

CHARACTERIZATION OF A NEW VARIANT OF *APPLE SCAR SKIN VIROID* ASSOCIATED WITH PEAR FRUIT CRINKLE DISEASE

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SUMMARY

A new variant of *Apple scar skin viroid* (ASSVd) was isolated from Chinese pear with fruit crinkling symptoms. The pear fruit crinkle variant of ASSVd is 333 nucleotides in length and shows 95% identity with ASSVd prototype. It also shows 92% and 98% identity with the viroid variants associated with pear rusty skin disease in China and pear fruit dimple disease in Japan, respectively. This viroid was detected in all fruit crinkle-diseased samples examined. *Pear blister canker viroid* (PBCVd) was detected in less than 20% of the same diseased samples. The Chinese variant of PBCVd is 315 nucleotides in length and shows 97% identity with PBCVd prototype.

Key words: ASSVd, detection, RT-PCR-ELISA, PBCVd, nucleotide sequencing.

INTRODUCTION

Pear trees are natural hosts of three viroids: *Apple scar skin viroid* (ASSVd), *Pear blister canker viroid* (PBCVd) and *Peach latent mosaic viroid* (PLMVd) (Hadidi and Yang, 1990; Hernandez *et al.*, 1991; Yang *et al.*, 1992; Zhu *et al.*, 1995; Kyriakopoulou *et al.*, 2001, 2003; Flores *et al.*, 2003). None of these viroids, however, has been reported to be associated with crinkling symptoms of pear fruit. Pear fruit crinkle disease was first observed in 1995 by one of the authors, X. Yang, in Chinese pear grown in the He Bei Province, China. The disease causes fruit disorder, thus lowering its marketability and quality. To investigate the possible involvement of a viroid or a viroid-like agent in pear fruit crinkle etiology, total nucleic acids were extracted from diseased fruits, analyzed for the presence of circular

RNA molecules and known viroids, which were then cloned and sequenced. Here, we report the characterization of a new variant of ASSVd that is associated with pear fruit crinkle disease in China. This variant is 333 nucleotides in length and differs from ASSVd variants that were reported from infected apple or pear trees. We also report the detection of PBCVd in less than 20% of the fruit crinkle-diseased samples examined.

MATERIALS AND METHODS

Source of pear crinkle-diseased fruits. Pear fruits showing symptoms of pear fruit crinkle disease and healthy fruits were obtained from orchards in the suburbs of Boarding, Hei Bei province, China. The diseased fruits were from Chinese pear cultivars 'Xuehuali' and 'Yali' with fruit crinkling symptoms.

Sources of known ASSVd-infected and PBCVd-infected pear tissues. ASSVd-infected pear leaves were provided by Ms. Suzanne Hurtt, USDA, ARS, Beltsville, MD. PBCVd-infected leaves were obtained from Mr. Joseph Postman, USDA, ARS, Corvallis, OR.

Nucleic acid extraction from pear crinkle-diseased fruits. Total nucleic acids were extracted from 40 g of pear crinkle-diseased fruits (cv Xuehuali or cv Yali) as essentially as described (Hadidi and Yang, 1990). The procedure includes sodium dodecyl sulfate (SDS), phenol, and chloroform extraction, and DEAE-cellulose column (1 x 15 cm) purification. The fraction containing nucleic acids was eluted from DEAE-cellulose with 20-30 ml of 1.5 M NaCl in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na₂EDTA, pH 8.0) and then precipitated by the addition of 2 vol. of cold ethanol and 0.1 vol. of 3 M sodium acetate at -20°C overnight. Nucleic acids were collected by centrifugation at 10,000 g for 15 min and the resulting pellet was rinsed once with 70% ethanol, re-pelleted, dried *in vacuo*, and dissolved in 1xTAE buffer. Nucleic acids were re-precipitated as described above and resuspended in sterile H₂O. Nucleic acid concentration was determined by measuring the optical density at 260 nm.

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RNA extraction from ASSVd-infected or PBCVd-infected tissues. One-gram samples of ASSVd- or PBCVd-infected pear leaves or uninfected leaves were powdered with liquid nitrogen in a mortar with a pestle. Total RNA was then extracted from the powdered tissue using the Qiagen RNeasy plant kit according to the manufacturers recommendation (Qiagen, Valencia, CA, USA).

Return gel and two-dimensional gel electrophoresis. Purified nucleic acids from pear crinkle-diseased fruit (30 µg/sample) were analyzed by return gel and two-dimensional gel electrophoresis and gels were stained with silver nitrate (Schumacher *et al.*, 1983, 1986; Bostan *et al.*, 2004).

Standard and multiplex reverse transcription - polymerase chain reaction (RT-PCR). One microliter of total nucleic acids from pear crinkle-diseased fruit tissue or 1 µl of total RNA from ASSVd-infected or PBCVd-infected leaf tissue was added to 10 µl of H₂O containing 1 µg ASSVd complementary primer (ASSVd-C) (Table 1). cDNA synthesis was as described (Shamloul and Hadidi, 1999). The cDNA was kept at -20°C prior to PCR amplification.

Five microliters of each cDNA were mixed with PCR reaction mixture containing 5 µl of 10xPCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, and 0.01% (w/v) gelatin], 1 µl 10 mM dNTPs, 0.2 µM of ASSVd-C and ASSVd homologous primer (ASSVd-H) for standard PCR or ASSVd-H and PBCVd homologous (PBCVd-H) primers for multiplex PCR (Table 1), one unit of AmpliTaq™ Gold DNA polymerase and sterile water to a total volume of 50 µl. The reaction was placed in a programmable DNA Thermocycler (9600, Perkin Elmer, Norwalk, USA) preheated at 95°C for 10 min to activate the enzyme. The cycling parameters were: denaturation at 94°C for 30 seconds, primer annealing at 62°C for 30 seconds, and extension at 72°C for 45 seconds, for 35 cycles, with a final extension step at 72°C for 7 minutes.

Analysis of RT-PCR amplified products. Aliquots of 5 µl of PCR-amplified products were analyzed on 5%

or 6% polyacrylamide gels as previously described (Shamloul *et al.*, 1995; Hadidi *et al.*, 1997). Gels were stained with silver nitrate.

PCR-DIG labeling. DIG labeling and PCR amplification of viroid cDNAs were essentially as described (Shamloul and Hadidi, 1999; Shamloul *et al.*, 2001, 2002). DIG-labeled amplified products were analyzed on 5% polyacrylamide gels as described above.

Preparation of biotin-labeled viroid cDNA capture probe. DNA oligonucleotides (21-25 nt in length, Table 2) were synthesized and biotinylated at Life Technologies, Inc. (Gaithersburg, MD, USA). The DNA sequence of each capture probe was complementary to the internal nucleotide sequence of the amplified viroid DNA. The sequence of each probe was selected by using the primer analysis software 'rawprimer' from University of Wisconsin (Madison, USA; <http://alces.med.umn.edu/rawprimer.html>).

Microwell capture hybridization assay. The detection of DIG-labeled amplified ASSVd DNA and PBCVd DNA with 5'-biotinylated ASSV cDNA and PBCVd cDNA capture probe, respectively, was carried out using a PCR-ELISA detection system (Roche, Indianapolis, IN, USA). It was essentially as described (Shamloul and Hadidi, 1999; Shamloul *et al.*, 2002; Youssef *et al.*, 2002) except that 5'-biotinylated DNA capture probe was used at 50 ng ml⁻¹, wells were washed 6 instead of 5 times, and microplates were incubated in the dark with agitation for only 30 min.

Cloning and Sequencing. Pear fruit crinkle disease (PFCD)-derived PCR products amplified using 3 different pairs of ASSVd primers (Table 1) were cloned into p^{CRUI-TOPO} vector as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). The nucleotide sequences of five clones of each product amplified with one pair of primers were determined. The cloned DNAs were sequenced using ABI-PRISM™ 373 A Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The obtained sequence was aligned with the sequences

Table 1. Viroid primers for RT-PCR amplification.

Viroid	Primers	Number of bases	Sequence	Position	Reference
ASSVd	ASSVd-C	16	5'-CCTTCGTCGACGACGA-3'	82-97	Hadidi and Yang, 1990
	ASSVd-H	25	5'-CCGGTGAGAAAGGAGCTGCCAGCA-3'	98-121	
ASSVd	PFC-C1	17	5'-GTCCGCTCGACTAGCGG-3'	226-242	This work
	PFC-H1	19	5'-TCCGGGTGTAGCCCCCTGT-3'	243-261	
ASSVd	PFC-C2	24	5'-ACCAATTGTGTTTACCCTGTAGA-3'	302-325	This work
	PFC-H2	24	5'-GTTTCCCCGAAAACACCGTGCGG-3'	326-16	
PBCVd	ASSVd-C	16	5'-CCTTCGTCGACGACGA-3'	81-96	Hadidi, unpublished
	PBCVd-H	25	5'-GTCTAGAAGCCTGGGCGCTGGCTGG-3'	97-121	

of ASSVd prototype from apple in Japan (Hashimoto and Koganezawa, 1987), pear rusty skin variant of ASSVd from China (Zhu *et al.*, 1995), and pear fruit dimple variant of ASSVd from Japan (Osaki *et al.*, 1996). The secondary structure of the agent associated with PFCD was constructed using online *mfold* computer program described by Mathews *et al.* (1999) (<http://www.bioinfo.rpi.edu>). Similarly, PFCD-derived PCR products amplified using PBCVd primers (Table 1) were also cloned into p^{CRII-TOPO} vector and the nucleotide sequences of several DNA clones were determined as described above.

Dot blot and Northern blot hybridization. About 20 µl of each sample of DEAE cellulose-purified total nucleic acids from pear crinkle-diseased fruits were denatured, spotted on a Nytran membrane and nucleic acids were cross-linked to the membrane as described (Kyriakopoulou *et al.*, 2001). Membranes were stored in a plastic bag at 4°C until used.

Total nucleic acids from pear fruit crinkle-diseased fruits were separated by electrophoresis in polyacrylamide gel containing 7% urea. The denatured gels were neutralized and nucleic acids were electro-transferred and cross-linked to Nytran membranes as previously described (Shamloul *et al.*, 1995). Each dot or Northern blot membrane was pre-hybridized in hybridization solution (6xSSC, 50% formamide, 1% SDS, 10xDenhardt's reagent, and 1 µg of calf thymus DNA per ml) for 1 h at 55°C and then hybridized in the same buffer with a DIG-labeled ASSVd cRNA (50 ng ml⁻¹) at 55°C overnight as described (Hadidi *et al.*, 1997; Kyriakopoulou *et al.*, 2001). The hybridized membranes were developed with chemiluminescence reagent as recommended by the manufacturer (Roche Diagnostic, IN, USA) (Hadidi *et al.*, 1997).

RESULTS

Pear fruit crinkle disease symptoms. The Pear fruit crinkle disease causes wrinkling of Chinese pear fruits cultivars Xuehuali (Fig. 1A, left) and Yali (Fig. 1B, left). Healthy pear fruits of the same cultivars lack disease symptoms (Fig. 1A and B, right). Both healthy and diseased fruits were found in the same orchards.



Fig. 1. Symptoms of pear fruit crinkle disease in Chinese pear cv Xuehuali (A, left) and cv Yali (B, left). Healthy Chinese pear fruits (A and B, right).

Return gel and two-dimensional gel electrophoresis analyses. Figure 2 shows analysis of nucleic acid extracts from pear crinkle-diseased fruit by return gel electrophoresis. One band in the region of circular molecules in the gel was detected from extracts of diseased pear cvs Xuehuali and Yali (Fig. 2A, lanes 1 and 2, respectively). This band is about the same size as that in the ASSVd control (Fig. 2A, lane 3). The circularity of the RNA molecules from pear fruit crinkle-diseased fruit cv Xuehuali was confirmed by two-dimensional gel electrophoresis analysis of total nucleic acids from diseased tissue (Fig. 2B).

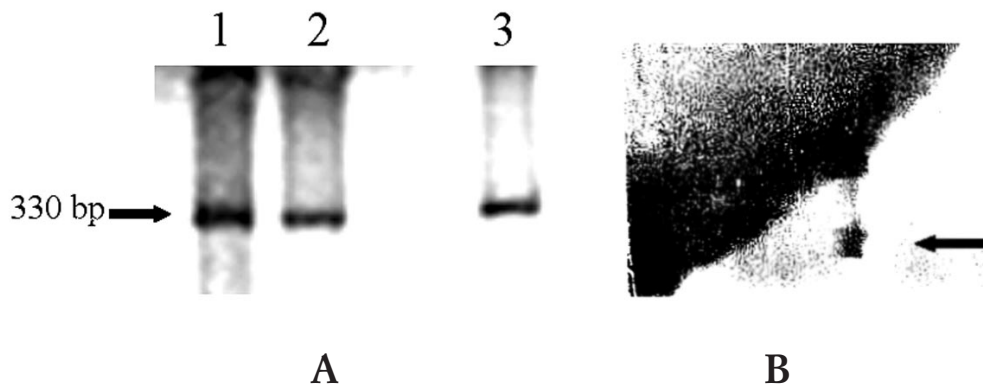


Fig. 2. Return gel electrophoretic analysis (A) and two dimensional gel electrophoretic analysis (B) of DEAE cellulose-purified nucleic acid extracts from pear fruit crinkle-diseased tissue. **A)** Lanes 1 and 2, samples from pear fruit crinkle-diseased cv Xuehuali and cv Yali, respectively. Lane 3, ASSVd standard control. **B)** Arrow indicates the circular RNA of viroid.

Dot blot and Northern blot hybridization. Figure 3 shows dot blot (A) and Northern blot (B) hybridization analyses of nucleic acid extracts from pear fruit crinkle-diseased fruit and from healthy pear fruit with DIG-labeled ASSVd cRNA probe. Positive hybridization signals were obtained with diseased fruit of pear crinkle cv Xuahueli and Yali in dot blot hybridization (Fig. 3A lanes 2 and 3, respectively) and in Northern blot hybridization (Fig. 3B, lanes 2 and 3, respectively). No hybridization was observed with extracts from healthy pear fruit cv Xuahueli (Fig. 3A and B, lane 1) or cv Yali (result not shown). All samples from diseased pear fruits with crinkle symptoms tested positive for ASSVd.

Most diseased samples tested negative for PBCVd when DIG-labeled PBCVd cRNA probe replaced the ASSVd cRNA probe for hybridization. Weak hybridization signals were obtained with 1-2 samples.

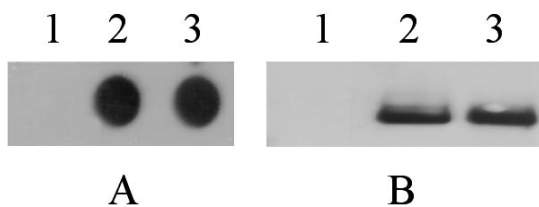


Fig. 3. Chemiluminescent detection of dot blot (A) and Northern blot (B) hybridization of DIG-labeled ASSVd cRNA probe with DEAE cellulose-purified total nucleic acids from pear crinkle-diseased fruits of cv Xuahueli (A and B, lane 2) and cv Yali (A and B, lane 3) and with total nucleic acids from healthy pear fruit of cv. Xuahueli (A and B, lane 1).

Standard and multiplex RT-PCR analyses. In a standard RT-PCR analysis, a cDNA fragment of the expected size of unit length ASSVd (330 bp) was amplified from nucleic acid extracts of the ASSVd-infected tissue positive control (Fig. 4A, lane 1). Under the same RT-PCR conditions, a cDNA fragment similar in size to that from ASSVd was amplified from nucleic acid extracts of pear crinkle-diseased fruit cv Xuahueli (Fig. 4A, lanes 2 and 3) and cv Yali (Fig. 4A, lanes 4 and 5). No amplified product was obtained from healthy pear fruit (Fig. 4A, lane 6). The ASSVd cDNA fragment was detected from each nucleic acid sample of diseased pear fruits.

When DNA primers for PBCVd (Table 1) were used in standard RT-PCR assays, a cDNA fragment of 315 bp (unit-length of PBCVd) was amplified from nucleic acid extracts of 2 out of 16 pear fruit crinkle-diseased samples tested. No amplification was observed from healthy samples (data not shown).

In multiplex RT-PCR analysis, the complementary primer of both ASSVd and PBCVd (ASSVd-C, Table 1) was used to synthesize cDNA; the homologous primers for ASSVd and PBCVd (ASSVd-H and PBCVd-H, respectively, Table 1) were used for PCR amplification. PBCVd of the expected size was clearly observed in one out of three samples of nucleic acid extracts of pear fruit crinkle-diseased tissue (Fig. 4B, lane 4). A faint band in the position of PBCVd was also observed in the sample in lane 3. An amplified product similar in size to that from ASSVd was observed in the three diseased samples analyzed (Fig. 4B, lanes 2-4). No amplification product of the expected size was observed with nucleic acids of healthy fruit tissue (data not shown). Multiplex RT-PCR

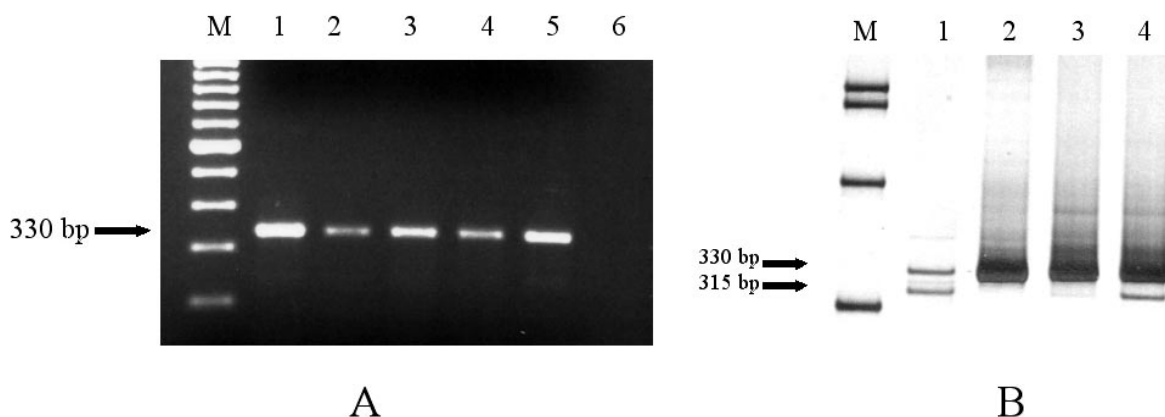


Fig. 4. A) Polyacrylamide gel electrophoretic analysis of RT-PCR amplified cDNAs with ASSVd primers from nucleic acid extracts of ASSVd-infected tissue control and from pear fruit crinkle-diseased tissue. Lane M, DNA marker; lane 1, ASSVd-infected tissue control; lanes 2 and 3, pear fruit crinkle-diseased cv Xuahueli; lanes 4 and 5, pear fruit crinkle-diseased cv Yali; lane 6, healthy pear fruit; B) Polyacrylamide gel electrophoretic analysis of multiplex RT-PCR of amplified cDNAs with ASSVd and PBCVd primers from nucleic acid extracts of samples from fruit crinkle-diseased tissue. Lane M, DNA marker; lane 1, a mixture of ASSVd and PBCVd positive control; lanes 2-4, three different samples from fruit crinkle-diseased tissue with ASSVd (lanes 2-4) and PBCVd (lane 4).

assays of other diseased samples tested were positive for ASSVd but only one sample tested positive for PBCVd.

Analyses of DIG-labeled ASSVcDNA and PBCVd cDNA in RT-PCR-ELISA assays. Amplified ASSV cDNA or/and PBCVd cDNA were successfully labeled with DIG in standard or multiplex RT-PCR. In standard RT-PCR, the electrophoretic mobility of the DIG-labeled cDNA of each viroid was relatively slower than that of its respective unlabeled cDNA. In multiplex RT-PCR the mixture of DIG- labeled ASSVcDNA and PBCVd was slower in its electrophoretic mobility than the mixture of the unlabeled two viroid cDNAs.

The mixture of DIG-labeled ASSVd cDNA and PBCVd cDNA was analyzed by capture hybridization assays using biotinylated ASSV cDNA or PBCV cDNA probe (Table 2). In experiments designed to test probe specificity during hybridization, biotinylated ASSV cDNA and PBCVd cDNA capture probes hybridized only to their respective, complementary DIG-labeled RT-PCR amplified product (Fig. 5). The detection of DIG-labeled ASSVd cDNA and PBCV cDNA in assays of pear fruit crinkle-diseased tissue showed that all samples were infected with ASSVd. PBCVd, however, was detected in very few samples. Less than 20% of samples (3 out of 16 samples) were infected with PBCVd.

Nucleotide sequence and secondary structure. The nucleotide sequence of the majority of pear fruit crinkle-associated viroid RNA clones consists of 333 nucleotides (accession # Ay783357) and the sequence can be arranged into a rod-like secondary structure (Fig. 6A), characteristic of the family *Pospiviroidae*. It shares 95% identity with the 330 nucleotide Japanese prototype strain of ASSVd from apple (accession # M36646; Hashimoto and Koganezawa, 1987). Thus it is a variant of ASSVd. The pear fruit crinkle variant of ASSVd differs from the prototype strain of ASSVd at 19 sites: four nucleotides were inserted (U₄₅, G₁₃₄, A₃₀₅, and C₃₃₃); 15 nucleotides were changed (U₃·A, C₁₀₄·G, C₁₅₈·U, C₁₅₉·U, C₁₆₂·G, C₁₈₂·U, C₂₂₆·G, U₂₂₇·C, C₂₂₈·U, C₂₃₅·G, C₂₆₈·G, C₃₀₂·U, A₃₁₉·U, C₃₂₀·G, C₃₂₁·G). It shares 92% identity with the 334 nucleotides pear rusty skin variant of ASSVd (Fig. 6B) and differs at 27 sites. As compared with this variant of ASSVd: three nucleotides were deleted (U₅₀, G₁₄₈, and A₂₅₄); two nucleotides were inserted (A₃₀₅, and C₃₃₃); 22 nucleotides were changed (U₃·A, U₃₂·C, A₃₉·C, U₄₃·G, A₄₅·U, U₄₇·G, U₄₈·A, C₁₅₉·U, G₁₇₀·A, C₁₇₉·U, U₁₈₁·C, A₂₄₈·G, G₂₅₃·U, U₂₅₇·C, U₂₅₈·C, A₂₆₃·G, C₂₈₁·U, U₂₈₂·C, U₂₈₆·G, G₂₈₇·A, A₂₉₈·G and C₃₀₇·U).

ASSVd-pfc differs from ASSVd-pear fruit dimple variant from Japan in 6 sites. The ASSVd-pfc has three nucleotides inserted (G₁₃₄, A₃₀₅, and C₃₃₃) and three nucleotides were changed (A₃·U, U₃₀₄·C, and U₃₂₃·A).

Table 2. Viroid capture cDNA probes.

Viroid	Probe	No. of bases	Sequence	Position	Reference
ASSVd	ASSVd-cap	22	5'-BIO-CGCCTACAAGAACGTACGGTGT-3'	162-183	Shamloul and Hadidi, 1999
PBCVd	PBCVd-cap	25	5'-BIO-TTTACCGCGGACCCCCGAGAGGAGG-3'	280-304	Shamloul <i>et al.</i> , 2002

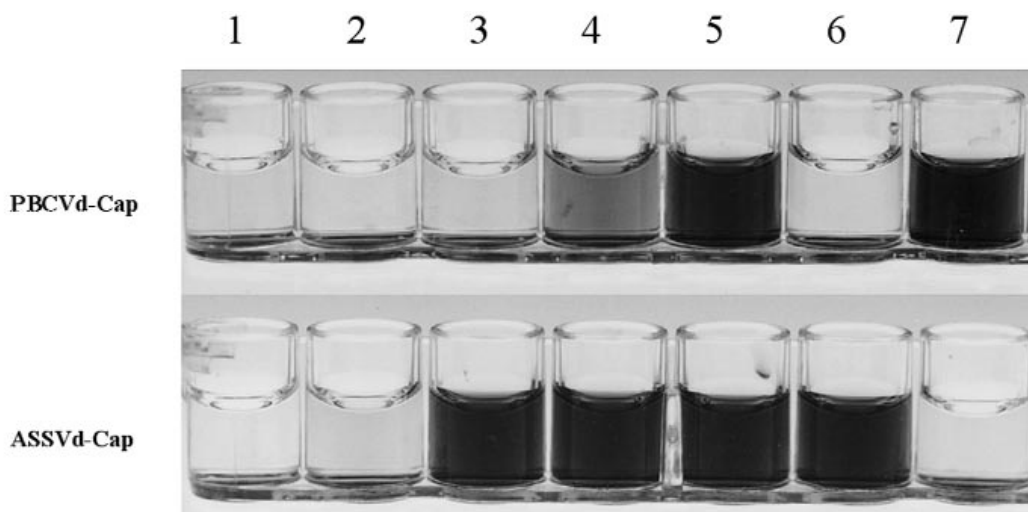


Fig. 5. Colorimetric detection of DIG-labeled PBCVd cDNA or ASSVd cDNA product as shown visually by RT-PCR-probe capture hybridization (ELISA) of samples from nucleic acid extracts of pear fruit crinkle-diseased tissues. PBCVd cDNA capture probe (upper samples); ASSVd cDNA capture probe (lower samples). Sample: 1, buffer control; 2, uninfected pear fruit control; 3-5 pear fruit crinkle-diseased tissue; 6, ASSVd-infected positive control; 7, PBCVd-infected positive control.

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10      20      30      40      50      60      70      80      90      100     110     120     130     140     150     160
- U | ---- CGGU- U UUCGC AA- AGAUAGA C ----- GA AA- - UC - - GAA- CG A- A- U - ACUAAC C C - A--- CGC U
GG AAACACC GUG UCCUG GG CCCGCC CGC UAAAGAAA GAG GAGAA AGG CUC ACCUG GUC G UCGAC GGC GUGAGA AGG GC GC CAGC CGGACGG GCC UCG CACC GUUC UGUGGG U
CC UUUGUGG CAC GGGAC CC GGGUGG GCG GUUUUUUUU CUC CUCUU UCC GAG UGGGC CAG C AGCUG CCG CGUCUU UCC CG CG GUCC GCCUGUC CGG AGU GUGG CAAG ACAUCC C
C - ^ AUAA AAAAU - UU--- GCG ACGCA-- U GCACU G- CCC G CU G G AUCG -- CA AC U C C----- C - U CAUG A-- G
320 310 300 290 280 270 260 250 240 230 220 210 200 190 180 170

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A

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ASSVd-pfc    GAAAAACACCGTGC GTTCTCTGTGGTTCGCCCCGCAACGCAGATAGAT-AAAGAAAACG 59
ASSVd-prs    GGTAACACCGTGC GTTCTCTGTGGTTCGCCTCGCCAAAGCATAAATTTAAAGAAAACG 60
*****

ASSVd-pfc    AGGAGAAGAAGGAAC TACCTGTCTGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAG 119
ASSVd-prs    AGGAGAAGAAGGAAC TACCTGTCTGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAG 120
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ASSVd-pfc    CACTAAGCCGGACGG CGCCCTCGCACC-AGTTCGGCTGTGGGTTCGCCTACAAGAACGTA 178
ASSVd-prs    CACTAAGCCGGACGG CGCCCTCGCACCAGTTCCGCTGCGGGTTCGCCTGCAAGAACGCA 180
*****

ASSVd-pfc    CGGTGTTGAGGCCCT GTCCGCCGCTGCGCTGCCACCTACTCTTCGCGCCGCTAGTCGAGC 238
ASSVd-prs    TGGTGTGAGGCCCT GTCCGCCGCTGCGCTGCCACCTACTCTTCGCGCCGCTAGTCGAGC 240
*****

ASSVd-pfc    GGACTCCGGGTGTAG -CCCCTGTCTCTCACGCTCTTTTCTTTTGACGCAGCGGGGGT 297
ASSVd-prs    GGACTCCAGGTGGAG ATCCCTATTCTCTCACGCTCTTTTCTTTTGGCAGCGGGGAGT 300
*****

ASSVd-pfc    GGGTCTACAGGGTAA AACACAATTGGTGTTCCTCC 333
ASSVd-prs    GGGTTC-CAGGGTAA AACACAATTGGTGTTCCTCC- 334
*****

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B

Fig. 6. Nucleotide sequence and secondary structure of pear fruit crinkle variant of ASSVd (ASSVd-pfc) (A) and its alignment with that of pear rusty skin variant of ASSVd (ASSVd-prs) (B).

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PBCVd-Ch     CTTTCCTGAGGTTCTCTGTGGTGTCTCCCTGACCTGCGTTCCAAAAAGCGAAAAAGTGAG 60
PBCVd-EU     CTTTCCTGAGGTTCTCTGTGGTGTCTCCCTGACCTGCGTTCCAAAAAGCGAAAAA-GTGAG 59
*****

PBCVd-Ch     AGGCCCTAGGGGCTTCTCGGCTCGTCTGACGAAGGGTCTAGAAGCCTGGGCCTGGCT 120
PBCVd-EU     AGGCCCTAGGGGCTTCTCGGCTCGTCTGACGAAGGGTCTAGAAGCCTGGGCCTGGCT 119
*****

PBCVd-Ch     GG-GCGCGCGGCTGTGAGTAATTGCTCCTCTGGAGAAGAAAACGAGCTTGCTTCCCGCC 179
PBCVd-EU     GGAGCGCGCGGCTGTGAGTAATCGCTCCTTTGGAGAAGAAAACGAGCTTGCTTCCCTGCC 179
** *****

PBCVd-Ch     TGAGCCTCGTCTTCTGTCCCCTAGTCGAGCGGACAACCCGAGCACC CGCGAAGCGCTTT 239
PBCVd-EU     TGAGCCTCGTCTTCTGTCCCCTAGTCGAGCGGACAACCCGAGCACC CGCGAAGCGCTTT 239
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PBCVd-Ch     TTTCTTTTATAGCAGCTTGGCTTCGCGCGAGGGTGAAGTTTACC GCGGACCCCGAGA 299
PBCVd-EU     TTTCTTTTATAGCAGCTTGGCTTCGCGCGAGGGTGAAGTTTACC GCGGACCCCGAGA 299
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PBCVd-Ch     GGAGGCCCTCGGGTCC 315
PBCVd-EU     GGAGGCCCTCGGGTCC 315
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Fig. 7. Nucleotide sequence of PBCVd from China and its alignment with that of PBCVd prototype.

Nucleotide sequence of the Chinese PBCVd. This viroid is 315 nucleotides in length (Fig. 7) and shares 97% identity with PBCVd prototype (Hernandez *et al.*, 1991). It differs from the prototype in 7 sites: one nu-

cleotide inserted (A_{55}), one nucleotide missing (A_{123}), and 5 nucleotides were changed ($C_{142}\cdot U$, $U_{149}\cdot C$, $U_{176}\cdot C$, $C_{234}\cdot G$, $G_{235}\cdot C$).

DISCUSSION

Molecular evidence presented in this investigation demonstrated the close association of ASSVd with pear fruit crinkle disease from China. We have also shown that the viroid is a new variant of ASSVd based on the complete nucleotide sequence of the pathogen and its comparison with nucleotide sequences of known variants of ASSVd. It is interesting to note that the ASSVd variants that cause pear rusty skin (Zhu *et al.*, 1995) and pear fruit crinkle diseases in China have undergone extensive mutation (changes in 25 and 19 sites for rusty skin and fruit crinkle, respectively) while the ASSVd variant that causes pear fruit dimple in Japan (Osaki *et al.*, 1996) has little mutation (changes in only 3 sites) when compared to the ASSVd prototype that infects apple in Japan. These analyses may suggest that the Chinese and Japanese pear variants of ASSVd may have the same origin and the observed sequence divergence may be the result of molecular evolution from a common ancestor under different selection pressures. Alternatively, the Chinese and Japanese pear variants of ASSVd may have originated in different locations. There are 10-12 wild pear species found in China, 3-4 are native to Japan, and at least one is found in Korea (Hedrick, 1921; Bailey, 1927). Detection of ASSVd in wild pear has been reported from Greece (Kyriakopoulou *et al.*, 2001). Thus it is possible that the Chinese pear variants of ASSVd may have originated in the Chinese wild pear species and the Japanese pear variant of the viroid may have originated in the Japanese and/or Korean wild pear.

Crinkling and dimpling disease symptoms on apple fruits have been reported. Apple fruit crinkle disease reported from Japan (Koganezawa and Ito, 2003) and apple dimple fruit disease reported from Europe (Di Serio *et al.*, 2003) are caused by *Apple fruit crinkle viroid* (AFCVd) and *Apple dimple fruit viroid* (ADFVd), respectively. On the other hand, a third viroid species, ASSVd, is highly associated with pear fruit crinkle disease in China, as shown in this investigation, and is the causal agent of pear fruit dimple disease in Japan (Osaki *et al.*, 1996). The above three viroid species belong to the same genus, *Apscaviroid* (Di Serio *et al.*, 2003; Koganezawa and Ito, 2003; Kyriakopoulou *et al.*, 2003), however, none of the three viroids is the causal agent or closely associated with the diseases caused by the other two.

PBCVd was detected in less than 20% of pear fruit samples with crinkle symptoms. The sequence of this viroid shares 97% identity with PBCVd from Europe. Thus the Chinese and European variants of the viroid are very similar. PBCVd in Chinese pear fruits with crinkle disease symptoms is found in trace amounts as it was detected only by RT-PCR and RT-PCR-ELISA. PBCVd usually causes a disease in the stem and bark of infected pear trees and the disease has been reported from European countries, New Zealand, and the U.S. (Atkinson,

1971; Thomsen *et al.*, 1989; Kyriakopoulou *et al.*, 2001; Flores *et al.*, 2003). In addition to the stem and bark, PBCVd was detected in infected pear leaves (Flores *et al.*, 1991). To our knowledge, however, there is no published report about PBCVd detection in fruits of infected pear trees. For this reason we suggest that PBCVd is not involved in pear fruit crinkle disease. The finding of PBCVd in less than 20% of pear fruit crinkle-diseased samples was most likely due to double infection of pear trees with ASSVd and PBCVd. The detection of trace amounts of PBCVd in infected Chinese pear fruit may be due to the fact that the viroid is located mainly in the stem, bark, and leaves of the infected trees. This is the first report of the presence of PBCVd in China.

Natural infection of pear trees in Greece with ASSVd and PBCVd has been reported previously (Kyriakopoulou *et al.*, 2001). Two different viroid species of the genus *Apscaviroid* may co-exist in the same plant host. Our work demonstrates that mixed infections of two species of *Apscaviroid* can also occur naturally in China.

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