Detection and identification of *Apple stem pitting virus* and *Apple stem grooving virus* affecting apple and pear trees in Egypt

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Abstract

Apple stem pitting virus (ASPV) and *Apple stem grooving virus* (ASGV) are economically important and infect either individually or in mixed infection commercial apple and pear cultivars causing yield loses. Young green bud and/or base of petiole were collected from naturally infected apple and pear trees from different locations in Egypt. Both viruses were detected frequently in apple and pear samples. A total of 420 trees from 9 different orchards were tested using one-step RT-PCR; 13% and 17% of these samples were infected with ASPV and ASGV, respectively. Mixed infection with both viruses occurred in 4% of the tested trees. ELISA was reliable for detection of ASGV but not ASPV. Total RNA for one-step RT-PCR was isolated from 100 mg fresh affected apple and pear leaf tissue using Qiagen RNeasy plant mini-kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. The one step-RT-PCR method was performed using ASPV and ASGV-specific primers for each virus. A 316 bp fragment for ASPV and 524bp fragment for ASGV were amplified and detected by gel electrophoresis analysis which indicated the presence of ASPV and ASGV in affected apple and pear cultivars. Southern blot hybridization of the amplified products to digoxigenin (DIG)-labeled cDNA probe for ASPV or ASGV confirmed the results obtained by electrophoresis analysis. No product was detected in amplified extracts of uninfected apple and pear samples. The detection of ASPV and ASGV by one step-RT-PCR assay was successful and appears useful for testing pome fruit germplasm in quarantine and budwood in certification programs.

Keywords: apple and pear, ASPV, ASGV, virus detection, One step RT-PCR, Southern blot hybridization

Introduction

Apple stem pitting virus (ASPV) and *Apple stem grooving virus* (ASGV) belong to the genera *Foveavirus and Capillovirus,* respectively. Both viruses have elongated filamentous particles and are latent in most commercial apple cultivars, graft transmissible, world-wide distributed in pome fruit trees, and often occur in mixed infection (Nemeth, 1986; Kummert et al., 1998; Kundu, 2003a). ASGV symptoms are expressed when an infected apple cultivar is grafted on a sensitive rootstock such as *Malus pumila* cv. Virginia crab. ASPV-infected plants grafted on the sensitive apple rootstock "Spy 227" cause epinasty and decline and stem pitting. The virus also causes stem pitting of Virginia crab. ASPV may cause characteristic symptoms on some susceptible apple cultivars. Also, it causes different diseases in pear such as pear vein yellows and pear stony pit. Biological indexing by grafting on woody indicators constitutes the baseline test for certification of fruit tree planting material, but it is cumbersome, lengthy and expensive to perform (time, space, and qualified manpower required) and does not respond to the actual constraint of production of certified material in a world where volume, distances, and rapidity of the exchange of material have increased dramatically (Kummert et al., 1998). Under these conditions, reliable and rapid detection protocols for latent viruses is thus important in the implementation of sanitary control of propagative material of fruit trees. Enzyme linked immunosorbent assay (ELISA)-based detection is routinely used for the detection of ASGV and ASPV (Fuchs, 1981; Kummert et al., 1998; Gugerli and Ramel, 2004) and several commercial kits are available, but ELISA is not sensitive enough to detect low concentration of these viruses in infected trees during summer months or in dormant tissues. The molecular amplification-based assay, reverse-transcription polymerase chain reaction (RT-PCR) has contributed to increase the detection sensitivity providing a means for detecting viruses in woody plants throughout the year, even during seasons of low concentration. Moreover, PCR primers are more easily produced than the virus-specific antiserum needed for ELISA. This paper presents results obtained for detection of ASGV and ASPV infection in apple and pear orchards in different locations in Egypt.

Materials and methods

Source of Samples: Leaf samples were collected randomly from different cultivars grown in commercial orchards during June and July of 2008 for ASGV and ASPV, respectively. Totally 420 samples were collected; 224 apple samples were collected from 2 verities (Anna, Dourest gold) and 196 pear samples were collected from 3 verities

(Lecont, Hod and Florida home). The samples were collected from 9 commercial orchards located in 4 Governorates in Egypt.

ELISA: All collected samples were subjected to DAS-ELISA (Clark and Adams,1977) using the ASGV detection kit of Loewe Biochemica (GmbH,Germany), and ASPV detection kit of Bioreba (AG,Switzerland) following recommendations of the manufacturer.

Total RNA extraction: One hundred mg of fresh apple or pear leaves were ground under liquid nitrogen to a fine powder using a mortar and pastel, then applied for RNA extraction using Qiagen RNeasy Plant Mini Kit (Qiagen) according to the instruction manual.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR): RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen, Inc.). The master mix typically contained all the components required for RT-PCR except the RNA template. The mix prepared in a thin-walled 0.2 µl PCR tube, by combining 10 µl of 5x QIAGEN OneStep RT-PCR buffer, 2.0 µl of dNTP mix (containing 10mM of each dNTP), 1µl of each viral and complimentary primers (Table 1) to a final concentration of 0.6µM, 2.0 µl of QIAGEN OneStep RT-PCR Enzyme Mix (Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase and HotStartTaq™ DNA Polymerase). To this mix 5 µl of total RNA were added and RNase-free water to a final volume of 50 µl. First strand cDNA synthesis was done using the following parameters: 50ºC for 30 min at 1 cycle. Second strand cDNA synthesis and PCR amplification were done in the same tube using DNA Thermal Cycler (DNA Engine, Bio-Rad) with the following parameters: 95ºC for 15 min at 1 cycle to activate HotStarTaq DNA Polymerase and to inactivate simultaneously the reverse transcriptases. The amplification parameters: denaturation at 94ºC for 30s, primer annealing at 54ºC (ASPV) and 60ºC (ASGV) for 45s, and extension at 72ºC for 60s, for 35 cycles with a final extension at 72ºC for 7 min.

Tab. 1 DNA primers used for reverse transcription-polymerase chain reaction (RT-PCR) amplification of ASGV or ASPV

Virus	Primer	Sequence	(bp)	Reference
ASGV	C6396	5'-CTGCAAGACCGCGACCAAGTTT-3'	524	Serghini et al., 1990
	H5873	5'-CCCGCTGTTGGATTTGATACACCTC-3'		
ASPV	C8849	5'-TGCCTCAAAGTACACCCCTCAGT-3'	316	Jelkmann, 1994
	H8534	5'-CGCCAAGAAATGCCACAGC-3'		

Electrophoresis Analysis: Five-microliter aliquots of RT-PCR product were analyzed on 1.5 % agarose gels (6 x 8 cm), in TBE buffer (89 mM Tris-HCL, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 120 volts. 100bp DNA molecular weight markers were used to determine the size of RT-PCR products. Gels were stained with ethidium bromide 10 µg/ml and visualized by UV illumination (Bio-Rad) (Sambrook et al., 1989). About 75 min were required for running the agarose gels and staining with ethidium bromide.

Probe Preparation and Southern Blot Hybridization Analysis of PCR-Products: PCR products for ASPV and ASGV were used as the template to synthesize a cDNA probes. Digoxigenin-ll-dUTP (Dig-ll-dUTP), (Boehringer Mannheim, Indianapolis, IN) was incorporated into the newly synthesized cDNA during 35 cycles of PCR with melting, annealing, and extension as described above. The cDNA was amplified in a Bio-Rad Engine, Thermal Cycler (Bio-Rad). For each reaction final concentrations were 0.2 µM of each primer, 10 mM dATP, dCTP, dGTP; 0.65 mM dTTP; 0.35 mM Dig-ll-dUTP labelled and 1.2 U *Taq* DNA polymerase (Promega Corporation, Madison, WI) in 50 µ1 containing 200 ng target cDNA. The expected molecular weight of the PCR-amplified product was confirmed by agarose gel electrophoresis as mentioned above (Sambrook et al., 1989). For Southern hybridization analysis (Southern, 1975), agarose gel containing separated cDNA fragments were soaked twice in 0.5M NaOH/1.5 M NaCl for 10 min each to denature the DNA, the denatured agarose gel was placed on a Saran Wrap covered glass plate and overlaid with the positively charged Hybond N+ membrane, 3 layers of Whatman 3MM paper, followed by 2 cm thick paper towels or blotting paper and glass plate. All were cut to the gel size. This was covered with Saran Wrap to prevent dehydration; a 250 g weight was placed on top of the covered gel and left for 6 h to overnight to allow transfer the DNA from the gel to the membrane. Pre-hybridization, hybridization and immunological detection were carried out using the "Genius II DNA Labeling and Detection Kit" (Boehringer Mannheim, IN) according to the manufacturer's recommendation.

Results and discussion

ELISA and RT-PCR: ELISA absorbance values for ASGV detection allowed the differentiation between positive and negative samples. ELISA-ASPV detection, however, yielded not always clear results and some of the results were difficult to interpret. Moreover, the background values were high and it was not possible to clearly distinguish in some samples virus-positive from virus-negative. The RNA Extraction protocol described in this paper has facilitated the routine RT-PCR detection of a number of different plant viral pathogens in their woody hosts. The method was based

on the commercially available RNase kit and was superior to other methods, in that it mitigated the effects of phenolic compounds and other inhibitory substances, avoided the use of organic solvents and phenol, and was extremely rapid. A modification of the manufacturer's tissue lysis buffer, which included addition of PVP and pH buffering to 5.0 with 0.2 M sodium acetate, was instrumental in improving the reproducibility of RNA extractions from diverse host species (Mackenzie et al., 1997). High-quality RNA suitable for use in One-step RT-PCR could be obtained from apple and pear allowing detection of ASGV and ASPV infection in these hosts (Table 2).

Tab. 2 Incidence of ASGV and ASPV among pome fruit samples from 9 commercial orchards in Egypt.

			Infected trees		
Species	**Variety**	**Tested trees**	**ASGV**	**ASPV**	**Mixed infection**
	Anna	200	47	30	11
Apple	Dorset Gold	24	3	00	00
Pear	Le Conte	100	12	20	4
	Hood	65	3	5	2
	Florida Home	31	7	00	00
Total		420	72 (17%)	55(13%)	17 (4%)

One-step RT-PCR was elaborated using primers targeting amplification a part of the coat protein of ASGV or ASPV genome. Primer pairs specifically detected ASGV or ASPV are presented (Fig.1 and Fig.2).

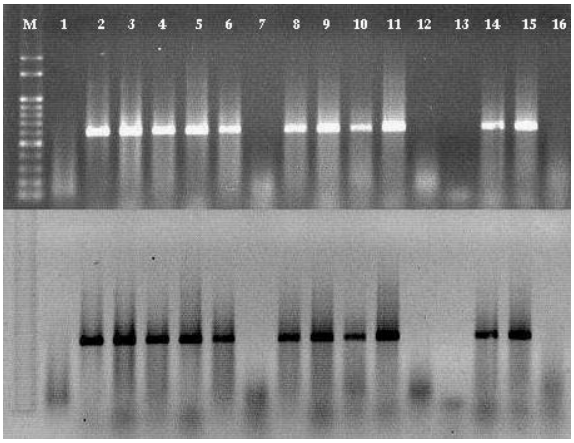

Fig. 1 **a)** Agarose gel electrophoresis of RT-PCR products of different *Apple stem grooving virus* (ASGV)-infected apple and pear trees. Lane M, 100 bp DNA molecular weight marker; Lanes 1, 7,12 and 13 non-infected apple and pear samples; lane 16 negative control; the remaining lanes represent some infected samples from apple and pear trees. **b)** An autoradiograph of Southern blot hybridization of a DIG-11-labeled ASGV cDNA probe to RT-PCR products of nucleic acid extracts of naturally infected apple and pear trees. A DNA fragment of the expected size (524 bp) from infected apple and pear leaves hybridized to ASGV- specific probe. No hybridization was obtained with extracts from uninfected apple and pear samples and from the negative control (lanes 1,7,12,13 and 16) .

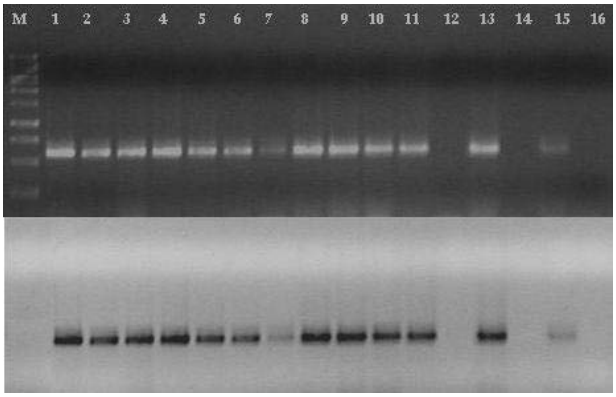

Fig. 2 **a)** Agarose gel electrophoresis of RT-PCR products of different *Apple stem petting virus* (ASPV)-infected apple and pear trees. Lane M, DNA molecular weight marker ; Lanes 12 and 14 non-infected apple and pear samples; lane 16 negative control; the remaining lanes represent some infected samples from apple and pear trees. **b)** Autoradiograph of Southern blot hybridization of a DIG-11-labeled ASPV cDNA probe to RT-PCR products of nucleic acid extracts of naturally infected apple and pear trees. A DNA fragment of the expected size (316 bp) from infected apple and pear leaves hybridize to the ASPV specific probe. No hybridization was obtained with extracts from uninfected apple and pear samples and from the negative control (lane 12, 14 and 16).

One–step RT-PCR confers a highly sensitive tool for the detection of these viruses, in which the entire reaction is carried out in a single tube, thus the risk of contamination, is significantly lower (Kundu, 2003b).

Autoradiographs were obtained for Southern blots of hybridization DIG-11-labeled ASGV or ASPV cDNA probe to RT-PCR products of nucleic acid extracts of naturally infected apple and pear trees from different commercial orchards in Egypt. A DNA fragment of the expected size (524 bp for ASGV) from infected apple and pear leaves hybridized to ASGV specific probe (Fig. 1b). Another DNA fragment (316 bp) hybridized to ASPV specific probe (Fig. 2b). These fragments were similar in size to those amplified from ASGV and ASPV infected apple and pear samples. No hybridization was obtained with extracts from uninfected apple and pear samples and from the negative control (Fig. 1, lanes 1,7,12,13 and 16) for ASGV and (Fig. 2, lanes 12,14 and 16) for ASPV.

For many viruses such as ASGV and ASPV that cause significant diseases in *Malus* and *Pyrus* species, the most commonly used diagnostic methods rely on biological indexing by either woody or herbaceous indicator plants (Leone and Lindner, 1995). While serological reagents are available for these viruses, the use of ELISA for routine screening of viruses has been limited to their detection in herbaceous plants, or to specific times of the year from young leaf tissue or flower blossoms. It is anticipated that the RT-PCR method described in this paper will facilitate the development of rapid and sensitive diagnostic techniques that can be used year-round for specific detection of these and other important viral pathogens directly in their woody hosts.

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