Plant Pathol. J. 17(5) : 300-304 (2001)

The Plant Pathology Journal

Occurrence of *Apple scar skin viroid*-Korean strain (ASSVd-K) in Apples Cultivated in Korea

Ju Hee Lee, Jean Kyung Park, Dong Hyuk Lee[1], Jae Youl Uhm[1], Sa Youl Ghim and Jai Youl Lee*

Department of Microbiology, Kyungpook National University, Daegu 702-701, Korea

[1]Department of Agricultural Biology, Kyungpook National University, Daegu 702-701, Korea

(Received on October 9, 2001)

Apple is the most economically important fruit in Korea. The suspected viroid disease of dapple apple was found in apple fruits cultivated in Kyungpook province. Symptoms begin in mid-July as small circular spots, which stand out against the background color on the young fruit. Dappling of the fruit becomes more intense and easier to detect as the fruit approaches maturity; the affected spots remain yellowish as the background color develops. Spots enlarge as the fruit matures. No leaf or bark syndromes have been associated with this disease. The infected fruits are downgraded considerably during quality grading. The low molecular weight RNA containing viroid RNA molecules were extracted from the peels of the apples with dapple symptoms. The RNA molecules were extracted from the apples using Qiagen column chromatography. The purified RNAs were used for the synthesis of cDNA with RT-PCR. The PCR products were then ligated into a pGEM-T Easy vector, cloned and sequenced. The sequence of the viroid RNA molecule shows 331 nucleotides with one base difference ("G" insertion between the position of 133 and 134) compared with that of the *Apple scar skin viroid* (ASSVd) reported by Hashimoto and Koganezawa in Japan. This is the first report on the occurrence of the ASSVd in apple trees cultivated in Korea, as well as the identification of a new Korean strain of the ASSVd.

Keywords **:** *Apple scar skin viroid* (ASSVd), reverse transcription polymerase chain reaction (RT-PCR), viroid RNA molecules.

Viroids are the smallest plant pathogens of single stranded, covalently closed circular RNA molecules with sizes ranging from 246 to 371 nucleotides. Viroids may cause significant damage to crops and fruit trees (Diener, 1987; Di Serio et al., 1996; Hashimoto and Koganezawa, 1987; Sano et al., 1989). Currently, about 27 different viroids have been detected (Flores et al., 1998). *Apple scar skin viroid* (ASSVd), dapple apple viroid (DAVd), and pear rusty skin viroid (PRSVd) are economically important in apples and pears in China and Japan.

Dapple apple, a fruit-blemishing disease, was first described in 1956 from apple orchards in New Hampshire. The disease has been reported to occur in Canada, Japan, the United Kingdom, and Italy. Another graft-transmissible disease of apple that causes fruit disorder is apple scar skin. This disease has been described in the United States, China, and Japan (Hashimoto and Koganezawa, 1987; Puchta et al., 1990). The host range of the agents of dapple apple and apple scar skin disease is limited, which is restricted to pome fruit trees (Ito et al., 1993; Hernandez et al., 1992).

Recently, the causal agent of apple scar skin disease in Japan has been identified as a viroid, and has been cloned and sequenced (Hashimoto and Koganezawa, 1987). The Japanese isolate of ASSVd contains 330 nucleotides, is not related to other known viroids, and has been categorized into a new viroid group assigned as the genus *Apscaviroid* and ASSVd as the type species in the genus. In this study, we have found the viroid-like symptoms in apple trees (*Malus pumila* var. *dulcissima* Koidz) var. Miegie Life cultivated in the south part of Kyungpook province, Korea. The Japanese variety of Miegie Life was improved as a hybrid of Senshu and Tsugaru in 1990. In this report, the viroid-like RNA molecules were cloned by the direct RT-PCR methods, and the complementary DNA (cDNA) sequences of the viroid-like RNA molecules were analyzed by using the Sanger method (Sanger et al., 1977).

Materials and Methods

Viroid RNA extraction. Samples with dapple apple symptoms were collected in mid August (Fig. 1). The apple skins were peeled off and then stored in the deep-freezer at -70°C or promptly used to extract the nucleic acids. The discolored apple skins (5-10 g of fresh sample) were homogenized with liquid nitrogen in sterilized mortars. The diluted samples with 5 volumes of extraction buffer (50 mM Tris-HCl [pH 7.6], 20 mM NaCl, 10 mM EDTA, 10 mM DTT) were centrifuged by 10,000 rpm at 4°C for 10 min. The supernatant was treated with 1% SDS and proteinase K (100

*Corresponding author.
Phone) +82-53-950-5375, FAX) +82-53-955-5522
E-mail) jaylee@knu.ac.kr

Fig. 1. Viroid-like dapple symptoms in apples (*Malus pumila* var. *dulcissima* Koidz.) var. Miegie Life cultivated in Korea.

μg/ml) and was incubated at 42°C for 15 min. After the addition of equal volume of phenol/chloroform (1 : 1), the homogenate was incubated at 60°C for 10 min or 72°C for 5 min. After centrifugation (12,000 rpm at 4°C for 10 min), the same volume of chloroform was added to the supernatant. The recovered upper aqueous phases were precipitated with the addition of 1/10 volume of sodium acetate solution and 2.5 volumes of 95% cold ethanol at -70°C for 30 min. The RNA samples recovered with centrifugation (12,000 rpm for 20 min) were applied to the RNA extraction by the use of the Qiagen kit.

Slab gels of 6% polyacrylamide, 0.12% bisacrylamide containing 10% ammonium persulfate were ran in 1/20 of 10X TBE. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml). The viroid RNA band was identified under UV transilluminator by standard size marker.

RT and PCR. Two pairs of primers were applied for the reverse transcription and PCR amplification of viroid RNA molecules; AS1 and AS2 primers were designed from previously published ASSVd (Hashimoto and Koganezawa, 1987) and AS3 and AS4 were designed from the primer program of Brick to give partial length products. Primers AS1 and AS3 contain complementary sequence of ASSVd, while primers AS2 and AS4 contain homologous sequence of ASSVd (Table 1). The first strand of cDNA was synthesized with the ASSVd-specific primer complementary to the AS2 and the M-MLV reverse transcriptase. The second strand of cDNAs was synthesized with the PCR-amplification. The RT-PCR products were electrophoresed on the native polyacrylamide gels and stained with ethidium bromide.

Cloning of Viroid RNA. The PCR products were electroeluted from 2% agarose gels and recovered by ethanol precipitation. The purified PCR products were ligated into pGEM-T Easy vector, and then the vector was transformed into *Escherichia coli* JM 109 by the $CaCl_2$ method based on Cohen et al. (1972). Each transformation was cultured onto LB/ampicillin/IPTG/X-Gal (LB agar medium, 50 μg/ml of ampicillin, 0.1 mM IPTG, 40 μg/ml of X-Gal) overnight (16-24 h) at 37°C. After transformation, the plasmid DNA was purified by Qiagen column chromatography from a white bacterial colony. The plasmid DNAs were incubated with *Eco*RI restriction enzyme and the size of inserted DNA was measured.

Sequence analysis. PCR products cloned into pGEM-T Easy vector were sequenced using the standard dideoxynucleotide chain termination method (Sanger et al., 1977) employed in the OmniBase™ DNA cycle system. The appropriate primers of M13 universal primer, T7 primer, SP6 primer, and reverse primers were applied for the sequencing analysis. DNA insert cloned into the vector was purified as a template using Qiagen column chromatography ideally suited for sequencing. The reaction tubes in a thermal cycler were preheated to 95°C for 2 min, before the cycling program started: denaturation at 95°C for 30 sec, annealing at 42°C for 30 sec, extension at 70°C for 1 min, 30 cycles total, then 4°C.

The alignment of the sequences was carried out by means of a multiple sequence algorithm for the phylogenic analysis. The most thermodynamically stable secondary structure of ASSVd variant was obtained by using the algorithm of Zuker and Stiegler (1981).

Dot blot hybridization. Five microliters of purified low molecular RNA (6.7 μg) was mixed with 11.63 μl of denaturing solution (1.5 μl of 5X formaldehyde gel running buffer, 2.63 μl of formaldehyde, 7.5 μl of formamide), incubated for 15 min at 65°C, then quickly chilled. Denatured nucleic acid samples were serially diluted with 10 SSC, 24.6% formamide, 2.9% formaldehyde, 10 mM MOPS, 24.75 mM sodium acetate, 0.49 mM EDTA by 2, 10 and 100-fold-dilution, and 5 μl aliquots were spotted onto a hybridization transfer membrane (GeneScreen™). The RNA was then fixed to the membrane by UV irradiation with 1800 Stratalinker (Stratagene) and prehybridized for 4 h at 65°C in prehybridization solution (5X SSC, 5X Denhardt's solution 0.5% SDS, salmon sperm DNA at 200 μg/ml). The denatured radiolabeled probe was directly added to the prehybridization fluid and incubation was continued for 16-24 h at the appropriate temperature. After hybridization, the filter was washed twice for 20 min at room temperature in 1×SSC, 0.1% SDS, followed by three washes of 20 min each at 68°C in 0.2×SSC and 0.1% SDS. An autoradiograph was established by exposing the filter for 24-48 h to X-ray film at -70°C.

Table 1. Synthetic primer sequence for RT-PCR used in this study

Primer name	Sequence	No. of bases
AS1	5'-TCGTCGACGACGACAGGTGAGTTCC-3'	25-mer
AS2	5'-GTCGTCGACGAAGGCCGGTGAGAAAG-3'	26-mer
AS3	5'-CCCGGTAAACACCGTGCGGT-3'	20-mer
AS4	5'-ACCGGGAAACACCTATTGTG-3'	20-mer

Nucleotide sequence accession number. The ASSVd-K DNA sequence determined from Korean apples has been deposited in the GenBank under the accession no. ASSVd-K AF421195.

Results

The viroid RNA band was identified from the low molecular weight RNAs extracted from apple skin with the application of the spectrophotometer and the 6% polyacrylamide nondenaturing gel (Fig. 2). Viroid RNA molecules show a single band between 220 bp and 500 bp of a known sequence ladder in the nondenaturing polyacrylamide gel electrophoresis (PAGE).

Low molecular RNAs purified by Qiagen kit were applied as templates for RT-PCR. The cDNA of viroid-like RNA was synthesized using reverse transcriptase with the two pairs of primers AS1, 2 and AS3, 4 composed of the central conserved region (CCR) region in the ASSVd. The amplified PCR products were detected between the known size markers of 298 bp and 344 bp in the gel (Fig. 3).

The PCR products amplified with two pairs of primers were ligated into pGEM-T Easy vector and transformed into *E. coli* JM109. Transformants were incubated at 37°C overnight and the plasmid DNA was purified by Qiagen plasmid midi kit and then digested with *Eco*RI restriction enzyme. The inserted DNA fragments were identified as the same size as the PCR products of the viroid RNA molecules (Fig. 4).

Nucleotide sequences of the cDNA were analyzed by the Sanger method (Sanger et al., 1977) and compared with homology of other viroids. The sequence of the viroid RNA molecule showed 331 nucleotides with one base difference ("G" insertion between the position of 133 and 134) compared with that of ASSVd reported in 1987 in Japan. Sequence analysis showed that identified nucleotide sequences were over 99% homologous with reported ASSVd sequences (Hernandez et al., 1992; Hashimoto and Koganezawa, 1987; Puchta et al., 1990; Zhu et al., 1995). Our results confirmed that the causal agent from the diseased apple is one of the strain of ASSVd.

The secondary structure representing the lowest free energy of ASSVd was determined by the program (http://mfold2.wustl.edu/~mfold/rna/form1.cgi) developed by Zuker. The most stable secondary structure for ASSVd is a

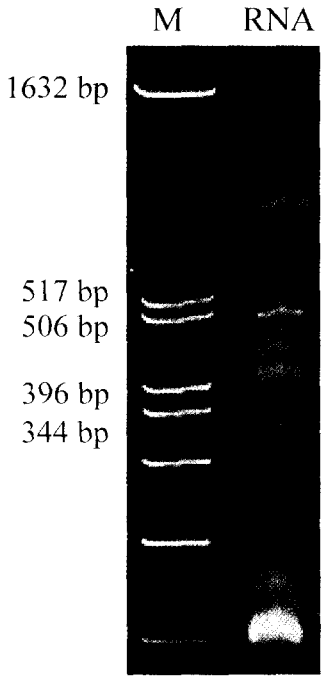

Fig. 2. Electrophoresis separation of viroid-like RNA from dapple apple under non-denaturing condition. Markers were pBR322 digested with *Hin*fI restriction enzyme.

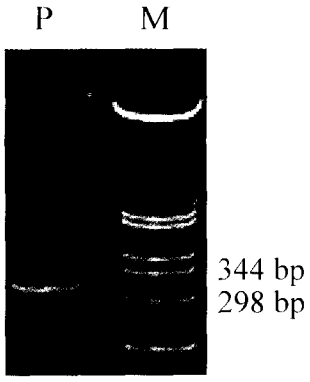

Fig. 3. PCR products of the viroid-like RNA molecules were migrated to the position of size markers of 298 and 344 base pairs under 6% PAGE.

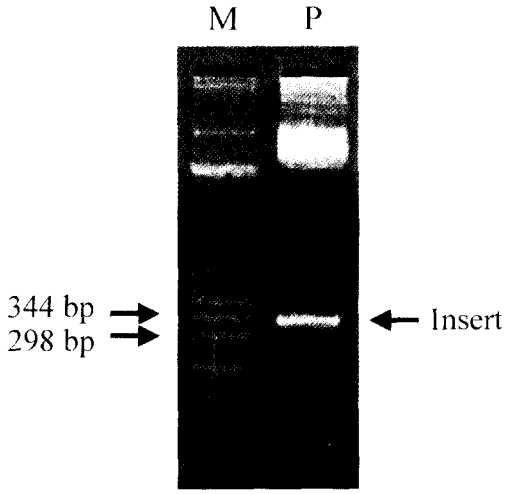

Fig. 4. Inserted DNA fragments were migrated to the position of size markers of 298 and 344 base pairs on the 2.0% agarose gel.

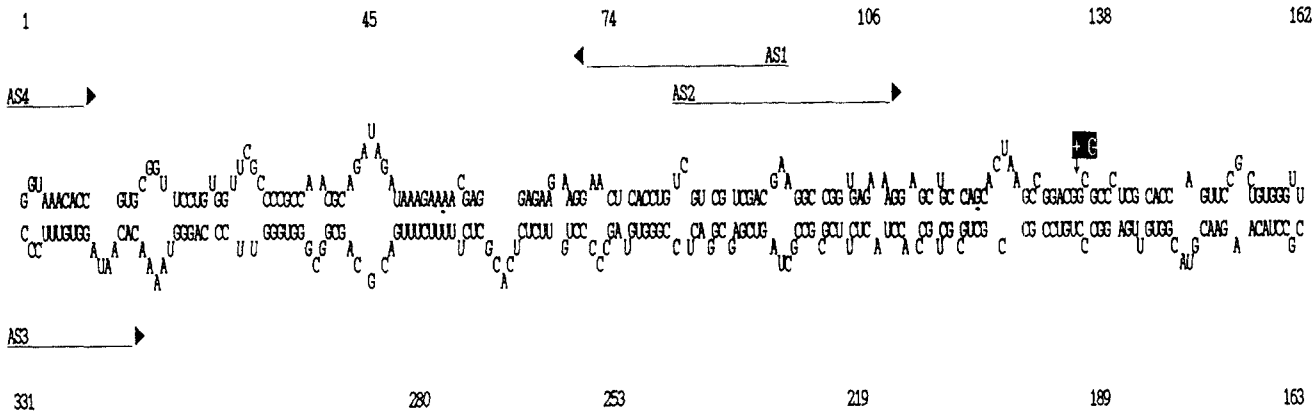

Fig. 5. Secondary structure of the *Apple scar skin viroid*-Korean strain (ASSVd-K). AS1, 2, 3, and 4 means the primers used for RT-PCR.

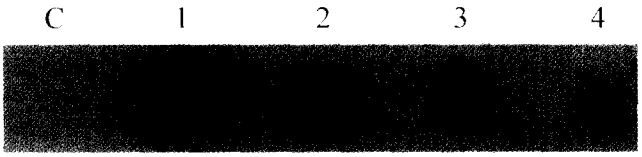

Fig. 6. Autoradiograph of dot blot hybridization of 32P-labeled *Apple scar skin viroid* (ASSVd) cDNA with the different dilution of RNA molecules at increasing extraction from the dapple apple symptoms: 1, the RNAs extracted from ASSVd-infected tomato leaf tissue; 2, the undiluted RNA samples extracted from ASSVd-infected apple fruit skin; 3, RNA samples diluted to 1:5; and 4, RNA samples diluted to 1:10. C, RNA samples extracted from apple skin with no symptoms were treated as control.

rod-like conformation with a free energy of -125.2 kcal/mole (Fig. 5).

The viroid-like RNA molecule causing dapple apple symptom was considered as a new variant of ASSVd included in the ASSVd subgroup from PSTVd type. For the phylogenic analysis (data not shown), the alignment of the nucleotide sequence was carried out by means of a multiple sequence algorithm with hierarchical clustering.

To confirm the identity of the viroids from the dapple apple, a dot blot hybridization assay was done with the ^{32}P-labeled ASSVd cDNA and the total RNAs extracted from ASSVd infected apple skin, as well as from uninfected tomato leaf tissue as negative control (Fig. 6)

The intensity of hybridization was correlated with sample RNA concentrations (Fig. 6). As shown by the intensity of the hybridization signals, the highest and lowest concentrations of the viroid RNA molecules were found in the infected fruit skin.

Discussion

Several viroids have been reported from hop, cucumber, and fruit trees, including citrus and grapevine. *Apple scar skin viroid* (ASSVd), dapple apple viroid (DAVd), and pear rusty skin viroid (PRSVd) are economically important in apples and pears in East Asia. Currently, however, while the pathogens of ASSVd and DAVd widely occur in Asia, Europe and USA, the ASSVd is the only known viroid detected from apple trees. The Japanese type strain of ASSVd contains 330 nucleotides. ASSVd has no relation to other known viroids and has been categorized into a new viroid group.

It has been reported that the viroid-like RNA isolated from apples of 'Indo' variety, which were grafted onto 'Starking Delicious' and 'Meguni' varieties, was that of dapple and scar skin viroids. It has also been reported that symptoms such as fruit crinkling, dappling and bark blister have been detected in varieties Jonathan, Ohrin, Starking Delicious, Mutsu, Senshu, Yoko, Fuji, and Hokuto. Disease suspected to be dapple apple viroid with a typical viroid symptom was found in a variety of Miegie Life apple (a hybrid from varieties Senshu and Tsugaru developed in Japan in 1990) in the south part of Kyungpook province. Dappling of the fruit becomes more severe and easier to detect as the fruits are maturing; the affected round spots remain yellowish or colorless as the background develops with red color.

Low molecular RNA was extracted from the apple skin with dapple symptom. Electrophoresis and RT-PCR were then applied for the identification of viroid RNA molecules. The RT-PCR assay is the more sensitive method for detecting both the purified viroid RNA molecules and the viroids in total nucleic acids extracted from infected plant tissues, suggesting that there is no inhibitory effect on RT-PCR by contaminants (Hurtt et al., 1996). In addition to RT-PCR, dot and northern blot analysis were used for the detection of viroids in a total RNA fraction (Yan et al., 1997). A gene diagnosis based on nucleic acid hybridization for the detection of viroids is a highly reliable method as compared with polyacrylamide gel electrophoresis.

The present study demonstrated that the agent associated

with the Korean isolate of dapple apple disease is a viroid that shows highly close homologous nucleotide sequence of *Apple scar skin viroid* in Japan (Hadidi et al., 1990; Hernandez et al., 1992). The molecular hybridization method for the detection of viroids has been developed. However, the ASSVd type Korean isolate was detected on the variety of Miegie Life, and that the viroid could have infected other varieties of apples being cultivated in Korea. In the application of molecular hybridization techniques, viroid diseases should be screened from the various foreign apple cultivars and the many varieties of apples being cultivated in the country.

Viroid diseases such as ASSVd, DAVd and PRSVd are economically important in apples and pears in the United States, China and Japan. In Korea, there is a risk of introducing these pathogens from other countries referred to as international movement of plant pathogens. Two different methods of heat therapy and meristem-tip culture have often been used to eliminate viruses from infected plants. Paludan (1985) reported that the combination of prolonged low temperature and meristem-tip culture was effective for production of viroid-free chrysanthemum upto 50 to 70% inactivation ratio against *Chrysanthemum stunt viroid* and *Chrysanthemum chlorotic mottle viroid*. However, the elimination of viroid RNA molecules by the application of heat-therapy and/or meristem-tip culture methods has been unsuccessful in practice. The prevention of viroid has not been achieved throughly around the world until now. Future studies should consider the development of a viroid detection method to prevent the introduction of new viroid RNA molecules from foreign countries and the propagation of viroid diseases in Korea.

Acknowledgment

This work was partly supported by grant No. 981-0603-016-2 from the Basic Research program of the Korea Science and Engineering Foundation.

References

Cohen, S. N., Chang, A. C. Y. and Hsu, L. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* 69:2110-2114.

Diener, T. O. 1987. The Viroids. Plenum Press: New York and London.

Di Serio, F., Aparicio, F., Alioto, D., Ragozzino, A. and Flores, R. 1996. Identification and molecular properties of a 306 nucleotide viroid associated with apple dimple fruit diease. *J. Gen. Virol.* 77:2833-2837.

Flores, R., Randles, J. W., Bar-Joseph, M. and Diener, T. O. 1998. A proposed scheme for viroid classification and nomenclature. *Arch. Virol.* 143:623-629.

Hadidi, A., Huang, C., Hammond, R. W. and 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.

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

Hernandez, C., Elena, S. F., Moya, A. and Flores, R. 1992. Pear blister canker viroid is a member of the apple scar skin subgroup (apscaviroids) and also has sequence homology with viroids from other subgroups. *J. Gen. Virol.* 73:2503-2507.

Hurtt. S. S., Podleckis, E. V. and Howell, W. E. 1996. Integrated molecular and biological assays for rapid detection of apple scar skin viroid in pear. *Plant Dis.* 80:458-462.

Ito, T., Kanematsu, S., Koganezawa, H., Tsuchizaki, T. and Yoshida, K. 1993. Detection of a viroid associated with apple fruit crinkle disease. *Ann. Phytopath. Soc. Japan* 56:520-527.

Paludan, N. 1985. Inactivation of viroids in chrysanthemum by low-temperature treatment and meristem-tip culture. *Acta Hort.* 164:181-186.

Puchta, H., Luckinger, R., Yang, X., Hadidi, A. and Sanger, H. L. 1990. Nucleotide sequence and secondary structure of apple scar skin viroid (ASSVd) from China. *Plant Mol. Biol.* 14: 1065-1067.

Sanger, F., Nicklen, S. and Coulson, A. R. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.

Sano, T., Hataya, T., Terai, Y. and Shikata, E. 1989. Hop stunt viroid strains from dapple fruit plum and peach in Japan. *J. Gen. Virol.* 70:1311-1319.

Yan, F., Wah, W. C. and Symons, R. H. 1997. A high sensitivity RT-PCR assay for the diagnosis of Grapevine viroids in field and tissue culture samples. *J. Virol. Methods* 63:57-69.

Zhu, S. F., Hadidi, A., Hammond, R. W., Yang, X. and 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 Hort.* 386:554-559.

Zucker, M. and Stiegler P. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* 9:133-148.