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Pospiviroidae viroids in naturally infected stone and pome fruits in Greece

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Abstract

Viroid research on pome and stone fruit trees in Greece is important, as it seems that such viroids are widespread in the country and may cause serious diseases. Our research dealt with three *Pospiviroidae* species infecting pome and stone fruit trees, namely *Apple scar skin viroid* (ASSVd), *Pear blister canker viroid* (PBCVd) and *Hop stunt viroid* (HSVd). Tissue-print hybridization, reverse transcription-polymerase chain reaction (RT-PCR), cloning and sequencing techniques were successfully used for the detection and identification of these viroids in a large number of pome and stone fruit tree samples from various areas of Greece (Peloponnesus, Macedonia, Thessaly, Attica and Crete). The 58 complete viroid sequences obtained (30 ASSVd, 16 PBCVd and 12 HSVd) were submitted to the GenBank. Our results showed the presence of ASSVd in apple, pear, wild apple (*Malus sylvestris*), wild pear (*Pyrus amygdaliformis*) and sweet cherry; HSVd in apricot, peach, plum, sweet cherry, bullace plum (*Prunus insititia*), apple and wild apple; and PBCVd in pear, wild pear, quince, apple and wild apple. This research confirmed previous findings of infection of Hellenic apple, pear and wild pear with ASSVd, pear, wild pear and quince with PBCVd and apricot with HSVd. Our findings also revealed for the first time the natural mixed infection of apple and wild apple with (ASSVd+PBCVd+HSVd), of apple and pear with (ASSVd+PBCVd), and of wild apple with (ASSVd+HSVd), as well as the natural infection of Hellenic sweet cherry, peach, bullace plum and plum with HSVd. To our knowledge, this is the first published report of detecting HSVd and PBCVd in infected apple and wild apple, and ASSVd in sweet cherry.

Keywords: ASSVd, PBCVd, HSVd, stone fruit, pome fruit, Greece

Introduction

Apple scar skin viroid (ASSVd), *Hop stunt viroid* (HSVd) and *Pear blister canker viroid* (PBCVd) are members of the family *Pospiviroidae* (Flores et al., 2003) and have been reported to infect wild and cultivated pome and stone fruit trees in Greece (Kyriakopoulou and Hadidi 1998; Amari et al. 2000; Kyriakopoulou et al. 2001; Boubourakas *et al*. 2006, 2008). The present study has focused on specifying the host range and geographical extent of viroid infections in Greece and obtaining complete nucleotide sequences of the viroids detected in Rosaceous species from various parts of the country.

Materials and methods

Sample collection: During 2006-2009, 947 field samples of cultivated and wild pome and stone fruit trees with various symptoms (Fig. 1-4) were collected in different regions of Greece (Macedonia, Peloponnesus, Thessaly, Attica and Crete).

Fig. 1 Pear fruit cv 'Kontoula' with ASSVd (scar skin, Argolis, Peloponnesus)

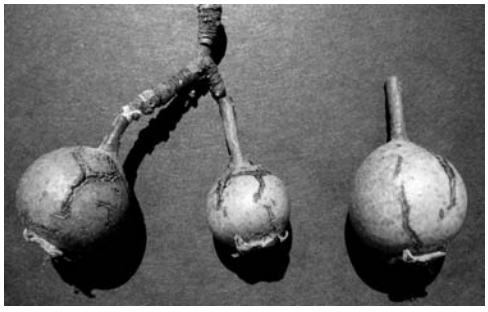

Fig. 2 Wild pear fruit (*Pyrus amygdaliformis*) with ASSVd (scar skin, Achaia, Peloponnesus)

Fig. 3 Pear twig cv 'Kontoula' with PBCVd (pear blister canker, Argolis, Peloponnesus).

Fig. 4 Apple fruit cv. 'Royal Gala' found to be infected with ASSVd, HSVd and PBCVd (red and white dappling on premature fruit, Pella, Macedonia).

Tissue-print hybridization: Tissue-print hybridization was conducted for all samples, using a modified imprint hybridization protocol of Palacio-Bielsa et al. (1999) and DIG-labelled DNA (ASSVd, PBCVd, HSVd) and RNA (ASSVd) probes.

Total RNA extraction and RT-PCR: Total RNA phenol extracts of 120 Rosaceous samples were used in a one tube/two step RT-PCR protocol, employing two different primer pairs per viroid, as described by Faggioli et al. (2001).

Cloning and sequencing: Properly-sized RT-PCR products were either sequenced directly or cloned into pGEM-T and pCR® II plasmid vectors, according to the pGEM-T Easy (Promega, Madison, WI, USA) and TOPO-TA (Invitrogen, Carlsbad, CA, USA) cloning kit instructions, and then sequenced. The sequences obtained were compared with others in the NCBI database and those identified as complete sequence viroid genomes were submitted to the GenBank.

Results

Tissue print hybridization: ASSVd, PBCVd and HSVd were found in 29.3%, 30.1% and 38.9% of field samples tested by tissue print hybridization, respectively. High frequencies were found for: ASSVd in pear and wild pear from Argolis, Achaia and Corinthia (Peloponnesus), in apple and wild apple trees from Pella (Macedonia) and in sweet cherry trees from Florina (Macedonia). PBCVd was found mainly in pear and wild pear trees from Argolis, Achaia and Corinthia, and in quince trees from Argolis; and HSVd in apricot and peach trees from Argolis and Corinthia, as well as in almond and wild almond (Prunus communis) trees from Achaia, Argolis and Arcadia (Peloponnesus) (Table 1a). Mixed infections by 2 or 3 viroids, (ASSVd+PBCVd), (ASSVd+HSVd) or (ASSVd+HSVd+PBCVd) were detected in 63 trees from Argolis, Achaia, Corinthia (Peloponnesus) and Pella (Macedonia) (Table 1b).

Tab. 1a Viroid-positive fruit tree samples in Greece

Viroid	**Tissue print hybridization**			**RT-PCR**			**Sequences**			**Complete sequences**		
Host species	**ASSVd 117/400**	**PBCVd 114/272**	**HSVd 232/596**	**ASSVd 33/120**	**PBCVd 23/120**	**HSVd 39/120**	**ASSVd 44**	**PBCVd 21**	**HSVd 21**	**ASSVd 30**	**PBCVd 16**	**HSVd 12**
Apple	Pella 40%	Pella 26%	Pella 20%	8	7	4	10	7	3	4	7	2
Pear	Argolis 51% Achaia 40%	Argolis 55% Corinthia 36% Achaia 40%		9	8		9	9		5	5	
Wild apple (*Malus sylvestris*)	Pella 77%	Pella 67%	Pella 77%	3	3	3	6	2	5	3	2	3
Wild pear (*Pyrus amygdaliformis*)	Achaia 30%	Argolis 89% Corinthia 71% Achaia 30%		6	4		3	1		2	1	

Viroid	Tissue print hybridization			RT-PCR			Sequences			Complete sequences		
Host species	**ASSVd 117/400**	**PBCVd 114/272**	**HSVd 232/596**	**ASSVd 33/120**	**PBCVd 23/120**	**HSVd 39/120**	**ASSVd 44**	**PBCVd 21**	**HSVd 21**	**ASSVd 30**	**PBCVd 16**	**HSVd 12**
Quince		Argolis 75%		1	1			2			1	
Almond			Argolis 100% Arcadia 60%									
Apricot			Argolis 60% Corinthia 63%			11			1			1
Bullace plum						1			1			
Cherry			Florina 100%	6		11	16		5	16		4
Peach			Argolis 70% Corinthia 52%						5			2
Plum						6			1			
Wild almond (*Prunus communis*)			Achaia 54%			2						
Wild plum (*Prunus spinosa*)			Corinthia 80%									

Tab. 1b Viroid in mixed infectious

Mixed infections	Imprint hybridization						Completely sequenced					
63 Trees	**Apple**	**Wild apple**	**Pear**	**Wild pear**	**Quince**	**Cherry**	**Apple**	**Wild apple**	**Pear**	**Wild pear**	**Quince**	**Cherry**
ASSVd+PBCVd	10		25	10	2		2	1	2			
ASSVd+HSVd		1				6						
ASSVd+PBCVd+HSVd	4	6					1	1				

RT-PCR: RT-PCR amplified products of the expected size were obtained in 70 out of the 120 field samples tested (Table 1a).

Cloning and sequencing: Cloning and sequencing or direct sequencing (using at least 2 different primers) were completed for 39 RT-PCR products from 31 trees (5 apple, 3 wild apple, 5 pear, 3 wild pear, 1 quince, 7 sweet cherry, 3 apricot, 2 peach, 1 bullace plum (Prunus insititia), 1 Japanese plum), resulting in 44 ASSVd, 21 HSVd and 21 PBCVd sequences. Viroids in mixed infections (ASSVd+HSVd+PBCVd) or (ASSVd+PBCVd) were completely sequenced in 7 trees (Table 1b). Fifty-eight complete viroid sequences, 30 ASSVd, 16 PBCVd and 12 HSVd were deposited in the GenBank under the accession numbers FJ974062-FJ974104, EU925587-EU925591, EU978462-EU978464, GQ249347-GQ249350, GQ141739-GQ141740 and FN376408-FN376409.

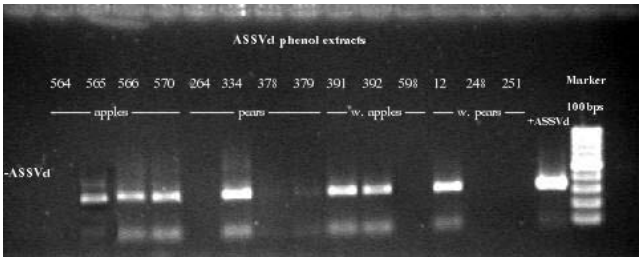

Fig. 5 RT-PCR test on 4 apple, 4 pear, 3 wild apple and 3 wild pear total RNA phenol extracts using ASSVd primers. From left: Lane 1, healthy control; lanes 2-15, pome fruit samples; lane 16, positive control; lane 17, Marker 100 bps (Fermentas, LTU).

Discussion

The data obtained in this study indicate that ASSVd, HSVd and PBCVd, previously reported in Greece, are widely spread in cultivated pome and stone fruit trees, including local varieties, especially in areas of great arboricultural importance, such as Central-Western Macedonia and Peloponnesus, as well as wild pears and wild apples and other wild rosaceous species, in the mountains and foothills of the above areas. Our findings also revealed for the first time the natural mixed infection of apple and wild apple with (ASSVd+HSVd+PBCVd), of apple and pear with (ASSVd+PBCVd), and of wild apple with (ASSVd+HSVd), as well as the natural infection of sweet cherry, plum, bullace plum and peach with HSVd in Greece. To our knowledge, this is the first published report of detecting HSVd and PBCVd in apple and wild apple and ASSVd in sweet cherry (Kaponi et al. 2009).

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