First detection and molecular characterization of new *Apple scar skin viroid* variants from apple and pear in Iran

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Received: 24 September 2011 / Accepted: 3 May 2012 / Published online: 28 May 2012
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Abstract Apple scar skin viroid (ASSVd) is one of the most destructive viroids of pome fruit. We report 12 new sequence variants of ASSVd from apple and pear trees in north-eastern Iran. The Iranian variants ranged in size from 329 to 334 nucleotides. They formed a cluster distinct from other reported ASSVd isolates, and showed sequence variations in specific regions of the viroid RNA. This is the first report of the identification and molecular characterization of ASSVd in Iran.

Keywords ASSVd · Cloning · Sequence variants · Iran

Viroids are infectious, naked, single-stranded, circular, non-coding RNAs which depend on host plant factors for their replication (Daros et al. 2006). *Apple scar skin viroid* (ASSVd; *Pospiviroidae*; *Apscaviroid*) has been reported infrequently in Europe but is widespread in East Asia (Desvignes et al. 1999; Behl et al. 1998; Kyriakopoulou et al. 2003; Koganezawa et al. 2003). Possible ASSVd-associated symptoms such as dapppling, dimpling, cracking and rusty skin in some apple cultivars were observed in Khorasan Razavi province, Iran. We report here the detection of ASSVd in one apple and one pear cultivar. We describe and compare the sequences of 12 isolates with several previously described ASSVd isolates from other countries and host plant species.

Sampling was done in mid summer of 2009 and 2010 from five regions of Khorasan Razavi province: Dargaz, 37° 44'N 59°10'E; Quchan, 37°10'N and 58°27'E; Chenaran, 36°39'N and 59°06'E; Mashhad, 36°20'N and 59°35'E; Neyshabur, 36°10'N and 58°50'E. In total, 261 samples were collected. Sampling was done from different cultivars of pear and apple in these regions: Dargaz, 10 samples from Red Delicious apples and 10 samples of Shekari pears; Quchan, 35 samples of Golden Delicious and 24 samples of Abbasi apples and 15 samples of each of the Spadana and Dargazi pear cultivars; Chenaran, 15 samples from each of the Mashhad, Golab and Golden Delicious apple cultivars, 10 samples of Spadana and 16 samples of Dargazi pear cultivars; Mashhad, 15 samples of each of the Golab and Abbasi apple cultivars and 11 samples of Dargazi pear; Neyshabur, 10 samples of each of the Red and Golden Delicious apple cultivars and 10 samples of each of the Spadana and Shekari pear cultivars. Symptoms of possible ASSVd infection were seen in Red Delicious and Golab apple cultivars. The pears did not show specific symptoms of ASSVd, although some rusty skin symptoms were seen in the Dargazi cultivar. Negative controls were obtained from the Australian apple cv Northern Spy.

Total nucleic acids were extracted from leaves using the silica capture method of Foissac et al. (2005). First strand cDNA was synthesized using primers AS1 and ADAS36 (Table 1) with Transcriptor Reverse Transcriptase (Roche Diagnostics) according to the manufacturer's directions. PCR was carried out using KAPA Taq DNA polymerase (KAPA Biosystems, South Africa) with either of the primer pairs shown in Table 1. RT-PCR products using AS1 and AS3 primers were purified with the QIAquick gel extraction kit (Qiagen, Australia) and cloned using the pGEM®-T Easy Vector System II (Promega, Australia). Plasmid DNA extraction was done with the HiYield mini kit (Biotech Corporation,

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Table 1 ASSVd specific primers used for RT-PCR

Primer	Sequence	Position	Product size (nt)	Reference
AS1	CCGGCCTTCGTCGACGACGA	101-82	330	Hashimoto and Koganezawa 1987
AS3	TGAGAAAGGAGCTGCCAGCAC	102-122		
ADAS36	GCCTTCGTCGACGACGACAG	98-79	330	Di Serio et al. 2002
AS37	CGGTGAGAAAGGAGCTGCCAG	99-119

Fig. 1 Multiple sequence alignment of 12 Iranian variants of ASSVd compared with isolates selected from different hosts and countries. The orange (nucleotide position 46–75) and red boxes (nucleotide position

258–286) indicate the P domain. The blue (nucleotide position 76–107) and green boxes (nucleotide position 224–257) show the C domain

Taiwan). Recombinant plasmids were sequenced in both directions using universal M13 forward and reverse primers at the Australian Genome Research Facility (Adelaide, Australia).

Six apple trees (*Malus domestica* cv Golden delicious) from 35 tested samples and three pear trees (*Pyrus communis* cv Dargazi) from 16 tested samples were positive with the RT-PCR assay. The ASSVd positive trees were located in Quchan and Chenaran. Eight and four sequence variants from the 6 apple and the 4 pear trees were cloned and sequenced, respectively. These variants have GenBank accession numbers HQ326087-HQ326098 (Figs. 1 and 2). These ASSVd variants were found in symptomless cv Golden

Delicious and the symptoms observed in the other cultivars possibly are not caused by ASSVd.

Sequence variations such as deletions, insertions and nucleotide changes were found to be distributed mainly in the T1, P and T2 domains of the viroid RNA. These are known to be regions of sequence variation (Kyriakopoulou et al. 2003). We can not exclude the possibility that some of the point mutations identified in single clones have been introduced during the amplification step because a polymerase lacking proof reading activity was used. This could be the case for variant HQ326087 showing mutations at position 92 and 95. These nucleotide changes are quite unexpected because they greatly alter the overall folded structure

Fig. 2 Condensed version of phylogenetic tree indicating relationships among the Iranian ASSVd variants and selected ASSVd isolates from different hosts and countries. Two isolates of *Apple dimple fruit viroid* (ADFVd) (EF08866 and EF088664) were used as the out-group

and in particular, the terminal palindromic tetraloop, and the adjacent 3-bp stem, which are strictly conserved in all viroid species of the family Pospiviroidae (Gas et al. 2007). Multiple sequence alignments (Fig. 1) were done with the Clustal W algorithm (Larkin et al. 2007). Phylogenetic analysis by the neighbour joining method within MEGA 4.0 software (Kumar et al. 2008) after bootstrapping of 1000 replicates showed that some of the Iranian isolates were distinct from other isolates of ASSVd. The Iranian isolates fell into two neighbouring clusters (Fig. 2). Pear and apple isolates occurred in both clusters.

While this study has confirmed the occurrence of ASSVd in Iran, further surveys will be required to determine incidence and distribution of the viroid in pome fruit orchards in this region.

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