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Multiplex RT-PCR Assay for the Detection of *Apple stem grooving virus* and *Apple chlorotic leaf spot virus* in Infected Korean Apple Cultivars

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To develop the diagnostic method for the viral infection in apple, the partial genes corresponding to the N-terminal region of RNA polymerase of *Apple stem grooving virus* (ASGV) and coat protein of *Apple chlorotic leaf spot virus* (ACLSV) were characterized from the infected apple cultivars in Korea. Based on the nucleotide sequences of the characterized partial genes, the virus gene-specific primers were designed for the detection of ASGV and ACLSV infected in species of *Malus*. The RT-PCR using the primers for the genes of ASGV and ACLSV successfully gave rise to 404 and 566 bp DNA fragments, respectively. Using those viral gene-specific primers, the multiplex RT-PCR assays were also established to diagnose the mixed infection by ASGV and ACLSV simultaneously. Furthermore, the control primers, which have to be included for the RT-PCR as an internal control, were designed using the nucleotide sequence of the gene encoding elongation factor 1α (EF1 α). This multiplex RT-PCR including the control primers provides more reliable, rapid and sensitive assay for the detection of ASGV and ACLSV infected in Korean apple cultivars.

Keywords : ACLSV, Apple virus, ASGV, Multiplex RT-PCR

Apple is widely infected by various viruses and its yields are significantly reduced by the infections. The major viruses infecting apple in Korea are *Apple stem grooving virus* (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV). ASGV is a member of the genus *Capillovirus* and is widespread in rosaceous fruit trees, particularly species of *Malus* and *Pyrus* (Nemeth, 1986). ASGV has a single stranded RNA genome, whose size is about 6.5 kb (Ohira et al., 1995; Yoshikawa and Takahashi, 1992; Yoshikawa et al., 1993). ACLSV is a member of the genus *Trichovirus* from the *Closterovirus* group (Martelli et al., 1994) and is known to infect a wide range of fruit tree species such as apple, pear, cherry and many ornamental rosaceous species. ACLSV has a single stranded RNA genome, whose size is about 7.56 kb (German et al., 1992; Sato et al., 1993).

The diagnosis of the viral infection in the early stage is economically important for crop production. The most common techniques for plant virus diagnosis are based on serological method using the specific antibodies. However, these methods are not sensitive enough for detection of viral RNA targets in some woody plant tissue due to the low viral titers (Kinard et al., 1996). In addition, ELISA tests often fail due to the inhibitory effects of compounds in the sap of plants. Therefore, the diagnosis of the viral infection by ELISA test is very difficult in the early stage of infection. Furthermore, the use of viral antibodies is expensive. Recently, nucleic acid-based diagnostic techniques such as RT-PCR are being used for the detection of virus (Kinard et al., 1996). RT-PCR assay is known to be more sensitive than ELISA, but RT-PCR assay can have false negative results due to RNA degradation or the presence of inhibitors of the reverse transcriptase or polymerase.

In this study, we characterized the partial genes corresponding to the N-terminal region of RNA polymerase of ASGV and coat protein of ACLSV and developed a multiplex RT-PCR method for the detection of ASGV and ACLSV infection in *Malus domestica* in order to provide a quick, reliable and cost effective method for routine diagnosis of the mixed viral infection.

To develop the diagnostic method for the viral infection, ASGV and ACLSV were isolated from the infected hosts of *Malus* and *Pyrus* grown in Korea, after confirming the presence of ASGV and ACLSV by the ELISA tests using the viral specific antibodies. Each virus was extracted from the infected leaves and transmitted to *Chenopodium quinoa* by mechanical inoculation using inoculation buffer (0.01 M phosphate buffer, pH 7.2). *C. quinoa* plants inoculated with ASGV showed chlorotic and mottle leaves, which gradually developed into wider necrotic areas (Fig. 1). *C. quinoa* plants inoculated with ACLSV also showed irregular chlorotic line, mosaic and mottle patterns and prematurely small leaves shed (Fig. 2). Each virus was further propa-

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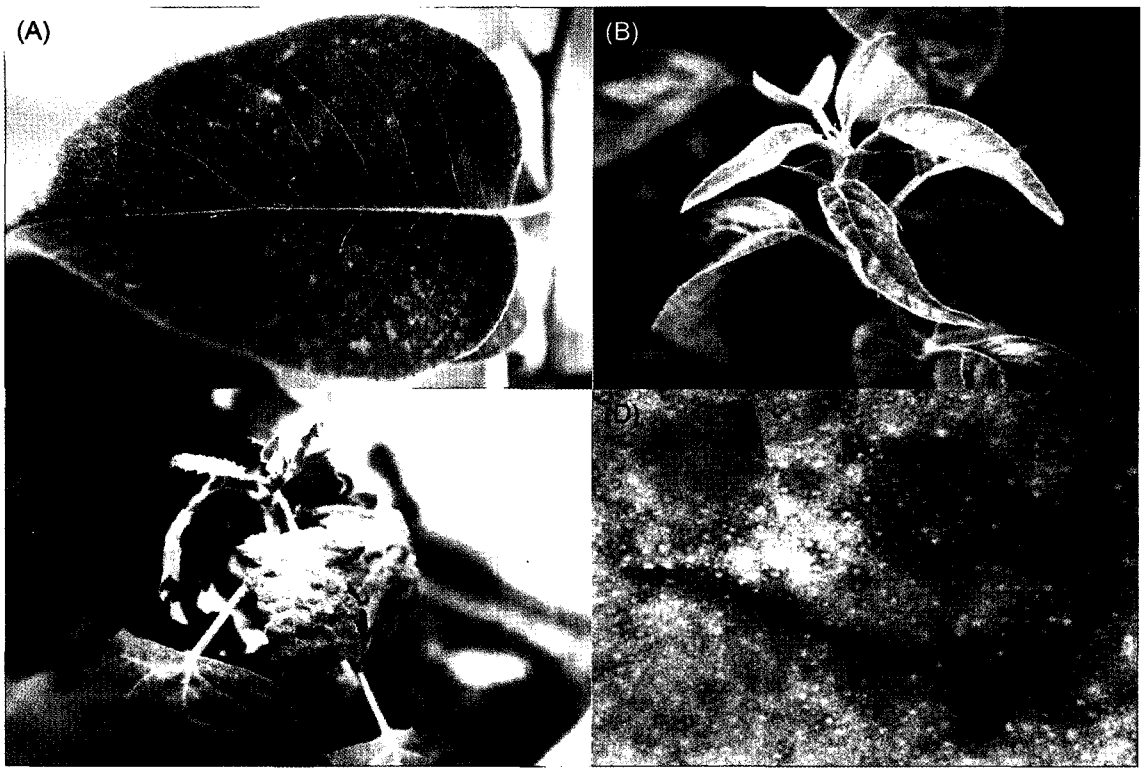

Fig. 1. Typical symptoms caused by *Apple stem grooving virus* (ASGV). (A) Symptoms showed black spots of infected *Pyrus pyfolia Nakai* leaf. (B) Spy227 leaf infected with ASGV. (C) *Chenopodium quinoa* plant infected with ASGV. (D) Preliminary electron microscopy observations of dips from symptomatic leaves.

Fig. 2. Typical symptoms caused by *Apple chlorotic leaf spot virus* (ACLSV). (A) Comparison of the symptoms of normal fruit (left) and viral infected small fruits of *Malus domestica Borkh.* (B) *Chenopodium quinoa* plant infected with ACLSV. (C) Preliminary electron microscopy observations of dips from symptomatic leaves.

gated using *C. quinoa* as host for the isolation of viral particles. The viral particles were purified using the PEG-bentonite method (Dunn and Hitchborn, 1965). The typical shape of ASGV and ACLSV particles were observed by electron microscopy as shown in Fig. 1D and 2C.

To design the primers for RT-PCR diagnosis of viral infection, we selected the N-terminal regions of the coat proteins of ASGV and coat protein of ACLSV, which were known to be highly conserved and thus suitable for primer design. To determine the nucleotide sequences of the N-terminal region of RNA polymerase of ASGV and coat protein of ACLSV found in Korean apple cultivars, theses regions were amplified from the purified virus particles by RT-PCR using the virus specific primers (ASGV primer set: 5'-ACAGGCTTAATTTCCGCGC-3', 5'-TCAAAAG-GGCTAAGAAAGTG-3', ACLSV primer set: 5'-GAGAG-TTTCAGTTTGCTAGACA-3', 5'-GCAAATTCAGTCTG-TAAAAG-3'). The amplified DNAs were cloned into the pGEM-T Easy vector systems (Promega Corp, USA), and their nucleotide sequences were determined and compared with those of ASGV and ACLSV previously reported as shown in Fig. 3. and Fig. 4, respectively. The comparison of the nucleotide sequences reveals that the amplified 573 bp and 566 bp cDNA from ASGV and ACLSV isolated in

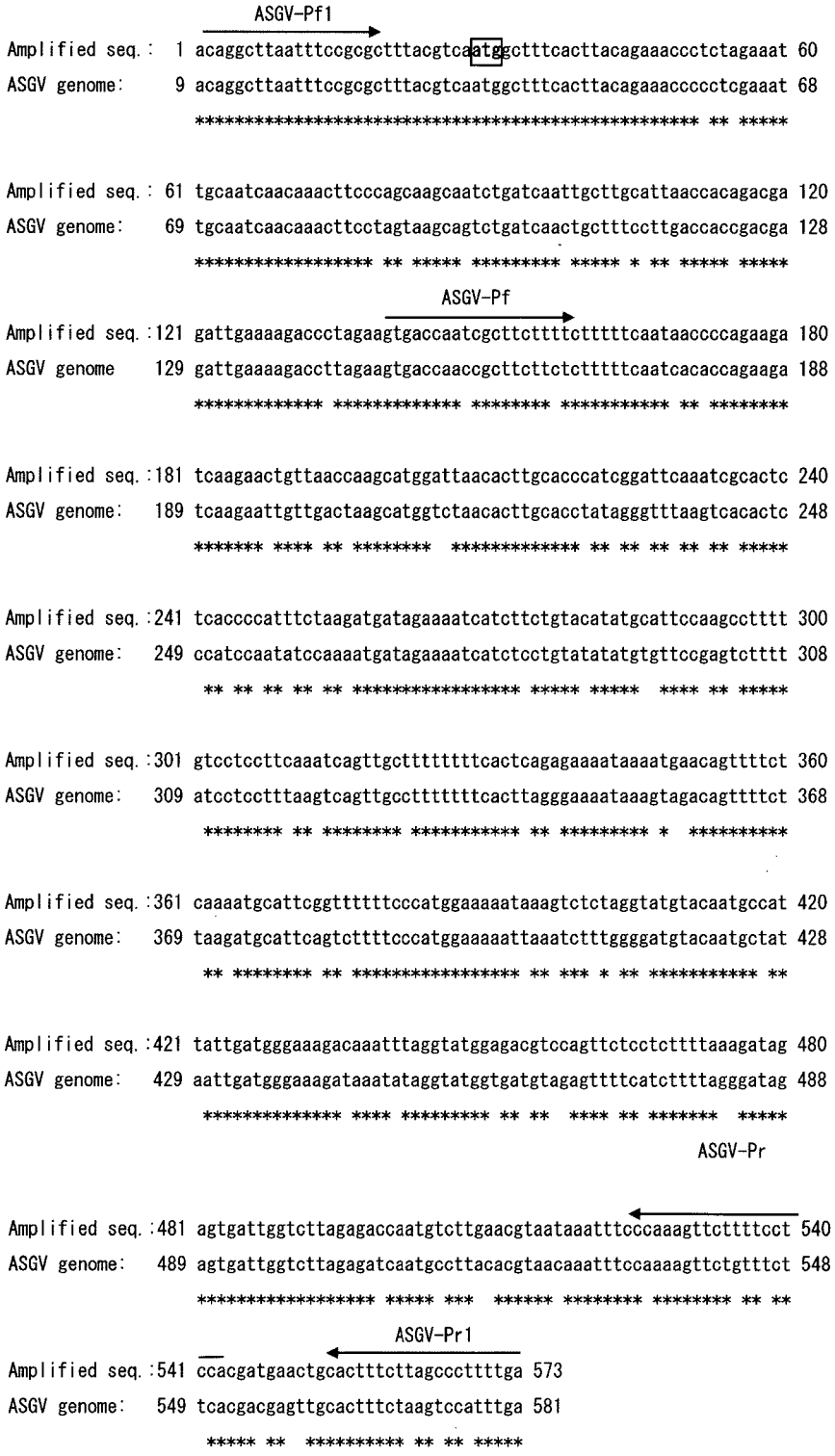

Fig. 3. Nucleotide sequence comparison of the N-terminal region of RNA polymerase from ASGV. Amplified sequence represents the sequence of the N-terminal region of RNA polymerase obtained from ASGV infected in Korean apple cultivar and the sequences of the ASGV genome are from the GeneBank (Accession number D14995). ASGV-Pf1 and ASGV-Pr1 are primers used in the amplification of the region and ASGV-Pf and ASGV-Pr are primers used in the detection of virus. The conserved nucleotides are represented by asterisk (*). The start codon for the RNA polymerase gene is designated in box.

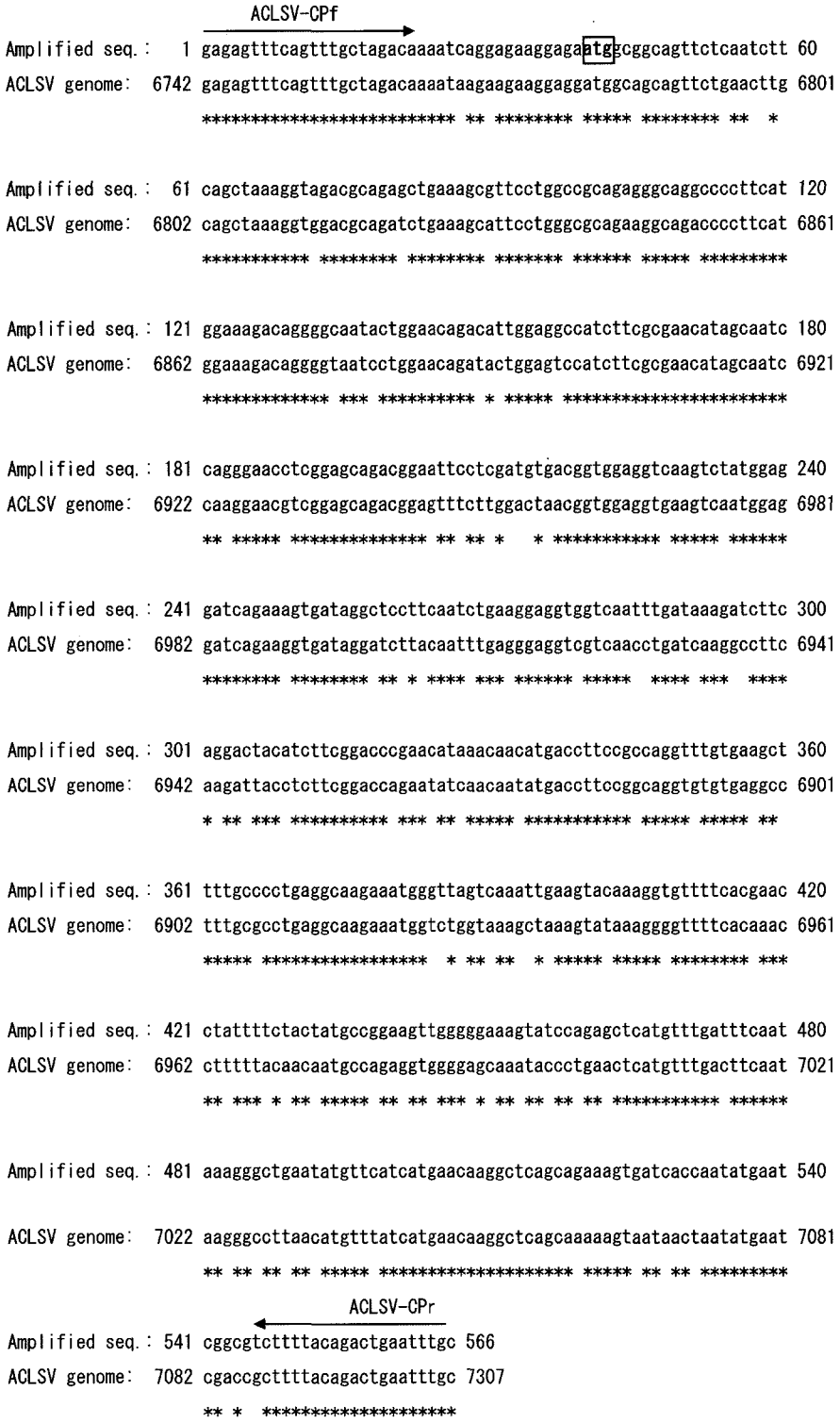

Fig. 4. Nucleotide sequence comparison of the N-terminal region of coat protein from ACLSV. Amplified sequence represents the the sequence of the C-terminal region of coat protein obtained from ACLSV infected in Korean apple cultivar and the sequences of the ACLSV genome are from the GeneBank (Accession number AJ243438). ACLSV-Pf1 and ACLSV-Pr1 are primers used in the amplification of the region and also in the detection of virus. The conserved nucleotides are represented by asterisk (*). The start codon for the coat protein gene is designated in box.

Table 1. Primer sequences and expected size of RT-PCR products for each specific primer pair

Primer	Primer sequence in 5'-3' orientation	Primer position[a]	Product size
Apple stem grooving virus			
Sense	GTGACCAATCGCTTCTTTTCT	148-168	404bp
Antisense	TGGAGGAAAAGAACTTTGGG	531-551	
Apple chlorotic leaf spot virus			
Sense	GAGAGTTTCAGTTTGCTAGACA	6742-6763	566bp
Antisense	GCAAATTCAGTCTGTAAAAG	7288-7307	
EF1-a			
Sense	ACCAACCTTGACTGGTACAAGG	710-731	236bp
Antisense	TGGTGCATCTCAACAGACTT	926- 945	

[a]The reference GenBank accession numbers for the determination of the primer positions are D14995 for *Apple stem grooving virus*, AJ243438 for *Apple chlorotic leaf spot virus* and AJ223969 for the *EF1-α* gene of *Malus*.

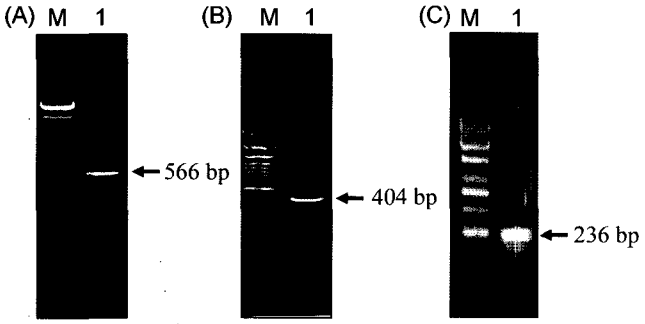

Fig. 5. Agarose gel electrophoresis of RT-PCR products. (A) The RT-PCR product amplified from total RNA preparation of *Chenopodium quinoa* leaves infected in ASGV (Lane 1). Lane M, *Hind* III digested-λ DNA. (B) The RT-PCR product amplified from total RNA preparation of *C. quinoa* leaves infected in ACLSV (Lane 1). Lane M, 100bp DNA ladder (MBI Fermentas, Burlington, Ontario, Canada). (C) The RT-PCR product amplified from total RNA preparation of and *Malus domestica* leaves using the internal control primers (Lane 1). Lane M, 1 kb DNA ladder (MBI Fermentas, Canada).

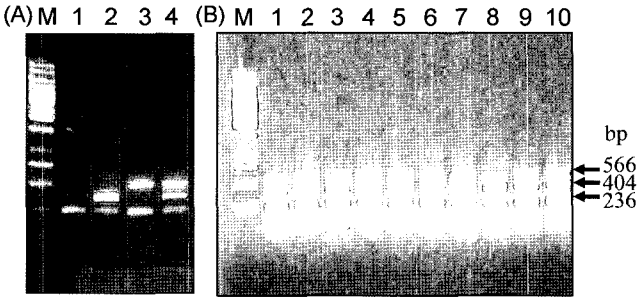

Fig. 6. Detection of ASGV and ACLSV by multiplex RT-PCR. (A) Agarose gel electrophoresis analysis of Multiplex RT-PCR amplification products, obtained with specific primer sets including internal control from total RNA preparation of *Malus domestica* leaves containing no viral RNA (Lane 1), ASGV RNA (Lane 2), ACLSV RNA (Lane 3), and ASGV and ACLSV RNA (Lane 4). Lane M, 1 kb ladder marker. (B) Agarose gel electrophoresis analysis of Multiplex RT-PCR amplification products (Lane 1-10), obtained from total RNA preparation of collected apple leaves showing the symptoms of viral infection. Lane M, 1 kb ladder marker (MBI Fermentas, Canada).

Korea have 86% and 85% homology with the sequences of ASGV and ACLSV previously reported, respectively. From the sequence alignments, the highly conserved regions were selected and used for the design of the virus specific primers (Table 1), which will be used for the detection of viruses by RT-PCR. As shown in Fig. 5, the RT-PCR, using the designed primers, amplified the 404 bp and 566 bp DNA fragments from ASGV and ACLSV genomic RNA, respectively, as expected. Furthermore, to ensure the reliability and minimize the risk of obtaining false negative result, the primer pairs for positive internal control were also designed from the highly conserved plant gene encoding elongation factor 1α (EF1 α), which is absolutely required for translation in all the plant. The RT-PCR, using the internal control primers, successfully amplified the 236 bp DNA fragments, irrespective of viral infection (Fig. 5).

Since the primer sets for the viruses and internal control were designed to amplify different sizes of DNA fragments (236 bp, 404 bp, and 566 bp), multiplex RT-PCR assay would be possible. To test the possibility of multiplex RT-PCR assay, total RNA extracted from healthy apple leaves was mixed with ASGV and ACLSV RNA and tested for multiplex RT-PCR. The multiplex RT-PCR assays were optimized according to Henegariu et al. (1977) and carried out in thermal cycler (Bio-Rad, USA). The reaction mixture contained 1 μl of total nucleic acid extract, 10 μl of 5 X RT-PCR buffer (Titan™ one tube RT-PCR system, Roche Applied Science, Germany), 4 μl of 2 mM dNTP, 2.5 μl of 100 mM DTT solution, 1 μl of each ACLSV primer (10 mmol/l), 1 μl of each ASGV primer (10 mmol/l), 1 μl of each control primer (10 mmol/l), and 1 μl of Enzyme mix (5 U/μl). Sterile deionized water was added to a final volume of 50 μl. The cycling parameters were: reverse transcription at 50°C for 30 min, activation of the *Taq* polymerase at 94°C for 2 min followed by 10 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1

Table 2. Comparison of virus detection in apple leaves by ELISA and multiplex RT-PCR

Collected Areas of Apple Leaves	Number of infected plants/tested plants					
	ELISA			Multiplex RT-PCR		
	ASGV positive	ACLSV positive	Mixed Infection of ASGV and ACLSV	ASGV positive	ACLSV positive	Mixed Infection of ASGV and ACLSV
Bonghwa	3/6	3/6	3/6	6/6	4/6	4/6
Yeongju	2/6	4/6	1/6	2/6	4/6	1/6
Andong	0/6	0/6	0/6	1/6	1/6	0/6
Yesan	0/3	0/3	0/3	1/3	0/3	0/3
Okcheon	1/3	1/3	1/3	2/3	1/3	1/3
Chungju	0/3	0/3	0/3	2/3	0/3	0/3
Suwon	1/24	10/24	1/24	4/24	16/24	4/24
Total	7/51	18/51	6/51	18/51	26/51	10/51

min, elongation at 68°C for 1 min, 94°C for 2 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 68°C for 1 min (+5 s/ cycle). The final extension step was 68°C for 7 min. As shown in Fig. 6A, the multiplex RT-PCR produces all the expected DNA fragments, which can be clearly identified, suggesting that the multiplex RT-PCR assay can be employed to diagnose the mixed infections by ASGV and ACLSV. To test whether the multiplex RT-PCR assay is really suitable and reliable for diagnosis of ASGV and ACLSV infection in apple tree, various apple trees showing the symptoms of viral infection were tested (Fig. 6B). All tested samples produced one or two bands of 404 bp and 566 bp DNA for the indication of viral infection as well as the band of 236 bp DNA for internal control. In addition, 51 apple trees were randomly selected from the orchards in various areas of Korea and examined for viral infection by the multiplex RT-PCR. As showed in Table 2, the result from the multiplex RT-PCR shows that 18 and 26 apple trees out of 51 are positive for ASGV and ACLSV infection, respectively. Among those infected apple trees, 10 are positive for mixed infection of the two viruses. Thus, 34 apple trees are at least infected by one of the two viruses and only 17 apple trees are free of those viruses. However, when these same apple trees were analyzed by ELISA using virus specific antibodies (Bioreba, Switzerland), only 18 and 7 apple trees are positive for ASGV and ACLSV infection, respectively, and 6 are positive for mixed infection, which means that 32 apple trees are free of those viruses. These results suggest that 15 apple trees, diagnosed to be positive for viral infection by the multiplex RT-PCR, are negative by ELISA test. Thus, the multiplex RT-PCR assay proved to be more sensitive and efficient than ELISA test. This multiplex RT-PCR assay using the virus specific primers and internal control primers can provide more sensitive, reliable, and rapid method for the parallel detection of two economically important viruses in apple. These advantages can be regarded as a valuable alternative for large-scale testing for apple trees.

Acknowledgement

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