REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) ASSAY FOR RAPID DETECTION OF COTTON LEAFROLL DWARF VIRUS (CLRDV) IN COTTON

Bisho Ram Lawaju
Marina Nunes Rondon
Kathy S. Lawrence
Auburn University
Auburn, AL

Abstract

Cotton leafroll dwarf disease caused by Cotton leafroll dwarf virus (CLRDV) is an emerging viral disease in cotton in the US. Infection with the virus shows varying degrees of symptoms making proper early diagnosis difficult. Conventional reverse transcription-polymerase chain reaction (RT-PCR) from symptomatic tissues is the most common diagnostic method for CLRDV but it is relatively complex and time consuming. To overcome these shortcomings, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for the detection of CLRDV and its specificity and sensitivity were compared with conventional RT-PCR assay. For this assay, a set of six primers to amplify the partial P1 (ORF1) and P0 (ORF0) region of the viral genome were designed. The primers were used for amplification at isothermal temperature with total RNA extracted from CLRDV infected cotton leaf tissues, and the optimum primer concentrations, reaction temperature, and assay time were determined. The amplified products were visualized by gel electrophoresis. The primers concentration of 0.4 µM internal primers, 0.1 µM external primers, and 0.2 µM loop primers at 65^0C for 45 minutes were determined as the optimized RT-LAMP reaction condition for successful amplification of the target region. The RT-LAMP specificity was similar to RT-PCR; however, RT-LAMP was able to detect viral dilutions up to 1 x10^{-5} which was 100 times more sensitive than the RT-PCR assay. These results suggest that CLRDV RT-LAMP is a simple, rapid, and sensitive diagnostic tool suitable for easy detection of CLRDV.

Introduction

Cotton leafroll dwarf virus (CLRDV) is an emerging cotton pathogen in North America. The virus is a member of the family *Luteoviridae* and genus *Polerovirus.* It is transmitted by the cotton aphid, *Aphis gossypii* in a persistent-circulative manner (Correa et al. 2005). It was first observed in the cotton fields in Alabama in 2017 growing season, but it has now been reported from most the southern cotton-producing states (Aboughanem-Sabanadzovie et al. 2019; Avelar et al. 2019; Tabassum et al. 2019; Faske et al; 2020).

When the virus infects cotton plants, it induces a wide range of symptoms that include stunted growth, foliar deformation, curling, reddening or bronzing of leaves, yellowing, wilt, and reduction in boll set (Aboughanem-Sabanadzovic et al. 2019; Avelar et al. 2019). These symptoms vary based on cotton cultivars, cotton growth stages, plant nutritional status, location, as well as other environmental factors (Brown et al. 2019). Since, it is a recently recognized pathogen in the US, very little information is available on its host range, transmission vectors, and the effect on yield; however, infection with closely related CLRDV genotypes in South American countries have reported to cause significant yield loss (Correa et al. 2005; Distefano et al. 2010; Galbieri et al. 2017). Thus, it should be considered as a potential threat to the cotton growers even though CLRDV was estimated to cause only 0.01% of cotton yield loss in the US in 2020 (Lawrence et al. 2021).

Early detection and accurate identification of the pathogen is essential for managing any disease. Reverse transcription-polymerase chain reaction (RT-PCR) is the only confirmatory test documented to detect the virus at present. RT-PCR is sensitive and most reliable but relatively expensive and time-consuming (Brown et al. 2019). Thus, researchers are working to develop an alternative diagnostic technique that is simple and rapid without compromising the reliability of the test. Loop-mediated isothermal amplification (LAMP) technique promises to fulfil these requirements. LAMP is a newer molecular technique for nucleic acid amplification in isothermal condition. It uses 4-6 primers targeting different regions of the gene of interest in combination with self-strand displacement DNA polymerase (Notomi et al, 2000). Because of its rapidity, simplicity, sensitivity, and low cost, the LAMP and RT-LAMP techniques have been adapted for detection of wide range of pathogens including virus (Zhao et al., 2015), fungi (Li et al, 2013), and nematodes (Zhang and Gleason, 2019). The current study was aimed to develop an RT-LAMP assay to detect CLRDV on cotton that is simple and give result in shorter time with considerable reliability compare to the conventional RT-PCR.

Materials and Methods

Plant materials and RNA extraction

The youngest leaves were collected from both symptomatic and asymptomatic cotton, corn, and soybean plants grown in the greenhouse at Plant Science and Research Center, Auburn University. Total RNA was extracted from the leaf petioles and veins using TRI reagent according to the manufacturer's instructions. (Direct-zol™ RNA Miniprep Plus, Zymo Research, USA). Total RNA was treated with DNase I before elution with nuclease free water and the concentration was determined using Qubit fluorometer (Qubit Fluorometer 2.0, ThermoFisher Scientific, USA).

RT-LAMP primer design

The complete genome sequence of CLRDV deposited in GenBank (accession number MN071395) was used to obtain the nucleotide sequence of ORF0/ORF1 of the CLRDV genome. A set of LAMP primers for CLRDV was then designed using the LAMP primer design software Primer Explorer version 5.0 (available at http://primerexplorer.jp/lampv5e/index.html) that will amplify a region of partial ORF0 and ORF1. The primers included two outer primers (F3 and B3), two loop primers (LF and LB), and two inner primers (FIP and BIP). A detailed description of the primers is provided in Table 1.

Table 1. Primers used for RT-PCR and RT-LAMP

Primer name	Nucleotide sequence
RT-PCR	
CLRDV3675F	CCACGTAGRCGCAACAGGCGT
Pol3982R	CGAGGCCTCGGAGATGAACT
RT-LAMP	
Forward outer (F3)	TTCGTTCTCTTCTTTTTCTCCTTCCATTC
Backward outer (B3)	GAGTGCAGAGATGCTCGAACTCAA
Forward inner primer (FIP)	TTCGGTAACCGTGGGCATATACTTGGTGGCCACGATTCGCTCTTCCCCGT
Backward inner primer (BIP)	TATCGTTGCCTTGCATCTGCCCTCCTGTTGTTTTCGACCAGAGAGCGAGTA
Loop forward (LF)	TGAGAGAGCCATAAAGTCTGCTCGAAGCC
Loop backward (LB)	GAGCCACTGGCCGAGCGAC

Initial RT LAMP

Total RNA isolated from plant tissues was directly used for RT-LAMP. Reaction was performed according to the manufacturer's instructions (WarmStart LAMP Kit (DNA&RNA), New England Biolabs, USA) with a slight modification. In brief, 5 µl of RNA, 0.2 µM of F3 and B3 each, 1.6 µM of FIP and BIP each, 0.4 µM of LF and LB each, 12.5 µl of WarmStart LAMP 2x master mix, 0.5 µl of WarmStart RTx reverse Transcriptase (15 U/µl) was taken in 0.2 µl PCR tube and adjusted the volume to 25 µl with nuclease free water. Reaction mixture was incubated at 65^0C for 60 minutes followed by 5 min at 80^0C to inactivate the enzyme in a PCR thermocycler (MyCycler Thermal cycler, BioRad Laboratories, USA). The amplified product (5 µL) was run on gel (1% agarose, TBE buffer) added with GelRed nucleic acid gel stain (Biotium, Inc., USA) at 70V for 45 min and observed under UV light (Azure c300, Azure Biosystems, USA).

Optimization of RT-LAMP conditions

The optimization reactions were performed using RNA isolated from symptomatic plant leaves as a template. The initial concentration of the RNA was set to 10 ng/µl. To optimize the RT-LAMP condition, the reactions were performed using two different concentrations of primers (1.6 µM and 0.4 µM for internal primers, 0.2 µM and 0.1 µM for external primers, 0.4 µM and 0.2 µM for loop primers), eight different reaction temperatures (50, 60, 61, 62, 63, 64, 65, 75^0C), and 3 different reaction times (30 min, 45 min, and 60 min).

RT-PCR

Complementary DNA (cDNA) from total RNA (5 µl) was prepared using SuperScript IV 1st strand synthesis system (ThermoFisher Scientific, USA) with specific reverse primer (Brown, 2019; Conner, 2019), and the resulting cDNA was used for PCR amplification. The PCR reaction was carried out in a thermocycler (MyCycler Thermal cycler, BioRad Laboratories, USA) following the protocol from Sharman et al. (2015). The amplified products were examined in agarose gel under UV light.

Specificity and sensitivity of RT-LAMP

Total RNA isolated from symptomatic and asymptomatic cotton leaves, corn, and soybean leaves were used to evaluate the specificity of the RT-LAMP assay following the optimized reaction conditions. For sensitivity assay, CLRDV-positive total RNA sample (10 ng/µL) was serially diluted in 10–fold up to 1 x 10^{-7} and cDNA was prepared from each dilution for RT-PCR. For RT-LAMP, diluted RNA was directly used for the reaction. The amplified products were detected by agarose gel electrophoresis.

Results and Discussion

Optimization of RT-LAMP condition

For the successful detection of CLRDV by RT-LAMP, the optimal primers concentration, reaction temperature, and reaction time were explored. The successful reactions produce ladder-like bands in the gel. The application of kit recommended concentrations of primers were unable to amplify the target gene; however, the external, inner, and loop primers in the concentration of 0.1 µM, 0.4 µM, and 0.2µM, respectively resulted successful amplification (Fig.1.A). Among the different temperatures evaluated for RT-LAMP reaction, 50^0C and 75^0C produced no products whereas gradual increase in the concentration of the products were observed from 60^0C to 65^0C (Fig.1.B). The product concentrations were similar at 45 min and 60 min of reaction times, though relatively lower but clearly observable amount of product was formed even with 30 min of reaction time (Fig.1.C). Based on these optimizing reactions, 65^0C for 45 min reaction time with external (0.1 µM), inner (0.4 µM), and loop (0.2µM) primers concentration were set as the optimum temperature, time, and primers concentration for successive reactions.

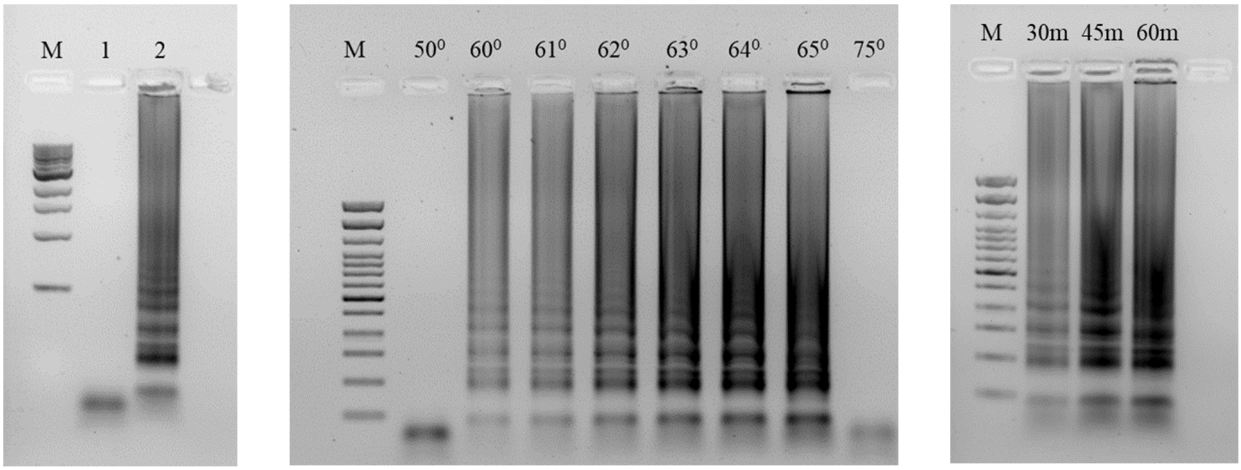

Fig.1. Gel electrophoresis of RT-LAMP products for optimization of RT-LAMP reaction conditions. (A) RT-LAMP products with different primers concentrations at 65^0C for 60 min; 1 kb DNA ladder (M), kit recommended primers concentrations (1), and optimized primers concentrations (2). (B) RT-LAMP products carried out at different temperatures for 60 min., M is the 100 bp ladder. (C) RT-LAMP products carried out for different reaction time at 65^0C, M: 100 bp ladder.

Specificity of RT-LAMP

Total RNA extracted from plant tissues previously identified as CLRDV positive or negative by RT-PCR method were used to evaluate the specificity of the RT-LAMP reaction. The amplified products in the agarose gel (Fig.2) showed that characteristic ladder-like bands were formed only with symptomatic cotton sample whereas no bands were formed with corn, soybean, asymptomatic cotton, and no-template control (NTC). The results were consistent with the RT-PCR results (data not shown).

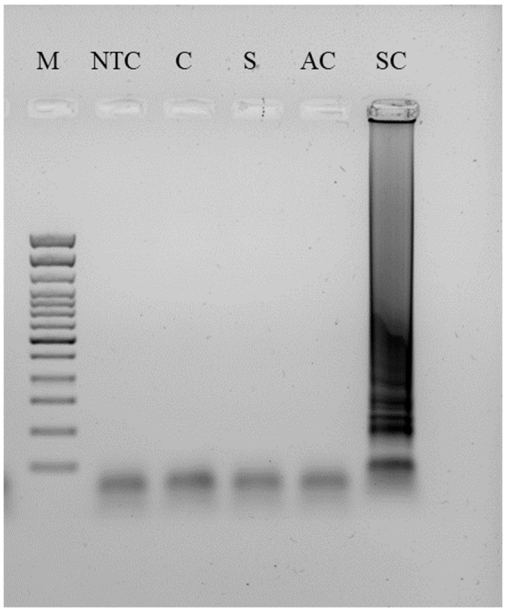

Fig.2. Specificity determination of RT-LAMP assay using RNA from different samples. Lane M:100 bp DNA ladder, NTC: no template control, C: corn, S: soybean, AC: asymptomatic cotton, SC: symptomatic cotton leaves.

Sensitivity of RT-LAMP detection

Ten-fold serially diluted RNA samples from CLRDV- positive cotton leaves were used for RT-LAMP reactions and the results were compared with the RT-PCR reactions from respective dilutions. The result demonstrated that RT-PCR could detect RNA diluted up to 10^{-3} fold while RT-LAMP could distinctly detect up to 10^{-5} dilution though a faint ladder like band of the amplified product was observed in 10^{-6} dilution as well (Fig.3). The result clearly indicated that RT-LAMP has higher sensitivity than the RT-PCR.

A

B

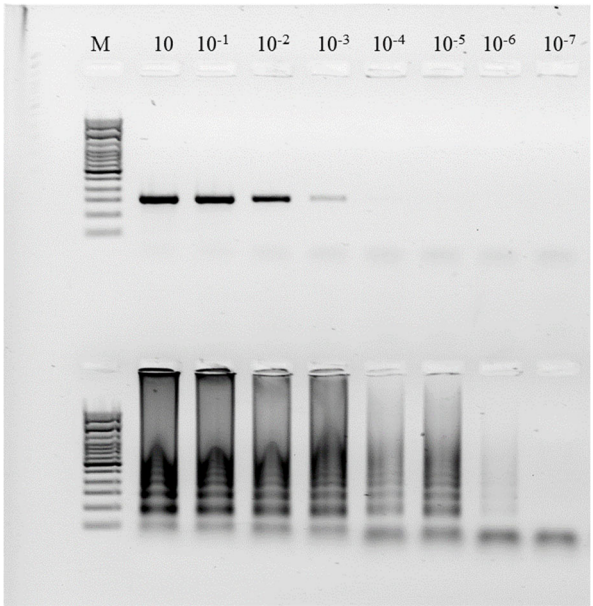

Fig.3. Sensitivity determination of RT-LAMP assay using 10-fold serial dilution of 10 ng/µl RNA from CLRDV positive sample. (A) Gel electrophoresis of products from RT-PCR. (B) Gel electrophoresis of RT-LAMP products. Lane M: 100 bp ladder, 10: 10 ng/ µl RNA, 10^{-1}-10^{-7}: serially diluted RNA samples from 10 ng/µl RNA stock.

Summary

RT-PCR is undoubtedly an invaluable tool in diagnosis of CLRDV. It gives results with high specificity and sensitivity. In fact, it is the only diagnostic method currently followed by all laboratories in diagnosis of CLRDV, though alternative methods are under development process. This study has demonstrated that RT-LAMP could be an alternate to the conventional RT-PCR. Considering the sample preparation and observation times after amplification to be the same for both methods, RT-PCR usually takes 2-3 hours for completion the reaction whereas RT-LAMP took only 45 min. Even with 30 min of RT-LAMP reaction, a weaker ladder like band was observable, which suggest the shorter reaction time for RT-LAMP. This shorter reaction time did not affect the specificity and sensitivity of the assay. In fact, RT-LAMP assay exhibited higher degree of sensitivity compared to RT-PCR. This added advantage in diagnosis of CLRDV. Because, CLRDV is a phloem restricted virus, usually the titer remains lower that can be challenging to detect even with the RT-PCR (Lotos et al. 2014). As the sensitivity is increased, the result of RT-LAMP will be more precise and reliable than the RT-PCR. In conclusion, the RT-LAMP assay developed in this study can detect CLRD in shorter time than conventional RT-PCR with higher degree of sensitivity. It can be a good alternate to RT-PCR in detection of CLRDV.

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