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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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. coco) [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., Petroselinum crispum, Vitis spp., Fritillaria imperialis, Humulus lusus, Euonymus europaeus, Pastinaca sativa, Laminum amplexicaule, Ribes spp., Rubus spp., Delphinium spp., Fragaria ananassa, Muscari spp., Lilium spp., Paeonia spp., Apium graveolens, Narcissus spp., Robinia pseudacacia, 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 Ilarivirus (TSV), Raspberry Ringspot Nepovirus (RbpRSV) and Prune Dwarf Ilarivirus (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 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

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 BfaI, 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: BfaI; LAMP; SLRSV (Strawberry Latent Ring-spot Virus)
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 μl buffer (1 x; 20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 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), and 1.5 ul of Bacillus stearothermophilus (Bst.) DNA polymerase (8 U/ul) in the presence of 7.5 mM MgCl₂ (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 expected single band size of cDNA fragment (446 bp) for SLRSV from 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 10⁻⁹ 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 BfaI (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 were purified using the

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 10⁻⁹ 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 BfaI (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 were purified using the
AccuPrep® PCR purification kit (Bioneer, Korea), and sequencing was performed by Macrogen Co. (Korea). Sequencing data were analyzed by Sequencer software version 5.0 (Gene Codes Corporation, USA). 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).

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### References


