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A rapid silica spin column-based method of RNA extraction from fruit trees for RT-PCR detection of viruses

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ABSTRACT

Efficient recovery of high quality RNA is very important for successful RT-PCR detection of plant RNA viruses. High levels of polyphenols and polysaccharides in plant tissues can irreversibly bind to and/or co-precipitate with RNA, which influences RNA isolation. In this study, a silica spin column-based RNA isolation method was developed by using commercially available silica columns combined with the application of a tissue lysis solution, and binding and washing buffers with high concentration guanidinium thiocyanate (GuSCN, 50% w/v), which helps remove plant proteins, polysaccharides and polyphenolic compounds. The method was successfully used to extract high quality RNA from citrus (*Citrus aurantifolia*), grapevine (*Vitis vinifera*), peach (*Prunus persica*), pear (*Pyrus spp.*), taro (*Colocosia esculenta*) and tobacco (*Nicotiana benthamiana*) samples. The method was comparable to conventional CTAB method in RNA isolation efficiency, but it was more sample-adaptable and cost-effective than commercial kits. High quality RNA isolated using silica spin column-based method was successfully used for the RT-PCR and/or multiplex RT-PCR amplification of woody fruit tree viruses and a viroid. The study provided a useful tool for the detection and characterization of plant viruses.

1. Introduction

Reverse transcription polymerase chain reaction (RT-PCR) technique has been widely used for the identification, detection and molecular characterization of plant viruses due to its high specificity and sensitivity (Henson and French, 1993; Mumford et al., 2006). However, successful application of RT-PCR for the detection of plant RNA viruses requires efficient recovery of high quality RNA. The quality and quantity of RNA recovered is strongly influenced by the extraction methods and the plant species (MacKenzie et al., 1997). Woody fruit trees are economically important crops. Virus infection in fruit trees is very common and usually can decrease the tree growth. Virus detection is necessary for the production of certified virus-free propagation materials of fruit trees. The leaves and/or bark tissues of most fruit trees contain some amount of polysaccharides and polyphenolic compounds (Demeke and Adams, 1992; Gambino et al., 2008), which usually are difficult to be removed and inhibit reverse transcription and PCR reactions (Asif et al., 2000; Demeke and Adams, 1992; Jones and McGavin, 2002; Korschineck et al., 1991; Li et al., 2008; Loomis, 1974; Nassuth et al., 2000; Pandey et al., 1996; Salzman et al., 1999; Staub et al., 1995). Moreover, fruit tree viruses usually occur at low titers and with uneven distribution in infected plants, their detection is difficult, which is also a challenge for high-quality RNA recovery.

For plant virus detection, different RNA extraction methods have been developed by using different denaturing organic solvents (phenol and chloroform), reducing agents (β-mercaptoethanol and dithiothreitol), or denaturing agents (guanidinium isothiocyanate salts) and different detergents, such as sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB) or Sarcosyl (Chang et al., 1993; Chomczynski and Cincinnati, 2011; Gehrig et al., 2000; Iandolino et al., 2004; Li et al., 2008; Manning, 1990; Tattersall et al., 2005; Wang et al., 2008). TRIzol − and CTAB −based methods are most widely used in plant RNA extraction (Gambino et al., 2008; MacRae, 2007; Kansal et al., 2008; Kolosova et al., 2004; Zhou et al., 2009). However, TRIzol is not suitable for the isolation of high quality RNA form some woody plant species (e.g., peach, pear and grapevine) (Malnoy et al., 2001; Meisel et al., 2005; Tattersall et al., 2005). Recent research also found that TRIzol showed a bias in mRNA extraction from young tissues of arabidopsis plant (Box et al., 2011). To improve their efficiency for virus detection and suitability for specific plants, some of these methods have been modified (Gambino et al., 2008; Portillo et al., 2006; Kalinowska et al., 2012). It is noted that some of these improved

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methods have been mostly employed for individual crop (Gambino et al., 2008). RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) is the most popular commercial kit for plant RNA extraction. The kit contains a column unit employed to reduce co-purification of polysaccharides or other substances which can interfere in RT-PCR reactions.The spin column-based protocol is convenient and safe, and does not employ toxic organic solvents. However, it is relatively expensive, in some cases, results in low yield and poor quality RNA for plants, which are rich in polyphenolic and polysaccharide compounds (Asif et al., 2000; Gasic et al., 2004; Gambino et al., 2008). Recently, magnetic particles based nucleic acid extraction methods have been reported being effective for different samples (Zhu et al., 2008; Intorasoot et al., 2009; Sun et al., 2014).

To meet requirement for production of certified virus-free propagation materials of fruit trees, it is important to develop a more universal, quick, and reliable RNA extraction method suitable for vast woody plant types. This paper reports the development of a simple, reliable, and rapid silica spin column-based method for RNA extraction from plant tissues in less than 1 h. The efficiency of this rapid protocol is compared with that of five other RNA extraction methods that were previously used for different plants. The suitability of extracted RNA for RT-PCR detection of viruses and a viroid infecting fruit trees is evaluated.

2. Materials and methods

2.1. Plant materials

Young leaf samples collected from tobacco (*Nicotiana benthamiana*), taro (*Colocosia esculenta*), citrus (*Citrus aurantifolia*), peach (*Prunus persica*), grapevine (*Vitis vinifera*) and pear (*Pyrus bretschneideri*) grown in a greenhouse were used to evaluate the efficiency of different RNA extraction protocols. Tobacco and taro represent herbaceous plant species with normal and high content of polysaccharides, respectively. Citrus, peach, grapevine and pear are worldwide grown woody fruit trees. Among these species, peach, grapevine and pear are rich in polysaccharides and polyphenolic compounds, which are relatively less in citrus.

Leaves collected form potted plants of pear infected by *Apple stem pitting virus* (ASPV), citrus infected by *Citrus exocortis viroid* (CEVd) and peach infected by *Cherry green ring mottle virus* (CGRMV), and stem bark tissues of grapevine infected by *Grapevine leaf roll associated virus 3* (GLRaV-3), and were used for virus detections by RT-PCR. *In vitro* cultured pear (*Pyrus communis*) plants were used for the multiplex RT-PCR (mRT-PCR) detection of *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV) and ASPV. The corresponding tissues of virus-free plants of each species were used as negative controls in RT-PCR and mRT-PCR tests.

All samples were collected immediately before being used for RNA extraction.

2.2. RNA extraction

Two silica spin column (SSC)-based RNA extraction methods were optimized and their efficiency were compared with that of five previously reported RNA extraction methods, including TRIzol (Invitrogen, USA), RNeasy plant kit (Qiagen, German), CTAB-LiCl, SDS-phenol and phenol precipitation. TRIzol and RNeasy Plant Mini Kit-based procedures were carried out as indicated in the manufacturer's handbook. CTAB-LiCl (Li et al., 2008), SDS-phenol (Gandía et al., 2007) and phenol precipitation (Chomczynski and Cincinnati, 2011) methods were performed as described previously. For each method, 0.1 g of each sample was powdered in liquid nitrogen and the resulted RNA extract was dissolved in a total of 70 µl RNase-free water.

For silica spin column based RNA extraction methods, all the centrifuge steps were performed at room temperature. Buffer solutions used in the protocols included sample lysis buffer, binding and washing buffers. For binding and washing buffers, initially three chaotropic salts, including guanidine thiocyanate (GuSCN), guanidine hydrochloride (GuHCl) and sodium iodide (NaI), were tested for their effects on the yield and quality of RNA. Then, the dosages of all inorganic salts were optimized. Silica columns were purchased from three manufacturers, including Sangon Co., Ltd (Shanghai, China), More Biotech (Wuhan, China) and Aidlab Biotechnologies Co., Ltd (Beijing, China). The optimized solutions and protocols for SSC-based RNA extraction methods were as follows.

(1) SSC-based protocol I (SSC-PI). The sample powdered in liquid nitrogen was quickly transferred into a tube and 700 µl of lysis buffer [2% SDS (w/w), 4% PVP-40 (w/w), 0.5 M NaCl, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 12.5% β-mercaptoethanol] was added. After vigorous vortex, the sample was incubated at 65 °C for 10 min and centrifuged at 12000*g* for 5 min. Equal volume (700 µl) of binding buffer [50% Guanidine thiocyanate (GuSCN) (w/w), 1.5 M KCl, 0.3 M NH_4Cl, 0.3 M KAcO (pH 5.5)] was added into the tube. The tube was immediately inverted for several times, and then was kept standing at room temperature for 2 min to let detergent be precipitated. After centrifugation at 12000*g* for 10 min, the supernatant was transfer to a new tube, and mixed with 0.5 vol of 100% ethanol. Next, the mixture was added into a silica spin column (Sangon, Shanghai, China. Catalogue number: SD5005) and centrifuged at 15000*g* for 1 min. The column was washed with 700 µl wash buffer wash buffer [50% GuSCN (w/w), 1.5 M NaCl, 50 mM KAcO (pH 5.5), ethanol 37% (v/v)] for one time and with 700 µl 80% ethanol (v/v) for two times. Then, the column was moved into a new tube, and 70 µl RNase-free water preheated at 65 °C was directly loaded onto the silica membrane in the column. After standing at room temperature for 3 min to let RNA be dissolved in the water, and followed by centrifugation at 12000*g* for 1 min, the obtain RNA solution was stored at −80 °C until to be used.

(2) SSC-based protocol II (SSC-PII). SSC-PII was a simplified protocol from SSC-PI by omitting the lysis buffer at the first step, and the sample powder was directly transferred into a tube containing 700 µl binding buffer.

It should be noted: (1) β-mercaptoethanol should be added into the lysis buffer just before it was used. (2) To avoid the reactions between GuSCN and other components, during the preparation of binding buffer and wash buffer, KAcO and ethanol should be added at last when the solution was completely cool to room temperature. (3) All reagents were prepared with DEPC-treated water.

2.3. Quantification and qualification of RNA

The quality and yield of obtained RNA extracts from different plant species was assessed by the spectrophotometric absorbance ratios of A260/A280 and A260/A230. RNA integrity was evaluated on 1.2% agarose gels stained with ethidium bromide.

2.4. Simplex and multiplex RT-PCR

First strand cDNA was synthesized by using 0.5 µM of the reverse random primers (Takara, Dalian, China), M-MLV reverse transcriptase (Promega, Madison, USA) and 4 µl RNA extract in a 10 µl reaction solution at 37 °C for 1 h. Primers used for the detection of ASPV, CEVd, GLRaV-3 and CGRMV by simplex RT-PCR and the simultaneous detection of ASPV, ASGV and ACLSV of pear samples by multiplex RT-PCR were listed in Table 1. PCR reaction was performed using 2 µl cDNA template, 0.1 mM of each dNTP, 0.2 µM of each primer, one unit of *Taq* DNA polymerase (TaKaRa, Dalian, China), in a 20 µl reaction solution. The thermal cycling condition consisted of initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s (the temperatures required were listed in Table 1), and extension 45 s at 72 °C followed by a final incubation at 72 °C for 10 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized

Table 1
Primers used for virus detection by simplex or multiplex RT-PCR.

Virus	Primer	Sequence (5′−3′)[a]	Tm (°C)	Target gene	Product size (bp)
Simplex RT-PCR					
ASPV	ASPV-F	ATGTCTGGAACCTCATGCTGCAA	55	CP	370
	ASPV-R	TTGGGATCAACTTTACTAAAAAGCATAA			
GLRaV-3	GLRaV-F	CAGGAAACCGATATAGGGGTAG	50	CP	315
	GLRaV-R	TCGAACTCTTTGAACTCTGTCG			
CGRMV	CGRMV-F	TGCGGGAAATCAACTCTTGTC	54	TGB1	363
	CGRMV-R	TGTGCCACCAAACACCTTAC			
CEVd	CEVd-F	CCGGGGATCCCTGAAGGACTT	60	genome	371
	CEVd-R	GGAAACCTGGAGGAAGTCGAG			
Multiplex RT-PCR					
ASPV	ASPV-mF	CAGTATTGTGCCTTYTAYGCRAAGC	60	CP	260
	ASPV-mR	CCATAGAACGGATGCGGTACATYTG			
ASGV	ASGV-F	CCCGCTGTTGGATTTGATACACCTC	60	CP	499
	ASGV-R	GGAATTTCACACGACTCCTAACCCTCC			
ACLSV	ACLSV-F	CAGACCCTTATTGAAGTCGAA	60	CP	358
	ACLSV-R	GGCAACCCTGGAACAGA			
Actin[b]	Actin-F	CTCCCAGGGCTGTGTTTCCTA	60	actin	172
	Actin-R	CTCCATGTCATCCCAGTTGCT			

[a] Degenerate base: Y = C/T, R = A/G.
[b] Actin gene used as amplification internal control.

under UV illumination after being stained with ethidium bromide (0.5 μg/ml).

Multiplex RT-PCR test was carried out according our previously reported method (Yao et al., 2014). An actin gene (GenBank ID: CF837237) was used as an internal control.

3. Results

3.1. The comparison of RNA extraction efficiency by different protocols

Total RNA was extracted from the leaf samples of tobacco, citrus, pear, peach GF305 seedling, grapevine and taro using two silica spin column-based protocols, and other five methods, namely, CTAB-LiCl, SDS-phenol, phenol precipitation, and commercial TRIzol and RNeasy kits (Table 2). The silica spin column-based protocol I (SSC-PI), CTAB-LiCl and SDS-phenol methods successfully extracted RNA from all tested plant samples. It was noticed that in all tested samples, the RNA isolated using SSC-PI was relatively less contaminated by protein, polysaccharide and phenolic compounds or other reagents as indicated by the A260/A280 ratios ranging from 1.79 ± 0.03 to 2.08 ± 0.03 and A260/A230 ratios ranging from 1.57 ± 0.08 to 2.47 ± 0.06, respectively, with RNA yields ranging from 210 ± 30.7 to 994 ± 102.4 μg/g sample (Table 2). The RNA extraction efficiency of other four methods, including PII, TRIzol, RNeasy plant kit and phenol precipitation, was highly affected by the conditions of plant samples. The standard TRIzol method performed following the manufacturer's instructions failed in RNA extraction from pear, peach, grapevine and taro samples. RNeasy plant kit, acid phenol precipitation and SSC-PII failed in RNA extraction or produced low RNA yields from pear and grapevine samples. Among six tested plant species, RNAs from tobacco and citrus samples were successfully extracted by all methods used in the study.

RNA integrity was further confirmed by agarose gel electrophoresis, which showed that the bands of 28S and 18S ribosomal RNAs (rRNA) extracted using SC-PI were clear and the intensity of 28S rRNA band was about 1.5-fold higher than that of 18S rRNA. However, low molecular weight smears were observed in the agarose gel for RNA samples extracted using CTAB-LiCl and SDS-phenol protocols, indicating that the RNA was not intact (Fig. 1). Among tested samples, tobacco and citrus samples always gave the best RNA quality and quantity.

3.2. Qualification for RNA extracts by simplex RT-PCR detection for viruses and a viroid of woody fruit trees

Firstly, total RNAs were extracted from bark tissue of seven grapevine plants using SSC-PI, CTAB-LiCl and TRIzol, and subjected to RT-PCR detection of GLRaV-3. Among these plants, two plants were GLRaV-3 negative and five plants showing leaf roll symptom were known to be positive for GLRaV-3. Results showed that the expected product of GLRaV-3 was amplified from all GLRaV-3 infected samples when RNA extracts obtained by using SSC-PI and CTAB-LiCl methods were used as templates (Fig. 2). However, when the RNA extracts obtained using TRIzol reagent were used as templates, RT-PCR failed in the detection of GLRaV-3. Then, the RT-PCR result for virus detection was in consistent with that quantified with 260/280 values and agarose gel electrophoresis.

To determine efficiency of the optimized rapid protocol SSC-PI for the detections of viruses and a viroid infecting other fruit trees, leaf samples of six pear plants, seven peach plants, and leaf and bark samples of two citrus plants were subjected to RNA extraction. RT-PCR tests using virus- and viroid-specific primers (Table 1) showed that all ASPV-infected pear and CGRMV-infected peach samples produced expected viral products of 370 bp and 363 bp (Fig. 3), respectively. Similarly, RT-PCR successfully detected CEVd in leaf and bark samples of citrus. Moreover, consistent band density of RT-PCR products of ASPV, CGRMV and CEVd in different samples of each plant species as visualized on the gels indicated steady RNA quality and quantity obtained with SSC-PI.

3.3. Efficiency of RNA extracts for multiplex RT-PCR detection of pear viruses

Furthermore, the efficiency of the optimized RNA extraction protocol SSC-PI was tested for the simultaneous detection of ASPV, ASGV and ACLSV infecting plants of an *in vitro*-cultured pear line by multiplex RT-PCR (mRT-PCR) and compared with that of TRIzol and CTAB-LiCl (Fig. 4). Results showed that RNA extracted using SSC-PI was suitable for mRT-PCR detection of pear viruses (Fig. 4A) and its efficiency was comparable with that of CTAB-LiCl (Fig. 4B). The similar product intensity of either these viruses or an actin gene from individual plant of an *in vitro*-cultured pear line, no matter obtained by RT-PCR or mRT-PCR, indicated a good stability of the methods. However, TRIzol reagent failed for both RT-PCR and mRT-PCR detections of the three

Table 2
Purity and yield (mean ± SD) of total RNA extracted from leaves of tobacco, citrus, grapevine, peach, taro and pear plants by seven protocols.

Protocol	Time required	Source[a]	A260/A280	A260/A230	RNA yield (μg/g)
SSC-PI	45 min	tobacco	1.79 ± 0.03	2.13 ± 0.07	689 ± 67.5
		citrus	1.83 ± 0.04	2.47 ± 0.06	994 ± 102.4
		pear	2.08 ± 0.03	1.65 ± 0.09	485 ± 50.4
		peach	1.98 ± 0.05	1.62 ± 0.04	697 ± 68.7
		grapevine	1.88 ± 0.06	1.69 ± 0.05	783 ± 82.4
		taro	1.9 ± 0.04	1.57 ± 0.08	210 ± 30.7
CTAB-LiCl	4 h	tobacco	1.9 ± 0.16	1.65 ± 0.04	394.6 ± 52.34
		citrus	1.85 ± 0.18	1.44 ± 0.06	185.3 ± 41.3
		pear	1.81 ± 0.08	1.32 ± 0.04	129.5 ± 49.3
		peach	1.76 ± 0.11	2.24 ± 0.03	358.2 ± 62.6
		grapevine	1.72 ± 0.07	2.60 ± 0.05	760 ± 101.3
		taro	1.87 ± 0.13	2.00 ± 0.04	218 ± 55.4
SDS-phenol	3.5 h	tobacco	1.74 ± 0.07	2.68 ± 0.03	436.5 ± 72.5
		citrus	1.76 ± 0.09	2.69 ± 0.05	1133.8 ± 101.3
		pear	1.76 ± 0.1	2.32 ± 0.03	155.7 ± 58.3
		peach	1.74 ± 0.04	2.22 ± 0.11	210 ± 46.3
		grapevine	1.54 ± 0.08	0.77 ± 0.03	873.1 ± 101.2
		taro	1.81 ± 0.13	2.11 ± 0.06	317 ± 32.4
TRIzol	40 min	tobacco	1.93 ± 0.9	1.74 ± 0.05	756.8 ± 114.3
		citrus	1.71 ± 0.06	2.50 ± 0.18	2352 ± 159.4
		pear	1.03 ± 0.04	0.20 ± 0.05	–
		peach	1.56 ± 0.03	0.33 ± 0.04	–
		grapevine	0.91 ± 0.05	0.09 ± 0.01	–
		taro	1.84 ± 0.03	0.51 ± 0.07	–
Phenol precipitation	1 h	tobacco	1.87 ± 0.04	1.63 ± 0.08	615 ± 68.3
		citrus	1.74 ± 0.02	1.87 ± 0.04	1608 ± 102.0
		pear	1.37 ± 0.05	0.45 ± 0.06	–
		peach	1.54 ± 0.05	0.52 ± 0.04	742.5 ± 89.4
		grapevine	1.33 ± 0.03	0.20 ± 0.04	–
		taro	1.92 ± 0.04	1.23 ± 0.06	904.5 ± 129.0
RNeasy plant kit	30 min	tobacco	1.9 ± 0.04	1.24 ± 0.02	388.7 ± 58.5
		citrus	2.03 ± 0.06	0.77 ± 0.03	690.6 ± 73.3
		pear	1.08 ± 0.02	0.18 ± 0.03	–
		peach	2.33 ± 0.05	0.84 ± 0.02	460 ± 78.3
		grapevine	3.41 ± 0.06	0.36 ± 0.04	–
		taro	1.85 ± 0.04	0.5 ± 0.06	243 ± 58.8
SSC-PII	35 min	tobacco	1.95 ± 0.06	1.81 ± 0.05	360 ± 48.3
		citrus	1.92 ± 0.03	1.87 ± 0.07	596 ± 67.7
		pear	1.84 ± 0.04	0.43 ± 0.05	32.8 ± 13.4
		peach	1.96 ± 0.06	0.65 ± 0.07	217 ± 53.6
		grapevine	2.00 ± 0.14	0.73 ± 0.08	–
		taro	2.28 ± 0.07	0.74 ± 0.04	93 ± 35.7

[a] For each plant species, one test consisted of 10 leaves, and three independent tests were taken.

viruses (Fig. 4C).

4. Discussion

The routine applications of RT-PCR techniques for the detection of plant RNA viruses largely depend on the isolation of sufficient and high-quality RNA preparations from a large number of samples in a short period. In this study, a highly reliable method (SSC-PI) for RNA preparation from woody fruit trees was developed. The method was based on the commercially available silica columns and the utilization of optimized RNA lysing and binding reagents. The optimized protocol had the advantage of RNeasy kit in that it avoided the use of organic solvents and phenol, and was rapid. Moreover, the yield and quality of RNAs from all tested samples was good and superior to RNeasy plant kit. RNA extracts from woody fruit trees were adequate for the applications in RT-PCR detection of viruses in these host plants. The efficiency of RNeasy kit for RNA extraction was highly affected by the conditions of samples. It failed in RNA recovery from pear and grapevine samples, which represented fruit tree species with difficulty for RNA extraction. The efficiency of silica spin column-based method SSC-PI was comparable with CTAB- and SDS-based methods, but more time and labor saving than that of the last two methods. Previously, silica capture of viral RNA and antibody-capture of virus particles were described to be effective for subsequent RT-PCR tests of some viruses and viroids infecting fruit trees (Rott and Jelkmann, 2001; Sun et al., 2014; Wetzel et al., 1991). Silica capture was a fast method, but it was not effective for viral RNA isolation from grapevine tissues (Gambino et al., 2008). The protocol SSC-PI described here was based on commercially available column with silica conjugated into a membrane. Therefore, SSC-PI did not need to prepare the silica solution (Rott and Jelkmann, 2001) or to encapsulate magnetic particles (Sun et al., 2014) and provided a convenient tool for RNA extraction. On the other hand, the successful RNA extraction from tobacco and citrus samples with all tested methods, but the failure from other four species with one or more methods confirmed that the rich polysaccharides and polyphenols in pear, peach, grapevine and taro strongly interfered in the RNA extraction efficiency (Demeke and Adams, 1992; Gasic et al., 2004).

The oxidization products of polyphenol and quinine compounds can irreversibly bind to RNA (Loomis, 1974; Salzman et al., 1999), and polysaccharides can co-precipitate with RNA in the presence of ethanol, and subsequently inhibit enzymatic reaction in subsequence RT-PCR (Asif et al., 2000; Levi et al., 1992; Lopez-Gomez and Gomez-Lim, 1992; Schneiderbauer et al., 1991). To remove these substances form RNA solution, some modifications were adopted for conventional CTAB-based method and also for RNeasy kit. Additional extraction buffer or step before using the RNeasy kit increased the binding capacity of RNeasy columns by removing some contaminants, thus improved the efficiency of RNA isolation from grapevine tissues (Gambino et al.,

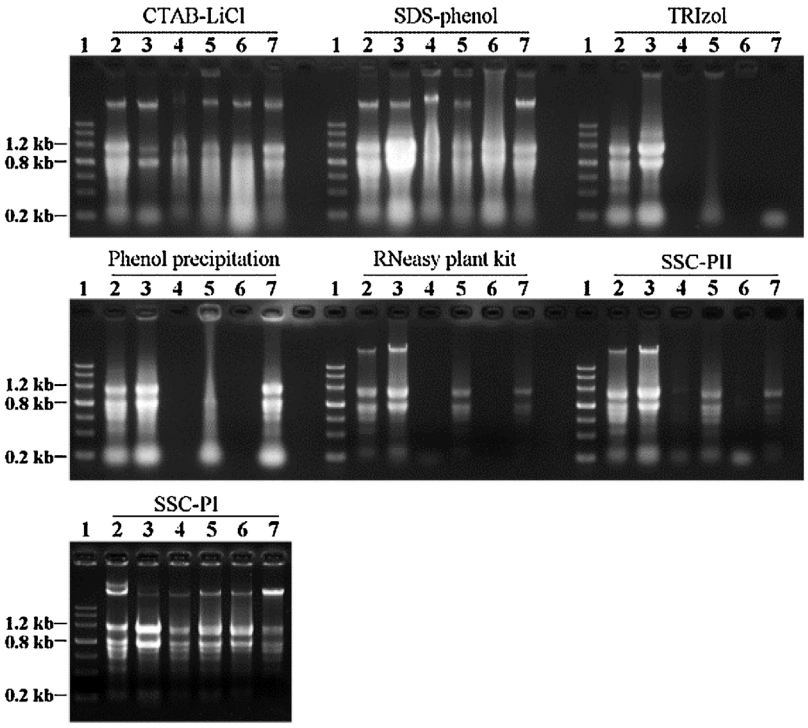

Fig. 1. Agarose gel electrophoresis of total RNA extracted by CTAB-LiCl, SDS-phenol, TRIzol, phenol precipitation, RNeasy plant kit and two silica spin column (SSC-PI and SSC-PII) based methods from tobacco (lane 2), citrus (lane 3), pear (lane 4), peach (lane 5), grapevine (lane 6) and taro (lane 7) samples. Lane 1: DNA marker.

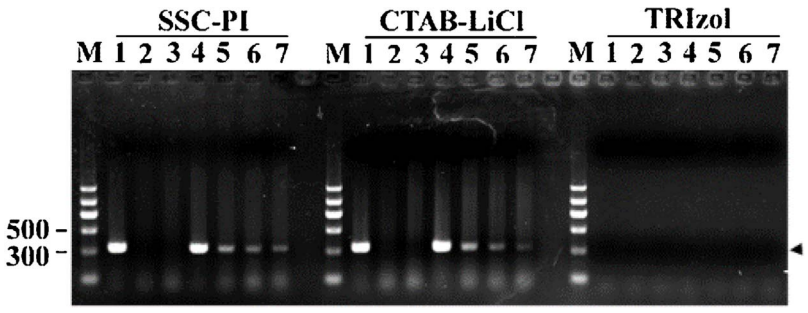

Fig. 2. Comparison of total RNA extracted by SSC-PI, CTAB-LiCl and TRIzol methods for the reverse transcription-polymerase chain reaction (RT-PCR) detection of *Grapevine leafroll-associated virus 3* (GLRaV-3) in bark tissues of seven grapevine plants. Lane M, DNA ladder; Lane 1 and lanes 4–7, GLRaV-3 infected grapevine plants; Lanes 2–3, healthy grapevine plants. The target band of GLRaV-3 was indicated by bold arrows.

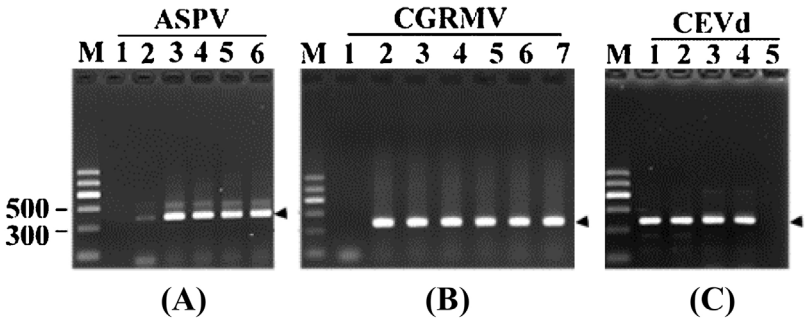

Fig. 3. Evaluation of total RNA extracted using the SSC-PI method for the reverse transcription-polymerase chain reaction (RT-PCR) detection of *Apple stem pitting virus* (ASPV) in pear (A), *Cherry green ring mottle virus* (CGRMV) in peach (B) and *Citrus exocortis viroid* (CEVd) in citrus (C) samples. Lanes A1, B1 and C5, healthy pear, peach and citrus samples used as negative controls. Target band of each virus or viroid was indicated by bold arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2008; MacKenzie et al., 1997). In this study, the application of a tissue lysis solution containing 2% SDS (w/v) combined with 4% PVP-40 (w/v) in a Tris-HCl and EDTA buffer system (pH 8.0) was an important and indispensable step of the silica column-based method for enhancing the reproducibility of RNA extractions from diverse host species. The SDS and PVP removed most proteins, polysaccharides, polyphenolic compounds and other inhibitors from plant samples as indicated by high RNA quality and yield from grapevine and pear samples, which were highly rich in these compounds. The absent of the tissue lysis buffer in a similar silica column based protocol II (SSC-PII) resulted in very low yield and poor quality of RNA extracts from pear, grapevine and taro samples, suggesting that the existence of those inhibition compounds in tissue lysates could decrease the efficiency of RNA banding into silica membrane in the spin columns. The application of high concentration GuSCN (w/v 50%) in subsequent binding and washing buffers also enhanced removing denatured proteins and other inhibitors. Notably, recent reports indicated that silica-coated magnetic particles was efficient for RNA or DNA extraction due to removing inhibitor from different samples (Zhu et al., 2008; Intorasoot et al., 2009; Sun et al., 2014). Whether it is comparable with SSC-PI needs further evaluation.

The successful amplification of CGRMV, GLRaV-3 , ASPV and a viroid CEVd by using RNA extracts obtained with the SSC-PI indicated that the method was efficient in removing RNA inhibitors in these plants. The efficiency of SSC-PI applied in GLRaV-3 detection by RT-PCR and simultaneous detection of three pear viruses by mRT-PCR was comparable to that of CTAB-LiCl method (Li et al., 2008). Our result

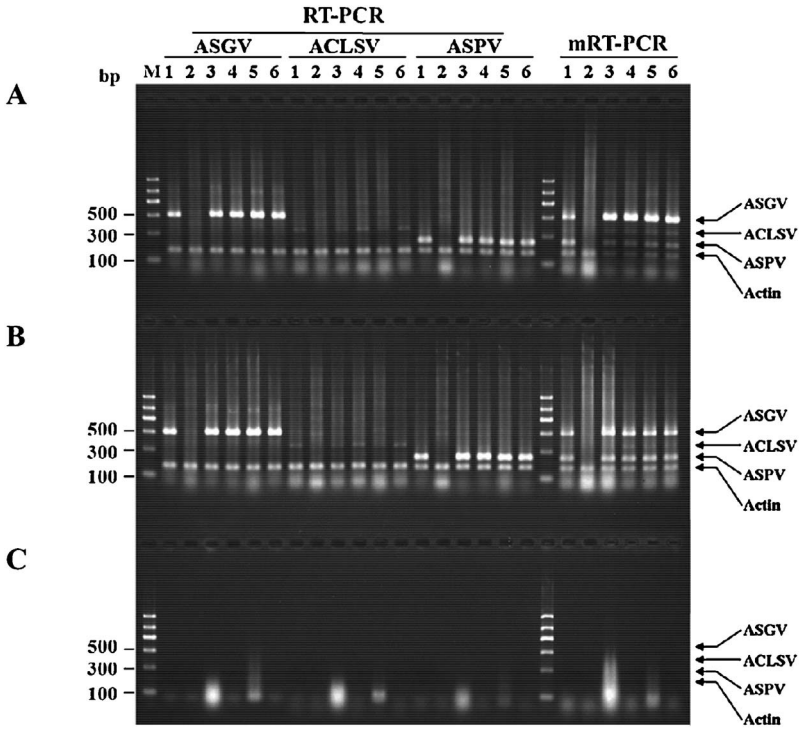

Fig. 4. Comparison of total RNA extracted by CTAB-LiCl (A), SSC-PI (B) and TRIzol (C) methods for the simplex and multiplex reverse transcription-polymerase chain reaction (RT-PCR) detection of ASPV, ASGV and ACLSV infecting *in vitro*-cultured pear plants. Lane M, DNA ladder; Lane1 and lines 3–6, ASPV, ASGV and ACLSV positive pear plants; Lane 2, healthy pear sample.

also confirmed that TRIzol reagent was not suitable for RNA isolation from grapevine and pear samples as indicated by very low A260/A280 and A260/A230 values and the failure in the RT-PCR detection of viruses (Malnoy et al., 2001; Meisel et al., 2005; Tattersall et al., 2005). Then, to resolve the problem, some modifications for the TRIzol procedure will be necessary to remove inhibitors as reported previously (Chomczynski and Mackey, 1995). Pear tissues contained considerable amounts of polysaccharides and polyphenolics (Malnoy et al., 2001; Wilson and Blunden, 1983), which made the isolation of high quality RNA from pear tissues particularly difficult and strongly interfered in the PCR amplification efficiency (Demeke and Adams, 1992; Pandey et al., 1996; Staub et al., 1995). SSC-PI produced high-yield and good-quality RNA extracts from pear leaf samples were suitable not only for the individual detection of ASPV, ASGV and ACLSV in pear plants by simplex RT-PCR, but also for the simultaneous high through-put detection of these viruses in pear samples by multiplex RT-PCR. SSC-PI method described here was suitable for dealing a large amount of samples in a limited period (less than one hour). The method has already been adopted for the routine RT-PCR or RT- LAMP detections and genome amplification of viruses and viroids in various fruit trees in our laboratories. In fact, the method reported here has been successfully used for RT-LAMP analysis of pear viruses in our lab. Additionally, the steady quality and quantity of RNA extracts obtained with SSC-PI allowed the consistent amplification of ASPV, CGRMV and CEVd in different samples suggested that the method should be applicable for further qPCR analysis.

In conclusion, this paper reports an optimized silica spin column-based RNA extraction protocol SSC-PI, which is fast and has good reliability and repeatability for the application in RT-PCR detection of viruses and viroids of different fruit tree species.

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