Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Kim et al., J Plant Pathol Microbiol 2016, 7:9
DOI: 10.4172/2157-7471.1000377

Short Communication Open Access

Development of Simple and Rapid Diagnostic Method for *Strawberry Latent Ring-spot Virus* in Plants Using Loop-Mediated Isothermal Amplification Assay

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Abstract

Strawberry Latent Ring-spot Virus (SLRSV) is seed or nematodes-transmitted viruses, and causes quantitative and qualitative loss of various crops. SLRSV is a non-reported, potentially control able virus, which is managed at the national level. Currently, RT-PCR and nested PCR system are the standard methods of detecting SLRSV, but more effective methods are required. In this study, loop-mediated isothermal amplification (LAMP) assay was used for detection of SLRSV. As a result, the LAMP assay showed sensitivity similar to that of the currently used method, but is more rapid (approximately 8 hrs), simple and specific. In addition, results can be verified by restriction fragment length polymorphism (RFLP) using *Bfa*I, or sequencing after the LAMP reaction. Therefore, we have shown that the LAMP assay developed in this study is a potential marker for the facilitation of rapid and simple screening of SLRSV in plants, which will ultimately be useful for the diagnosis of SLRSV infected plants and quarantine.

Keywords: *Bfa*I; LAMP; SLRSV *(Strawberry Latent Ring-spot Virus)*

Introduction

Strawberry Latent Ring-spot Virus (SLRSV) was first discovered in Scotland and classified in the genus Nepovirus as a plant pathogen of the Group IV positive-sense ssRNA viruses [1,2]. SLRSV is classified into the family *Secoviridae* (International Committee on Taxonomy of Viruses; ICTV) and is transmitted by seeds and soil-inhabiting nematodes (*Xiphinema diversicaudatum* and *X. coxi*) [3]. The infection by SLRSV forms tubule-like structures, facilitating the mechanism of cell-cell translocation through plasmodesmata [4,5]. Some of the typical symptoms after infection include chromatic and/or morphological alteration of leaves. This virus has a wide host range, attacking many economically cultivated crops [6,7]. For example, SLRSV was recently found to infect olive in Syria, Oriental hybrid lily in Northern India, strawberry fields in United States, and black locust (*Robinia pseudacacia* L, family *Fabaceae*) in Poland [8-11]. The hosts of this virus in Korea are *Rosa* spp., *Aesculus* spp., *Trifolium* spp., *Peteroselinum crispum*, *Vitis* spp., *Fritillaria imperialis*, *Humulus lulus*, *Euonymus europaeus*, *Pastinica sativa*, *Laminum amplexicaule*, *Ribes* spp., *Rubus* spp., *Delphinum* spp., *Fragaria ananassa*, *Muscari* spp., *Lilium* spp., *Paunus* spp., *Apium graveolens*, *Narcissus* spp., *Robinia peudoacacia*, *Asparagus densiflorus*, *Prunus avium*, and *Prunus* [12]. Despite its wide geographical distribution, SLRSV has not been reported in Korea; however, the possibility of its emergence and significant economic damage has been raised [12].

In Korea, SLRSV has been detected using reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR [13]. However, the reaction time for these PCR methods is time-consuming and laborious, requiring 10 hrs to obtain results, due to the post-PCR reaction and gradient of cycling temperature between three amplification steps. As a consequence, they are inconvenient for rapid diagnosis and point-of-care applications [14,15]. In recent year, nucleic acid-based amplification methods are demanded low-cost, rapid, specific and easy in comparison with traditional methods. Loop mediated isothermal amplification (LAMP) does not require a thermal cycler or analysis software and can be performed using an oven and/or water bath [14,16-21]. Moreover, LAMP is more specific and rapid than PCR-based methods because four specially designed primers (forward inner primer [F1c, F2], reverse inner primer [B1c, B2], outer primers [F3, B3]) bind to six specific regions on the target DNA [22]. These features of LAMP assay have been found to be efficient for detecting many pathogenic organisms including virus, bacteria, and fungi [23]. Therefore, LAMP will be useful both for the diagnosis of SLRSV infected plants and quarantine. In this study, we developed a LAMP assay for rapid detection SLRSV with high specificity as compared to RT-PCR and nested PCR.

Samples of SLRSV and reference viruses [*Cucumber Mosaic Virus* (CMV), *Carnation Ringspot Dianthovirus* (CRSV), *Cherry Leaf Roll Nepovirus* (CLRV), *Grapevine Fanleaf Nepovirus* (GFLV), *Little Cherry Virus* (LchV), *Tomato Black Ring Nepovirus* (TBRV), *Tomato Ringspot Nepovirus* (ToRSV), *Tomato Spotted Wilt Tospovirus* (TSWV), *Tobacco Streak Ilarvirus* (TSV), *Raspberry Ringspot Nepovirus* (RpRSV) and *Prune Dwarf Ilarvirus* (PDV)] were collected with approval for import of prohibited goods. For RNA extraction from these samples, we used an RNA-spin™ II P RNA extraction kit (iNtRon, Korea) and cDNA was synthesized using a ReverTra Ace-α-® (TOYOBO, Japan) [24,25]. RNA of 170-200 ng/ul was extracted from the samples, and used for synthesis of 100 ul cDNA. To design LAMP primers, the sequences of three SLRSV strains (NCBI accession numbers NC006965, X77466 and X75165) and 101 reference virus strains with high sequence similarity or the family *Secoviridae* were collected from the National Center for Biotechnology Information (NCBI). The sequences of the collected viruses underwent multiple alignments using the BioEdit version 7.0.0 software, and six sets of LAMP primers for detection of SLRSV were designed using the PrimerExplorer software (Table 1). SLRSV template

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Received September 08, 2016; **Accepted** September 29, 2016; **Published** September 30, 2016

Citation: Kim J, Lee S, Choi J, Kim SK, Jang W (2016) Development of Simple and Rapid Diagnostic Method for *Strawberry Latent Ring-spot Virus* in Plants Using Loop-Mediated Isothermal Amplification Assay. J Plant Pathol Microbiol 7: 377. doi: 10.4172/2157-7471.1000377

Set	Primer	Sequence (5'→3')	Length (bp)	G+C (%)	T_m (°C)
SLRSV_1	F3	GGTTAGAACTGCTATTGTGG	20	45.0	50.7
	B3	CCTCGAGATCCTCTTCAA	18	50.0	50.5
	FIP	CGTCAGAATCAAATCGGTACTTACCAACCTTCGATTACACT	41		
	BIP	TTAACATTGCAGGACGTTGATAAACCCATTCAGCCCAC	38		
SLRSV_2	F3	CAGTTGGTGTGGATTTCC	18	50.0	50.9
	B3	AAAGCCAAATGCAAGCAG	18	44.4	51.8
	FIP	CTCTTAAGACGCTCTTGAGGACGTTCTGGAGCTAACAACAG	43		
	BIP	ACACATTTCAATAGCCTTCTGCCTCCATTTACCAACGGAA	40		
SLRSV_3	F3	CTGGTAGCTTGCTCACAA	18	50.0	52.2
	B3	CCCGATTGCTGATGATGG	18	55.6	53.5
	FIP	AACCCGATGCCTGAATACGGGAGGCTAGTTTGCTGCTC	38		
	BIP	GCCAGGCCACAAGTAGTGAGCTCTTCTCTCCAACCAGC	38		
SLRSV_4	F3	ATTCGCATGCTCTCTTTG	18	44.4	50.4
	B3	TCTCTGCAGCTTGGAAAT	18	44.4	51.4
	FIP	GAGAAGGTGGTAAGCAACTGTACGCCTGAATATTCACTACG	41		
	BIP	GATGCACACTTTCTCCCTGAAACCCTTTTAACCCCGAG	38		
SLRSV_5	F3	CTCTTAAGGATACAGAGTGG	20	45.0	49.1
	B3	TGACAACTTTAAAGGCGC	18	44.4	50.8
	FIP	CTTAGCTTGAATGGGAGCTGAAAAGTTACACCTTCATGCG	40		
	BIP	CTTGGTTTATGCCTGGTAGACTCATTATGGAGTAGCCAGAC	41		
SLRSV_6	F3	GTGGTAGTGGTTCTCTGT	18	50.0	51.0
	B3	GTAAGCGGAAAAGAGGTG	18	50.0	50.6
	FIP	CGACCCCTTCTTTGTGGTGTTGATTATGGGCAATCTCTGA	40		
	BIP	GGAGATTGAATCCGGCAACTAGTTCCAACTTGAACAACAGG	40		

Table 1: Loop-mediated isothermal amplification primer sequences to quickly detect Strawberry Latent Ring-spot Virus (SLRSV).

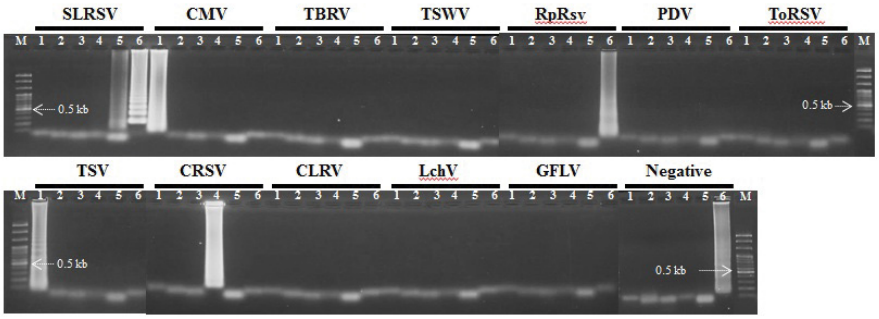

Figure 1: Amplification of SLRSV and reference viruses using the designed LAMP primer sets. Lane M, 100 bp marker (Genepia, Korea); lanes 1 to 6, number of LAMP primer sets for detection of SLRSV. [*Strawberry Latent Ringspot Virus*, (SLRSV); *Cucumber Ringspot Dianthovirus*, (CMV); *Tomato Black Ring Nepovirus*, (TBRV); *Tomato Spotted Wilt Tospovirus*, (TSWV); *Raspberry Ringspot Nepovirus*, (RpRSV); *Prune Dwarf Ilarvirus*, (PDV); *Tomato Ring-spot Nepovirus*, (ToRSV); *Tobacco Streak Ilarvirus*, (TSV); *Carnation Ring-spot Dianthovirus*, (CRSV); *Cherry Leaf Roll Nepovirus*, (CLRV); *Little Cherry Virus*, (LchV); *Grapevine Fanleaf Nepovirus*, (GFLV)].

cDNA was reacted for 1 hr at three different temperatures (60, 62, and 65°C) to determine the optimum LAMP conditions for detection of SLRSV after 10 min at 95°C and 1 min at 4°C. The LAMP reaction was conducted in 2 ul buffer (1 x; 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8), 1.5 ul of template DNAs (100 ng/ul), 2.0 ul of 10 mM dNTP mix (2.5 mM each), 0.6 ul of F3 (forward) and B3 (backward) primers (10 pmoles/ul), 1.4 ul of FIP (forward inner primer) and BIP (backward inner primer) (10 pmoles/ul), 1.5 ul of *Bacillus stearothermophilus* (Bst.) DNA polymerase (8 U/ul, New England Biolabs, USA).

We confirmed the specificity of the LAMP assay using the designed primers for SLRSV. A specific reaction occurred at all temperatures and detected that the optimal reaction condition was at 62°C. Among a total of 6 primer sets, the amplified DNA product with a primer set 5 did not show nonspecific amplification and the negative control for all reference viruses (CMV, CRSV, CLRV, GFLV, LchV, TBRV, ToRSV, TSWV, TSV, RpRSV and PDV) (Figure 1). Thus, the subsequent LAMP reactions were conducted using the primer set 5. Instead of an expected single band size of cDNA fragment (446 bp) for SLRSV from nested PCR, the LAMP product of the positive sample visualized by gel electrophoresis displayed multiple bands of different sizes like a ladder of DNA fragments because of the formation of stem-loop DNAs of various stem lengths (Figure 2). As shown in Figure 1, the primer set 5 appeared as a typical ladder-like pattern which had high specificity to SLRSV. Both LAMP and nested PCR cDNA products were equivalent and able to detect up to 10^{-2} dilution (Figure 2) [13].

To confirm the specificity of the LAMP products, 10 ul of LAMP amplicons were digested with 10 U of the restriction enzymes *Bfa*I (5'-C/TAG-3') (New England Biolabs, USA) at 37°C for 2 hrs. Fragments of restriction fragment length polymorphism (RFLP) products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide (EtBr) [digestion fragments (159 and 138 bp)]. In addition, the product of conventional PCR using outer primers (F3 and B3) was used to Sanger sequencing. PCR products ware purified using the

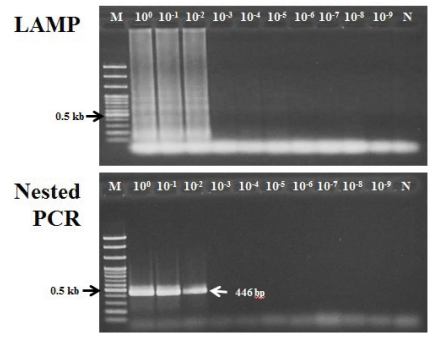

Figure 2: Sensitivity of the LAMP and nested PCR assay for detecting SLRSV. Lane M, 100 bp marker (Genepia); lanes 10^0 to 10^{-9}, amplification of ten-fold serial dilutions of total cDNA including SLRSV; lane N, negative control.

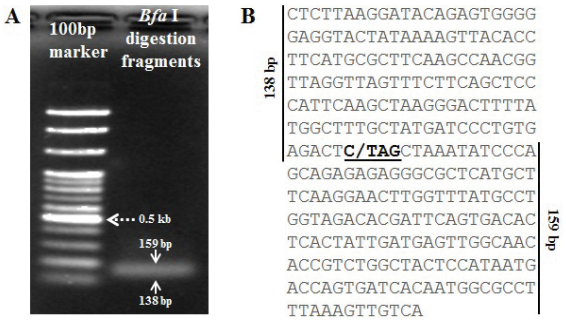

Figure 3: Restriction fragment length polymorphism (RFLP) analysis of the LAMP assay amplicon with *Bfa* I enzyme. (A) Result of RFLP through 1.5% agarose gel. (B) Sequence information of *Bfa* I activation site in the amplicon.

Steps	Reaction time	
	Nested PCR	LAMP
Extraction	2 h	2 h
cDNA synthesis	30 min	30 min
RT-PCR	3 h	N/A
Amplification	3 h	1 h
RFLP	N/A	2 h
Electrophoresis	2 h*	30 min

N/A: Not association; *Electrophoresis is repeated PCR reaction steps, respectively.

Table 2: Processing time of Nested PCR and Loop-mediated isothermal amplification (LAMP).

AccuPrep® PCR purification kit (Bioneer, Korea), and sequencing was performed by Macrogen Co. (Korea). Sequencing data were analyzed by Sequencher software version 5.0 (Gene Codes Corporation, USA). Our results showed that the LAMP assay can detect SLRSV in imported samples within 1.5 hrs (30 mins for cDNA synthesis, 60 min for LAMP) after RNA extraction, and as described here, it is easier, simpler and more specific than PCR-based methods. Thus, this method reduces the detection time compared to the previous 10 hrs necessary for RT-PCR, nested PCR, and electrophoresis steps. After LAMP assay, we performed RFLP using the restriction enzyme *Bfa*I [digestion fragments (159 and 138 bp)], and direct sequencing. Our results confirmed that the LAMP products used in this study had correct digestion fragments and sequence of SLRSV (Figures 3A and 3B).

The PCR based methods combined with RFLP has been used as effective tools for the identification and differentiation of plant viruses such as tobamoviruses [26]. Standard molecular biological detection method of SLRSV has involved RT-PCR and nested PCR since 2010 in Korea. However, no modified-plasmid positive control at quarantine sites can be used with these methods. A modified-plasmid positive control has since 2012 been applied to full-scale standard tests [13]. Accordingly, LAMP assay required to develop a positive control, as well as PCR –based methods. Previously, we reported that developed a LAMP assay for detection of *Wheat* Streak Mosaic Virus (WSMV) that enabled rapid detection during quarantine inspections [27]. In future, we plan to develop rapid, simple, and user-friendly LAMP assays for detection of non-reported, latent, harmful viruses, because the LAMP assay is a powerful diagnostic assay for screening various pathogens.

Conclusion

In conclusion, SLRSV is non-reported potential controlled virus that has problems about economic, yield and quality damage to various crops. Therefore, fast, easy handle, and commercial method is demanded in society, and consequently, we developed a LAMP assay to detect SLRSV in this study, which was more rapid and simple than RT-PCR and nested PCR (Table 2). The LAMP assay showed sensitivity similar to the PCR-based methods; however, this method had high specificity because of four primers targeting six distinct regions on the target DNA when compared to PCR primers that recognize two regions. This specificity allows accurate diagnosis through verification of specific amplicons by RFLP and sequencing after the LAMP reaction Moreover, LAMP can be evidently reduced the processing time more than 6 to 8 hrs because it alleviates the time for gel electrophoresis (Table 2).

Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (Grant number: 2009-0093829).

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