SHORT COMMUNICATION

NATURAL INFECTION OF SWEET CHERRY TREES WITH *APPLE SCAR SKIN VIROID*

M.S. Kaponi[1*], T. Sano[2] and P.E. Kyriakopoulou[1]

[1]*Laboratory of Plant Pathology, Department of Crop Science, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece*
[2]*Plant Pathology Laboratory, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki-shi, Aomori-ken 036-8561, Japan*
* Present address: *Plant Pathology Laboratory, Department of Agricultural Development, Democritus University of Thrace, 193 Pantazidou Str, 68200 N. Orestiada, Greece*

SUMMARY

Apple scar skin viroid (ASSVd) is a fruit-damaging pathogen that causes significant economic losses to pome fruit trees. In the context of a survey on fruit tree viroids in Greece, ASSVd was initially detected by RT-PCR in two sweet cherry trees of cv. Tragana Edessis in an apple orchard in Florina (Macedonia, Greece). This finding was confirmed by direct viroid sequencing of the amplified RT-PCR products. In order to verify this finding, we further examined four sweet cherry trees cvs Tragana Edessis and Biggareau Burlat, two sweet cherry trees of undetermined cultivar, and fifteen neighboring apple trees in the same orchard for possible infection with ASSVd. The viroid assay was done by tissue print hybridization using an ASSVd-specific DIG-labeled probe at stringent hybridization conditions and by RT-PCR using two different ASSVd-specific primer pairs. ASSVd was detected in the six sweet cherry trees, including symptomatic samples, but not in any of the 15 apple trees. Purified ASSVd-positive RT-PCR products from sweet cherries were sequenced either directly or after cloning into pGEM-T or pCR II plasmid vectors. Sixteen ASSVd sequences obtained from five trees were 327-340 nucleotide long and shared 96-99% identity with ASSVd isolates from Indian apples. There was no cherry-specific nucleotide changes in the ASSVd sequences obtained. The viroid was graft transmitted successfully from cherry trees to cherry rootstocks and the newly developed rootstock leaves were ASSVd-positive by RT-PCR. To our knowledge, this is the first molecular and biological analyses of ASSVd infecting sweet cherry trees.

Key words: ASSVd, sweet cherry, tissue print hybridization, sequence analysis, graft transmission, Greece.

Corresponding author: M. Kaponi
Fax: +30.2106841062
E-mail: mkaponi@yahoo.gr

Apple scar skin viroid (ASSVd), the type species of the genus *Apscaviroid* (family *Pospiviroidae*) (Hashimoto and Koganezawa, 1987; Flores *et al.*, 2003; Hadidi and Barba, 2011), is the causal agent of fruit-damaging diseases such as apple scar skin, dapple apple, pear rusty skin and pear dimple fruit reported from Asia, Europe, and North America (Hashimoto and Koganezawa, 1987; Hadidi *et al.*, 1990; Zhu *et al.*, 1995; Osaki *et al.*, 1996; Kyriakopoulou and Hadidi, 1998; Koganezawa *et al.*, 2003; Kyriakopoulou *et al.*, 2003; Shamloul *et al.*, 2004; Hadidi and Barba, 2011). It also infects apple (*Malus domestica*), pear (*Pyrus communis, P. pyrifolia*), wild apple (*M. sylvestris*), and wild pear (*P. amygdaliformis*) (Kyriakopoulou and Hadidi, 1998; Kyriakopoulou *et al.*, 2001, 2003; Koganezawa *et al.,* 2003; Shamloul *et al.*, 2004; Boubourakas *et al.*, 2008). Recently, ASSVd was also detected in stone fruits, i.e peach (*Prunus persica*) and apricot (*Prunus armeniaca*) in China (Zhao and Niu, 2008a, 2008b), sweet cherry in Greece (Kaponi, 2009), and Himalayan wild cherry (*Prunus cerasoides*) in India (Walia *et al.*, 2011), but its biological properties were not characterized.

In the course of a survey for pome and stone fruit viroids in Greece, leaves, bark and/or fruit samples were collected in early summer from eleven 4-year-old apple trees, originating from a state-certified nursery in Hemathia (Macedonia, Greece), and cultivated in a newly established apple orchard in Florina (Macedonia, Greece). In addition, samples were collected from two 17-year-old sweet cherry (*Prunus avium* cv. Tragana Edessis) trees grown at the edge of the same orchard, which showed mosaic symptoms on several leaves (Fig. 1a) and white spots on most of the fruit (Fig. 1b). The samples were tested by RT-PCR, using ASSVd-specific primers (Hadidi and Yang, 1990). ASSVd-positive results were obtained from the two symptomatic sweet cherry trees, whereas the eleven apple trees were all ASSVd-negative. The finding that sweet cherry trees naturally infected with ASSVd showed leaf and fruit disease symptoms needs to be further investigated to establish the relationship between ASSVd infection and disease symptoms.

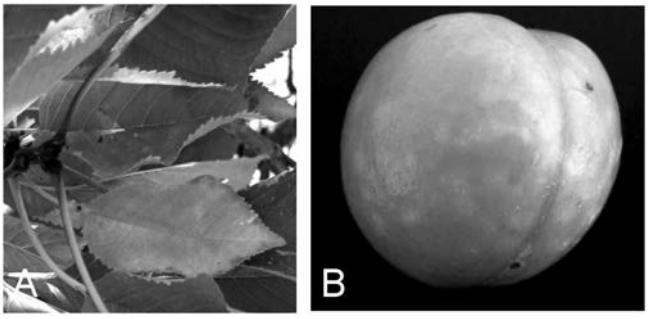

Fig. 1 Symptoms associated with natural infection of sweet cherry trees, cv. 'Tragana Edessis' with ASSVd: a, mosaic symptoms on cherry leaves; b, white spots on cherry fruit.

A second sampling of six sweet cherry trees (four 17-year-old sweet cherry trees cvs Tragana Edessis and Biggareau Burlat including the two aforementioned trees, and two 10-year-old sweet cherry trees of undetermined cultivar) and fifteen 4-year-old neighboring apples, including the first 11 samples (a possible ASSVd source of sweet cherry trees infection), was done a few weeks later in the same orchard. Tree samples were tested by tissue print hybridization, using an ASSVd-specific DIG-labeled riboprobe at stringent hybridization conditions (50% formamide, T=60°C), performed according to Palacio-Bielsa *et al.* (1999) with slight modification. Samples (fruit skin, bark and/or pedicel) of sweet cherry trees were ASSVd positive (Fig. 2) whereas apple samples were negative. Tissue-print hybridization has been used for the detection of several viroids such as *Potato spindle tuber viroid* (Podleckis *et al.*, 1993), *Chrysanthemum stunt viroid* (Hooftman *et al.*, 1996; Torchetti *et al.,* 2012), pome fruit viroids, including ASSVd (Podleckis *et al.*, 1993; Di Serio *et al.*, 2010), and other viroids (Muhlbach *et al.*, 2003; Pallas *et al.*, 2011).

Additional samples (leaf, bark, fruit skin) were collected from the above six sweet cherry trees later in the same summer and total nucleic acid extracts were used for ASSVd amplification by one tube-two steps RT-PCR (Faggioli *et al.*, 2001). Samples from five trees were assayed using two different ASSVd-specific primer pairs (Hadidi and Yang, 1990; Di Serio *et al.*, 2002) (Fig. 3), and samples from the sixth tree were assayed with the second ASSVd-specific primer pair (Di Serio *et al.*, 2002). Each of the six sweet cherry ASSVd-amplified amplicons was about 330 bp. The amplicons were directly sequenced individually with both primers of the reaction and found to be 96-97% homologous to ASSVd. Then, five of the six RT-PCR products were gel-purified, then successfully sequenced after cloning into pGEM-T Easy (Promega, USA) or pCR II (TOPO-TA, Invitrogen, USA) plasmid vectors, according to the manufacturer's instructions. A total of 20 complete ASSVd sequences were obtained, which were analyzed in NCBI database using BLASTn.

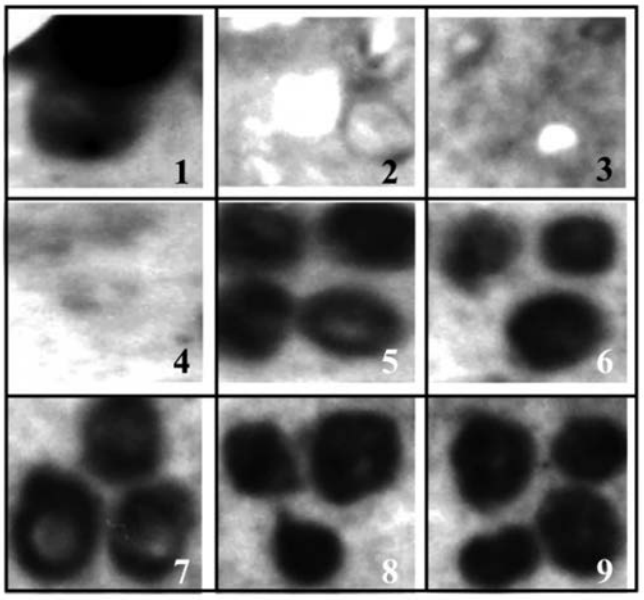

Fig. 2 Detection of ASSVd infection in sweet cherry trees by tissue printing hybridization using ASSVd DIG-labeled riboprobe. Positions: 1 and 2, ASSVd-positive and negative controls from apple, respectively; 3 and 4, apple trees adjacent to cherry trees; 5-9, sweet cherry trees nos. 659, 683, 681, 680, and 660, respectively (5, cv. Tragana Edessis, 6, cv. Biggareau Burlat, 7 and 8, undetermined cultivar, 9, cv. Tragana Edessis).

Sixteen different variants 327-340 nt in length were identified and deposited in GenBank under accession Nos. FJ974062-FJ974074, FN376408-FN396409 and GQ249350. The heterogeneity among the sequences ranged from 0 to 10% (0-29 nt), and all formed rod-like secondary structures as known ASSVd variants (Hadidi and Barba, 2011). They differed from the prototype sequences of ASSVd (ASSCS and Y00435, Hashimoto

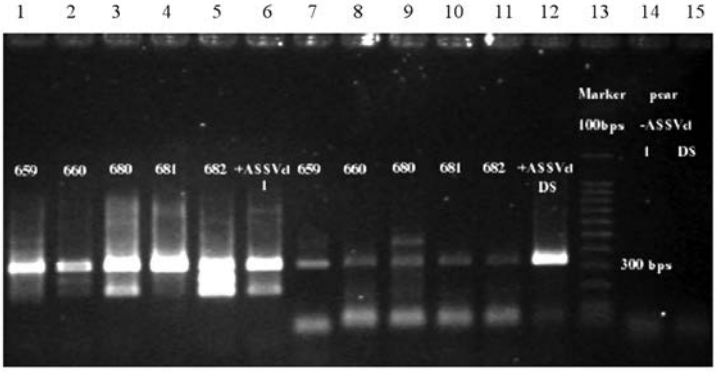

Fig. 3 Gel analysis of RT-PCR products of total nucleic acid extracts (leaf, bark, fruit) from the original sweet cherry tree samples (trees nos. 659, 660, 680, 681, 682) with two different primer sets for ASSVd. Lanes 1-6 and 14, Hadidi and Yang (1990) primers; lanes 7-12 and 15, Di Serio *et al.* (2002) primers; lane 13, DNA marker 100 bps (Invitrogen, UK); lanes 6 and 12, ASSVd-positive controls from infected apple; lanes 14-15, ASSVd-negative controls (healthy pear).

and Koganezawa, 1987) at 4-29 sites, and shared 93-99% identity (1-20 nt changes) with the Indian (Walia *et al.,* 2010) and Chinese (Zhao and Niu, 2006) apple isolates. In particular, they shared higher identity 96-99% (1-13 nt changes) with the Indian ASSVd isolates Y5, Y7 and Y8. and varying sequence identity (91-98%) with the Hellenic (Greek) ASSVd variants from pome fruit trees (1-30 nt changes).

There were 15 nt differences with the ASSVd prototype ASSCS (Hashimoto and Koganezawa, 1987), that were common to all Hellenic ASSVd variants, including sweet cherry and pome fruit tree variants (Kaponi, 2009). There were no sweet cherry-specific nucleotide changes in the ASSVd sequences obtained, and these sequences did not form a separate cluster in phylogenetic trees (Fig. 4). The variation (total number of nucleotide changes/total number of nts X%) among all 16 sweet cherry ASSVd sequences obtained was 9%, whereas the overall variation between all the Greek ASSVd-cherry sequences and other ASSVd sequences did not exceed 10%. Sequence variation in the same tree in two out of five trees ranged from 3 to 11 nts. This variation was located mostly in the variable domain (V) of the viroid molecule (nt 110-150), and secondarily in the terminal right (TR) domain (nt 150-190). Only one clone (FJ974062) of the 16 had mutations (5 nt) at primer annealing positions, the upper central conserved region (UCCR) and part of the upper variable domain (nt 82-122); these mutations were not considered in the analysis.

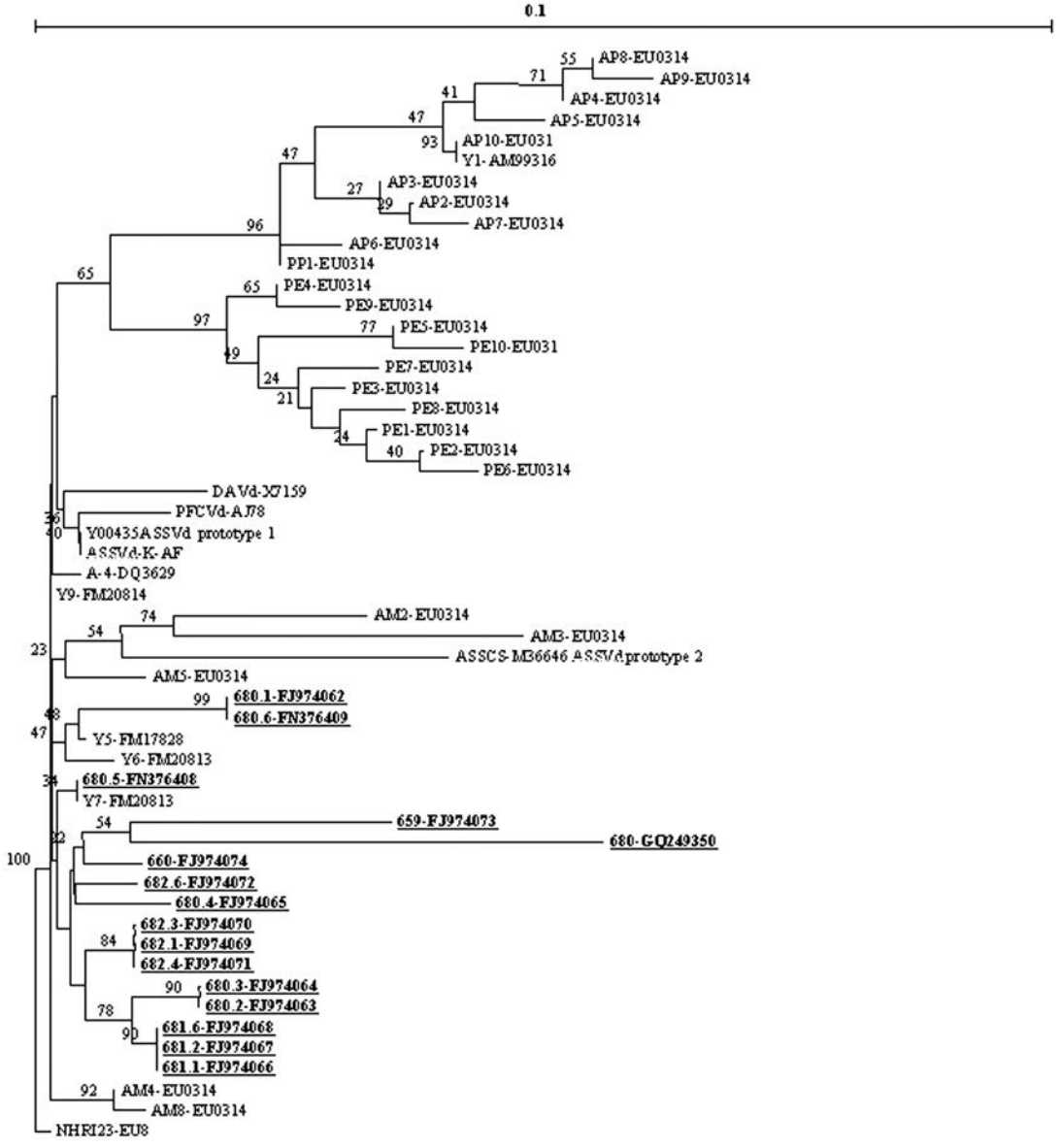

Fig. 4 Phylogenetic tree comprising 16 ASSVd Hellenic cherry sequences (bold, underlined), the prototype ASSVd sequences Y00435 and ASSCS, as well as additional 35 ASSVd sequences from GenBank. The Hellenic ASSVd cherry sequences do not form a coherent separate cluster.

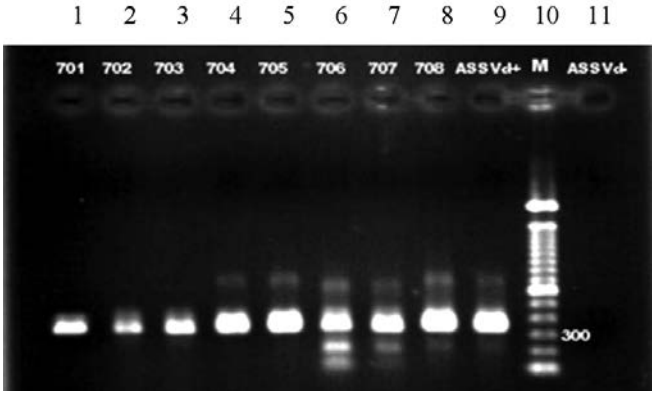

Fig. 5. Gel analysis of RT-PCR products of nucleic acid extracts of symptomatic leaves developed on cherry rootstocks grafted with buds from sweet cherry trees naturally infected with ASSVd. ASSVd-positive RT-PCR DNA fragments of the expected size (330 bp) were detected. Lanes 1-4, rootstock Gisella 6 (no. 701-704) grafted with buds from sweet cherry cv. Tragana Edessis; lanes 5-8, rootstock Maxma 14 (no.705-708) grafted with buds from sweet cherry cv. Tragana Edessis; lane 9, positive ASSVd control; lane 10, DNA marker 100 bp ladders, 300 indicates the position of 300 bp; lane 11, ASSVd-negative control.

Eight EU-certified (virus-free) cherry rootstocks (4 Maxma-14, *Prunus mahaleb* x *P. avium*, and 4 Gisella-6, *P. cerasus* x *P. canescens*) were grafted with sweet cherry buds from two ASSVd-infected trees of cv. Tragana Edessis at the beginning of autumn. Eight months later, in May (2 months after bud breaking) grafted rootstocks showed mosaic symptoms on several fully-developed leaves which tested ASSVd-positive by RT-PCR (Fig. 5).

Desvignes *et al.* (1999) reported that *Prunus avium* and other *Prunus* species, such as *P. armeniaca*, *P. insititia* and *P. persica*, are not experimental hosts of ASSVd. Zhao and Niu (2008a, 2008b) and Walia *et al.* (2011), however, showed that this viroid infects stone fruit species in nature, but they did not perform transmission experiments. We now report natural ASSVd infection of sweet cherry and provide experimental evidence that this viroid can be graft-transmitted to widely used cherry rootstocks such as Gisella-6 or Maxma-14. This finding confirms the need that sweet cherry, apricot, and peach germplasm be included in certification and quarantine programs in Europe and other countries to prevent ASSVd spread.

ACKNOWLEDGEMENTS

Research project co-financed by the European Social Fund (75%) and the Greek Ministry of Development - GSRT (25%).

REFERENCES

Boubourakas I.N., Arambatzis C., Dovas C., Kyriakopoulou P.E., 2008. Amelioration of a reverse transcription polymerase chain reaction (RT-PCR) for the detection of ASSVd, PBCVd and PLMVd viroids, and their presence in cultivated and wild pome and stone fruits in Greece. *Acta Horticulturae* **781**: 519-527.

Desvignes J.C., Grasseau N., Boye R., Cornaggia D., Aparicio F., Di Serio F., Flores R., 1999. Biological properties of *Apple scar skin viroid*: isolates, host range, different sensitivity of apple cultivars, elimination, and natural transmission. *Plant Disease* **83**: 768-772.

Di Serio F., Afechtal M., Attard D., Choueiri E., Gumus M., Lolic B., Matic S., Navarro B., Myrta A., 2010. Detection by tissue printing hybridization of pome fruit viroids in the Mediterranean basin: Incidence and biodiversity. *Proceedings 21st International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops, Neustadt, Germany*, *Julius Kühn Archiv* **427**: 357-360.

Di Serio F., Malfitano M., Alioto D., Ragozzino A., Flores R., 2002. *Apple dimple fruit viroid*: sequence variability and its specific detection by multiplex fluorescent RT-PCR in the presence of *Apple scar skin viroid*. *Journal of Plant Pathology* **84**: 27-34.

Faggioli F., Ragozzino E., Barba M., 2001. Simultaneous detection of stone or pome fruit viroids by single tube RT-PCR. *Acta Horticulturae* **550**: 59-63.

Flores R., Randles J.W., Owens R.A., 2003. Classification. In: Hadidi A., Flores R., Randles J.W., Semancik J.S. (eds.). Viroids, pp. 71-75. CSIRO Publishing, Collingwood, Australia.

Hadidi A., Barba M., 2011. *Apple scar skin viroid*. In: Hadidi A., Barba M., Candresse T., Jelkmann W. (eds.). Virus and Virus-Like Diseases of Pome and Stone Fruits pp. 57-62. APS Press, St. Paul, MN, USA.

Hadidi A., Huang C., Hammond R. W., Hashimoto J., 1990. Homology of the agent associated with dapple apple disease to *Apple scar skin viroid* and molecular detection of these viroids. *Phytopathology* **80**: 263-268.

Hadidi A., Yang X., 1990. Detection of pome fruit viroids by enzymatic cDNA amplification. *Journal of Virological Methods* **30**: 261-270.

Hashimoto J., Koganezawa H., 1987. Nucleotide sequence and secondary structure of *Apple scar skin viroid*. *Nucleic Acids Research* **15**: 7045-7052.

Hooftman R., Arts M.J., Shamloul A.M., Van Zayan A., Hadidi A., 1996. Detection of *Chrysanthemum stunt viroid* by reverse transcription-polymerase chain reaction and by tissue blot hybridization. *Acta Horticulturae* **432**: 120-128.

Kaponi M.S., 2009. Pome and stone fruit viroids in Greece-molecular characterization and phylogenetic relationships. Ph.D. Thesis. Agricultural University of Athens, Athens, Greece.

Koganezawa H., Yang X., Zhu S.F., Hashimoto J., Hadidi A., 2003. *Apple scar skin viroid* in apple. In: Hadidi A., Flores R., Randles J.W., Semancik J.S., (eds.). Viroids, pp. 137-141. CSIRO Publishing, Collingwood, Australia.

Kyriakopoulou P.E., Giunchedi L., Hadidi A., 2001. *Peach la-*

tent mosaic and pome fruit viroids in naturally infected cultivated pear *Pyrus communis* and wild pear *P. amygdaliformis*: implications on possible origin of these viroids in the Mediterranean region. *Journal of Plant Pathology* **83**: 51-62.

Kyriakopoulou P.E., Hadidi A., 1998. Natural infection of wild and cultivated pears with *Apple scar skin viroid* in Greece. *Acta Horticulturae* **472**: 617-625.

Kyriakopoulou P.E., Osaki H., Zhu S.F., Hadidi A., 2003. *Apple scar skin viroid* in pear. In: Hadidi A., Flores R., Randles J.W., Semancik J.S. (eds.). Viroids, pp. 142-145. CSIRO Publishing, Collingwood, Australia.

Muhlbach H.P., Weber U., Gomez G., Pallas V., Duran Vila N., Hadidi A., 2003. Molecular hybridization. In: Hadidi A., Flores R., Randles J.W., Semancik J.S. (eds.). Viroids, pp. 103-114. CSIRO Publishing, Collingwood, Australia.

Osaki H., Kudo A., Ohtsu Y., 1996. Japanese pear fruit dimple disease caused by *Apple scar skin viroid* (ASSVd). *Annals of the Phytopathological Society of Japan* **62**: 379-385.

Palacio-Bielsa A., Foissac X., Duran-Vila N., 1999. Indexing of citrus viroids by imprint hybridization. *European Journal of Plant Pathology* **105**: 897-903.

Pallas V., Faggioli F., Apartico F., Sanchez-Navarro J.A., 2011. Molecular hybridization techniques for detecting and studying fruit tree viruses and viroids. In: Hadidi A., Barba M., Candresse T., Jelkmann W. (eds.). Virus and Virus-Like Diseases of Pome and Stone Fruits pp. 333-339. The American Phytopathological Society Press, St. Paul, MN, USA.

Podleckis E.V., Hammond R.W., Hurtt S.S., Hadidi A., 1993. Chemiluminescent detection of potato and pome fruit viroids by digoxigenin-labelled dot blot and tissue blot hybridization. *Journal of Virological Methods* **43**: 147-148.

Shamloul A.M., Yang X., Han L., Hadidi A., 2004. Characterization of a new variant of *Apple scar skin viroid* associated with pear fruit crinkle disease. *Journal of Plant Pathology* **86**: 249-256.

Torchetti E.M., Navarro B., Trisciuzzi V.N., Nuccitelli L., Silletti M.R., Di Serio F., 2012. First report of *Chrysanthemum stunt viroid* in *Argyranthemum frutescens* in Italy. *Journal of Plant Pathology* **94**: 451-454.

Walia Y., Dhir S., Bhadoria S., Hallan V., Zaidi A.A., 2012. Molecular characterization of *Apple scar skin viroid* from Himalayan wild cherry. *Forest Pathology* **42**: 84-87.

Walia Y., Kumar Y., Rana T., Ram R., Hallan V., Zaidi A.A., 2010. Variability assessment and construction of infectious clone of Indian *Apple scar skin viroid*. *Proceedings 21st International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops, Neustadt, Germany*. *Julius Kühn Archiv* **427**: 96-100.

Zhao Y., Niu J., 2006. Cloning and sequencing of Sinkiang isolate of *Apple scar skin viroid* (ASSVd). *Journal of Fruit Science* **23**: 896-898.

Zhao Y., Niu JX., 2008a. Apricot is a new host of *Apple scar skin viroid*. *Australasian Plant Disease notes* **3**: 98-100.

Zhao Y., Niu JX., 2008b. Detection and sequencing of *Apple scar skin viroid* from peach in Xinjiang. *Journal of Fruit Science* **25**: 274-276.

Zhu S.F., Hadidi A., Yang X., Hammond R.W., Hansen A.J., 1995. Nucleotide sequence and secondary structure of pome fruit viroids from dapple apple diseased apples, pear rusty skin diseased pears and apple scar skin symptomless pears. *Acta Horticulturae* **386**: 554-559.

Received November 5, 2012
Accepted December 22, 2012