Simplification of RNA Preparation Procedure for RT-PCR in Detection of Pome Fruit Tree Viruses

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Abstract

A rapid, easy to handling and sensitive RNA preparation procedure, RNA release protocol was described here for the detection of *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV) by RT-PCR. Comparing total RNA extraction protocol, RNA release protocol give raised similar rate of ASPV and ASGV detection within the field-grown apple cultivars. Among sampling plant tissues, the bud leaf and leaf (during blossom) were showed efficient tissues for the routine detection, regardless the using RNA preparation procedures.

Keywords: RT-PCR; ASPV; ASGV; RNA release

INTRODUCTION

The molecular amplification-based assay, The Reverse Transcription Polymerase Chain Reaction (RT-PCR) has been widely used for the detection of fruit tree viruses. RT-PCR was previously used for the detection of *Apple stem pitting virus* – ASPV (*Foveavirus*) (SCHWARZ & JELKMANN 1998) and *Apple stem grooving virus* – ASGV (*Capillovirus*) (MACKENZIE *et al.* 1997). Nevertheless, purification of total RNA from fruit tree tissues remains a major difficulty and causes the limitation of RT-PCR application in virus detection. Plant polyphenolic components and polysaccharides are usually co-purified with total nucleic acid (RNA or DNA) and inhibit the sensitivity of PCR (resp. RT-PCR) reaction. A good number of modifications of phenol extraction procedure and commercial purification kits were used with more and less success. Some approaches of viral RNA and DNA released procedure were previously used (THOMSON & DIETZGEN 1995). The protocol of RT-PCR without using total RNA was previously applied for the detection of these viruses, which included immunocapture/RT-PCR for ASPV (JELKMANN & KEIM-KONRAD 1997) and immunocapture/RT-PCR, tube capture/RT-PCR (JAMES 1999), and the use of crude extracts as template for RT-PCR (MARINHO *et al.* 1998) for ASGV detection.

This paper reports a rapid and sensitive RNA preparation protocol, RNA-Release Procedure (RNA-RP) for the detection of ASPV and ASGV in field-grown apple trees. The protocol RNA-RP was compared with total RNA extraction procedure for the successful detection of these viruses. Suitability of plant tissues for sampling prior to RT-PCR is also reported.

MATERIALS AND METHODS

The tested trees and virus isolates

Leaf bud, leaf during blossom and flower of four cultivars of field-grown *Malus domestica* Borkh including Idared, Spartan, Vista Bella, and Stark Earliest without any artificial infection were used for the test. The orchard and cultivars were selected according to previous detection of these viruses (KUNDU 2002). As positive control ASPV apple isolate (IPO, Wageningen) maintained in *Nicotiana occidentalis* 37B and ASGV apple isolate (University of Halle-Wittenberg, Germany) maintained in *Chenopodium quinoa* were used.

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RNA-RP protocol

The apple tissues (leaf bud, leaf and flower) were ground with a mortar and pestle and diluted in DEPC treated sterile water (1:10), and transferred to sterile centrifuge tubes. The tubes were centrifuged at 10 000 g for 10 min and the supernatants were transferred to new sterile tubes, made a final dilution 1:100 with DEPC-treated sterile water and added 20% M-MLV reaction buffer (50mM Tris-HCl pH 8.3 buffer containing 75mM KCl, 3mM $MgCl_2$ and 10mM DTT) or 1% Triton X-100. The tubes were then incubated at 60°C for 5 min with time-to-time mixing. The tubes were put in chilled ice for 5 min, and thus treated sap was used as template for RT-PCR.

Phenol extraction of total RNA protocol

A modified protocol, the Proteinase K-phenol: chloroform:isoamyl alcohol and ethanol precipitation procedure as described KUNDU (2002) was used for total RNA isolation from apple tissues (leaf bud, leaf during blossom and flower).

Primers

The primer pair ASP-A & ASP-C (JELKMANN & KEIM-KONRAD 1997) specific for ASPV and primer pair ASGV-U & ASGV-2 (JAMES 1999) specific for ASGV was used prior to RT-PCR.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Two-step RT-PCR

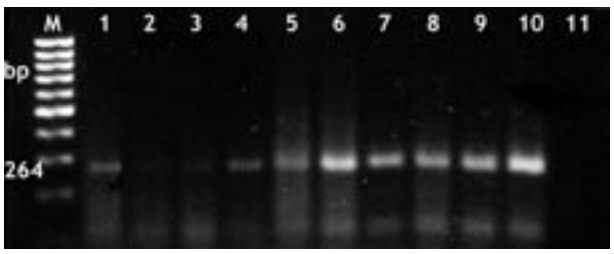
Synthesis of cDNA and its amplification were done according to KUNDU (2002).

One-step RT-PCR

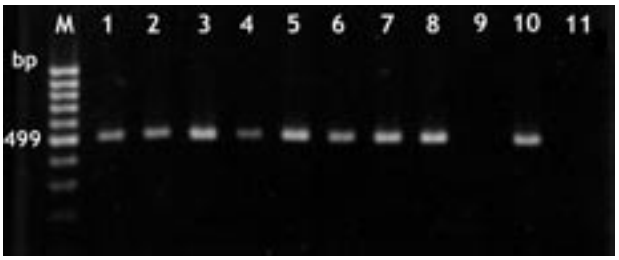
One-step RT-PCR was performed with one-step RT-PCR kit (Qiagen) as follows: One-step RT-PCR mixtures, containing 5 μl 5× Qiagen one-step RT-PCR buffer, 1 μl dNTP mix (10mM of each dNTP), 1 μl Qiagen one-step RT-PCR enzyme mix, 1 μl Q-solution, 10 pmol of upstream and downstream primers (ASP-A/ASP-C for ASPV or ASGV-2/ASGV-U for ASGV) were prepared in micro-tubes and 5 μl of sap with released RNA or 1 μl of total RNA 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 reverse transcription step at 50°C for 30 min and an initial PCR activation step at 95°C for 15 min then 33 cycles of 94°C for 30 s (denaturation), 55°C for 45 s (annealing), 72°C for 80 s (extension), and after the last cycle a final extension step at 72°C for 10 min was added.

RESULTS AND DISCUSSION

The RNA preparation protocols, RNA-release procedure and total RNA extraction procedure described here, were successful for the detection of ASPV and ASGV in apple cultivars. The specific fragment of 264 bp (primer pair ASP-A/ASP-C) of ASPV genome and that of 499 bp (primer pair ASGV-U/ASGV-2) of ASGV genome was amplified with apple trees of all tested cultivars, except with the healthy control (Figures 1 and 2). Similar rate of ASPV and ASGV were detected using RNA-RP protocol in comparing with total RNA extraction procedure. No significant differences of detection efficiency were recorded among the sampling plant tissues (bud leaf, leaf during blossom and flower), using total RNA. But in RNA-RP protocol the flower was showed less efficiency in virus



1. ASPV, Lane M – molecular marker 100 bp (MBI Fermentas), Lane 1–10 RT-PCR products of leaf bud of cultivar Spartan tree 1 to tree 10, Lane 11 – healthy control



2. ASGV, Lane M – molecular marker 100 bp (MBI Fermentas), Lane 1–10 RT-PCR products of leaf bud of cultivar Spartan tree 1 to tree 10, Lane 11 – healthy control

Figures 1 and 2. Agarose gel (1.5%) electrophoresis of RT-PCR product where RNA released sap was used as template for ASPV and ASGV detection, respectively

Table 1. Comparison of RNA-RP with total RNA extraction procedure in detection of ASPV and ASGV by RT-PCR

Apple cultivars	Plant tissue	ASPV positive trees/total tested trees		ASGV positive trees/total tested trees	
		RNA-RP	Total RNA	RNA-RP	Total RNA
Idared	Leaf bud	8/10	8/10	6/10	6/10
	Leaf	8/10	8/10	6/10	6/10
	Flower	8/10	7/10	5/10	6/10
Spartan	Leaf bud	8/10	8/10	9/10	9/10
	Leaf	8/10	8/10	9/10	9/10
	Flower	0/10	5/10	3/10	9/10
Stark Earliest	Leaf bud	8/10	8/10	10/10	9/10
	Leaf	8/10	8/10	10/10	10/10
	Flower	0/10	3/10	0/10	10/10
Vista Bella	Leaf bud	7/10	7/10	2/10	2/10
	Leaf	6/10	7/10	2/10	2/10
	Flower	1/10	7/10	1/10	2/10

detection (Table 1). In RNA-RP an optimal dilution is needed for a successful detection, and sap dilution 1:100 gives satisfactory results. Previously, Triton X-100 was successfully applied for the detection of *Beet yellows virus* – BYV (RYŠÁNEK *et al*. 2000) and *Plum pox virus* – PPV (WETZEL *et al*. 1991). For RNA releasing approach both M-MLV buffer and Triton X-100 could be used for successful detection of ASPV and ASGV by RT-PCR. However, the reverse transcription buffer (M-MLV) seems to be more effective in our hand. The RNA release protocol either with M-MLV buffer or Triton X-100 is a reliable and effective approach to RNA preparation from fruit tree tissues without loss of sensitivity. Hence, the procedure could be suggested for routine detection of fruit tree viruses.

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