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Rapid and specific detection of apple chlorotic leaf spot virus in pear by reverse-transcription recombinase polymerase amplification

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Summary. – Apple chlorotic leaf spot virus (ACLSV) is an important virus infecting fruit trees. It causes serious economic losses in the global production of fruit trees belonging to the genera *Prunus* and *Malus* and can be vegetatively transmitted during propagation. In this study, an isothermal reverse transcription-recombinase polymerase amplification (RT-RPA) assay method was developed for detecting ACLSV in pear leaves. A set of RT-RPA primers showed high rapidity, sensitivity, and specificity in ACLSV detection. The RT-RPA assay was performed at a single, constant temperature of 42°C, could be completed in approximately 10 min, and did not exhibit cross-reactivity with other common pear viruses. This RT-RPA assay was 100-fold more sensitive than regular RT-PCR. The optimized RT-RPA assay was further used to detect ACLSV in field-collected pear samples. These advantages make RT-RPA a promising diagnostic tool for determining ACLSV infection in pear certification programs.

Keywords: apple chlorotic leaf spot virus; detection; pear; reverse transcription-recombinase polymerase amplification

The Asian pear (*Pyrus pyrifolia* [Burm.f.] Nakai) is an extensively cultivated and commercially popular temperate fruit across East Asia and other countries such as India, Australia, New Zealand, China, Korea, and Japan (Saito, 2016). The Asian pear is one of the six major commercial fruits of Korea; others include apple, citrus, persimmon, grape, and peach.

Apple chlorotic leaf spot virus (ACLSV), a species belonging to the genus *Trichovirus* in the family Betaflexiviridae, is distributed worldwide and exhibits a broad host range in the genera *Prunus* and *Malus*, including apple, pear, quince, cherry, peach, plum, and apricot (Nemeth, 1986). ACLSV was first isolated from the apple *Malus platycarpa* in the USA (Mink and Shay, 1959). This is a filamentous, flexuous virus, with a particle size of 680–780 nm and a single-stranded, positive-sense RNA molecule consisting of 7,474–7,561 nucleotides. It exhibits high molecular variability with numerous viral isolates expressing differential pathogenicity (Candresse *et al*., 1995; Chen *et al*., 2014). In general, ACLSV is a latent virus that generally does not produce clear visible symptoms in the leaves or fruits of most apple and pear cultivars, making it difficult to observe or identify in the field. However, some virus isolates cause bark splitting, severe fruit deformations, yield reduction, and bud necrosis. Severe symptoms may sometimes be caused by ACLSV in association with other viruses, such as in mixed infections with Apple stem grooving virus (ASGV) or Apple stem pitting virus (ASPV) (Yanase *et al*., 1979). ACLSV is transmitted primarily by grafting and propagation of infected materials and is not known to be transmitted through natural vectors, seeds or pollens. Thus, a strategy for producing virus-free plantlets is essential for the fruit industry, and a reliable ACLSV detection method is critical for the sustainable production of ACLSV-free materials for transplantation.

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Abbreviations: ACLSV = apple chlorotic leaf spot virus; ASGV = Apple stem grooving virus; ASPV = Apple stem pitting virus; RT-RPA = reverse transcription-recombinase polymerase amplification

Currently, serological assays, RT-PCR, and real-time TaqMan RT-PCR are used for ACLSV detection in pear and apple (Clark and Adams, 1977; Candresse *et al*., 1995; Kinard *et al*., 1996; Menzel *et al*., 2002; Watpade *et al*., 2012; Beaver-Kanuya and Harper, 2020). However, ELISA employs antibodies and is prone to yielding false-negative results because of low viral titers in the infected trees. RT-PCR is time-consuming and requires a thermal cycler. TaqMan RT-PCR requires expensive equipment, trained personnel, a sophisticated laboratory setting, and well-designed probes (Wang *et al*., 2009). Recently, reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays have been developed to detect ACLSV in apple and pear and overcome the disadvantages of PCR-based assays (Peng *et al*., 2017; Lu *et al*., 2018). However, RT-LAMP assays require relatively high reaction temperature and four or six primers, making it difficult to use in the field. Moreover, LAMP products have certain disadvantages in direct sequencing, cloning, and restriction analysis, because of their multimeric nature.

Recombinase polymerase amplification (RPA) is another relatively cost-effective, rapid, simple, and sensitive isothermal nucleic acid amplification technique (Piepenburg *et al*., 2006). RPA reactions require a constant low temperature (37–42°C), and amplification can be achieved in as little as 15 min. For RNA templates, a reverse transcription process is added for cDNA conversion prior to the RPA reaction (Euler *et al*., 2012). The amplicons obtained using RT-RPA are visualized by gel electrophoresis. In recent years, RT-RPA has been widely applied in the detection of viruses and viroids infecting fruit trees, such as ASGV, ASPV, little cherry virus 2, plum pox virus, peach latent mosaic viroid (Mekuria *et al*., 2014; Kim *et al*., 2018, Kim *et al*., 2019; Lee *et al*., 2020). In the present study, a quick, sensitive, and reliable RT-RPA diagnostic method was developed for ACLSV detection in field-grown pear leaves. We further compared RT-RPA and RT-PCR in terms of their sensitivity and specificity for ACLSV detection.

Fresh leaves were collected from ACLSV-infected pear trees from pear orchards in Naju City, South Korea, and stored at –80°C until total RNA extraction. Leaf samples (~100 mg) were homogenized using a mortar and pestle, and total RNA was extracted using IQeasy Plus Plant RNA Extraction Mini Kit (iNtRON, Daejeon, South Korea), according to the manufacturer's instructions. The presence of ACLSV in the samples was determined by RT-PCR using previously described diagnostic primers (Cho *et al*., 2010) and SuPrimeScript RT-PCR Premix (GeNet Bio, Daejeon, South Korea), according to the manufacturer's instructions. The RT-PCR products were visualized by gel electrophoresis on 1.5% agarose gel containing ethidium bromide (data not shown).

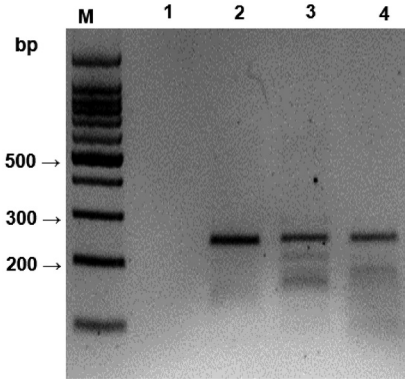

Fig. 1

Optimization of RT-RPA reaction time for ACLSV detection
Lane M, DNA marker; lanes 1–4, reaction times of 5, 10, 20, and 30 min, respectively.

To design optimal primers for ACLSV RT-RPA, 20 complete ACLSV coat protein (CP) gene sequences of isolates available from GenBank were used for multiple sequence alignment to identify highly conserved regions, using BioEdit version 7.0.5.3. As recommended for RPA reactions, primers were 30 to 35 nucleotides long, GC-rich at the 3'-end, included a pyrimidine at the 5'-end, and amplicons were less than 500 bp in length. Nine sets of primers were selected using PrimedRPA (TwistDX, Ltd., Cambridge, UK), according to the manufacturer's instructions. The oligonucleotides for RT-RPA were synthesized by Bionics Co., Ltd. (Daejeon, South Korea). We selected the optimal primer set ACLSV-RPA7 for RT-RPA after evaluating the specificity of all primer sets (data not shown). The forward and reverse primer sequences of the selected primer set were 5'-TTCATGGAAAGACAGGGGCAATACTGGAAC-3' and 5'-TGTTGTTTATATTCGGGTCCGAAGATGTAG-3', respectively.

RT-RPA using the primer set ACLSV-RPA7 generated a single amplicon band of 219 bp from ACLSV-infected leaf samples. The identity of the amplicons generated by RT-RPA was confirmed by cloning into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequencing in both directions.

RT-RPA assays were performed using the TwistAmp® Basic RT kit (TwistDx Limited, Cambridge, UK) in 50 µl total volume, according to the manufacturer's instructions. Briefly, for the reaction mixture, total RNA (0.5 µl), and 0.48 µM of each primer were added to the reaction pellet tube, followed by 29.5 µl of rehydration buffer and 2.5 µl of 280 nM magnesium acetate. cDNA was then synthesized using 1 µl of SuperScript III (Invitrogen, Carlsbad, CA, USA) and 1.2 µl of RNase inhibitor. The reaction mixture was incubated at 42°C for 5–30 min in a water bath. The RT-RPA products were then visualized by gel electrophoresis in

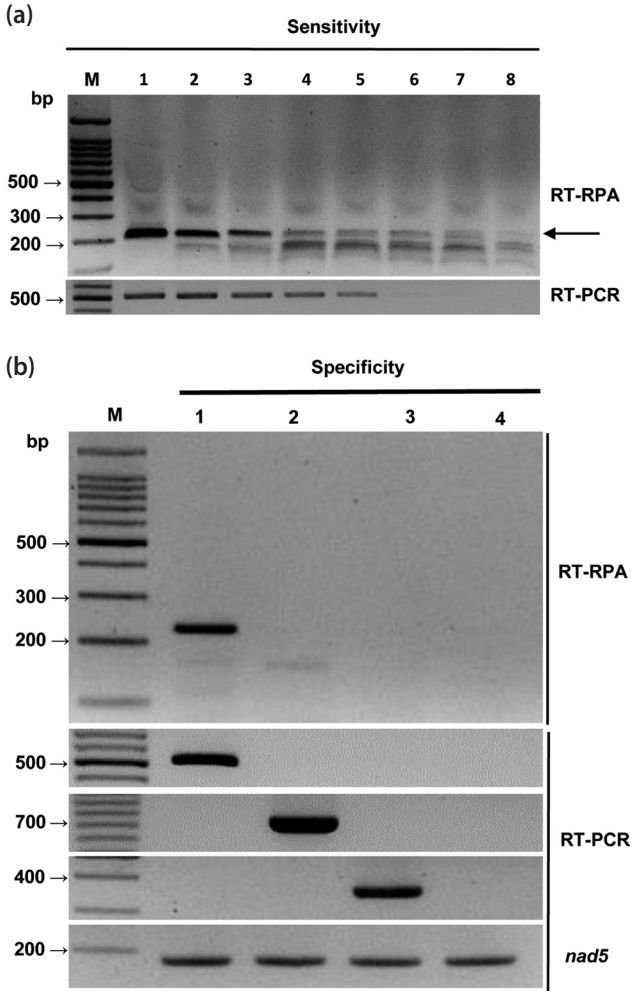

Fig. 2

Sensitivity and specificity of the RT-RPA assay for ACLSV detection

(a) Amplified products (219 bp from the RT-RPA assay and 509 bp from RT-PCR) were visualized on 2% agarose gels. M, DNA marker; lanes 1-8, 10-fold serial dilutions (10^{0}-10^{-8}) of ACLSV template (CP transcripts). **(b)** M, DNA marker; lanes 1–4, RT-RPA product amplified from ACLSV-, ASGV, and ASPV-infected (lanes 1–3) and healthy (lane 4) tissues, respectively. RT-PCR was performed to confirm the presence of ASGV (699 bp) and ASPV (370 bp) on these samples. *nad5* (181 bp) was used as the internal control.

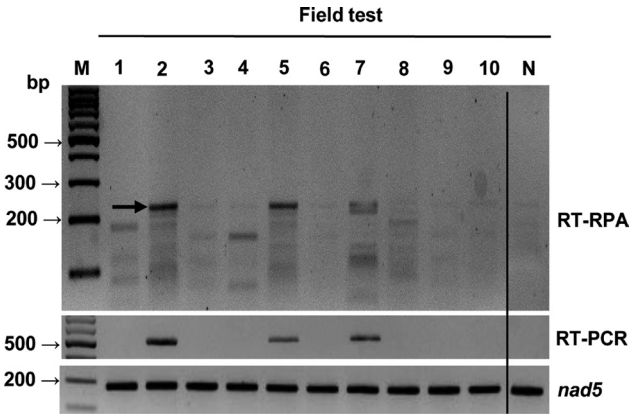

Fig. 3

Sample test of ACLSV by RT-RPA

M, DNA marker; lanes 1–10, products amplified from 10 symptomatic ACSLV-suspected leaves collected from the field; lane 11, ACLSV-free leave (negative control). *nad5* was used as the internal control.

2% gel containing ethidium bromide at a constant voltage (120 V) for 40 min.

The optimal incubation reaction time for the RT-RPA assay was determined by incubating the RNA samples extracted from ACLSV-infected pear leaves for 5, 10, 20, and 30 min. A clear DNA band with the expected size (219 bp) was observed after reaction time of 10 min, and no significant difference was observed in the products obtained by incubation time of 20 and 30 min (Fig. 1). All subsequent RT-RPA reactions were thus performed for 10 min. Four independent assays using different RNA templates were performed, which yielded similar results.

The sensitivities of the RT-RPA assay and RT-PCR were compared using serially diluted ACLSV-CP transcripts obtained from cloned plasmids using T7 RNA Polymerase (Promega). Transcript concentration was measured using a Nanodrop Spectrophotometer (BioDrop, Cambridge, UK), and the initial concentration of the transcript sample was 1,000 ng/µl. Ten-fold serial dilutions of the transcripts were prepared, with concentrations ranging from 10^{0} to 10^{-7}. The detection limit of the RT-PCR was 100 pg/µl (10^{-4}), as visualized by 2% agarose gel electrophoresis analysis. However, RT-RPA could detect ACLSV at 1 pg/µl (10^{-6}) (Fig. 2a). Four independent reactions were performed, and all of which yielded similar results. The results indicated that the sensitivity of the RT-RPA assay was 100-fold higher than that of RT-PCR.

To evaluate the feasibility of the developed RT-RPA assay, cross reaction assays were performed using two major pear-infecting viruses in Korea, i.e., ASGV and ASPV, and healthy pear leaves were used as a negative control. The results were further compared with those of RT-PCR (Cho *et al*., 2010). The RNA extracted from ACSLV-infected samples resulted in a positive reaction, whereas that from ASGV- and ASPV-infected samples and healthy pear leaves did not (Fig. 2b). These results indicated the high specificity of the optimized RT-RPA assay for ACLSV detection in pear leaves.

The reliability of the RT-RPA assay for detecting ACLSV in different pear samples was evaluated using 10 field pear leaf samples collected randomly from major local orchards in Naju, Namyangju, and Ulsan Provinces of South Korea, and the results were compared with those

of RT-PCR. The results showed that ACLSV was detected by both RT-RPA and RT-PCR in three of the ten field samples (lanes 2, 5, and 7) (Fig. 3), indicating that the RT-RPA assay and the designed primers could successfully detect ACLSV in field-collected samples. The faint bands in other lanes were non-specific bands.

ACLSV is an important virus infecting fruit trees and causing serious in the commercial production of apple, pear, peach, and plum (Song *et al*., 2011; Guo *et al*., 2016). More importantly, ACLSV frequently is detected in co-infection with other viruses (Yanase *et al*., 1979). In addition, ACLSV is genetically highly variable; infected pear and apple trees often yield low viral titers, making it difficult to select optimal primers and detect the virus in the field (Yoon, *et al*., 2014; Zhu *et al*., 2014). Because of its high pathogenicity and risk of transmission by grafting, ACSLV has been included in the diagnostic protocol for the national virus-free certification program for apple, pear, and peach in South Korea, which necessitates the development of a simple, sensitive, and reliable ACLSV detection method. RPA, an isothermal nucleic acid-based amplification assay supported by rapid kinetics, is an inexpensive, simple, rapid, and specific assay requiring constant low reaction temperature (37–42°C), presenting an ideal detection method that is more advantageous than other molecular diagnostic techniques. In this study, an optimal primer set was selected from conserved regions of the ACLSV-CP gene from various isolates worldwide, whose sequences are deposited in the NCBI database. Amplification could be achieved at 42°C (single temperature) and within 10 min. The RT-RPA was 10 times more sensitive than RT-PCR for ACLSV detection in pear leaves, thus presenting higher accuracy for detecting low ACLSV titers in plant materials. In addition, the RT-RPA assay did not exhibit cross-reactions with other pear viruses. The reliability of the RT-RPA assay was further validated using field samples.

In conclusion, this is the first application of RT-RPA assay for robust detection of ACLSV in pear. RT-RPA presents an inexpensive, rapid, sensitive, specific, and reliable detection method for ACLSV. This RT-RPA assay is suitable for detecting ACLSV in field-grown pear plants and can be successfully employed in sanitary certification programs for pear propagation materials.

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