Journal of Virological Methods 263 (2019) 105–110

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A sensitive nested multiplex RT-PCR assay for the simultaneous detection of three common viruses infecting pear plants

Bingyu Yao[a,c], Yongcan Lu[a,b], Liu Li[a], Yanxiang Wang[a], Qingyu Li[d], Guoping Wang[a,b,c], Ni Hong[a,b,c,*]

[a] State Key Laboratory of Agromicrobiology, Huazhong Agricultural University, Wuhan, Hubei 430070, China
[b] The Key Laboratory of Plant Pathology of Hubei Province, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, China
[c] Key Laboratory of Horticultural Crop (Fruit Trees) Biology and Germplasm Creation of the Ministry of Agriculture, Huazhong Agricultural University, Wuhan, Hubei 430070, China
[d] Yantai Academy of Agricultural Science, Yantai, Shandong 264000, China

ARTICLE INFO

Keywords:
Apple chlorotic leaf spot virus
Apple stem grooving virus
Apple stem pitting virus
NmRT-PCR assay
Pear

ABSTRACT

A highly sensitive nested multiplex reverse transcription-polymerase chain reaction (nmRT-PCR) assay was developed for the simultaneous detection of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) infecting pear trees. In the assay, a set of three forward primers specific to each of the three viruses and a universal reverse primer was used as external primers in the first-round PCR, which was followed by a second-round PCR developed previously. The nmRT-PCR assay was 10^4 times more sensitive than conventional mRT-PCR assay in detecting the three viruses in *in vitro* pear plantlets. This assay was subsequently used to detect these viruses in leaf and bark samples of cultivated and wild pear trees from orchards and demonstrated to be highly sensitive and reliable. This is the first report describing a use of nmRT-PCR for the sensitive and simultaneous detection of the three viruses infecting pear plants. The assay would be useful for the certification of pear planting materials and surveillance of nursery stocks.

Infections of apple stem grooving virus (ASGV), apple stem pitting virus (ASPV) and apple chlorotic leaf spot virus (ACLSV) are very common in commercially cultivated pear (*Pyrus* spp.) and apple (*Malus* spp.) trees worldwide (Németh, 1986). Although most apple and pear trees naturally infected by these viruses are asymptomatic, their growth can be reduced by the virus infection (Gella, 1988; Németh, 1986; Desvignes, 1999). Mixed infections of these viruses can induce a rapid decline of top-worked trees and reduce fruit yield (Posnette et al., 1963; Yanase, 1983; Desvignes, 1999; Cembali et al., 2003). A recent study showed that the co-infection of ASGV and ASPV significantly decreased the growth and proliferation of *in vitro* cultivated plants of *P. communis* cv. 'Confenence', and ASGV infection strongly inhibited the root development of *in vitro* cultivated plants of *P. pyrifolia* cv. 'Jinshui no. 2' (Chen et al., 2017).

Apple stem grooving virus, Apple stem pitting virus and Apple chlorotic leaf spot virus are the type species of the genera Trichovirus, Capillovirus and Foveavirus in the family Betaflexiviridae, respectively (Adams et al., 2012). Their single-stranded positive-sense genomic RNAs consist of two to five open reading frames (ORFs) and a poly (A) tail at their 3'-terminus. These viruses are commonly disseminated by utilization of virus-infected propagative materials (Yanase, 1983; Németh, 1986). Use of certified virus-free plants is currently considered to be the most effective way to manage viral diseases of apple and pear trees worldwide (EPPO, 1999; Cembali et al., 2003). Hence, it is important to develop effective and high throughput assays for the detection of these viruses in certification and quarantine programs. RT-PCR assays have been widely used for the detection and identification of pear and apple viruses (Kinard et al., 1996; Candesse et al., 1998; Mathioudakis et al., 2008; Lu et al., 2017). Multiplex RT-PCR assays have been developed for rapid and sensitive detection and identification of several pathogens simultaneously in a single test (Nassuth et al., 2000; Saade et al., 2000; Ito et al., 2002; Sánchez-Navarro et al., 2005; Li et al., 2012; Malandraki et al., 2015). We developed an mRT-PCR assay for the detection and differentiation of ACLSV, ASGV and ASPV in pear trees (Yao et al., 2014). However, the assay sometimes gave negative results when samples with low virus concentration were repetitively tested. Dormant pear shoots are important materials used in germplasm exchange and plant propagation programs, and the mRT-PCR frequently failed to detect the viruses in pear bark tissues due to the interference by polysaccharides and polyphenol components and

* Corresponding author at: State Key Laboratory of Agromicrobiology, Huazhong Agricultural University, Wuhan, Hubei 430070, China.
E-mail address: whni@mail.hzau.edu.cn (N. Hong).

https://doi.org/10.1016/j.jviromet.2018.10.021
Received 29 September 2017; Received in revised form 26 October 2018; Accepted 26 October 2018
Available online 29 October 2018
0166-0934/

Table 1
Primers used for the multiplex RT-PCR and the nest-multiplex RT-PCR detection of three pear viruses.

Primer [a]	Primer sequence (5′-3) [b]	Target gene /position[c]	Position (nt) [d]
Reverse transcription primer			
M4-T	GTTTTCCCAGTCACGAC$(T)_{15}$	poly(A)	–
External primer			
M4	GTTTTCCCAGTCACGAC	–	–
rdrp-F	TTCAA**Y**ACA**H**T**K**GCCAA**Y**ATG	RdRp	–
OUT-ASGV-F	GAGAGGATTTAGGTCCCT	MP	5576-5593
OUT-ASPV-F	CCTTATTACCACCCATTAGGT	CP	7839-7859
OUT-ACLSV-F	C**Y**CTTCATGGAAAGACAGG	CP	6839-6857
mRT-PCR primer			
ASGV-U-F	CCCGCTGTTGGATTTGATACACCTC	CP	5871-5895
ASGV-2-R	GGAATTTCACACGACTCCTAACCCTCC	CP	6344-6370
ASPV247-F	CAGTATTGTGCCTT**Y**TA**Y**GC**R**AAGC	CP	8809-8833
ASPV247-R	CCATAGAACGGATGCGGTACAT**Y**TG	CP	9032-9055
ACLSV-A53-F	GGCAACCCTGGAACAGA	CP	6875-6891
ACLSV-A52-R	CAGACCCTTATTGAAGTCGAA	CP	7212-7232

[a] F: forward primer ; R: reverse primer.
[b] Y = C/T, H = A/C/T, K = G/T, R = A/G.These nucleotides were hightlighted in bold.
[c] RdRp: RNA dependent RNA polymerase; MP: movement protein; CP: coat protein.
[d] Target positions correspond to the ASGV (HE978837.1), ASPV (KF915809.1) and ACLSV (KC935956.1) genome sequences.

low virus concentrations in the tissues (Yao et al., 2014). In previous studies, it was also found that some seedlings of P. betulifolia, a species widely used as the rootstock for pear plant propagation in China, were positive for the viruses ASGV, ASPV and ACLSV in RT-PCR. But their low concentrations in P. betulifolia might affect the detection reliability of conventional RT-PCR (unpublished data). Similarly, in virus elimination treatments using meristem culture combined with thermotherapy or chemotherapy (Hu et al., 2012), the virus concentrations are usually very low in the newly regenerated in vitro cultures (unpublished data), which strongly impedes the selection of virus-free pear plants. To overcome the deficiencies of the mRT-PCR, this study was undertaken to develop a reliable and highly sensitive nested mRT-PCR assay (nmRT-PCR) for the simultaneous detection of ACLSV, ASGV and ASPV in different pear propagating materials.

In this study, two sets of external forward primers (Table 1) were designed using the PrimerExplorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). One degenerated external forward primer rdrp-F was designed based on sequences conserved in the RNA dependent RNA polymerase (RdRp) encoding gene of the three viruses. Three forward primers, OUT-ASGV-F specific for ASGV, OUT-ASPV-F specific for ASPV and OUT-ACLSV-F specific for ACLSV, of another set were designed based on multiple alignments of available sequences. All these primers were targeted to the relatively conserved sites on the genomic sequences of each of the three viruses, respectively, and the first forward nucleotide of each primer was strictly anchored to a conserved genomic site of each virus (Figs. S1, S2 and S3). Specificity of each primer was evaluated by BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Both sets used the M4 (Table 1) as an external reverse primer in the first round of the RT-PCR.

Total RNA from pear plant samples was extracted according to a rapid silica spin column-based method described recently (Yang et al., 2017). In the mRT-PCR assay, cDNA synthesis was performed at 37 °C for 1 h by using random primers and M-MLV reverse transcriptase (Promega, Madison, USA). The three primer pairs ASGV-U/ASGV-2, ACLSV-52/ACLSV-53 and ASPV247-F/ACLSV247-R (Table 1) were used for the simultaneous detection of ASGV, ACLSV and ASPV by the mRT-PCR assay, and the PCR reaction solution and thermal cycling condition were the same as those reported previously (Yao et al., 2014). In the nmRT-PCR assay, cDNA was synthesized by using the universal primer M4-T (Table 1). The nmRT-PCR assay consisted of two rounds of amplifications. In the first round of amplification, PCR reaction mixture of 25 µl contained 2.5 µl of 10 × PCR buffer, 0.1 mM of each dNTP, 0.2 µM forward primer rdrp-F or 0.08 µM each of forward primers OUT-ASGV-F, OUT-ASPV-F and OUT-ACLSV-F, and 0.2 µM reverse primer M4, 1U *Taq* DNA polymerase (TaKaRa, Dalian, China), and 2 µl of cDNA template. The first-round PCR amplification parameters were as follows: 94 °C for 5 min, 35 cycles of 95 °C for 1 min, 56 °C for 1.5 min, and 72 °C for 1.5 min, and a final incubation for 10 min at 72 °C. In the second round of PCR reaction, 1 µl product from the first round of PCR reaction was used as template in a 25 µl reaction solution, and primers, reaction reagents and thermal cycling condition were the same as that of conventional mRT-PCR (Yao et al., 2014). The PCR products were electrophoresed on 2% agarose gels in TAE buffer, stained with ethidium bromide (0.5 µg/ml) and visualized under UV illumination.

The nmRT-PCR assays were evaluated by testing 12 individual *in vitro* plantlets generated from a single *P. communis* line that was known to be positive for ASGV, ACLSV and ASPV as tested by RT-PCR assay and mRT-PCR assay (Yao et al., 2014). A seedling of *P. betulifolia* was used as a negative control. Total RNA was extracted from each plantlet and subjected to comparative mRT-PCR and nmRT-PCR tests using either of the primer sets in the first round of amplification (Fig.1). When the products from the first round of RT-PCR were visualized in agarose gels, nonspecific bands were usually observed, and the target bands were frequently indeterminable, indicating a general lack of specificity and sensitivity in the first round of PCR (data not shown). However, the results of the second round of PCR were greatly improved (Fig. 1A and B). The amplification efficacy of the nmRT-PCR assays for the three viruses was much better than that of mRT-PCR (Fig. 1C), as indicated by the detection of intensive and specific bands from all plantlets (Fig. 1A and B). Cloning and sequencing of selected nmRT-PCR products also confirmed the amplification specificity (data not shown). Comparison of the two sets of external primers used in the nmRT-PCR assays also showed that the detection of ASGV and ACLSV were comparable to each other (Fig. 1A and B). However, the nmRT-PCR using rdrp-F and M4 primer set failed to detect ASPV from two ASPV-infected plantlets (Fig. 1A). The mRT-PCR assay failed to detect ASGV, ASPV and ACLSV from five, six and four of the 12 samples, respectively (Fig. 1C). These results also showed that the individual plantlets from the same *in vitro* culture line had different concentrations of the three viruses. Additionally, several follow up tests showed that although the nmRT-PCR assay using the rdrp-F and M4 produced stronger amplification for the three viruses than that of the conventional mRT-PCR, the detection efficacy was highly variable among the field samples tested (data not shown). Thus, based on these results, further evaluation studies were carried out using the OUT-ASGV-F/OUT-ASPV-F/OUT-ACLSV-F and M4 external primer set in the nmRT-PCR assay. Unexpectedly, a weak band with the expected size of the ACLSV specific PCR product was observed for the sample used as a negative control in all three PCR tests (Fig. 1,

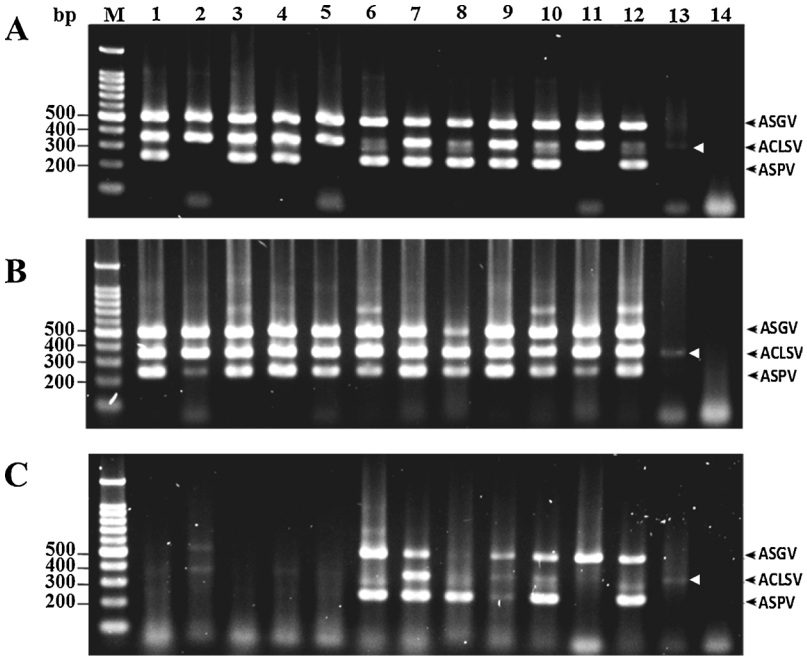

Fig. 1. Detection of viruses ASGV, ASPV and ACLSV in *in vitro* cultured pear (*P. communis*) plants by three RT-PCR assay methods. M, 100 bp plus DNA ladder; Lanes 1–12, *in vitro* cultures of pear (*P. communis*); Lanes 13 and 14, *P. betulifolia* seedling and H_2O, respectively. A, by nmRT-PCR assay using the rdrp-F/M4 external primer set in the first round of amplification; B, by nmRT-PCR using the OUT-ASGV-F + OUT-ASPV-F + OUT-ACLSV-F/M4 external primer set in the first round of amplification; C, by mRT-PCR assay. The faint bands with the expected size of the ACLSV specific PCR product from a *P. betulifolia* seedling control were indicated by white arrows.

lane 13). Sequencing of the amplicon and repeating the tests confirmed the presence of ACLSV infection in the plant. Therefore, another *P. betulifolia* seedling was used as a negative control for further tests.

To compare the analytical sensitivities of the nmRT-PCR and the conventional mRT-PCR, total RNA extracted from an in vitro culture of *P. communis* infected with the three target viruses was diluted in ten-fold series and used as template for cDNA synthesis. The cDNAs were then used as templates in mRT-PCR and nmRT-PCR assays, respectively. The detection limits of the mRT-PCR assays were 10^{-2} (equivalent to 5.52 ng/μl total RNAs) for ASGV and ACLSV, and 10° for ASPV (Fig. 2A). In contrast, nmRT-PCR detected all three viruses simultaneously at the dilution of 10^{-4} (equivalent to 0.055 ng/μl total RNA), with detection limits of 10^{-4} for ASPV, and 10^{-7} for ASGV and ACLSV (Fig. 2B). The results indicated that the sensitivity of the nmRT-PCR assay for the detection of each of the three viruses was 10^4 times higher than that of the mRT-PCR assay and was suitable for the simultaneous detection of the three viruses in pear plants with low virus concentrations.

Leaf samples were taken from 11 *P. betulifolia* field plants known to be infected by one or more of the three viruses. Results of the comparative tests showed that although mRT-PCR could amplify the three viruses from some samples (Fig. 3C), the resultant target product bands were very weak (Fig. 3A), making it difficult to judge the detection results. In contrast, nmRT-PCR detected all three viruses in three samples (Fig. 3B, lanes 3, 10 and 11), and either one or two of these viruses were detected in the remaining eight samples (Fig. 3B). Thus, compared to the mRT-PCR assay, the nmRT-PCR assay showed a higher efficiency for the detection of the three viruses in these samples (Fig. 3C). To validate the ability of the nmRT-PCR assay to detect molecular variants of each of the three viruses, the complete CP genes of ASGV, ACLSV and ASPV were amplified from three, one and two plants of *P. betulifolia* and 1–3 clones from each amplicon were sequenced, respectively (Tables S1, S2 and S3). Eight clones of the three ASGV isolates shared 90.8–99.7% nucleotide (nt) similarity with each other and the lowest nucleotide similarity of 89.6% with corresponding ASGV sequences referred from GenBank (Tables S1). Similarly, four clones of the ACLSV amplicon from one sample (pc2) shared 88.7–100% nt similarity, and 81.3–90.0% nucleotide similarity with CP gene of other ACLSV isolates represented by represented by NC_001409 and KC935956 (Tables S2). As reported previously, ASPV was highly variable (Ma et al., 2016). Three clones of the ASPV amplicon from a *P. betulifolia* plant (pb2) were highly divergent by sharing 65.9–72.2% nt

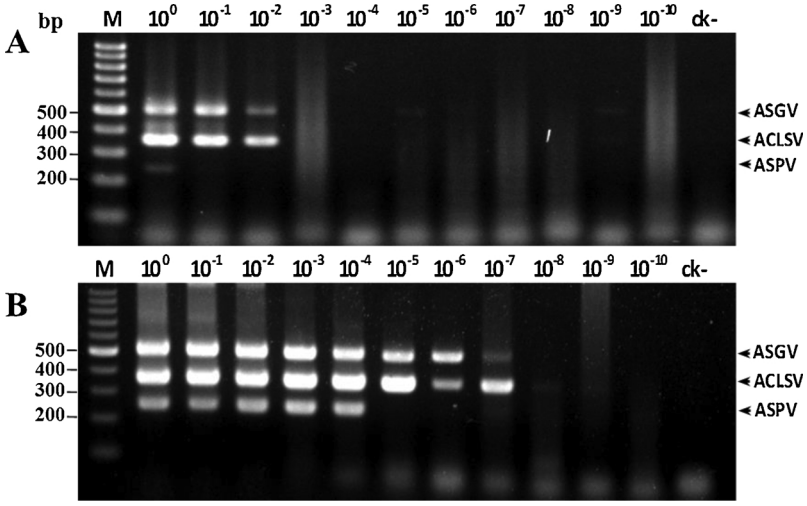

Fig. 2. Comparison of the sensitivities of mRT-PCR (A) and nmRT-PCR (B) assays. M, 100 bp plus DNA ladder ; Lanes10°-10^{-10}, ten-fold serial dilutions of an RNA extract from an *in-vitro* cultured pear sample infected with all three viruses; ck-, negative control (*P. betulifolia*). In the nmRT-PCR assay, the external primers of OUT-ASGV-F + OUT-ASPV-F + OUT-ACLSV-F/M4 were used.

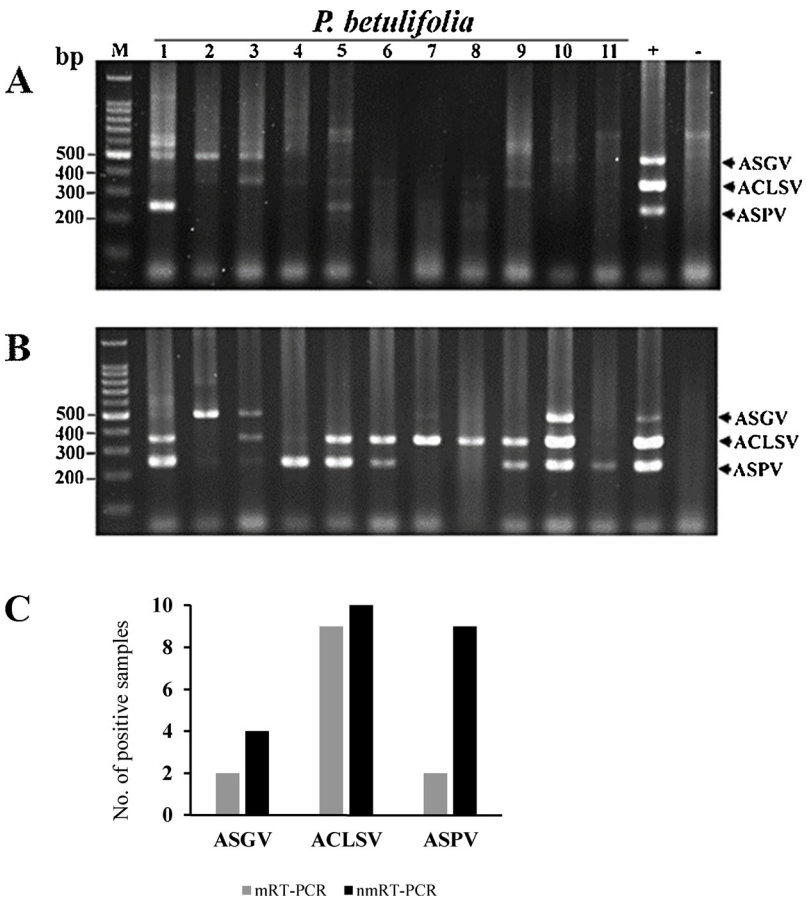

Fig. 3. Comparison of the nmRT-PCR and mRT-PCR assays for the detection of ASGV, ASPV and ACLSV in *P. betulifolia* field plants. A, mRT-PCR; B, nmRT-PCR; C, Total number of positive samples detected for each virus by the nmRT-PCR and mRT-PCR assays. M, 100 bp plus DNA ladder; +, positive control; -, negative control; Lanes 1–11, leaf samples of 11 *P. betulifolia* plants. In the nmRT-PCR assay, the external primers OUT-ASGV-F + OUT-ASPV-F + OUT-ACLSV-F/M4 were used.

similarity with each other, and 66.6–68.0 % nt similarity with the clone pc2, and these clones shared 66.6–83.8% nt similarity with the CP gene sequences of other ASPV isolates referred from GenBank (Tables S3). The result indicated that the primers used in the nmPCR assay could detect divergent molecular variants of the three viruses.

To validate the new nmRT-PCR assay for use in the detection of the three viruses in leaf and bark tissues of pear plants, dormant shoots were collected in early spring from five plants of *P. pyrifolia*, one of *P. betulifolia*, and one of *P. ussuriensis*. Among the three species, *P. pyrifolia* is widely grown in the central and southern China, *P. ussuriensis* is grown in the northern China, and the seedlings of *P. betulifolia* are widely used as pear rootstocks. The shoots from each plant were divided into two groups, one of which was stored at 4 °C to maintain its dormant status while the other was cultured in water-containing pots at room temperature to enable the sprouting of new leaves. After sprouting, total RNA was extracted from the young leaves and from the barks of the dormant shoots and subjected to the mRT-PCR and nmRT-PCR virus detection assays. Two *in vitro* cultures of *P. communis* co-infected with ASGV and ASPV, and co-infected with ACLSV and ASPV were used as positive controls. Compared with mRT-PCR assay (Fig. 4A), the nmRT-PCR assay usually generated intense bands of the three viruses in both bark and leaf samples (Fig. 4B). When the bark samples were tested, the mRT-PCR assay detected ASGV and ASPV in the *P. betulifolia* plant, the *P. ussuriensis* plant and two out of the five *P. pyrifolia* plants, but ACLSV was only detected in the *P. pyrifolia* bark sample with a weak product band (Fig. 4A, left panel lane 6). In contrast, the nmRT-PCR assay detected ASGV and ASPV from most of the bark samples (with one exception that sample 5 showed a negative result for ASGV and ASPV), and ACLSV in the *P. betulifolia* plant, the *P. ussuriensis* plant and two out of five *P. pyrifolia* plants (Fig. 4B, left panel). When the leaf samples were tested, nmRT-PCR assay detected ASGV and ASPV in all tested samples, and ACLSV in two out of five *P. pyrifolia* plants, and in the *P. ussuriensis* plant, and the three viruses were simultaneously detected in three plants (Fig. 4B, right panel lanes 2, 4 and 7). However, compared with the nmRT-PCR assay, the mRT-PCR assay missed the detection of ASGV in one *P. pyrifolia* plant and in the *P. betulifolia* plant (Fig. 4A, right panel lanes 5 and 6), ASPV in the *P. pyrifolia* plant (Fig. 4A, right panel lane 5) and ACLSV in one *P. pyrifolia* plant and in the *P. ussuriensis* plant, respectively (Fig. 4A, right panel lanes 2 and 7). The results indicated that the nmRT-PCR assay had higher positive detection rates for the three viruses in the all leaf samples as compared with mRT-PCR, and pear leaf samples were more suitable for the virus detection than bark samples in both mRT-PCR and nmRT-PCR assays (Fig. 4C). On the other hand, the bark sample of the *P. betulifolia* plant (Fig. 4B, lane 6) tested positive for ASGV and ASPV in the mRT-PCR assay and positive for ASGV, ACLSV and ASPV in the nmRT-PCR assay, but its leaf sample only tested positive for ASPV in the mRT-PCR assay and for ASGV and ASPV in the nmRT-PCR assay.

Nested multiplex RT-PCR has the advantage of the high sensitivity of nested RT-PCR and the capability of mRT-PCR for the simultaneous detection of multiple viruses (Ben Salem et al., 2010), but is rarely reported for the detection of plant viruses. The results presented in this study demonstrated that the nmRT-PCR employing multiplex external forward primers specific for each virus is a reliable and sensitive method for the surveillance of ASGV, ASPV and ACLSV infecting pear plants. The three viruses ASGV, ASPV and ACLSV belong to three different genera in the same family (Adams et al., 2012). There are high levels of genomic diversity among these viruses and within each virus, especially among the ASPV isolates (Ma et al., 2016), which causes difficulty to design a primer set capable of targeting all isolates of the three viruses (Magome et al., 1997; Mathioudakis et al., 2008; Komorowska et al., 2010). It might be the reason that the generic forward primer rdrp-out-F failed in detecting one or more viruses in some samples. In fact, this study represents the first successful application of

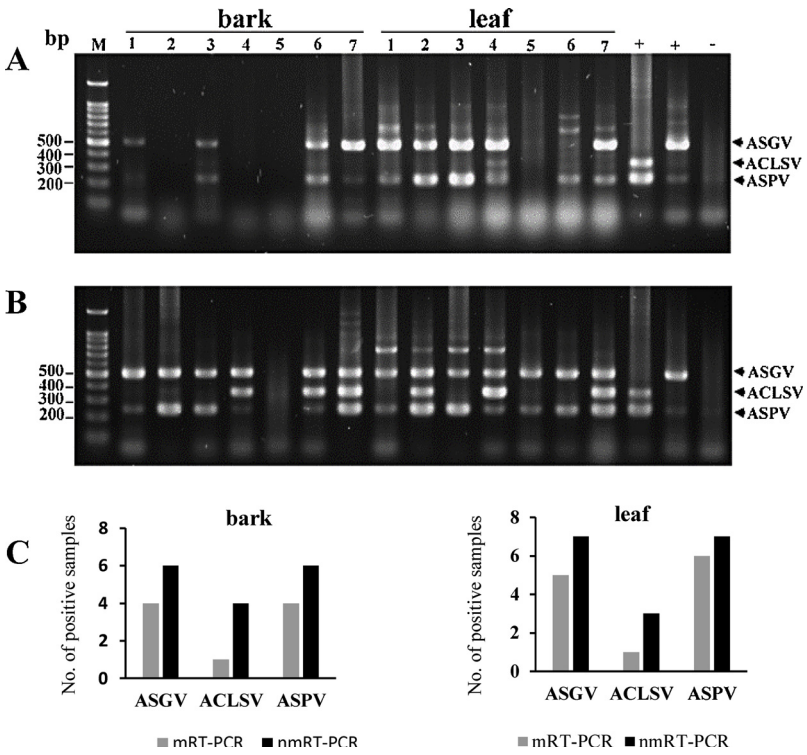

Fig. 4. Comparison of the nmRT-PCR and mRT-PCR assays for the detection of ASGV, ASPV and ACLSV in barks and leaves from plants of three pear species collected from the fields. A, mRT-PCR; B, nmRT-PCR; C, Total number of positive samples detected for each virus by the nmRT-PCR and mRT-PCR assays. M, 100 bp plus DNA ladder; +, positive control; -, negative control; Lanes 1–5, *P. pyrifolia*; Lane 6, *P. betulifolia*; Lane 7, *P. ussuriensis*. In the nmRT-PCR assay, the external primers OUT-ASGV-F + OUT-ASPV-F + OUT-ACLSV-F/M4 were used.

an nmRT-PCR method for the simultaneous and sensitive detection of the three common viruses in pear samples. The study provides new clues for designing PCR strategy. Some of these viruses remained undetected in some leaf samples, which could be attributed to an uneven distribution of the viruses in the plant, resulting in virus concentrations below the detection limits in some samples (Knapp et al., 1995). Despite the low virus concentrations and the presence of inhibitors (such as polysaccharides and polyphenols) in the leaf and bark samples of field-grown pear trees, the developed nmRT-PCR assay successfully amplified the three viruses from most of these samples. It was noted that non-specific products could be resulted in the nmRT-PCR assay due to its high sensitivity. However, it did not affect the visualization of positive results since nmRT-PCR assays could produce intense positive bands. Taking together, our results indicated that this nmRT-PCR assay was highly sensitive and reliable for virus detection in pear samples containing low virus concentration. Therefore, this assay would be very useful in the certification of pear propagation materials and in screening nursery stocks. The approach could be extended for simultaneous detection of common viruses in apple samples.

Acknowledgements

This work was financially supported by the programs for Key International S&T Cooperation Project (2017YFE0110900), Chinese Ministry of Agriculture with the earmarked fund for Pear Modern Agro-industry Technology Research System (CARS-29-10), and Fundamental Research Funds for the Central Universities (2662016PY107). We thank Hongju Hu at Institute of Fruit and Tea, Hubei Academy of Agriculture Sciences for kindly providing field pear samples.

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.2018.10.021.

References

Adams, M.J., Candresse, T., Hammond, J., Kreuze, J.F., Martelli, G.P., Namba, S., Pearson, M.N., Ryu, K.H., Saldarelli, P., Yoshikawa, N., 2012. Betaflexiviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus Taxonomy: Classification and Nomenclature of Viruses. Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, pp. 920–941.
Ben Salem, A.N., Chupin, S.A., Bjadovskaya, O.P., Andreeva, O.G., Mahjoub, A., Prokhvatilova, L.B., 2010. Multiplex nested RT-PCR for the detection of porcine enteric viruses. J. Virol. Methods 165, 283–293.
Candesse, T., Hammod, R.W., Hadidi, A., 1998. Detection and identification of plant viruses and viroids using polymerase chain reaction. In: Hadidi, A., Khaterpal, R.K., Koganezawa, H. (Eds.), Plant Virus Disease Control. APS Press, St. Paul, MN, USA, pp. 399–416.
Cembali, T., Folwell, R.J., Wandschneider, P., Eastwell, K.C., Howell, W.E., 2003. Economic implications of a virus prevention program in deciduous tree fruits in the US. Crop Prot. 22, 1149–1156.
Chen, J., Tang, H.H., Li, L., Qin, S.J., Wang, G.P., Hong, N., 2017. Effects of virus infection on plant growth, root development and phytohormone levels in *in vitro*-cultured pear plants. Plant Cell Tiss. Organ Cult. 131, 359–368.
Desvignes, J.C., 1999. Virus Diseases of Fruit Trees. Ctifl, Paris.
EPPO, 1999. Certification schemes-pathogen-tested material of *Malus*, *Pyrus* and *Cydonia*. Bull. OEPP 29, 239–252.
Gella, R., 1988. Effect of some virus diseases on the performance of two clones of 'Agua de Aranjuez' pear. Int. Symp. Pear Grow 256. pp. 137–142.
Hu, G.J., Hong, N., Wang, L.P., Hu, H.J., Wang, G.P., 2012. Efficacy of virus elimination from in vitro-cultured sand pear (*Pyrus pyrifolia*) by chemotherapy combined with thermotherapy. Crop Prot. 37, 20–25.
Ito, T., Ieki, H., Ozaki, K., 2002. Simultaneous detection of six citrus viroids and *Apple stem grooving virus* from citrus plants by multiplex reverse transcription polymerase chain reaction. J. Virol. Methods 106, 235–239.
Kinard, G.R., Scott, S.W., Barnett, O.W., 1996. Detection of *Apple chlorotic leaf spot* and *Apple stem grooving virus* using RT-PCR. Plant Dis. 80, 616–621.
Knapp, E., da Câmara Machado, A., Pühringer, Q., Wang, H., Hanzer, V., Weiss, H., Katinger, H., Laimer da Câmara Machado, M., 1995. Localization of fruit tree viruses by immuno-tissue printing in infected shoots of*Malus* sp. and *Prunus* sp. J. Virol. Methods 55, 157–173.
Komorowska, B., Malinowski, T., Michalczuk, L., 2010. Evaluation of several RT-PCR primer pairs for the detection of apple stem pitting virus. J. Virol. Methods 168, 242–247.
Li, F., Zuo, R., Abad, J., Xu, D., Bao, G., Li, R., 2012. Simultaneous detection and differentiation of four closely related sweet potato potyviruses by a multiplex one-step RT-PCR. J. Virol. Methods 186, 161–166.
Lu, Y., Yao, B., Wang, G., Hong, N., 2017. The detection of ACLSV and ASPV in pear plants by RT-LAMP assays. J. Virol. Methods 252, 80–85.
Ma, X.F., Hong, N., Moffett, P., Wang, G., 2016. Genetic diversity and evolution of *Apple stem pitting virus* isolates from pear in China. Can. J. Plant Pathol. 38, 218–230.
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.
Malandraki, I., Varveri, C., Olmos, A., Vassilakos, N., 2015. One-step multiplex quantitative RT-PCR for the simultaneous detection of viroids and phytoplasmas of pome fruit trees. J. Virol. Methods 213, 12–17.
Mathioudakis, M.M., Maliogka, V.I., Dovas, C.I., Paunović, S., Katis, N.I., 2008. Reliable RT-PCR detection of *Apple stem pitting virus* in pome fruits and its association with quince fruit deformation disease. Plant Pathol. 58, 228–236.
Nassuth, A., Pollari, E., Helmeczy, K., Stewart, S., Kofalvi, S.A., 2000. Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. J. Virol. Methods 90, 37–49.
Németh, M., 1986. Viruses, Mycoplasma and Ricketsia Diseases of Fruit Trees. Kluwer Academic Publishers, Boston.
Posnette, A.F., Cropley, R., Ellenberger, C.E., 1963. The effect of virus infection on the growth and crop of apple, pear, and plum trees. Phytopathol. Mediterranea 2, 158–161.
Saade, M., Aparicio, F., Sanchez-Navarro, J.A., Herranz, M.C., Myrta, A., Di Terlizzi, B., Pallaz, V., 2000. Simultaneous detection of three ilarviruses affecting stone fruit trees by nonisotopic molecular hybridization and multiplex reverse transcription polymerase chain reaction. Phytopathology 90, 1330–1336.
Sánchez-Navarro, J.A., Aparicio, F., Herranz, M.C., Minafra, A., Myrta, A., Pallás, V., 2005. Simultaneous detection and identification of eight stone fruit viruses by one-step RT-PCR. Eur. J. Plant Pathol. 111, 77–84.
Yanase, H., 1983. Back transmission of *Apple stem grooving virus* to apple seedlings and induction of symptoms of apple topworking disease in Mitsuba Kaido (*Malus sieboldii*) and Kobano Zumi (*Malus sieboldii* var. arborescens) rootstocks. Acta Hort 130, 117–122.
Yang, F., Wang, G., Xu, W., Hong, N., 2017. A rapid silica spin column-based method of RNA extraction from fruit trees for RT-PCR detection of viruses. J. Virol. Methods 247, 61–67.
Yao, B., Wang, G., Ma, X., Liu, W., Tang, H., Zhu, H., Hong, N., 2014. Simultaneous detection and differentiation of three viruses in pear plants by a multiplex RT-PCR. J. Virol. Methods 196, 113–119.