

SHORT COMMUNICATION  
DETECTION OF POME FRUIT VIROIDS  
BY RT-PCR USING A SINGLE PRIMER PAIR

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SUMMARY

*Apple scar skin viroid* (ASSVd), *Pear blister canker viroid* (PBCVd), and *Apple dimple fruit viroid* (ADFVd) are widely distributed and economically important pome fruit viroids, all belonging to genus *Apscaviroid*. For a rapid and efficient detection method of these viroids, we have developed a one-tube RT-PCR protocol that amplifies simultaneously ASSVd, PBCVd and ADFVd from their respective hosts. Multiple alignment and comparison of the nucleotide sequences of ASSVd, PBCVd and ADFVd allowed us to select a conserved region and design a primer pair for their polyvalent amplification in a single RT-PCR reaction. These newly designed primers amplify a specific DNA fragment which contains a major portion of each viroid: ASSVd 267 bp, PBCVd 261 bp and ADFVd 252 bp. The development of this RT-PCR protocol enabled us to identify different isolates of the three viroids and to evaluate their distribution in cultivated and wild pome fruit germplasm.

**Key words:** genus *Apscaviroid*, pome fruit viroids, polyvalent detection, single primer pair, RT-PCR.

Viroids are plant pathogens which may cause severe diseases (Garnsey and Randles, 1987). Viroids are infectious, low molecular weight single-stranded RNA molecules 246 to 399 nucleotides in size, have autonomous replication and are currently classified in two families and seven genera (Flores *et al.*, 2000).

Among important viroids in the genus *Apscaviroid*, *Apple scar skin viroid* (ASSVd), the type member of this genus, was the first viroid reported to infect pome fruit trees (*Malus*, *Pyrus* and *Cydonia* spp.) (Hashimoto and Koganezawa, 1982). The symptoms induced by ASSVd infections are restricted to fruits and include color dappling, cracking, scarring and distortion. Three other diseases, apple dapple (Hadidi *et al.*, 1990), pear rusty

skin (Chen *et al.*, 1987) and Japanese pear fruit dimple (Osaki *et al.*, 1996) have been attributed to ASSVd molecular variants (Zhu *et al.*, 1995).

The other two apscaviroids reported to infect pome fruit germplasm are *Pear blister canker viroid* (PBCVd) (Flores *et al.*, 1991), which infects 10% of European pear and quince germplasm causing blisters, splits and bark canker on susceptible varieties, and *Apple dimple fruit viroid* (ADFVd) (Di Serio *et al.*, 1996), which causes green depressed spots on the red surface of the fruits (Di Serio *et al.*, 2001).

Since these viroids cause only fruit and bark blemishes in pome fruit cultivars of commercial importance, infection is not detected visually until infected trees begin to bear. Therefore sensitive, reliable, rapid and little expensive molecular diagnostic to prevent the distribution of infected material are needed.

Conventional biological detection of apscaviroids is done by growing graft-inoculated pome fruit indicators in the open field for 2-5 years, or for 3-5 months in a greenhouse (Howell and Mink, 1992; Desvignes *et al.*, 1999). The availability of nucleotide sequence data for pome fruit viroids has resulted in the development of molecular assays for more rapid and sensitive method. In particular, the RT-PCR technique has been successfully applied for detecting ASSVd (Hadidi and Yang, 1990), PBCVd (Loreti *et al.*, 1997) and ADFVd (Di Serio *et al.*, 1996).

Pome fruit indexing requires testing for all apscaviroids, which would be greatly facilitated if a method was available for the simultaneous detection of all viroids.

This paper describes the application of a previously described polyvalent RT-PCR assay (Faggioli *et al.*, 2001) to detection of apscaviroids in wild and cultivated pome fruits. This technique was used to identify different isolates of the three viroids and to evaluate their distribution in cultivated and wild pome fruit germplasm. Moreover, we used mixtures of two target viroidal RNAs to verify the ability of the protocol to detect possible natural mixed infections.

Leaves and fruits were collected from donors, each infected by a different isolate of ASSVd (three), PBCVd

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(three), ADFVd (three) and from non-inoculated (healthy) quince seedlings. Moreover, field plants with viroid-like symptoms or apparently healthy were sampled in Latium and Campania (central and southern Italy) and tested, *i.e.*, 15 samples from apple of cvs 'Annurca', 'Stark Delicious' and 'Red Delicious', 15 from pear (cvs 'Coscia', 'Abate Fetel', and 'William'), 5 from azerole (*Crataegus azarolus* L.) and 5 from wild pear (*Pyrus amygdaliformis*).

Total nucleic acids were extracted from frozen and fresh leaves and fruit skin samples as described (Loreti *et al.*, 1999). Homologous nucleotide sequences of ASSVd, PBCVd, and ADFVd were selected by multiple alignment to design a pair of primers suitable for the polyvalent amplification of these viroids. A primer pair (VirPom-H: 5'-0TCGTCGTCGACGAAGG-3'; VirPom-C: 5'-GAGCACACAGGAACCTCACGG-3') that would allow the amplification of a specific DNA fragment containing a major portion of each viroid (ASSVd 267 bp, PBCVd 261 bp and ADFVd 252 bp) was selected (Faggioli *et al.*, 2001). RT-PCR reaction was carried out utilizing a single tube protocol (Faggioli *et al.*, 2001). Amplified products were analyzed by electrophoresis in 7% polyacrylamide gel (PAGE) followed by silver nitrate staining.

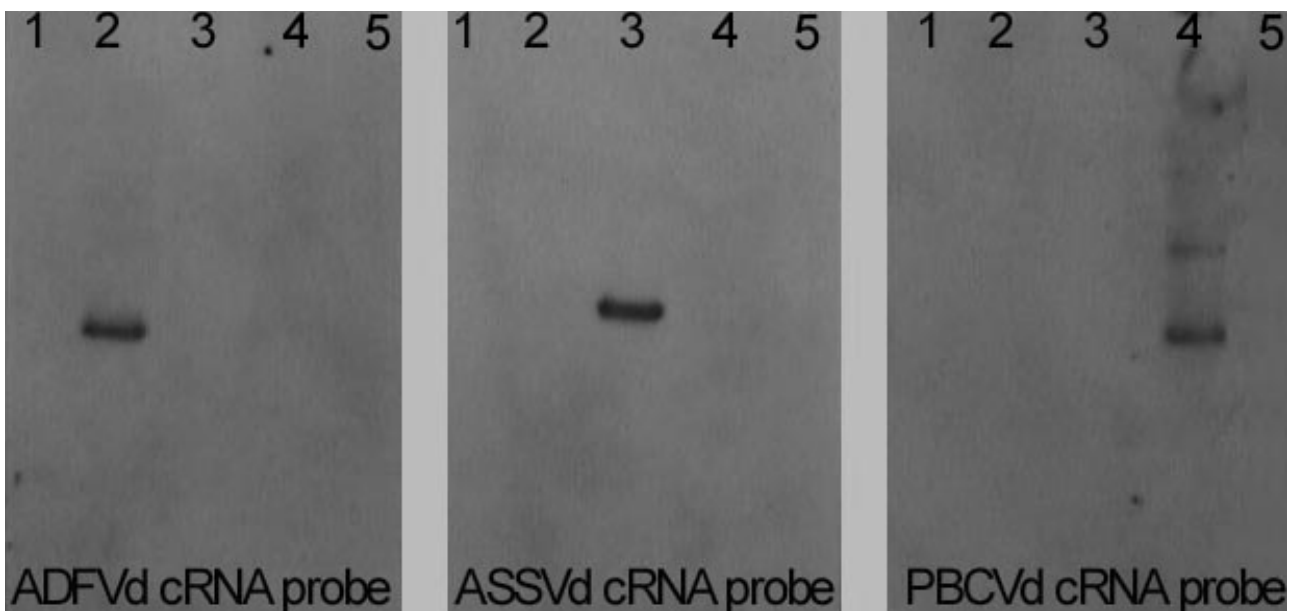
VirPom primers gave the expected cDNA fragments of 267 bp for ASSVd, 261 bp for PBCVd and 252 bp for ADFVd. In fact positive results were obtained by

polyvalent one-tube RT-PCR with all extracts from infected pears and apples. VirPom primers did not amplify healthy and water controls (data not shown). To verify PCR amplicons were from the expected viroids, control samples were analyzed by Southern blot using the corresponding Dig-labelled viroid-specific full-length riboprobes. Signals were obtained in the expected lanes. In fact, each probe hybridized only with the specific amplified product, confirming the nature of the amplicons (Fig. 1).

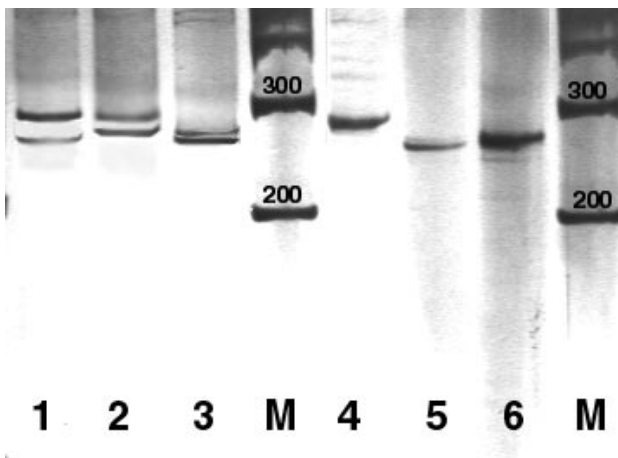
Analysis of different isolates of ASSVd, PBCVd and ADFVd from experimentally and naturally infected pear and apple trees confirmed that the selected primer pair allowed the polyvalent detection of the three viroids separately or in artificial preparations with a mixture of two target viroid-RNA's. Furthermore, the very narrow size range of the specific amplified cDNAs (252-267), it was possible to discriminate each viroid by running the PCR products on vertical 7% PAGE for two hours at 100 V (Protean II apparatus – BioRad) (Fig. 2).

The distribution of the three apscaviroids, in field-grown pome fruits, seems to be very limited in central and southern Italy. Only one cv. 'Annurca' sample out of 40 was infected by ADFVd, whereas all other samples were negative for all apscaviroids (Table 1).

In conclusion, the present work demonstrates the ability of this RT-PCR protocol to detect and distinguish, in a single PCR reaction, the most widely distrib-



**Fig. 1.** Autoradiograph of Southern blot hybridization of Dig-labelled ADFVd (left), ASSVd (centre) and PBCVd (right) cRNA probes to RT-PCR products amplified from: water control (lane 1), ADFVd-infected control (lane 2), ASSVd-infected control (lane 3), PBCVd-infected control (lane 4), and uninfected quince leaves (lane 5).



**Fig. 2.** Polyacrylamide gel electrophoresis of RT-PCR amplified products obtained using the polyvalent VirPom primer pair. Lane 1, artificial co-infection of ASSVd+ADFVd; lane 2, artificial co-infection of ASSVd+PBCVd; lane 3, artificial co-infection of PBCVd+ADFVd; lane 4, ASSVd-infected control; lane 5, ADFVd-infected control; lane 6: PBCVd-infected control; lanes M, molecular weight markers.

**Table 1.** Results of RT-PCR for the detection of three apscaviroids.

Samples	ASSVd	ADFVd	PBCVd
Annurca	0/5*	1/5	0/5
Stark Delicious	0/5	0/5	0/5
Red Delicious	0/5	0/5	0/5
Coscia	0/5	0/5	0/5
Abate Fetel	0/5	0/5	0/5
William	0/5	0/5	0/5
Azerole	0/5	0/5	0/5
<i>P. amygdaliformis</i>	0/5	0/5	0/5
Controls			
PK13 ASSVd infected control	+	-	-
X4843 ASSVd infected control	+	-	-
X4573 ASSVd infected control	+	-	-
P1914 PBCVd infected control	-	-	+
P10433 PBCVd infected control	-	-	+
P2098 PBCVd infected control	-	-	+
Annurca 1 ADFVd infected control	-	+	-
Annurca 2 ADFVd infected control	-	+	-
Stark delicious ADFVd infected control	-	+	-
Quince seedling healthy control	-	-	-

\* no. of positive samples/no. of analyzed samples.

uted and important viroids affecting pome fruit germplasm. Compared with a monospecific RT-PCR protocol that requires several reactions to obtain the same results, our method saves both time and reagents.

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