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ORIGINAL ARTICLE

Rapid detection of tomato leaf curl Bengaluru virus through loop mediated isothermal amplification assay

R. Arutselvan[1] · M. Krishna Reddy[2] · T. Makeshkumar[1]

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Abstract A loop-mediated isothermal amplification (LAMP) technique was employed to develop a simple and rapid method for the detection of tomato leaf curl Bangalore virus (ToLCBaV) in diseased plants of tomato (*Solanum lycopersicum*). Six sets of primers were designed for LAMP technique targeting the conserved AC1 region and successfully detected ToLCBaV. No reaction was detected in the tissues of healthy plants by either the LAMP or the polymerase chain reaction (PCR). The LAMP products can be visualized by presence or absence of turbidity and staining (0.2 µL for 25 µL LAMP product) directly in the tube with nucleic acid stain dye which allowed easy detection. Sensitivity of LAMP assay is 100 times of conventional PCR technique. Although, both the LAMP and the PCR methods were capable of detecting ToLCBaV in infected tissues of tomato, the LAMP method would be more useful than the PCR method for detection of ToLCBaV infection in tomato plants because it is more rapid, simple and accurate method.

Keywords LAMP · ToLCBaV · Turbidity · Nucleic acid stain · Sensitivity

✉ R. Arutselvan
rarutselvan@gmail.com

[1] ICAR, Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala 695 017, India

[2] ICAR, Indian Institute of Horticultural Research, Bengaluru 560 089, India

Introduction

Tomato is one of the most important widely grown vegetable crops in India and severely infected with leaf curl disease. The members of the genus *Begomovirus* (family *Geminiviridae*) cause leaf curling disease in tomato worldwide. However, the virus constantly evolving and threaten horticulture in many of the world's tropical and subtropical regions. Tomato leaf curl New Delhi virus (ToLCNDV) and tomato leaf curl Bangalore virus (ToLCBaV) are the most damaging in India and they are transmitted by *Bemisia tabaci* [3]. Epidemics of ToLCV is associated with an upsurge of whiteflies has been frequently reported with up to 100% yield losses [8].

In 1948, Vasudeva and Sam Raj first reported tomato leaf curl disease in northern parts of India [27]. Currently, the disease is a major problem and widespread throughout the country, causing yield loss up to 27–100%, during maturity stage [10]. The symptoms of the disease are leaf curling, vein clearing, reduction in leaf lamina, vein enation and, stunted growth of plants. At least 14 distinct indigenous tomato-infecting begomoviruses that cause major constraints on tomato production in tropical and subtropical regions of the world [6], of which ToLCBaV is widely prevalent in the southern and south-western parts of the country [7, 10].

Several methods have been reported for the detection of the genomic DNA of ToLCV [14, 15, 22, 24]. PCR is the most widely used method for detection of ToLCV DNA from infected plants tissues [14, 15]. However, the PCR method has disadvantages, such as the requirement of thermal cycling and liable to contamination.

Loop mediated isothermal amplification (LAMP) is a novel gene amplification method [11, 12, 18]. The method is

functioned by employing a DNA polymerase with strand-displacement activity, along with two inner primers (FIP, BIP) and two outer primers (F3, B3) to form auto-cycling amplification by loop primers. The new assay is quite simple, requiring only a conventional water bath or heat block for incubation under isothermal conditions. In addition, the reaction process is rapid, usually taking 60 min or even less, and low-cost. Generally, four to six primers are designed that recognize several regions of the target sequence, which gives high specificity in LAMP assay. Recently, LAMP assay been applied to the rapid detection of tomato torrado virus in tomato [2]. Several workers found that LAMP shows more sensitive than quantitative real-time PCR assays [19, 21, 25].

However, crop losses can be minimized, and specific treatments can be tailored to combat specific pathogens if plant diseases are correctly diagnosed and identified early. Without proper identification of the disease and the disease-causing agent, disease control measures can be a waste of time and money and can lead to further plant losses. LAMP is a powerful innovative nucleic acid amplification technique emerging as a simple rapid diagnostic tool for advanced detection and identification of virus [20]. While this helps to improve detection research and reduces time effectively for detecting viral pathogens of tomato during field analysis. The objective of this study was to develop a LAMP assay for rapid and efficient detection of ToLCBaV infection in tomato.

Materials and methods

ToLCBaV plants or virus samples

ToLCBaV infected leaf was collected from the vegetable orchard of ICAR—Indian Institute of Horticultural Research (IIHR), Bengaluru. Leaf samples showing foliar curling, shortening of internodes and stunted growth were collected in summer of 2016 and stored under −20 °C. These samples were screened for the presence of ToLCBaV using molecular techniques.

Total DNA isolation

Approximately 100 mg of diseased leaf tissue was placed into a thick-gauged plastic bag. The tissue was ground using pestle and mortar with 10 volumes (1 mL) of CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0.05 M EDTA, 0.2 M Tris–HCl with pH 6.5). About 750 µL of the sample was poured into a 1.5 mL eppendorf tube and the samples were heated at 60 °C for 30 min. The samples were mixed with an equal volume (750 µL) of chloroform: isoamyl alcohol (24:1) and centrifuged at 13,000 rpm for 10 min. The top aqueous phase was transferred to a new 1.5 mL eppendorf tube and DNA was precipitated by adding 0.6 volume (300 µL) of cold (−20 °C) isopropanol and incubated at –20 °C for 1 h. The samples were centrifuged at 13,000 rpm at 4 °C for 10 min and the supernatant was discarded. The pellet was washed in 0.5 mL of 70% ethanol by vortexing and then centrifuged for 5 min at 13,000 rpm. The ethanol was removed and the pellet was vacuum dried for 5 min and the dried pellet was suspended in 100 µL 1 × TE buffer and stored at –20 °C. All the DNA extracts were further diluted to ten-fold in single distilled water (SDW) before using for PCR amplification.

PCR amplification and gel electrophoresis

PCR reactions were performed in 25 µL of reaction mix containing 2 µL of template DNA (100 ng), 2 mM dNTPs, 25 mM $MgCl_2$, 3 units of *Taq* polymerase, 0.4µM of primer and 10X reaction buffer (20 mM (NH_4) SO_4, 75 mM Tris–HCl (pH 9.0). ToLCBaV primers were used are (5′-TCCCCTTCTTGGCTAACC-3′ Forward and 5′-AGCCAGTTCAAATTAAAGGAG-3′ Reverse). Single distilled water (SDW) control was included in all reactions as the control. The reaction condition consists of for one cycle of 2 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, followed by a final cycle of 10 min at 72 °C in PCR thermal cycler (Eppendorf). A 20 µL of PCR product was mixed with 4 µL of Orange G loading dye [15% (w/v) Ficoll type 400, 0.25% (w/v) Orange-G, 40 mM ethylenediaminete traacetic acid (EDTA), pH 8.0 in 100 mL of SDW] and electrophoresed at 5 V cm^{-1} through a 0.8% (w/v) agarose gel with ethidium bromide solution (0.5 µg mL^{-1}) in 0.5x TBE buffer (4.5 mM Tris–borate, 0.1 mM EDTA). The DNA size marker 1 kb Ladder (Fermentas) was included to allow the size of the DNA bands to be estimated. Amplified products then visualized and documented by Alpha Digi-Doc 1000 system (Alpha Innotech Corporation, USA).

LAMP based detection

Target gene and primer design

Target sequences that are traditionally used to identify ToLCBaV were selected. The LAMP primers were designed to target the AC1 gene from ToLCBaV. Complete set of LAMP primers including loop primers were designed using the software Primer Explorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). The LAMP primer sequences are listed in Table 1.

LAMP reaction and detection

ToLCBaV-LAMP assay were performed in a final reaction volume of 25 µL with 1.4 mM dNTPs (Promega, WI, USA),

Table 1 Details of LAMP primers designed for the amplification of ToLCBaV

LAMP assay (gene target)	Primer name	Primer sequence (5′–3′)	Length	Temp and time
AC1 gene	F3	TCCCCTTCTTGGCTAACC	18	60 °C for 60 min
	B3	AGCCAGTTCAAATTAAAGGAG	21	
	FLOOP	AAGAACCATTCTACTCAGGT	20	
	BLOOP	TCTTTATAGCTGCTGTTAGG	20	
	FIP	TGCGAGATTCATCACCCTCGTGTGTTGCACTTTGATTGGA	40	
	BIP	GCTTTAGTGCGTTGTTCTTGTCCTCCCATTATCTTCCTCTGC	42	

8 mM $MgSO_4$, 0.8 M betaine (Sigma-Aldrich, MO, USA) and 8 U of *Bsm* DNA Polymerase (New England Biolabs, MA, USA). The LAMP reactions also contained 1.6 μM of FIP and BIP primers, 0.2 μM of F3 and B3 primers, 0.4 μM of F Loop and B Loop primers and the corresponding DNA as the template. The templates consisted of 1 μL of high-quality DNA obtained by the rapid DNA extraction procedure as described earlier. The reaction tubes were incubated in equipment [MyCyclerthermocycler (Bio-Rad, CA, USA)]. The LAMP amplicons were visualized by different strategies: (a) 2% agarose gel electrophoresis, staining with 0.5 μg/mL of ethidium bromide and visualized and documented by Alpha DigiDoc 1000 system (Alpha Innotech Corporation, USA); (b) naked-eye inspection by turbidity and nucleic acid stains such as Ethidium bromide, Calcein staining and hydroxynaphthol blue (HNB). For staining, 1 μL of 1/10 nucleic acid stain solution was added directly to each reaction tube after incubation, and the DNA was visualized under naked eye.

Results

Detection of ToLCBaV from infected tomato samples by polymerase chain reaction (PCR)

Tomato samples showing viral infections were collected from ICAR-IIHR, Bengaluru, India and screened for the presence of ToLCBaV DNA through PCR technique. The most consistent amplification of 0.7 kb DNA fragment was obtained with the ToLCBaV specific primer with samples from ToLCBaV infected tomato plants. The result revealed that there is the presence of ToLCBaV DNA with this disease and the virus could be detected by using specific primers (Fig. 1a).

Visual examination of LAMP products

Turbidity

Tomato samples showing viral infection were collected from ICAR-IIHR, Bengaluru and screened for the presence of ToLCBaV DNA using LAMP technique. LAMP reaction was carried out using *Bsm* DNA polymerase and incubated for 60 min under isothermal condition @ 60 °C. Positive samples are visualized by the presence of turbidity caused by increasing the quantity of magnesium pyrophosphate in reaction tubes.

Nucleic acid stains

Viral nucleic acids were subjected to isothermal amplification to evaluate the assay's detection ability utilizing virus-specific LAMP primers-sets using nucleic acid stain. The reaction tubes having LAMP products were mixed with 1 μL of 1/10 nucleic acid stain solution like Ethidium bromide, HNB and Calcein and visualize the colour change under the naked eye in normal light (Fig. 2). The result revealed an intense colour change in tubes with amplified product of ToLCBaV but no colour change in the control tube.

Gel electrophoresis

ToLCBaV DNA was amplified from total genomic DNA extracts of the individual ToLCBaV-infected tomato plant by LAMP. The result revealed electrophoretic analysis of reaction products amplification of the targeted viral pathogen by their specific LAMP primer set and produced characteristic ladder-like banding patterns for ToLCBaV infected sample in the 2% agarose gel electrophoresis (Fig. 1b).

LAMP sensitivity assay

To determine the limit of the LAMP assay, total nucleic acids were extracted from both ToLCBaV- infected and healthy tomato cultivars and diluted from 10^0 to 10^{-4} and used in amplification reaction to investigate the sensitivity of both PCR and LAMP assay. Results of electrophoretic gel analysis revealed detection limit of ToLCBaV is 10^0 to 10^{-2} and sensitivity of the LAMP assay is 100 times that of PCR (Fig. 1c).

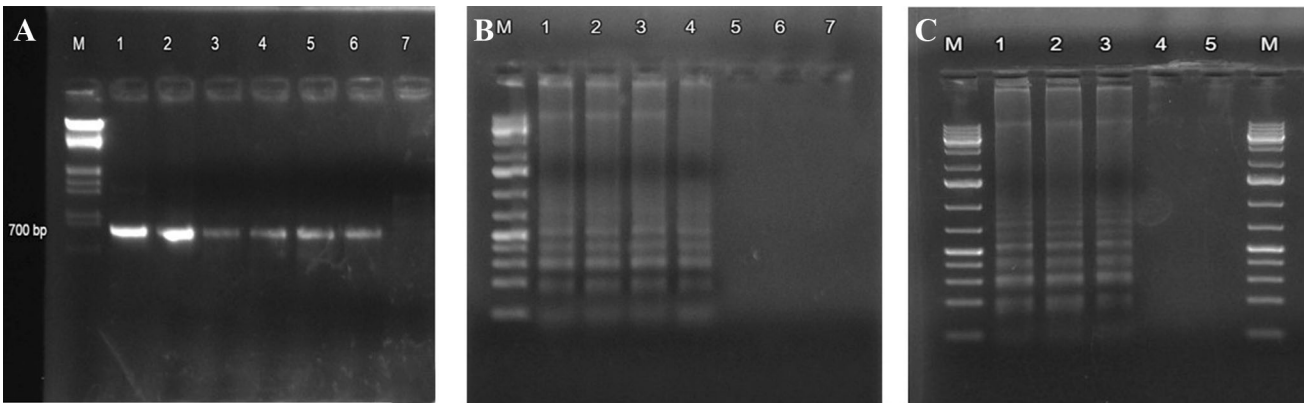

Fig. 1 **a** PCR detection of ToLCBaV specific 700 bp in virus infected tomato samples, electrophoresis on a 0.8% agarose gel and staining by ethidium bromide. *Lane M* Marker (1 kb DNA ladder, Fermentas), *Lane 1* & *2* positive sample for ToLCBaV, *3–6* virus infected tomato samples, *7* Healthy tomato leaf sample. **b** LAMP-PCR detection of ToLCBaV in virus infected tomato samples, electrophoresis on a 2.0% agarose gel and staining by ethidium bromide. *Lane M* Marker (1 kb DNA ladder, Fermentas), *Lane 1* positive sample for ToLCBaV, *2–4* virus infected tomato samples, *5* Healthy tomato leaf sample, *6* Control (Water). **c** Sensitivity of LAMP assay for detecting ToLCBaV in tomato sample. Dilutions of target viral nucleic acids, standards ranging from 10^{0} to 10^{-4}. *Lane M* 100 bp DNA ladder, (*Lane 1–5*) amplification products with ten-fold serially diluted total DNA of tomato from 10^{0} to 10^{-4}

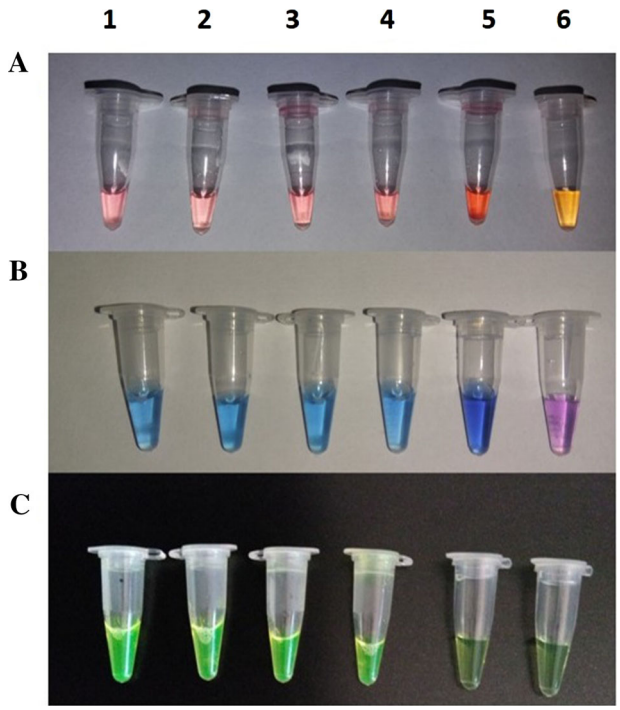

Fig. 2 Visualization of LAMP product using nucleic acid stains **a** Ethidium Bromide **b** HNB **c** Calcein. *Tube 1* positive sample for ToLCBaV, *2–4* virus infected tomato sample, *5* Healthy tomato sample, *6* control (water). The reaction products were visualized under naked eye and revealed *colour change* in tubes with amplified products (colour figure online)

Discussion

Several detection and diagnosis methods have been operated for the amplification of ToLCV [14, 16, 22, 23]. Among these techniques, LAMP method has the advantage of rapid amplification rate and high sensitivity. The LAMP method is functioned by four different sets of primer to recognize the six distinct regions of the target pathogen. In that F3 and B3 act as an outer primer for the strand displacing activity and FIP & BIP act as an inner primer which helps to form stem loop structure or dumbbell structures at the ends. These stem loop structure pay way to auto cyclic amplification by involving loop primers which shorten time and increase sensitivity. *Bst* and *Bsm* DNA polymerase is the enzyme play key role in LAMP amplification process [1]. The LAMP reaction process proceeds at isothermal conditions vary from 60 to 65 °C for 30–60 min [9, 12, 18]. LAMP reaction can be supervised by observing the white precipitation of magnesium pyrophosphate produced as outcome of combination with magnesium ion in the reaction solution [9, 26]. The turbidity in the reaction tubes shows the formation of magnesium pyrophosphate precipitate which indicates the presence of ToLCBaV DNA in the sample and switches the visual examination of the LAMP reaction product to replace the gel electrophoresis.

In this experiment, we have collected fifteen tomato leaf curl disease infected samples from ICAR-IIHR, Bengaluru, India for the amplification of genomic DNA of ToLCBaV by the use of LAMP method. Six sets of primers were designed for targeting the conserved AC1 region of ToLCBaV by using the primer explorer V4 software. Appropriate design of the primers is an important key in gene amplification using the LAMP method [26]. LAMP reaction was carried out using *Bsm* DNA polymerase and incubated @ 60 °C for 60 min. No reaction was detected in the healthy sample either by LAMP technique or PCR. The positive reaction tubes show the presence of turbidity in the

LAMP product. Colorimetric detection of LAMP using the metal ion-binding indicator dye HNB, which is known to bind the Mg^{2+} ion and changes its colour depending upon the pH and Mg^{2+} concentration present in the reaction master mix [4].

The visual examination was carried out using nucleic acid stains like Ethidium bromide, HNB and Calcein and colour differentiation was observed between healthy and ToLCBaV—infected LAMP products. A higher sensitivity of detection of LAMP products by the colour change method using HNB than by turbidity measurement has also been reported by others [4]. Previous studies revealed that 0.8 M betaine resulted in elevated sensitivity and increased effectiveness of the LAMP assay [13, 17]. To determine the limit of LAMP assay, total nucleic acid were extracted from both ToLCBaV—infected and healthy tomato cultivar and diluted from 10 to 10^{-6}. The sensitivity of the LAMP assay is ten-fold that of conventional PCR technique [5, 24, 28]. This result shows that LAMP assay for ToLCBaV will straighten the ability to diagnose the viral infection in tomato cultivar under various circumstances.

In summary, LAMP-PCR based detection of plant virus uses 4–6 primers recognizing 6–8 distinct regions of target DNA. A strand-displacing DNA polymerase initiates synthesis by (F3 & B3) outer primer and (FIP & BIP) inner primers to form loop structures which facilitate auto cyclic amplification. LAMP-PCR is simple, rapid, sensitive, and amplification is so extensive that the magnesium pyrophosphate produced during the reaction can be seen by naked eye, making LAMP well-suited for field diagnostics.

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