Molecular Diagnostic Assay for the Simultaneous Detection of Pome Fruit Viruses

In-Sook Cho
Gug-Seoun Choi
Sun-Jung Kwon

Horticultural & Herbal Crop Environment Division
National Institute of Horticultural & Herbal Science, RDA
100, Nongsaengmyeong-ro, Iseo-myeon, Wanju-gun, Jeollabuk-do, Korea

Abstract

Multiplex RT-PCR assay was developed for the simultaneous detection of internal controls and viruses infecting apple and pear trees. These viruses are the most commonly occurring and economically important pathogens in apple and pear commercial orchards in Korea: Apple stems grooving virus (ASGV), Apple stem pitting virus (APSV), Apple chlorotic leaf spot virus (ACLSV) and/or Apple mosaic virus (ApMV). Primer concentrations and PCR conditions were optimized for the effective application of the assay. The sensitivity of detection by multiplex RT-PCR assay was up to 10^0 or 10^{-1} dilution depending on the virus. The multiplex RT-PCR assay was able to successfully detect target viruses infecting apple and pear. In addition, positive control plasmid was developed as controls for multiplex RT-PCR assay. Positive control plasmid was constructed by cloning sequences of target viruses infecting apple tree into a single vector. The multiplex RT-PCR assay developed is rapid and cost-effective molecular diagnosis tools for simultaneous detection of pome fruit viruses. The positive control plasmid is a new approach to apply as alternatives to classically used control infected plants for the detection of pome fruit viruses.

Keywords: Multiplex RT-PCR, Positive control plasmid, Pome fruit viruses

1. Introduction

In fruit trees, the management of virus diseases is very important because they cause economic losses both in terms of quantity and quality of products. Unlike other plant pathogens, there are no direct methods available yet to control viruses and, consequently, the current measures rely on indirect tactics to manage the virus diseases (Naidu et al., 2003). The most effective management strategy for virus diseases is to use virus-free plant materials with good genetic background, which can help minimize disease impact on fruit trees (Thresh, 2006). Therefore sensitive and accurate virus diagnostic methods are critical in selection or production of virus-free plant materials for propagation. The diagnostic methods also help in prevention of spread of virus diseases and in routine screening to detect viruses at an early stage before they become major problem (Sastry, 2013).

The diagnostic methods for virus disease are being continuously improved. Commonly used assays for the detection of fruit tree viruses are bioassays such as graft inoculation of susceptible indicator plants, enzyme-linked immunosorbent assay (ELISA) and more recently, reverse transcription-polymerase chain reaction (RT-PCR). However, the bioassays are time consuming, usually spanning over a period of a minimum several months up to 1-2 years for symptom expression. The results are also not specific and expressed symptoms are often difficult to interpret (Akbas et al., 2010). ELISA is very appropriate assay for screening large numbers of samples but it has certain limitations of the sensitivity due to low virus titer and/or inhibitory effects of polysaccharides or phenolic compounds in extracts of fruit trees (Kinard et al., 1996; Menzel et al., 2002). The sensitivity of detection assay was greatly improved following the development of PCR based on nucleic acid analysis (Mullis et al., 1986). Although RT-PCR is used most widely for the detection of fruit tree viruses due to its high sensitivity, the assay is time consuming, labour intensive and costly especially when large numbers of samples with potential multiple infections need to be tested, as the simplex RT-PCR assay detects one gene at a time (Wei et al., 2008; Noorani et al., 2013).

Multiplex RT-PCR has been developed for simultaneous detection of several fruit tree viruses in a single reaction, thus providing rapid, sensitive and more cost-effective routine diagnosis (Menzel et al., 2002; Sanchez-Navarro et al., 2005; Hassan et al., 2006; Gambino et al., 2006; Youssef and Shalaby, 2009; Jarosova et al., 2010). In previous studies for pome fruit viruses, Menzel et al. (2002) developed multiplex RT-PCRs for the detection of virus combinations of *Apple stem grooving virus* (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV) or *Apple stem pitting virus* (APSV) and *Apple mosaic virus* (ApMV). Hassan et al. (2006) has set up a one tube pentaplex RT-PCR for the simultaneous detection of four pome fruit viruses. In addition, an internal control was incorporated into the multiplex RT-PCR to prevent false negative results that might be caused by RNA degradation or PCR inhibitors. In Korea, bioassays, ELISA and RT-PCR assays used for detection of pome fruit viruses and Park et al. (2006) developed a multiplex RT-PCR for detection of ACLSV and ASGV in infected apple trees. So, multiplex RT-PCR is highly effective diagnosis assay to control pome fruit viruses for the production of virus-free propagating materials and eradication of infected trees. In this work, multiplex RT-PCR assay with internal control was developed for the simultaneous detection of viruses infecting apple and pear trees. The viruses are the most commonly occurring and economically important pathogens in apple and pear commercial orchard in Korea: ASGV, ASPV, ACLSV and/or ApMV. In addition, positive control plasmid was developed and evaluated as positive controls for multiplex RT-PCR. A positive control plasmid was constructed by cloning sequences of four pome fruit viruses infecting apple trees into a single vector. This is a new approach for the simultaneous detection of pome viruses using positive control plasmid instead of infected plants as positive control.

2. Materials and Methods

2.1 Plant material

Leaf samples were obtained from commercial orchards or net screen house at the National Institute of Horticultural and Herbal Science (NIHHS, Korea) infected with ASGV, ASPV, ApMV and ACLSV in apple trees, ASGV, ASPV and ACLSV in pear trees. The viruses were confirmed by simplex RT-PCR and samples infected with known viruses were used for the optimization of the efficiency and specificity of simultaneous detection assays.

2.2 Virus specific primers

Previously published primers for ASPV, ACLSV, internal control (NADH dehydrogenase subunit 5 (*nad*5)) and newly designed primers for ASGV, ApMV are described in Table 1. The new primers were designed based on conserved regions of each virus in GenBank of the National Center for Biotechnology Information (NCBI) with similar annealing temperatures and different sizes using the software FastPCR 6.1. All primers were used for simultaneous detection of pome viruses by multiplex RT-PCR.

2.3 Multiplex RT-PCR

Total RNA was extracted from about 100mg of leaves using the NucliSENS easyMAG system (bioMérieux, The Netherlands) for the detection of pome fruit viruses. 20µl of reaction was set up for first-strand cDNA synthesis containing 9µl total RNA (100-500ng), 4µl M-MLV reverse transcriptase buffer (5X), 1µl (10pmol/µl) random primer, 2µl dNTP mix (10mM), 2 µl DTT (0.1M), 1µl (40u/µl) RNAse inhibitor and 1µl (200u/µl) M-MLV Reverse Transcriptase (Invitrogen, USA). RT reaction was incubated at 42°C for 52min, 70°C for 15min and then chilled on ice. The primers were tested by simplex RT-PCR prior to multiplex analysis. Multiplex RT-PCR was optimized to enable to perform simultaneous detection of all target viruses on apple and pear trees in one reaction using all of the relevant virus-specific primers.

Multiplex RT-PCR was performed with 5µl of cDNA prepared in a 20µl reaction with AccuPower PCR PreMix (Bioneer, Korea). Primer concentrations for apple and pear viruses were 1µl (20pmol/µl) of each ASGV primer, 1µl (15pmol/µl) of each ASPV primer, 1µl (30pmol/µl) of each ACLSV primer, 1µl (2pmol/µl) of each *nad*5 primer and/or 1µl (10pmol/µl) of each ApMV primer. Cycling conditions were as follows: initial denaturation at 94°C for 4min followed by 40 cycles of 94°C for 30sec, 55°C for 1min, 72°C for 1min 20sec, and a final extension step at 72 °C for 10min. PCR products were analyzed by electrophoresis in 1.5% agarose gels and stained with BLUE Mango (GENEPOLE, Korea) and visualized under UV light. To confirm the identity of the amplified products, the multiplex RT-PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, USA) and sequenced (Bionics, Korea) using specific primers for each virus. These sequences were verified by a BLAST search of the NCBI nucleotide database.

2.4 Evaluation of multiplex RT-PCR

To estimate the detection limits of multiplex RT-PCR, total RNA extracts from infected apple and pear trees were diluted serially 10-fold (10^0-10^{-6}) in total RNA extracts from RNase free water and used in multiplex RT-PCR. The multiplex RT-PCR was validated using field samples of apple and pear commercial orchards.

2.5 Construction of positive control plasmid

The amplified PCR products of viruses and internal control were obtained by simplex RT-PCR from infected apple trees using specific primers as described in Table 1 plus restriction enzyme sites (Table 2). The products were cloned individually into pGEM-T Easy vector. Positive control plasmid construct was generated by subcloning the ASGV, ASPV, ApMV and *nad*5 fragments into a single plasmid of the pGEM/ACLSV using SacII, SacI, PstI and SphI restriction sites, respectively.

2.6 Application of positive control plasmid

To test applicability of the positive control plasmid, all subcloned viruses and internal control sequences were amplified by PCR and confirmed by sequencing (Bionics, Korea). The PCR cycling conditions were as follows: initial denaturation at 94°C for 2min followed by 25 cycles of 94°C for 30sec, 55°C for 45sec, 72°C for 1min, and a final extension step at 72°C for 10min. The positive control plasmid was used as positive controls in the multiplex RT-PCR for detection of pome fruit viruses.

3. Results

3.1 Multiplex RT-PCR for simultaneous detection of pome fruit viruses

To develop multiplex RT-PCR for simultaneous detection of pome fruit viruses, ASGV, ASPV, ACLSV and/or ApMV with internal control (*nad*5), previously published and newly designed primers as described in Table 1 were tested in simplex RT-PCR using infected apple and pear trees. The amplified products of expected sizes were observed: 273bp for ASGV, 370bp for ASPV, 677bp for ACLSV, 181bp for *nad*5 and/or 450bp for ApMV (Fig. 1, Lane 1-5). Reaction components including primer concentrations, annealing temperature, extension time and number of cycles were optimized for the effective application of multiplex RT-PCR. The optimization of multiplex RT-PCR was achieved above those used in multiplex RT-PCR as well as optimal annealing temperature at 55°C. The PCR fragments of the expected size were successfully amplified and identified from infected apple and pear trees by multiplex RT-PCR (Fig. 1, Lane 6). The specificity of the PCR fragments was confirmed by sequencing separately. The sequences of three viruses and internal control showed the following nucleotide identities to the corresponding region: ASGV, 94% to 96%; ASPV, 86%; ALCSV, 95%; and *nad*5, 99% in infected apple samples and ASGV, 92%; ASPV, 91%; ACLSV, 94%; and *nad*5, 99% in infected pear samples.

3.2 Evaluation of multiplex RT-PCR

The detection sensitivity of multiplex RT-PCR was determined by testing 10-fold serial dilutions (10^0-10^{-6}) of cDNA generated from total RNA extracted from apple and pear trees infected by pome fruit viruses. The detection limits of multiplex RT-PCR from infected apple trees for ApMV was up to 10^{-6} dilution, ASPV and ASGV up to 10^{-2} dilution, and ACLSV up to 10^0 dilutions (Fig. 2A). The detection limits of multiplex RT-PCR from infected pear trees for ASPV and ASGV up to 10^{-2} dilution and ACLSV up to 10^{-1} dilution (Fig. 2B). All pome fruit viruses were well detected at dilution of 10^0 in infected apple trees and 10^{-1} in infected pear trees by multiplex RT-PCR. The virus combinations of ASGV, ASPV, ACLSV and/or ApMV with *nad*5 in apple and pear samples from commercial orchard were successfully identified simultaneously by multiplex RT-PCR (Fig. 3).

3.3 Positive control plasmid for simultaneous detection of pome fruit viruses

Positive control plasmid was used as controls for simultaneous detection of pome fruit viruses by multiplex RT-PCR assay. To construct positive control plasmid, PCR fragments of viruses and internal control were amplified by simplex RT-PCR from infected apple trees using specific primers described in Tables 2. The PCR products were cloned individually into pGEM-T Easy vector. Positive control plasmid was generated by subcloning the restriction enzyme digestion fragments of ApMV, ASPV, ASGV and *nad*5 shown into the pGEM/ACLSV plasmid, which were obtained by cloning ALCSV products into pGEM-T Easy vector (Fig. 4).

The positive control plasmid was tested for suitability as positive control for simplex or multiplex PCR. The specific amplification products of the target fragments were detected (Fig. 5) and confirmed by sequencing. The confirmed positive control plasmid was used as templates in place of infected apple and pear trees in the multiplex RT-PCR. The amplified PCR fragments were loaded onto the gel and analyzed (Fig. 5B, Lane 5 and 9).

4. Discussion

For effective control of fruit tree viruses, it is essential to develop a rapid, sensitive and cost-effective diagnosis assay to detect them. A multiplex RT-PCR has been developed as a suitable tool for the simultaneous detection of viruses in a large number of samples with multiple infections (Menzel et al., 2002; Sanchez-Navarro et al., 2005; Hassan et al., 2006; Gambino et al., 2006; Youssef and Shalaby, 2009; Jarosova et al., 2010). Recently developed real time PCR and LAMP (loop mediated isothermal amplification) methods were more sensitive than multiplex RT-PCR but were expensive to detect several viruses with large numbers of samples. The multiplex RT-PCR assays reported from previous studies cannot detect all of the viruses infecting apple and pear trees in Korea because of the viral genetic diversity (Magome et al., 1997; Fiore et al., 2008; Komorowska et al., 2011; Seah et al., 2012).

In this study, the multiplex RT-PCR assays were developed for the simultaneous detection of internal control and pome fruit viruses infecting apple and pear trees. These viruses are the most commonly occurring and economically important pathogens in commercial orchards in Korea. The internal control was used as an indicator of RNA quality and RT-PCR efficiency. For the effective application of multiplex PCR assays, several parameters including primer concentrations and PCR conditions were optimized with previously published and newly designed primers. In general the detection sensitivities of multiplex RT-PCR assay are known to be lower than those of simplex RT-PCR assays because the cocktail of primers in the multiplex assays compete for all the templates rather than just for one (Roy et al., 2005; Uga et al., 2005; Gambino et al., 2006). However, the sensitivity level of the multiplex RT-PCR assays is regarded as adequate for routine diagnosis. Because the specific bands for all viruses in infected pear trees were well amplified up to 10^{-1} dilution in multiplex RT-PCR, corresponding to 10mg of infected sample in 100mg total. Although apple viruses were well amplified up to 10^{0} dilutions, corresponding to 100mg of infected apple sample. The different detection limits of the multiplex RT-PCR in infected apple and pear trees is presumably a result of different viral RNA concentrations in the extracts and/or the degree of interference among primers (Menzel et al., 2002; Hassan et al., 2006). The positive control plasmid was also developed as positive controls for simultaneous detection of pome fruit viruses by multiplex RT-PCR. This tool is a simple, sensitive and more transferable method between laboratories than the classical use of known infected plants, which may be difficult to obtain.

In conclusion, the multiplex RT-PCR assay developed here were found useful for the simultaneous detection of pome fruit viruses in apple and pear samples with multiple infections. Additionally, the positive control plasmid developed can be utilized as positive controls for pome fruit viruses detection by multiplex RT-PCR.

Acknowledgement

This research was carried out with the support of research fund (Project No. PJ010077) from Rural Development Administration, Korea.

References

Akbas, B., & Degirmenci, K. (2010). Simultaneous detection of Apple mosaic virus in cultivated hazelnuts by one-tube RT-PCR. African Journal of Biotechnology, 9, 1753-1757.

Fiore, N., Fajardo, T. V. M., Prodan, S., Herranz, M. C., Aparicio, F., Montealegre, J., Elena, S. F., Pallas, V., & Sanchez-Navarro, J. A. (2008). Genetic diversity of the movement and coat protein genes of South American isolates of *Prunus necrotic ringspot virus*. Archives of Virology, 153, 909-919.

Gambino, G., & Gribaudo, I. (2006). Simultaneous detection of nine grapevine viruses by multiplex RT-PCR with coamplification of a plant RNA internal control. Phytopathology. 96, 1223-1229.

Hassan, M., Myrta, A., & Polak, J. (2006). Simultaneous detection and identification of four pome fruit viruses by one-tube pentaplex RT-PCR. Journal of Virological Methods, 133, 124-129.

Jarosova, J., & Kundu, J. K. (2010). Simultaneous detection of stone fruit tree viruses by one-step multiplex RT-PCR. Scientia Horticulturae, 125, 68-72.

Kinard, G. R., Scot, S. W., & Barnett, O. W. (1996). Detection of *Apple chlorotic leaf spot* and *Apple stem grooving virus* using RT-PCR. Plant Disease, 80, 616-621.

Komorowska, B., Siedlecki, P., Kaczanowski, S., & Hasiów-Jaroszewska, B. (2011). Sequence diversity and potential recombination events in the coat protein gene of *Apple stem pitting virus*. Virus Research, 158, 263-267.

Magome, H., Yoshikawa, N., Takahashi, T., Ito, T., & Miyakawa, T. (1997). Molecular variability of the genomes of capilloviruses from apple, Japanese pear, European pear, and citrus trees. Phytopathology, 87, 389-396.

Menzel, W., Jelkmann, W., & Maiss, E. (2002). Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. Journal of Virological Methods, 99, 81-92.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symposia on Quantitative Biology, 51 Pt 1, 263-273.

Naidu, R. A., & Hughes, J. D. A. (2003). Methods for the detection of plant viral diseases in plant virology in sub-Saharan Africa, Proceedings of plant virology, IITA, Ibadan, Nigeria. Eds. Hughes JDA, Odu B (pp. 233-260).

Noorani, M. S., Awasthi, P., Sharma, M. P., Ram, R., & Zaidi, A. A. (2013). Simultaneous detection and identification of four cherry viruses by two step multiplex RT-PCR with an internal control of plant *nad*5 mRNA. Journal of Virological Methods, 193, 103-107.

Park, P. H., Choi, Y. M., Lee, I. K., Kim, H. R., & Jeong, S. B. (2006). Occurrence of *Apple scar skin viroid* in pear (*pyrus* spp.) in Korea and diagnosis of virus, viroid on important genetic resources. The Plant Pathology Journal, 22, 406 E-61 (Abstract).

Roy, A., Fayad, A., Barthe, G., & Brlansky, R. H. (2005). A multiplex polymerase chain reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. Journal of Virological Methods, 129, 47-55.

Sanchez-Navarro, J. A., Aparicio, F., Herranz, M. C., Minafra, A., Myrta, A., & Pallas, V. (2005). Simultaneous detection and identification of eight stone fruit viruses by one-step RTPCR. European Journal of Plant Pathology, 111, 77-84.

Sastry, K. S. (2013). Plant virus and viroid diseases in the tropics. Volume-1: Introduction of plant viruses and sub-viral agents, classification, assessment of loss, transmission and diagnosis. Springer, New York.

Seah, Y. M., Sharma, A. M., Zhang, S., Almeida, R. P. P., & Duffy, S. (2012). A divergent variant of *Grapevine leafroll-associated virus* 3 is present in California. Virology Journal, 9, 235.

Thresh, J. M. (2006). Control of tropical plant virus diseases. Advances in Virus Research, 67, 245-295.

Uga, H., & Tsuda, S. (2005). A one-step reverse transcriptionpolymerase chain reaction system for the simultaneous detection and identification of multiple *Tospovirus* infection. Phytopathology, 95, 166-171.

Wei, T., Lu, G., & Clover, G. (2008). Novel approaches to mitigate primer interaction andeliminate inhibitors in multiplex PCR, demonstrated using an assay for detection of three strawberry viruses. Journal of Virological Methods, 151, 132-139.

Youssef, S. A., & Shalaby, A. A. (2009). Single-step multiplex reverse transcription-polymerase chain reaction (m-RT-PCR) for simultaneous detection of five RNA viruses affecting stone fruit trees. International Journal of Virology, 5, 100-108.

Table 1: List of primers used in multiplex RT-PCR for pome fruit viruses at an annealing temperature of 55°C

Virus	Primer	Sequence(5′- 3′)	(bp)	Reference
ASGV	F	GAAGACGTGCTTCAACTAGC	579	This study
	R	TTTTAGACCAGTGGCAAAGT		
ASPV	F	ATGTCTGGAACCTCATGCTGCAA	370	Menzel et al.(2002)
	R	TTGGGATCAACTTTACTAAAAAGCATAA		
ApMV	F	AGGGTCCTGAGCAGTCGAGA	264	This study
	R	GTTTGGAGGGGCTTCCCACT		
ACLSV	F	TTCATGGAAAGACAGGGGCAA	677	Menzel et al.(2002)
	R	AAGTCTACAGGCTATTTATTATAAGTCTAA		
*nad*5	F	GATGCTTCTTGGGGCTTCTTGTT	181	Menzel et al.(2002)
	R	CTCCAGTCACCAACATTGGCATAA		

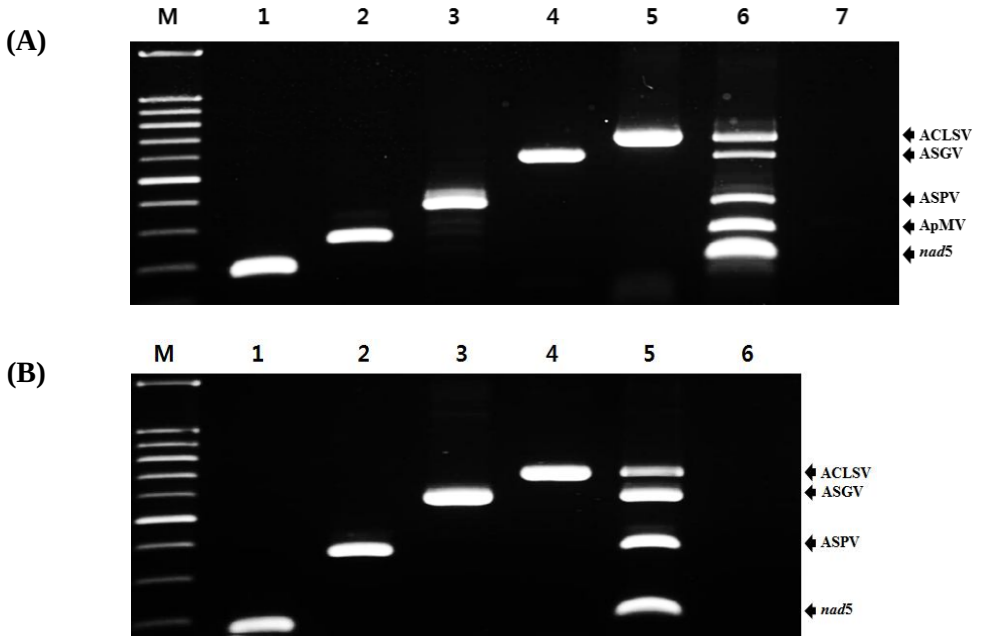

Fig. 1: Specificity of the simplex and multiplex RT-PCR for detection of pome fruit viruses from infected apple (A) and pear (B) trees. (A) Lane A-1 and B-1, simplex RT-PCR for *nad*5 (118bp); Lane A-2, simplex RT-PCR for ApMV (264bp); Lane A-3 and B-2, simplex RT-PCR for ASPV (370bp); Lane A-4 and B-3, simplex RT-PCR for ASGV (576bp); Lane A-5 and B-4, simplex RT-PCR for ACLSV (677bp); Lane A-6 and B-5, multiplex RT-PCR for *nad*5, ApMV, ASPV, ASGV and/or ApMV; Lane A-7 and B-6, water control. M: 100 bp ladder DNA marker. The specific amplified PCR products corresponding to individual viruses and *nad*5 are indicated on the right.

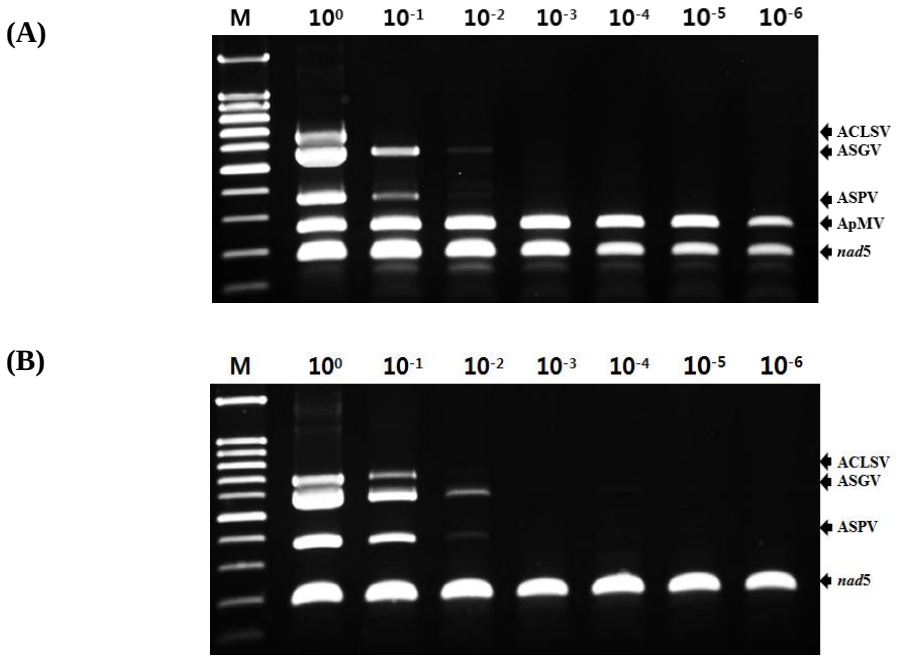

Fig. 2: Comparison of sensitivity limits of multiplex RT-PCR for detection of pome fruit viruses from infected apple (A) and pear trees (B). The cDNA was extracted from infected samples with ASGV, ASPV, ACLSV and/or ApMV was serially diluted (10-fold) in RNase free water. M, 100bp ladder DNA marker. The specific amplified PCR products corresponding to individual viruses and *nad*5 are indicated on the right.

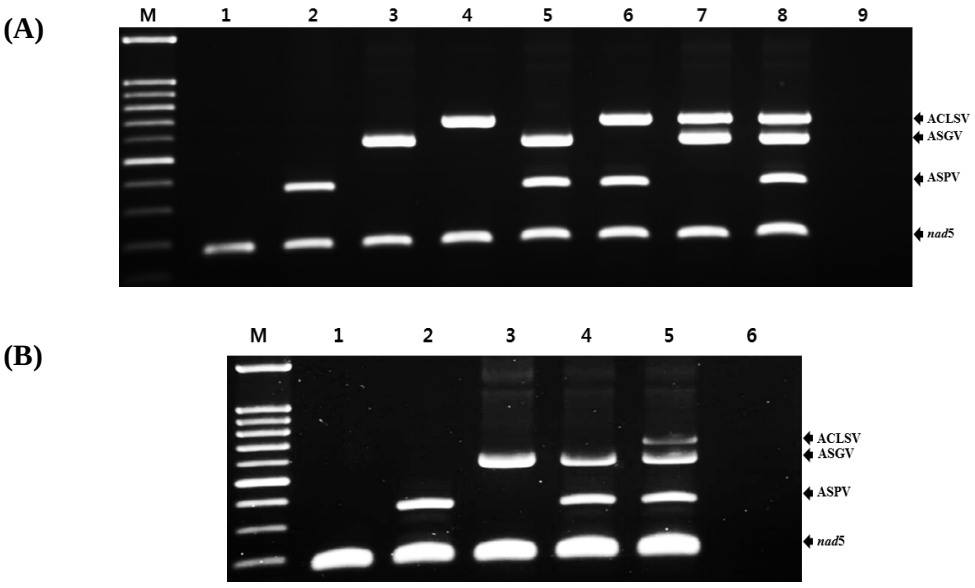

Fig. 3: Simultaneous detection of pome fruit viruses from field samples by multiplex RT-PCR from infected apple (A) and pear trees (B). Lane A-1, healthy apple control; Lane A-2 to 8, infected apple trees from commercial orchard; Lane B-1, healthy pear control; Lane B-2 to 5, infected pear trees from commercial orchard; Lane A-9 and B-6; water control. M, 100 bp ladder DNA marker. The specific amplified PCR products corresponding to individual viruses and *nad*5 are indicated on the right.

Table 2: List of primers used for the construction of positive control plasmid

Virus	Primer	Sequence(5′- 3′)	Restriction enzymes
ASGV	F	GCG**CTGCAG**GCCACTTCTAGGCAGAACTC	*Pst* I
	R	GCC**CTGCAG**AACCCCTTTTTGTCCTTCAGT	
ASPV	F	GCC**GAGCTC**ATGTCTGGAACCTCATGCTG	*Sac* I
	R	GCG**GAGCTC**TTGGGATCAACTTTACTAAA	
ApMV	F	GCG**CCGCGG**CGTAGAGGAGGACAGCTTG	*Sac* II
	R	GCC**CCGCGG**CCGGTGGTAACTCACTCGTT	
ACLSV	F	TTCATGGAAAGACAGGGGCAA	-
	R	AAGTCTACAGGCTATTTATTATAAGTCTAA	
*nad*5	F	GCG**GCATGC**GATGCTTCTTGGGGCTTCTT	*Sph* I
	R	GCG**GCATGC**CTCCAGTCACCAACATTGGCA	

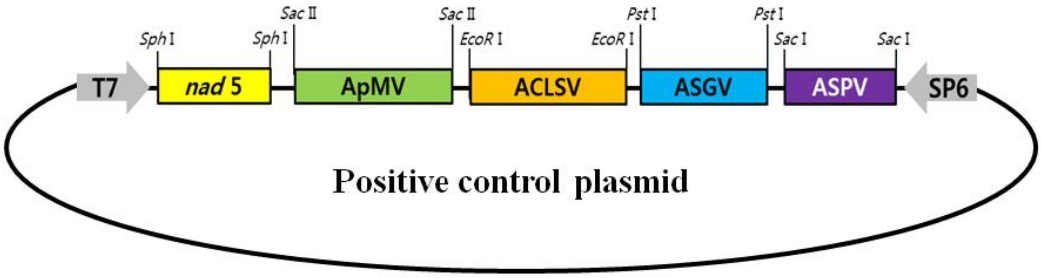

Fig. 4: Schematic representation of positive control plasmid for multiplex RT-PCR of apple viruses. Four pome fruit viruses and *nad*5 PCR products were individually cloned into pGEM-T Easy vector using the restriction enzymes shown. The positive control plasmid construct was the fusion of *nad*5, ApMV, ACLSV, ASGV and ASPV sequences in the order indicated.

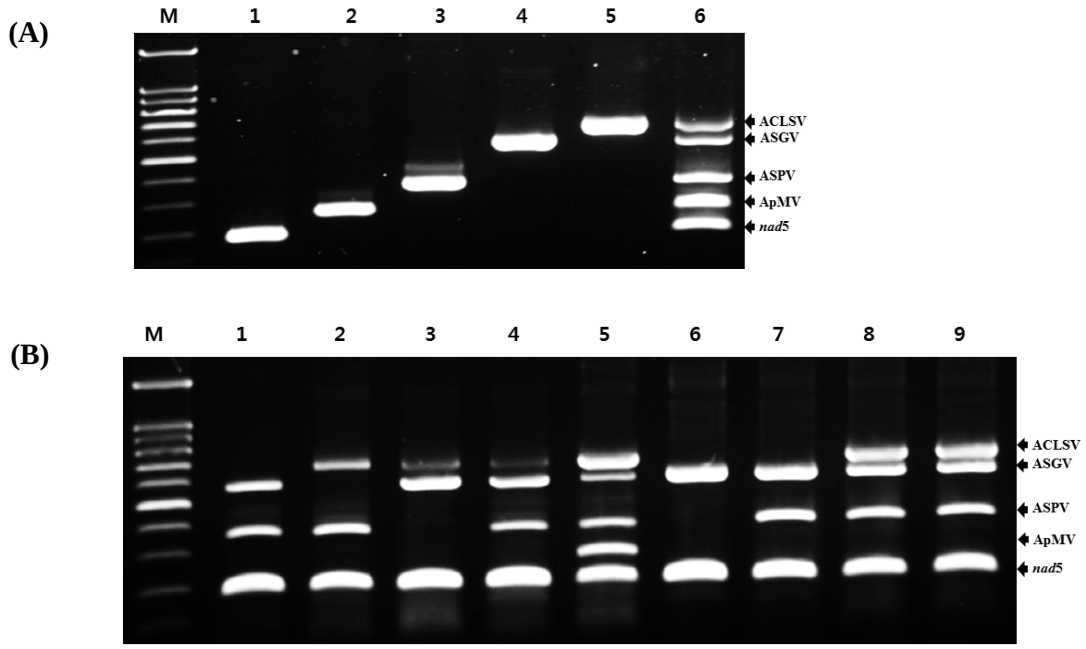

Fig. 5: (A) Molecular detection of pome fruit viruses and *nad*5 using positive control plasmid with simplex and multiplex PCR. Lane 1, simplex PCR for *nad*5; Lane 2, simplex PCR for ASGV; Lane 3, simplex PCR for ASPV; Lane 4, simplex PCR for ApMV; Lane 5, simplex PCR for ACLSV; Lane 6, multiplex PCR for ASGV, ASPV, ApMV, ACLSV and *nad*5. (B) Application of positive control plasmid as positive control on multiplex RT-PCR. Lane 1 to 4, naturally infected apple trees; Lane 6-8, naturally infected pear trees; Lane 5 and 9, synthetic positive control of multiplex PCR products using positive control plasmid. M, 100bp ladder DNA marker. The specific amplified PCR products for individual viruses and *nad*5 are indicated on the right.