A RAPID AND EFFECTIVE RNA RELEASE PROCEDURE FOR VIRUS DETECTION IN WOODY PLANTS BY REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

J.K. KUNDU

Department of Virology, Research Institute of Crop Production, Drnovská 507, 161 06 Prague 6, Czech Republic

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Summary. – A rapid and effective RNA release procedure (RNA-RP) for detection of the Apple stem pitting virus (ASPV) and the Apple stem grooving virus (ASGV) in woody plants by reverse transcription–polymerase chain reaction (RT-PCR) was developed. RNA-RP released RNA into crude homogenate. RNA-RP was compared with classical phenol/chlorophorm extraction of RNA. The RT-PCR detection of ASPV and ASGV was shown to be similar by both the RNA preparation procedures. RNA-RP in contrast to the classical phenol/chloroform procedure represents a reliable and easy-to hand protocol convenient for routine virus detection. Leaf tissues, especially dormant bud leaves and leaves during blossom, were shown to be the most optimal material for detection of these viruses, irrespective of the used RNA preparation procedure.

Key words: ASPV; ASGV; RT-PCR

Introduction

A molecular amplification-based assay, the RT-PCR has been widely used for detection of fruit tree viruses, namely ASPV (Schwarz and Jelkmann, 1998; Kummert *et al.,* 1998), the *Foveavirus* genus (Martelli and Jelkmann, 1998) and ASGV (Kinard *et al.,* 1996; MacKenzie *et al.,* 1997) the *Capillovirus* genus (Yoshikawa *et al.,* 1992). Nevertheless, extraction of total RNA from a fruit tree material remains a major difficulty, because woody plants contain high amounts of components like polyphenols and polysaccharides. These components usually co-purify with nucleic acids, RNA and DNA, and are believed to inhibit the sensitivity of PCR or RT-PCR (Mitra and Kootstra, 1993). It is likely that their amounts differ among different tissues at different time of the year (Stewart and Nassuth, 2001). A good number of modifications of phenol extraction procedures and commercial purification kits have been used for RNA preparation from fruit tree materials with more or less success. Also some RNA and DNA release procedures have been previously described (Thomson and Dietzgen, 1995). A few protocols of RT-PCR without using total RNA have been previously applied to detection of these viruses, which included immunocapture/RT-PCR for ASPV (Jelkmann and Keim-Konrad, 1997) and immunocapture/RT-PCR, tube capture/RT-PCR (James, 1999) for ASGV. Crude extracts of apple leaves have been also used in RT-PCR (Marinho *et al.,* 1998) for ASGV detection.

This paper reports on a rapid and sensitive RNA release procedure, RNA-RP for the detection of ASPV and ASGV by RT-PCR in field-grown apple trees. The RNA-RP was compared with the classical phenol-chloroform procedure for extraction of total RNA regards their sensitivity and

E-mail: jiban@vurv.cz ; fax: +4202-33311592.

Abbreviations: ASPV = Apple stem pitting virus; ASGV = Apple stem grooving virus; BYV = Beet yellows virus; DEPC = diethyl pyrocarbonate; DTT = dithiothreitol; EDTA = ethylenediamine tetraacetate; M-MLV = Moloney murine leukemia virus; RNA-RP = RNA release procedure; RT-PCR = reverse transcription–polymerase chain reaction; SDS = sodium dodecyl sulfate

efficiency. The suitability of different apple tissues as sample materials was also evaluated.

Materials and Methods

Trees, cultivars, samples and virus isolates. Four cultivars of field-grown *Malus domestica* Borkh including Idared, Spartan, Vista Bella and Stark Earliest without any artificial infection were tested. Dormant bark, dormant leaf buds, leaves during flowering, petals and leaves after flowering were sampled. The apple trees from the orchard Horoměřice were tested according to our previous virus testing of this orchard (Kundu, 2002a). An ASPV apple isolate (IPO, Wageningen, Germany) maintained in *Nicotiana occidentalis* 37B and another apple isolate of ASGV (Univerrsity of Halle, Wittenberg, Germany) maintained in *Chenopodium quinoa* were used as positive controls.

RNA-RP. Apple tree tissues, ASPV-infected *N. occidentalis* 37B leaves with symptoms and ASGV-infected *C. quinoa* leaves with symptoms were ground in a mortar with pestle, and diluted 10^{-1} in diethylpyrocarbonate (DEPC)-treated sterile water. The homogenates were transferred into 1.5 ml sterile centrifuge tubes, which were spun down at 10,000 x g for 10 mins and the supernatants were transferred into new tubes. The supernatants of apple tissues were diluted 10^{-2}. Dilutions of 10^{-1}–10^{-8} were prepared from the supernatants from *N. occidentalis* 37B and *C. quinoa* in a Tris-KCl-$MgCl_2$-DTT buffer (50 mmol/l Tris-HCl pH 8.3, 75 mmol/l KCl, 3 mmol/l $MgCl_2$ and 10 mmol/l DTT) or 1% Triton X-100 made in DEPC-treated sterile water. The tubes were then incubated at 60°C for 5 mins with time-to-time mixing. The tubes were put in chilled ice for 5 mins and thus treated saps were used in RT-PCR.

Phenol/chloroform extraction of total RNA. Apple tree tissues (*M. domestica* Borkh) and indicator host tissues (*N. occidentalis* 37B for ASPV or *C. quinoa* for ASGV) were ground in a mortar with pestle using an extraction buffer (20 mmol/l Tris-HCl pH 7.8, 200 mmol/l LiCl, 20 mmol/l EDTA and 1% SDS) diluted 1:20. The extract was transferred to sterile centrifuge tubes and centrifuged at 10,000 x g for 10 mins. The supernatants (500 µl) were removed into sterile centrifuge tubes; 5 µl of proteinase K (20 mg/ml) was added and incubated at 37°C for 1 hr. The supernatants (500 µl) were mixed with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) for 10 mins by vortexing and centrifuged at 10,000 x g for 10 mins. The aqueous phase (400 µl) was taken into new sterile centrifuge tube and the process was repeated twice.

The aqueous phase (400 µl) was then mixed with a triple volume of absolute ice-cold ethanol (99.8 % v/v) and with 80 µl of 10 mol/l LiCl and the mixture was stored at -20°C for 1 hr to precipitate nucleic acids. The precipitate was collected by centrifugation at 20,000 x g for 10 mins and washed twice for 30 mins in 70% ethanol (v/v) at room temperature. Then it was again centrifuged at 20,000 x g for 8 mins, ethanol was removed and the pellet was dried under vacuum, resuspended in 40 µl of sterile deionized DEPC-treated water and stored at -20°C until used.

Two-step RT-PCR was done according to Kundu (2002a).

One-step RT-PCR was performed with the one-step RT-PCR kit (Qiagen) as follows: the one-step RT-PCR mixture containing 5 µl of the 5x Qiagen one-step RT-PCR buffer, 1 µl of dNTP mixture (10 mmol/l dNTPs), 1 µl of the Qiagen one-step RT-PCR enzyme mixture, 1 µl of Q solution, 10 pmoles of upstream and downstream primers ASP-A/ASP-C for ASPV (Table 1) (Jelkmann and Keim-Konrad, 1997) or ASGV-2/ASGV-U for ASGV (James, 1999) were prepared in microtubes and 5 µl of the sap prepared by RNA-RP or 1 µl of RNA prepared by the phenol/chloroform method were added. The mixture was adjusted to 25 µl with RNase-free water. The reaction was carried out in a thermocycler (MJ Research) as follows: a RT step at 50°C for 30 mins and an initial PCR activation step at 95°C for 15 mins, then 33 cycles of 94°C for 30 secs (denaturation), 55°C for 45 secs (annealing), and 72°C for 80 secs (extension). After the last cycle a final extension step at 72°C for 10 mins was added.

Analysis of amplified products. Aliquots of the PCR products were analyzed by agarose gel (1.5% for ASGV and 2% for ASPV) electrophoresis in TBE buffer (90 mmol/l Tris-borate and 2 mmol/l EDTA) at 120 V for 45 mins. The RT-PCR products were stained with ethidum bromide (0.5 µg/ml), visualized under a UV transilluminator and photographed with Polaroid system. Size of fragments was determined using DNA size markers (MBI Fermentas).

Results and Discussion

The RNA preparation protocols, RNA-RP and total RNA extraction procedure by phenol/chloroform described here were successful for the detection of ASPV and ASGV in apple tree cultivars. The specific 264 bp fragment using the primer pair ASP-A/ASP-C for ASPV genome and that of 499 bp using the primer pair ASGV-U/ASGV-2 for ASGV genome were amplified from apple trees of all tested cultivars except the healthy control (Figs 1 and 2). ASPV and ASGV were detected with similar efficiency using RNA-RP and the phenol/chloroform extraction procedure.

The survey of sampled tissues showed detectable amounts of ASPV and ASGV in all tissues of infected apple trees. Dormant bark and petals showed lesser amounts of these viruses (Table 2). However, leaf tissues in general (especially leaf buds and leaves during flowering appeared to be appropriate sample materials irrespective of the RNA preparation procedure.

In ASGV detection protocols crude extracts have been used by Marinho *et al.* (1998) in the one-step Titan RT-PCR and it has not proved as reliable as the two-step RT-PCR (Kirby *et al.,* 2001). In contrast to the findings of Kirby *et al.* (2001), in detecting ASPV and ASGV using the RNA extracted by phenol/chloroform we did not observe any difference between the one-step RT-PCR (the Quiagen kit) and the two-step RT-PCR.

However, in detecting the viruses with RNA-RP we prefer the one-step RT-PCR over the two-step RT-PCR, because in the one-step RT-PCR, in which the entire reaction is carried out in a single tube the risk of contamination is significantly lower. The released RNA is ready for RT-PCR in less than

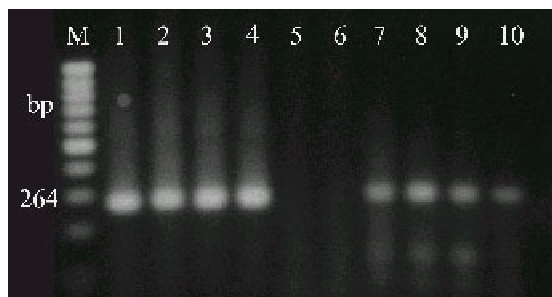

Fig. 1

Agarose gel electrophoresis of products of one-step RT-PCR of ASPV

DNA size marker, 100 bp ladder (lane M), apple tree leaves, the phenol/chloroform method (lanes 1–4), negative control, the phenol/chloroform method and RNA-RP (lanes 5 and 6), apple tree leaves, RNA-RP (lanes 7–10).

Fig. 2

Agarose gel electrophoresis of products of one-step RT-PCR of ASGV

DNA size marker, 100 bp ladder (lane M), apple tree leaves, the phenol/chloroform method (lanes 1–4), negative control, the phenol/chloroform method and RNA-RP (lanes 5 and 6), apple tree leaves, RNA-RP (lanes 7–10).

Table 1. Primers used in the study

Virus	Primer	Sequence (5'-3')	Position[a]	Product size
ASPV	ASP-C (sense)	CTCTTGAACCAGCTGATGGC	8993–9012	264 bp
	ASP-A (anti-sense)	ATAGCCGCCCCGGTTAGGTT	9237–9256	
ASGV	ASGV-U (sense)	CCCGCTGTTGGATTTGATACACCTC	5873–5897	499 bp
	ASGV-2 (anti-sense)	GGAATTTCACACGACTCCTAACCCTCC	6345–6371	

[a]The reference Acc. Nos. (NCBI) for the determination of the primer positions are NC 003462 for ASPV and NC 001749 for ASGV.

Table 2. Comparison of RNA-RP with phenol/chloroform RNA extraction for ASPV and ASGV detection by RT-PCR

Apple tree cultivar	Tissue	ASPV-positive/total		ASGV-positive/total	
		RNA-RP	Phenol/chloroform	RNA-RP	Phenol/chloroform
Idared	Dormant bark	5/10	5/10	4/10	3/10
	Dormant leaf buds	8/10	8/10	6/10	6/10
	Leaves during flowering	8/10	8/10	6/10	6/10
	Petals	8/10	7/10	5/10	6/10
	Leaves after flowering	8/10	8/10	6/10	5/10
Spartan	Dormant bark	4/10	3/10	7/10	8/10
	Dormant leaf buds	8/10	8/10	9/10	9/10
	Leaves during flowering	8/10	8/10	9/10	9/10
	Petals	0/10	5/10	3/10	9/10
	Leaves after flowering	7/10	7/10	9/10	8/10
Stark Earliest	Dormant bark	6/10	7/10	5/10	5/10
	Dormant leaf buds	8/10	8/10	10/10	9/10
	Leaves during flowering	8/10	8/10	10/10	10/10
	Petals	0/10	3/10	0/10	10/10
	Leaves after flowering	4/10	4/10	10/10	10/10
Vista Bella	Dormant bark	4/10	5/10	0/10	1/10
	Dormant leaf buds	7/10	7/10	2/10	2/10
	Leaves during flowering	6/10	7/10	2/10	2/10
	Petals	1/10	7/10	0/10	2/10
	Leaves after flowering	7/10	7/10	3/10	2/10

Fig. 3

Agarose gel electrophoresis of products of one-step RT-PCR of ASPV using RNA-RP

DNA size marker, 100 bp ladder (lane M), crude sap diluted 10^{-1}–10^{-8} (lanes 1–7), negative control (lane 8).

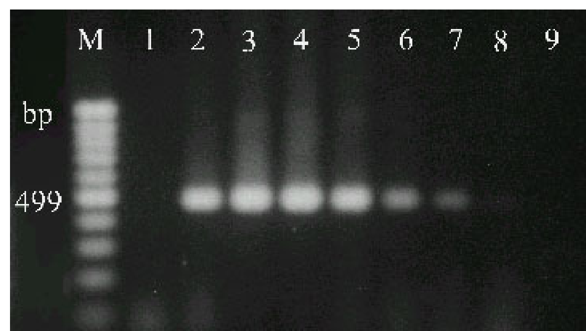

Fig. 4

Agarose gel electrophoresis of products of one-step RT-PCR of ASGV using RNA-RP

DNA size marker, 100 bp ladder, crude sap diluted 10^{-1}–10^{-8} (lanes 1–8), negative control (lane 9).

15 mins in comparison with much longer extraction of RNA with phenol/chloroform or with a commercial kit.

Using RNA-RP an optimal dilution of the sap was needed for a successful virus detection; a sap dilution of 10^{-2} gave satisfactory results. The detection end point was recorded up to the dilution of 10^{-6} for ASGV and 10^{-4} for ASPV in *C. quinoa* and *N. occidentalis* 37B leaf tissues, respectively (Figs 3 and 4). Similar results were obtained when infected apple tree leaves were used (data not shown). This difference in the detection efficiency between ASPV and ASGV seems to correlate with virus properties. ASPV is unstable in high ionic strength, which is common characteristics of elongated viruses. In the case of ASPV the end-to end aggregation of virus particles make them even more sensitive during sample preparation (Koganezawa and Yanase, 1990). RT-PCR using RNA-RP was usually negative at low sap dilutions (10^{-1}) probably due to the presence of high amounts of compounds inhibiting either RT or PCR. Similar results have been obtained with Triton X-100 in preparing RNA for detection of BYV by RT-PCR (Kundu and Rysanek, unpublished results). Triton X-100, as an RNA extraction means has had, in general, a positive effect on RT-PCR and immunocapture (IC) RT-PCR system (Wetzel *et al.,* 1992; Nolasco *et al.,* 1993; Glasa *et al.,* 2002). Incubation of plants extracts with the buffer used in RNA-RP described here or Triton X-100 for 5 mins at 60°C before transcription had a positive effect on product formation and usually increased the sensitivity of RT-PCR. Thomson and Dietzgen (1995) have demonstrated that RNA can be released from plant tissues also without homogenization.

We have showed that the clarification step after homogenization prevents RT-PCR inhibition (Kundu, 2002b). The Tris-KCl-$MgCl_2$-DTT buffer or Triton X-100 is a reliable and effective means of RNA preparation from woody plant tissues. RNA-RP has following advantages: (1) it does not require additional RNA purification for RT-PCR, (2) it does not include biohazardous steps such as dealing with phenol and chloroform, (3) it has a high detection efficiency comparable to the phenol/chloroform method, (4) it allows detection of viruses in low concentration, and (5) it is convenient for routine virus detection.

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