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Short communication

A portable detection assay for *Apple stem pitting virus* using reverse transcription-recombinase polymerase amplification

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ABSTRACT

A molecular diagnostic assay for the rapid, sensitive and specific detection of *Apple stem pitting virus* (ASPV) in infected samples, utilizing reverse transcription-recombinase polymerase amplification (RT-RPA) at an isothermal constant temperature of 42 °C and the designed target-specific primers, was developed. The RT-RPA assay was able to be used in ASPV-infected leaves, rootstocks and fruits. Sensitivity tests, using ASPV transcripts, showed that the RT-RPA with the ASPV-specific primers was more sensitive than the conventional RT-PCR, with a detection limit of 1 fg/μL of RNA. In addition, the reaction time for the amplification of ASPV was shortened to as little as 1 min. The assay was highly specific and did not give a positive reaction to other viruses infecting pears. Moreover, the amplified genomic fragment of ASPV produced by the assay could be determined within 4 min using a portable capillary gel electrophoresis system. The entire process, excluding the extraction of total RNA, could be completed in 5 min using portable equipment in the field. This is the first report of utilizing an RT-RPA assay to detect a pear tree virus and the assay could be used both in the laboratory and in the field for ASPV detection.

Asian pear [*Pyrus pyrifolia* (N. L. Burm.) Nakai] is the main commercial fruit tree in Korea. *Apple stem pitting virus* (ASPV), a member of the genus *Foveavirus* in the family *Betaflexviridae*, commonly occur in commercially cultivated pear and apple trees worldwide. ASPV occurs frequently in single or mixed infections and cause significant yield reduction in pear cultivars (Németh, 1986). ASPV infection causes pear vein yellows and necrotic spot (Martelli and Jelkmann, 1998). Mixed infections of ASPV with other viruses, such as *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV), are common in cultivated pear and apple plants, and can cause severe disease of pear and apple trees on susceptible rootstocks, resulting in significant losses in fruit productions (Desvignes, 1999; Cembali et al., 2003). The severity of the diseases caused by ASPV depends largely on the fruit tree cultivars and virus strains (Fridlund, 1989). ASPV can be transmitted by infected propagating materials, such as buds and cuttings.

Currently, no insect vector of ASPV has been reported. Therefore, the development of fast, inexpensive, sensitive and specific detection methods for pear-infecting viruses is urgently required for the virus-free certification and quarantine programmes in the pear industry. Currently, serological assays, and RT-PCR or multiplex RT-PCR assays, are the most widely used methods for the detection and diagnosis of viruses in pear and apple (Gugerli and Ramel, 2004; Komorowska et al., 2010; Yao et al., 2014). ASPV was also detected by real-time RT-PCR in pear and apple trees (Nickel and Fajardo, 2014). However, real-time RT-PCR assays are lengthy (approximately 100 min) and require expensive and specific equipment. A rapid and specific assay for ASPV, using reverse-transcription loop-mediated isothermal amplification (RT-LAMP), was reported for pear leaves (Lu et al., 2018). LAMP and RT-LAMP assays are isothermal reactions with high sensitivity and specificity, and avoid the disadvantages of PCR/RT-PCR by having a shorter amplification time than PCR, not requiring special equipment and can be carried out in a water bath (Notomi et al., 2000). However, RT-LAMP assays require four or six primers, and the larger number of primers per target increases the primer-primer interactions, posing limitations.

Recombinase polymerase amplification (RPA) is another type of isothermal amplification method which aims to overcome the limitations of the LAMP assay (Bakheit et al., 2008). It targets and amplifies DNA or RNA (via cDNA) from virus-infected tissues. RPA utilizes DNA binding proteins (two ATP-dependent recombinases, usvX and usvY, and the gp32 single-strand DNA binding protein) which bind to the primers and then scan for the primer-specific homologous sequences on the target DNA, as well as a mesophilic polymerase (*Bacillus subtilis* DNA polymerase I) which extends the 3′ end of the hybridized primers using the complementary strand on the target as a template (Piepenburg et al., 2006). The RPA product is amplified at a constant

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low temperature (25°C to 42°C) in as little as 15 min. In the case of RNA targets, such as plant RNA viruses, a reverse transcription step is included to convert the target RNA into cDNA prior to the RPA reactions (Euler et al., 2012). Gel electrophoresis or fluorescence-based probes are the most common methods used to detect the RPA products. Although the RT-RPA technology has been used to detect other plant viruses such as *Little cherry virus 2*, *Plum pox virus*, and *Yam mosaic virus*, to date, there is no RT-RPA assay developed for ASPV detection (Mekuria et al., 2014; Zhang et al., 2014; Silva et al., 2015; Londono et al., 2016). In this study, the development of a rapid, sensitive and specific assay for the easy detection of ASPV using RT-RPA technology is described.

Field samples used for validating the ASPV RT-RPA assay comprised of 158 leaf samples (exhibiting typical symptoms, including leaf chlorosis and necrotic spots) and 20 fruit samples (exhibiting malformation and pitting) collected from pear orchards located in five major production areas of Korea in 2017 (Sangju, Namyangju, Ulsan, Chonan, and Naju), and 35 root-stock samples purchased from the commercial markets. These samples were stored at – 80 °C before being extracted for their total RNA using the IQeasy Plus plant RNA extraction mini kit (iNtRON, Korea) according to the manufacturer's instructions. ACLSV- and ASGV-infected samples used for specificity tests of the ASPV RT-RPA assay were derived from a previous study (Kim et al., 2018).

To design suitable primers for the ASPV RT-RPA assay, additional complete ASPV CP coding sequences from Korean ASPV isolates were first obtained as follows. Total RNA was extracted from symptomatic and ASPV-free pear plant samples grown trees of certified virus-free stocks and tested for the presence or absence of ASPV using a previously described one-step RT-PCR and corresponding primers that amplified the partial ASPV CP coding sequence (367 bp) (Cho et al., 2010). The RT-PCR was performed using the SuPrimeScript RT-PCR Premix (GeNet Bio, Daejeon, Korea) according to the manufacturer's protocol. The complete ASPV CP amplicons generated from the RNA of the symptomatic trees were cloned into the pCR2.1 TOPO TA vector (Invitrogen, Carlsbad, Grand Island, NY) and sequenced using the M13 F primers to obtain the ASPV coding sequences. The nucleotide sequences of four of the cloned ASPV CP amplicons from the Korean viruses were deposited into the National Center for Biotechnology Information database (NCBI) (GenBank accession No. LC367338, LC367339, LC367342, and LC367341) and compared with those in the database using Blastn. A total of 11 ASPV CP sequences from the NCBI database, including those of the Korean isolates, (GenBank accession No. LC367338, LC367339, LC367341, LC367342, KF915809, AF345893, KP994194, KP994195, KP994196, D21828, and D21829) were aligned, and the highly conserved regions were identified using BioEdit version 7.0.5.3 to design the primers of the CP RT-RPA assay (Fig. S1). Based on the highly conserved regions, two forward primers and two reverse primers were designed for the ASPV CP RT-RPA assay according to the TwistDx RPA instruction manual and the PrimedRPA program (TwistDx, Ltd., Cambridge, UK; Higgins et al., 2018). The primer sequences, primer sets and the expected RT-RPA products sizes from these primers are indicated in Table 1. The oligonucleotide RT-RPA primers were synthesized by Bionics Co., Ltd (Korea).

ASPV CP RNA (367 bp) was transcribed from the plasmid containing the partial ASPV CP amplicons using T7 RNA polymerase (Promega, Madison, WI) according to the manufacturer's instructions. After removing the template DNA by digestion with RNase-free DNase I at 37 °C for 30 min, the transcript concentration was quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE).

All the RT-RPA assays were performed according to, and using the reagents in, the TwistAmp® Basic RT kit (TwistDx Limited, Cambridge, UK) in 50 µl reaction volumes, each containing 0.5 µg of total RNA from the test sample, 29.5 µl of 1x rehydration buffer, and 0.24 µM of each primer. The reactions were then incubated at 42°C in a water bath for 10 min. The total RNA from the confirmed ASPV-infected plant (source of LC367338 and designated as SJ isolate) was used as the positive control for both the RT-RPA and RT-PCR assays. Reactions without templates and those containing total RNA from the ASPV-free samples that had tested negative for ASPV by RT-PCR described above were included in the experiments as negative controls. The RT-RPA products were analyzed in 3% agarose gels containing ethidium bromide and their nucleotide sequences were confirmed by gel-purifying the products using the Qiagen PCR purification kit (Qiagen inc., Valencia, CA), cloned into the pCR2.1-TOPO TA vector and sequenced as described above.

The RT-RPA products were detected in the field using a portable capillary gel electrophoresis (CGE)-based biomolecule detection system (Qsep1 dna-CE, BiOptic Inc., Taiwan). To analyze the products of the RT-RPA assay, 0.5 µl of the reaction mixture was electrically injected at 4 kV for 15 s at 25 °C and electrophoresed at a constant voltage of 8 kV. The resultant electrophoresis profiles of the products were analysed at the ethidium bromide emission wavelength of 590 nm to determine the RT-RPA amplicon size, using DNA alignment markers (20 bp, 1.442 ng/µl and 1000 bp, 1.852 ng/µl) obtained from BiOptic Inc. The DNA peaks were visualized using the Q-Analyzer software (BiOptic Inc.; Jian et al., 2019). The analysis time for the detection of the ASPV amplicons by the CGE detection system was 1.5 min for each sample.

The conventional RT-PCR assays for ACLSV, ASPV, and ASGV were carried out using the respective virus-specific primers presented in Table S1, using the amplification products of the nad 5 gene as an internal control. The respective amplicon sizes of these templates are also shown in the Table. The reactions were conducted as follows: stage 1, 50 °C for 30 min; stage 2, 95 °C for 5 min; stage 3, 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 40 s; and stage 4, 72 °C for 5 min. The RT-PCR products were analyzed in 3% agarose gels (Cho et al., 2010).

The ASPV CP transcripts were diluted with RNase-free water in a 10-fold series down to 10^{-4} (equivalent to 1 fg/µL) for use in sensitivity tests of the RT-RPA and RT-PCR assays. Each sensitivity experiment was carried out using five replicated dilution series. Preliminary tests of the two ASPV CP primer sets in ASPV RT-RPA assays using the confirmed ASPV-positive and ASPV-negative controls showed that only the primer set ASPV1F/ASPV1R produced an ASPV CP-specific product of the expected size of ~146 bp (Table 1) only from the ASPV-infected samples while the other primer set (ASPV2F/ASPV2R) did not (data not shown). RT-RPA assays of pear leaves, rootstocks and fruits using the ASPV1F/ASPV1R primers also gave the expected amplification bands but not from the corresponding uninfected control samples (Fig. 1A). Experiments optimizing the reaction time of the RT-RPA assay by testing reaction times of 1, 3, 5, 10, 15, 20 and 30 min showed that the total RNA

Table 1
RT-RPA Primers used in these studies.

Target region	Primer pair	Sequence 5'-3'	bp	Tm (°C)	GC%	Target position[b]	Amplicon size (bp)
CP[a]	ASPV RPA 1F	TCAATGGAGGGTACCCAGGCTGTTAATTTT	30	71	43	9114-9143	146
	ASPV RPA 1R	TCAACTTTACTAAAAAGCATAAGTACTGAA	30	62	27	9229-9258	
CP[a]	ASPV RPA 2F	CAAAGAGTTTAAGTTTGAAACAAGGTATGC	30	64	33	8951-8980	258
	ASPV RPA 2R	TTAATCAATTATTTCCTAATGGATAGAACA	30	60	23	9179-9208	

[a] CP = Coat protein.
[b] Relative to the reference sequence (GenBank Accession No. D21829.2).

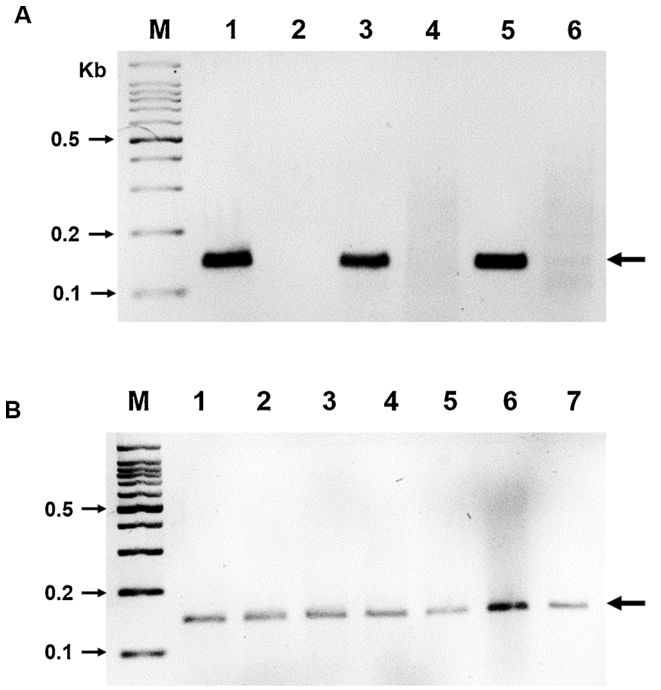

Fig. 1. Characterization of the CP RT-RPA assay for ASPV detection in pear trees. The ASPV-specific RT-RPA product of the expected size (~146 bp) were detected by agarose gel electrophoresis. (A) RT-RPA detection of ASPV in total RNA samples from leaves, rootstock, and fruits. M, DNA markers; lane 1, ASPV-infected leaves; lane 2, healthy leaves; lane 3, ASPV-infected rootstock; lane 4, healthy rootstock; lane 5, ASPV-infected pear fruits; lane 6, healthy fruits. (B) Optimization of the reaction time of the ASPV RT-RPA assay using total RNA from tissues infected with the ASPV SJ isolate. M, DNA markers; lanes 1–7, DNA products from reactions ran for 1, 3, 5, 10, 15, 20, and 30 min, respectively.

from the ASPV-infected control produced a clear RT-RPA product when the reaction time was only 1 min and there were no significant differences in the product yields when the reaction times were between the 1 min and 30 min (Fig. 1B). Five independent reaction time optimisation experiments using different RNA samples were performed and similar results were obtained.

Testing of the field samples using a reaction time of 10 min showed that the ASPV CP RT-RPA assay produced an ASPV positive reaction in 148 leaf samples, 25 rootstock samples and 10 fruit samples, and gave negative results for 10 leaf, 10 rootstock and 10 fruit samples. RT-PCR assays of these samples produced the same results (data not shown). The overall diagnostic agreement between the RT-RPA and RT-PCR assays for these samples was 100%. Sequence comparison of the RT-RPA CP amplicons derived from the Korean samples with the CP sequences of the ASPV isolates from other countries found in the NCBI database indicated nucleotide sequence identities of over 90% between them (Results not shown).

Comparison of the analytical sensitivities of the RT-RPA and RT-PCR assays using 10-fold serial dilutions of the CP RNA transcript preparation showed that the RT-RPA assay consistently detected the ASPV CP transcripts down to 1 fg/μL of RNA (10^{-4} dilution) (Fig. 2A). However, the RT-PCR assay detected the ASPV transcripts only down to 1 pg/μL of RNA (10^{-1} dilution) (Fig. 2A). These results indicated that the level of sensitivity of the RT-RPA method for ASPV CP transcript detection was 1000-fold higher than that of the RT-PCR assay. The same results were obtained when the sensitivity experiment was repeated using four independent dilution series of the same transcript preparation. Testing the specificity of the ASPV CP RT-RPA assay showed that the RT-RPA assay only amplified the RNA of ASPV and not those of ACLSV and ASGV from infected pear tissues, while the ASPV, ACLSV and ASGV RT-PCR assays each amplified its respective virus RNA. This indicated a high level of specificity for the ASPV CP RT-RPA assay (Fig. 2B).

Testing of the ASPV RT-RPA assay for field application using the portable CGE-based biomolecule detection system showed that the ASPV amplicons amplified from the total RNA of ASPV-infected leaf samples by the RT-RPA (Fig. 3A) and the RT-PCR (Fig. 3B) assays were both detected by the CGE system. In Fig. 3A, the blue peak in the chromatograph indicated the RT-RPA amplified ASPV-specific product (146 bp) while in Fig. 3B, the blue peak indicated the RT-PCR-amplified ASPV-specific product (378 bp). The ASPV amplicons were detected in 1.5 min using CGE detection system. The results also showed that the CGE system gave the correct ASPV amplicon sizes of the RT-RPA and

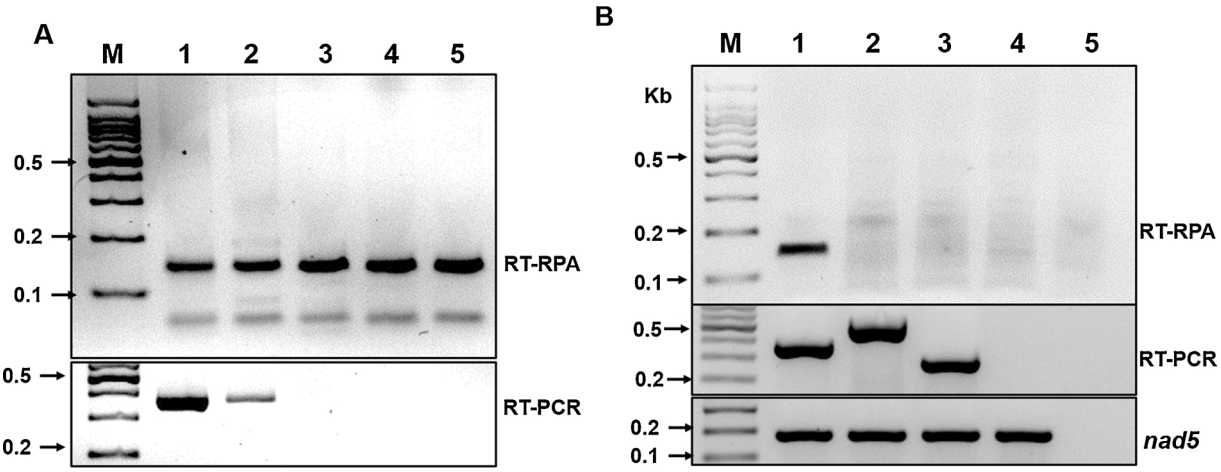

Fig. 2. Assessments of the analytical sensitivity and specificity of the ASPV CP RT-RPA assay. (A) The detection limits of the ASPV RT-RPA and ASPV RT-PCR assays were determined using ASPV RNA transcripts as the template. The reaction products (146 bp for the RT-RPA assay and 367 bp for the RT-PCR assay) were analysed in agarose gels. M, DNA markers; lanes 1–5, RT-RPA (top) and RT-PCR (bottom) products from reactions containing 10 pg/μL, 100 fg/μL, 10 fg/μL and 1 fg/μL of the transcript, respectively. (B) Testing the specificity of the RT-RPA assay using total RNAs from ASPV-, ACLSV-, and ASGV-infected tissues and analysis of the products were in agarose gels. M, DNA markers; lane 1, ASPV RT-RPA (top panel) and ASPV RT-PCR (middle panel) assays of ASPV-infected tissues (isolate SJ) showing amplification of the corresponding ASPV-specific products; lane 2, ASPV RT-RPA (top panel) and ACLSV RT-PCR (middle panel) assays of ACLSV-infected tissues; lane 3, ASPV RT-RPA (top panel) and ASGV RT-PCR (middle panel) assays of ASGV-infected tissues; lane 4, corresponding control assays of healthy tissues; lane 5, corresponding control assays with no template. Lanes 1–4, bottom panel, amplification products of the nad 5 gene used as an internal control. All the positive assays produced the correct-sized amplicon for each assay.

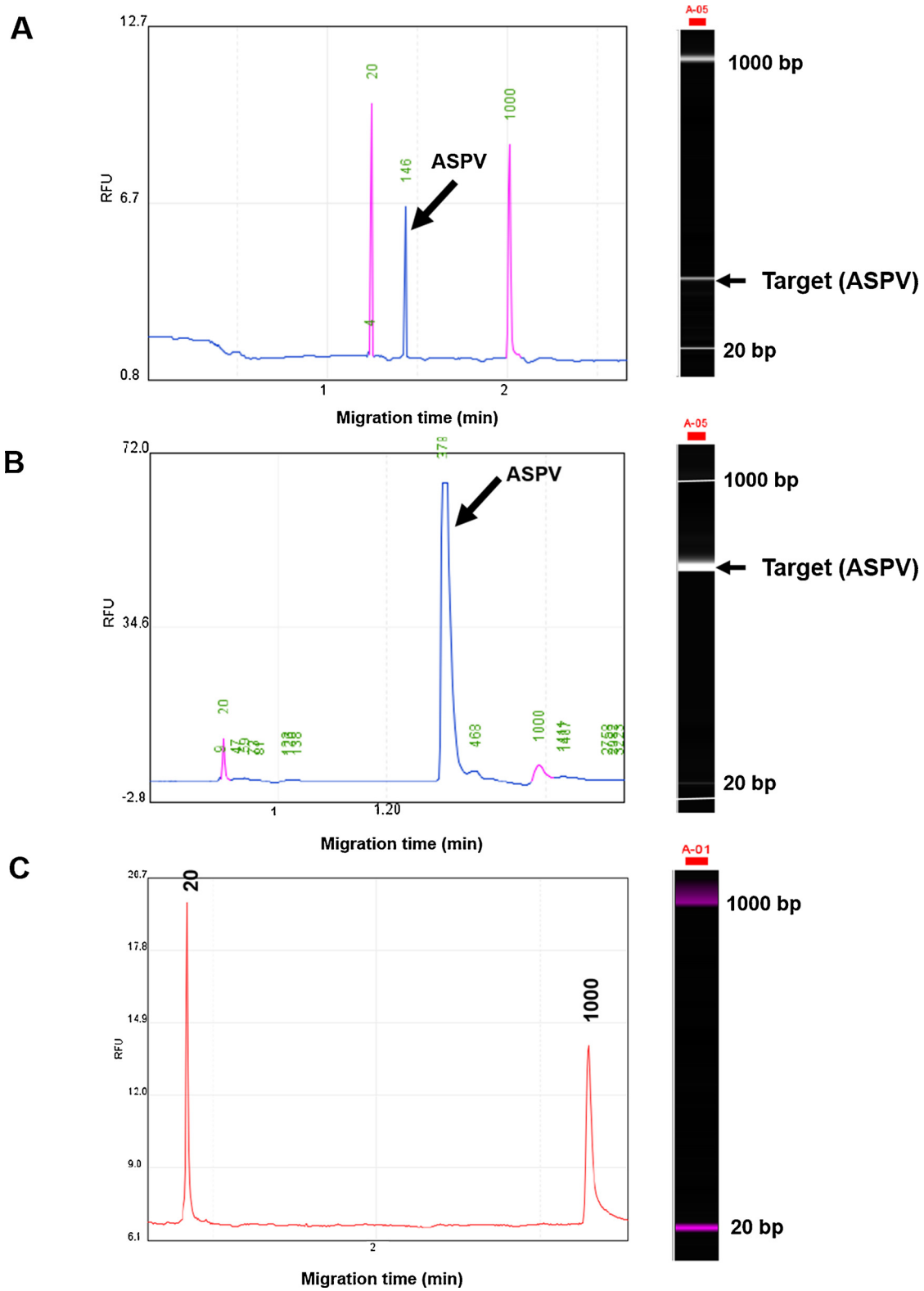

Fig. 3. Detection and visualization of the ASPV amplicons produced by the ASPV RT-RPA and RT-PCR assays of total RNA from ASPV-infected leaves using the CGE-based biomolecule detection system. (A) Detection of the ASPV amplicons produced by RT-RPA. The pink peaks in the chromatogram indicate the DNA markers (20 bp and 1000 bp) and the blue peak (146 bp) indicates the RT-RPA amplified ASPV-specific product (146 bp). The black bar at the right-hand side of the chromatogram indicates the gel profile of the RT-RPA reaction mixture produced by the detection system after capillary gel electrophoresis. (B) Detection of the ASPV amplicons produced by RT-PCR. The blue peak in the chromatogram indicates the RT-PCR amplified ASPV-specific product (378 bp) while the corresponding gel profile is shown on the right. (C) A chromatograph and gel profile of the ASPV-free negative control RT-RPA reaction mixture (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

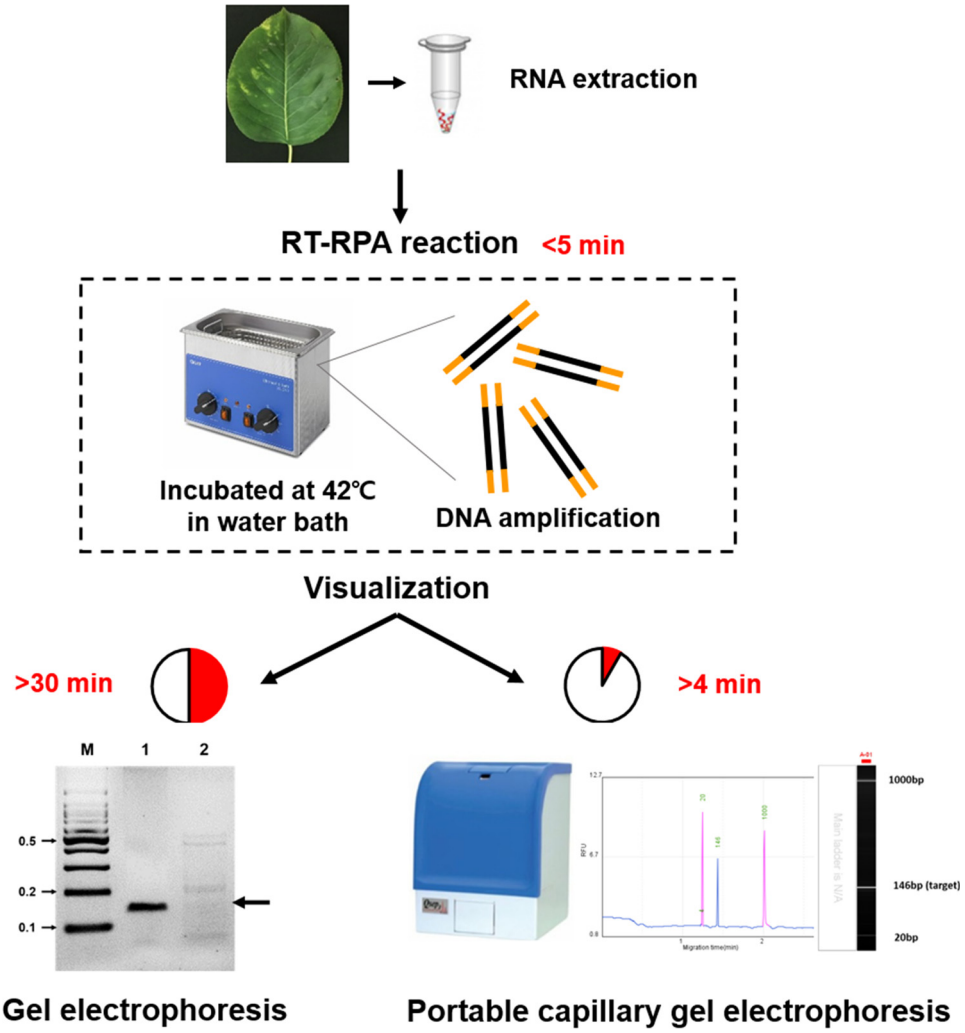

Fig. 4. Schematic representation of a portable system using the RT-RPA assay for the detection of ASPV in the field. The figure shows the steps, all of which can be performed in the field or in a low-resourced field laboratory as follows: RNA extraction (30 min); RT-RPA reaction (less than 5 min); visualization of the ASPV amplicon using a portable capillary gel electrophoresis system (within 4 min), compared to agarose gel electrophoresis (30 min).

RT-PCR products.

To our knowledge, this is the first report of using a RT-RPA assay for the detection of a pear virus and, specifically, for the detection of ASPV in infected pear leaf, fruits and rootstock tissues. The ASPV RT-RPA assay developed targeted the CP region of the ASPV genome which is the region of choice for detecting pear viruses (Ma et al., 2016). The primers did not produce any non-specific cross reactivity (false positive) when tested against two other pear-infecting viruses or RNA from healthy control pear plants, indicating the high specificity of the designed primer and the assay.

In general, a RPA or RT-RPA reaction takes less than 20 min to amplify an amplicon from a DNA or RNA template, respectively (Lobato and O'Sullivan, 2018). The reaction time required to perform the ASPV RT-RPA assay was considerably reduced compared to the ASPV RT-PCR assay. In fact, in this study the reaction time of the RT-RPA was less than 1 min compared to > 150 min required for the RT-PCR assay. The short reaction time, combined with the requirement for a single reaction temperature in the region of 42 °C, meant that the RT-RPA assay developed in this study is more efficient than the RT-PCR and ELISA currently used for ASPV diagnosis. A single, low heating temperature for the RPA assay is a major advantage because a PCR instrument is not required. In this assay, there was no significant difference in the product yields of reaction times between the 1 min to 30 min, suggesting that a 1 min reaction time could be sufficient for the routine ASPV RT-RPA assay. In addition, the amplicons generated by the RT-RPA assay were easily cloned and sequenced directly, as with the amplicons generated by PCR, while the products of some other isothermal reactions, such as LAMP, could not.

The ASPV RT-RPA assay was highly sensitive and the level of sensitivity for detecting the ASPV CP transcript was 1000-fold higher than that of the corresponding RT-PCR assay (Fig. 2A). Previous studies concerning the development of RPA assays for the detection of a variety of pathogens, such as the virus species *Yam mosaic virus* (Silva et al., 2015), the bacteria species *Francisella tularensis* (Euler et al., 2012), the virus species *Rose rosette virus* (Babu et al., 2017) and the virus species *Dengue virus* (Teoh et al., 2015), also reported comparable differences in sensitivities between their respective RPA- and PCR-based assays. The RPA assay could be combined with other amplicon detection methods for product visualization, for example, real-time fluorescence and lateral flow strips detection methods (Hou et al., 2017; Zhang et al., 2014). However, the former method requires a specific primer containing a fluorescent probe while the latter requires product-specific detection strips which are expensive and cannot be reused. In this study, a portable capillary gel electrophoresis (CGE)-based biomolecule detection system was successfully used to overcome these disadvantages.

In conclusion, the ASPV RT-RPA method developed in this study was specific for the detection of ASPV in pear leaves. The sensitivity of

the assay was 1000x higher than that of the corresponding RT-PCR-based assay for ASPV detection. The assay detected the virus in a one-minute reaction and the amplicon amplified could be visualized within 2 min when using a portable CGE-based biomolecule detection system in the field (Fig. 4). The developed ASPV RT-RPA assay should be useful in commercial nurseries and diagnostic laboratories for the rapid detection of ASPV, thereby preventing the risk of the spread of the pear virus.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113747.

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