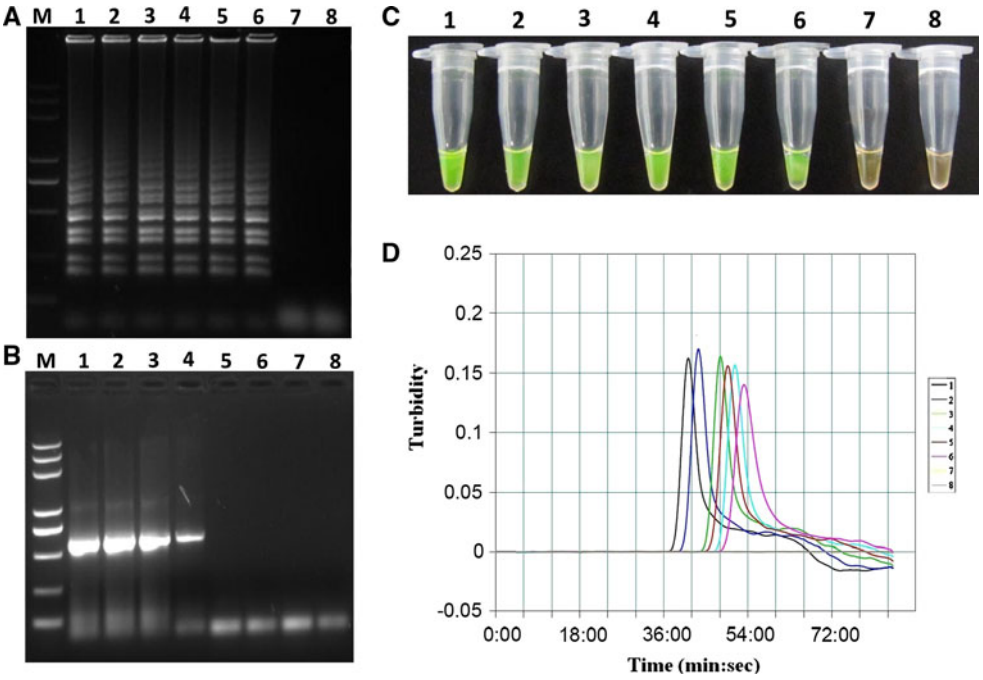

Fig. 2 Comparison of the sensitivity of RT-LAMP and RT-PCR. (**A**) Detection limit of the LAMP assay for the detection of pMD18-T-RP plasmid DNA. (**B**) Detection limit of the PCR assay for the detection of the pMD18-T-CMV plasmid DNA. (**C**) Visual inspection of the LAMP assay corresponding to agarose gel electrophoresis analysis. (**D**) Graph of turbidity over time for the CMV RT-LAMP reaction. Lanes 1-8 correspond to serial tenfold dilutions of pMD18-T-CMV plasmid (100 ng /μL) with healthy banana extract ranging from 1×10^0 to 1×10^7 copies. Lane M, Trans2K Plus II DNA Marker

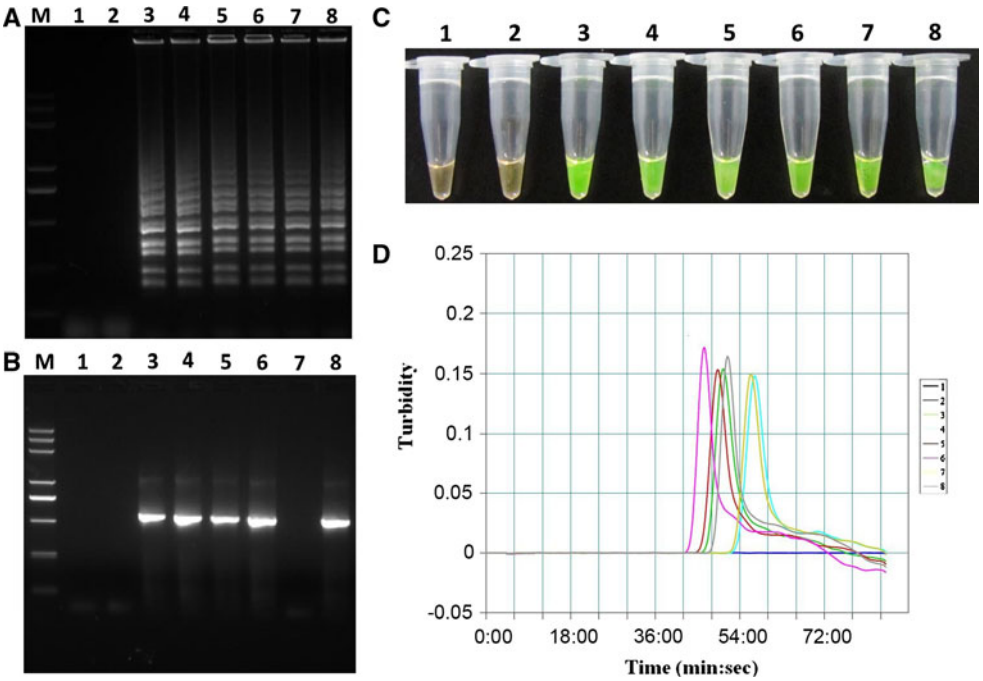

Fig. 3 Detection of selected samples by one-step RT-LAMP and RT-PCR methods. (**A**) Detection of CMV in field samples by simple one-step RT-LAMP. (**B**) Detection of CMV in field samples using conventional RT-PCR. (**C**) Visual inspection of the RT-LAMP assays corresponding to agarose gel electrophoresis analysis. (**D**) Graph of turbidity over time for the CMV RT-LAMP reaction. Lanes 1 and 2, water and healthy plant controls; lanes 3-8, random samples collected from different geographic locations; lane M, Trans2K Plus II DNA Marker

based on the conserved coat protein gene [22]. In this paper, the RT-LAMP assay is suitable for detection of a CMV isolate from banana using primers with six nucleotide differences. Moreover, the improved closed-tube detection system in which the fluorescent dye is added to the interior of the lid before amplification was developed for high-throughput application.

The specificity of the primers used is a key issue of the LAMP assay. The four primers were designed based on the sequences of highly conserved regions of the CMV genome, and there was no cross-reactivity with other templates after extraction, or with other banana-infecting pathogens (Fig. 1). The sensitivity of RT-LAMP was also demonstrated using field samples, and two positive samples detected by LAMP gave false negative results in the RT-PCR assay (Fig. 2).

SYBR Green I is one of the most sensitive general nucleic acid fluorescence dyes [23]; however, the LAMP reaction would be inhibited if the dye is directly added to LAMP reaction solutions at concentrations required for

visualization [24, 25]. In this study, a slight improvement was made by addition of 1 μl SYBR Green I to the inside of the lid prior to amplification. After the reaction, the SYBR Green I was added to LAMP reaction solution by gentle centrifugation at about $500 \times g$ for 10 s. The risk of cross-contamination is minimal when using the improved closed-tube visual detection system, which facilitates the rapid screening of samples without the use of gel electrophoresis, and this will be helpful for high-throughput applications.

In conclusion, a method for the detection of CMV using a basic one-step RT-LAMP method was developed, and this approach has the potential to become a valuable diagnostic tool in the banana industry. The method requires basic laboratory equipment and requires less time to obtain results using the SYBR Green I stain when compared with gel electrophoresis. The early detection of CMV suggests that the established one-step RT-LAMP method should be useful for both disease monitoring and mass propagation of virus-free banana plantlets.

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