

## Homology of the Agent Associated with Dapple Apple Disease to Apple Scar Skin Viroid and Molecular Detection of These Viroids

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### ABSTRACT

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Gel electrophoresis coupled with molecular hybridization analyses using <sup>32</sup>P-labeled SP6-generated apple scar skin viroid (ASSV)-specific cRNA probes demonstrated that the pathogen associated with dapple apple disease is a viroid that is closely homologous to ASSV. Dapple apple viroid (DAV) consists of fewer than 359 nucleotides and is systemically distributed in apple seed, fruit, bark, leaf, and root tissues of infected apple trees. Molecular hybridization assays using <sup>32</sup>P-labeled ASSV cRNA

probes have been developed and applied for the detection of DAV or ASSV in small amounts of infected apple tissue (0.2–2.0 g). These assays are accurate, easy to perform, and applicable for screening DAV or ASSV in imported apple cultivars. These viroids now can be positively identified from infected apple tissue in a few days instead of a few years by fruit symptoms on grafted woody indicators.

*Additional keywords:* Northern blot hybridization, nucleic acid extraction method, return gel electrophoresis, riboprobe.

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Dapple apple, a fruit-blemishing disease, was first described in 1956 from apple orchards in New Hampshire (18). In 1958, the causal agent was shown to be graft transmissible (1). Subsequently, the disease has been reported from Canada, Japan, the United Kingdom, and Italy (2,5,23,25). Another graft-transmissible disease of apple that causes fruit disorder is apple scar skin. This disease has been described from the United States, China, and Japan (4,13–15,21). The host range of the agents of dapple apple and apple scar skin diseases is limited and it is restricted to pome fruit trees (2,11,13,14,16,18,21). The average time required for positive identification of each disease by fruit

symptoms on grafted woody indicators is 3 yr (2,5,24). Reactions of apple cultivars to dapple apple and apple scar skin diseases have been studied, and a possible relationship between the causal agents of the two diseases has been suggested (19,24).

Two low-molecular-weight RNA species have been associated with nucleic acid extracts from apple scar skin-diseased fruit or bark tissue, but not from healthy tissue (11). The smaller RNA is circular (3). Apple seedlings inoculated with nonfractionated total nucleic acid from diseased tissue contain RNAs with the electrophoretic mobilities of the two disease-associated RNAs (11).

Recently, the causal agent of apple scar skin disease in Japan has been identified as a viroid, and it has been cloned and sequenced (9). The Japanese isolate of apple scar skin viroid (ASSV) contains 330 nucleotides, is not related to other known viroids, and has been allocated to a new viroid group (9). We have subcloned an ASSV cDNA into the SP6 transcription vector pSP65

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and synthesized an ASSV-specific cRNA probe. We have used this probe to determine the relationship of the American and Canadian dapple apple pathogens to the Japanese ASSV and to develop a molecular detection method for these pathogens. In this paper we report that nucleic acids from the North American dapple apple-diseased tissues tested positive with the ASSV cRNA probe and that the dapple apple viroid (DAV) is systemically distributed in apple seed, fruit, bark, leaf, and root tissues collected from infected trees. We also report the development and application of accurate hybridization assays using the SP6-generated ASSV cRNA probes for the rapid detection of DAV and ASSV from nucleic acid extracts of infected tissue. Only a few days are required for positive identification of the viroid from infected apple trees. A preliminary report of this work has been presented (7).

## MATERIALS AND METHODS

**Source of North American isolates of DAV and ASSV.** Apple fruits, leaves, and budwood infected or uninfected with the Canadian isolate of DAV were obtained from A. J. Hansen, Agriculture Canada, Summerland, B. C., Canada. Budwood was grafted by the T-double budding technique onto apple seedlings at Glenn Dale, MD, in September 1987 and then allowed to grow the following spring. Tissue samples from apple trees infected with the American isolates of DAV or ASSV were obtained from W. E. Howell and G. I. Mink of Washington State University and IR-2 Virus Free Fruit Tree Repository, Prosser, WA. These isolates were provided to Prosser by G. N. Agrios.

**Source of imported Asian apple cultivars.** Asian apple cultivars of undocumented entry into the United States were grown in greenhouses at the IR-2 Virus Free Fruit Tree Repository. Leaves of these cultivars were obtained from W. E. Howell and G. I. Mink. These cultivars were known to contain apple latent and other viruses.

**Nucleic acid extraction from plant tissue.** Total nucleic acids from North American DAV-infected or uninfected leaf, bark, or fruit tissue were isolated and fractionated into LiCl soluble and insoluble fractions as described by Koganezawa (11).

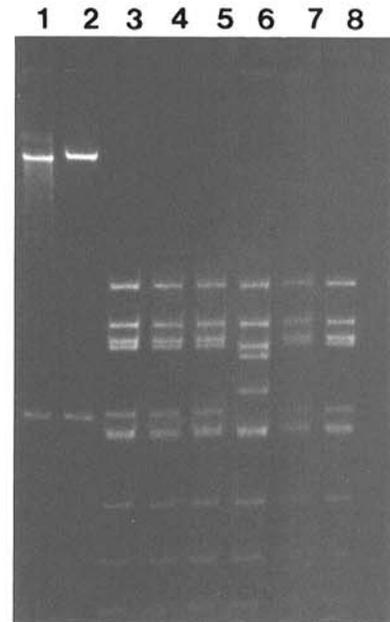
Alternatively, total nucleic acids from apple seed, fruit, bark, leaf, and root tissue were isolated by the following procedure, which was developed during the course of this investigation. Apple tissue (0.2–2.0 g) was powdered with liquid nitrogen using a mortar and pestle. An extraction buffer was added to the powder at 4 ml of buffer/1 g of tissue. The buffer consisted of 0.1 M Tris-base, 1.5 M NaCl, 0.5% sodium dodecyl sulfate (SDS), 0.25% diethyldithiocarbamate, 0.5% 2-mercaptoethanol, and 5% (w/v) polyvinylpyrrolidone, pH 8.5. After the slurry was mixed thoroughly, it was transferred to a beaker and stirred for 10 min. An equal volume of phenol saturated with 1 M Tris-HCl, pH 7.4, and containing 0.1% 8-hydroxyquinoline was added and allowed to stir for 10 min. An equal volume of chloroform was added and allowed to stir for an additional 10 min. The sample then was centrifuged at 7,650 g for 10 min in a low-speed, refrigerated Sorvall centrifuge (Sorvall Instruments, DuPont Co., Wilmington, DE). The aqueous phase was collected, to which 2 v of cold absolute ethanol and 0.1 v of 3 M sodium acetate, pH 5.4, were added. Samples were stored for at least 1 hr at  $-70^{\circ}\text{C}$  and then centrifuged at 12,000 g for 20 min as described above. The resulting pellet was air dried and resuspended in 500  $\mu\text{l}$  of 1 M NaCl in an Eppendorf tube and centrifuged at maximum speed for 5 min in an Eppendorf microcentrifuge (Brinkman Instruments, Sybron Co., Westbury, NY) at 4 C. The supernatant was collected, to which 0.1 v of 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 v of cold absolute ethanol were added. The supernatant was stored at  $-70^{\circ}\text{C}$  for 1 hr and centrifuged at maximum speed for 20 min in an Eppendorf microcentrifuge. The resulting pellet was allowed again to air dry and was resuspended in 200  $\mu\text{l}$  of 1 M NaCl. This suspension was centrifuged in an Eppendorf microcentrifuge for 5 min. To the collected supernatant, 0.1 v of 3 M sodium acetate, pH 5.4, and 2 v of cold absolute ethanol were added. Samples then were stored

at  $-70^{\circ}\text{C}$  for 1 hr and centrifuged for 20 min in a microcentrifuge as before. The pellet was vacuum dried and dissolved into 50  $\mu\text{l}$  of sterile distilled deionized water. The optical density at 260 nm was measured to determine the total nucleic acid content.

To obtain total RNA, the pellet obtained after vacuum drying was dissolved into 400  $\mu\text{l}$  of RQ1 DNase buffer (40 mM Tris-base, 10 mM NaCl, and 6 mM  $\text{MgCl}_2$ ). DNA was digested with RQ1 DNase (2 units/1,000  $\mu\text{l}$ , Promega Biotec, Inc., Madison, WI) at 37 C for 30 min. One-tenth volume of 3 M sodium acetate, pH 5.4, and 2 v of cold absolute ethanol were added, and the suspension was stored at  $-20^{\circ}\text{C}$  overnight. Samples then were centrifuged in a microcentrifuge for 20 min as above, and the pellet was vacuum dried. The pellet was resuspended in 50  $\mu\text{l}$  of 12 $\times$  standard saline citrate (SSC) (1 $\times$  SSC = 150 mM NaCl, 15 mM Na-citrate, pH 7.0) containing 6% formaldehyde. The optical density was measured at 260 nm to determine the total RNA content.

**Probe preparation.** A double-stranded cDNA clone, pUAS14, contains a 274-base-long cDNA copy of the Japanese isolate of ASSV inserted into the *EcoRI* site of plasmid pUC13, as previously described by Hashimoto and Koganezawa (9). The cDNA is derived from residues 286/330-1/228 in the sequence of ASSV. The *EcoRI* fragment containing the ASSV sequence was subcloned into the *EcoRI* restriction endonuclease site of the plasmid vector pSP65 and was propagated in strain JM83 of *Escherichia coli*. Plasmid DNAs were extracted as recommended (Promega Biotec, Inc.). Plasmid DNA was linearized with *PstI* before transcription with SP6 polymerase. High-specific-activity RNA probes ( $5 \times 10^8 - 1 \times 10^9$  cpm/ $\mu\text{g}$ ) were synthesized as described (Promega Biotec) using  $^{32}\text{P}$ -labeled uridine 5'-triphosphate (800 Ci/mmol) (New England Nuclear, Du Pont Co., Boston, MA).

**Gel electrophoresis, Northern blotting, and dot blotting.** Nucleic acids were analyzed by electrophoresis on nondenaturing 5% polyacrylamide slab gel (14  $\times$  15  $\times$  0.2 cm) at 10 V/cm for 3 hr in a running buffer containing 0.04 M Tris-acetate and 0.002 M EDTA, pH 8.0. Alternatively, nucleic acids were fractionated by return gel electrophoresis as described by Singh and Boucher (17). Nucleic acids were stained with ethidium bromide, then transferred to nylon membranes (Nytran) (Schleicher & Schuell, Inc., Keene, NH) using an electrotransfer apparatus. The membranes were baked in a vacuum oven at 80 C for 2 hr. Total RNA extracts from plant tissues in 12 $\times$  SSC containing 6%



**Fig. 1.** Polyacrylamide gel electrophoresis of *HaeIII*-restricted recombinants in plasmid pSP65. 1, pUAS14 digested with *EcoRI*; 2, pSP65-apple scar skin viroid (ASSV) digested with *EcoRI*; 3–5, 7, 8, pSP65 recombinants containing ASSV cDNA in the “plus” orientation; 6, pSP65 recombinant containing ASSV cDNA in the “minus” orientation.

formaldehyde (22) were vacuum blotted onto Nytran membranes that had been saturated with 20× SSC. Blotted membranes then were baked under vacuum at 80 C for 1 hr.

**Hybridization conditions.** Hybridization was carried out as suggested by the manufacturers of the nylon membrane (Schleicher & Schuell) with modifications. Briefly, prehybridization conditions were 50% formamide, 5× Denhardt's reagent, 5× SSC, 1% SDS, and 100 µg/ml of denatured, fragmented calf thymus DNA at 55 C for 1–2 hr. After prehybridization, 1 × 10<sup>6</sup> cpm/ml of <sup>32</sup>P-labeled cRNA probe was added, and hybridization was carried out for 18–20 hr at 55 C. Hybridized membranes then were washed three times in 1× SSC/0.5% SDS at 65 C for 15 min each and once in 0.1× SSC/0.5% SDS at 60 C for 15 min. The filters were treated with RNase A to reduce background and remove mismatched hybrids. The RNase treatments were 1 µg/ml of RNase A in 2× SSC at room temperature for 15 min followed by washing with 0.1× SSC/0.1% SDS at 50 C for 30 min. The membranes were air dried and autoradiographed with Kodak XAR or XRP film and intensifying screens. Autoradiography was for 6–48 hr at –70 C, depending upon the strength of the signal.

## RESULTS

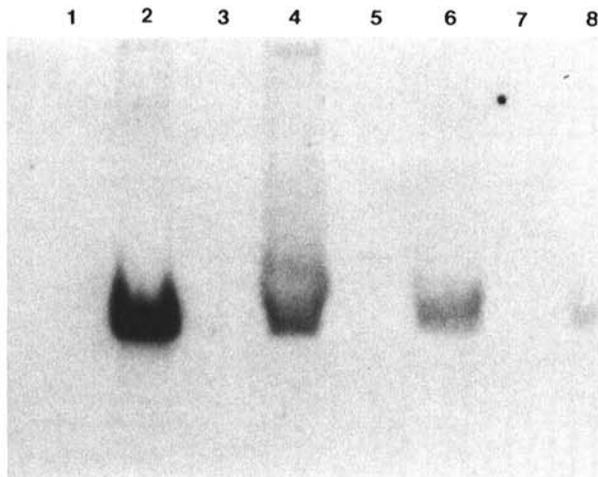
**Subcloning of ASSV cDNA into the SP6 vector pSP65 and preparation of SP6-generated ASSV cRNA probes.** Double-stranded ASSV cDNA from plasmid pUAS14 was subcloned into the *Eco*RI site of the SP6 vector pSP65 in “plus” and “minus” orientations. They were distinguished by restriction mapping of the recombinant plasmids using the restriction endonuclease *Hae*III (Fig. 1). <sup>32</sup>P-labeled ASSV cRNA probes then were synthesized from *Pst*I-linearized recombinants containing DNA template of ASSV with minus orientation using SP6 RNA polymerase.

**Isolation of DAV from infected apple tissue suitable for analysis by Northern blot hybridization.** RNAs from LiCl soluble and insoluble fractions were separated by polyacrylamide gel electrophoresis, transferred to a Nytran membrane, and hybridized with a <sup>32</sup>P-labeled ASSV cRNA probe. As shown in Figure 2, the hybridizing low-molecular-weight viroid RNA is present mainly in the LiCl soluble but not in the insoluble fraction from infected leaf or bark tissue (lanes 2, 4, 6, and 8). The amount of hybridizing RNA isolated from the bark, however, was much

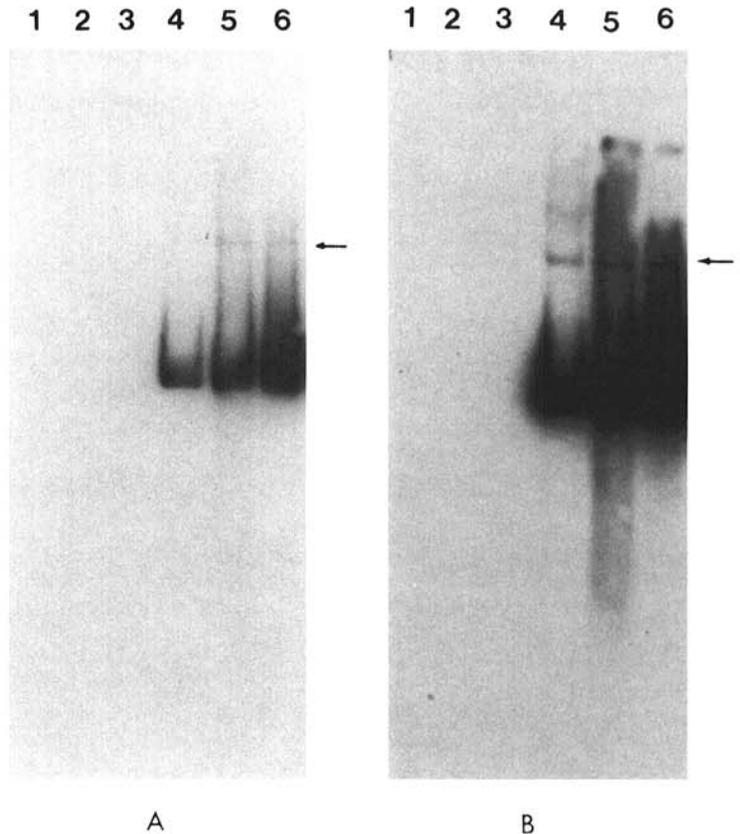
less than that isolated from the leaves. Nucleic acids isolated from uninfected tissue did not hybridize to the probe (Fig. 2, lanes 1, 3, 5, and 7). Weak hybridization signals also were obtained with nucleic acid preparations from DAV-infected fruit tissue (data not shown). Treatment of isolated total nucleic acids from infected tissues with RNase abolished hybridization (results not shown), which indicates that the hybridizing nucleic acid is an RNA.

Figure 3 shows Northern blot hybridization analysis of total nucleic acids extracted as described in the Materials and Methods section from American DAV-infected or uninfected apple bark, leaves, and roots. A hybridization signal corresponding to an RNA with the electrophoretic mobility of a viroid monomer was detected in all infected tissues, including that of roots (Fig. 3A and B, lanes 4–6). A minor RNA species whose electrophoretic mobility is slower than that of viroid monomer also was observed (Fig. 3A and B, arrow). <sup>32</sup>P-labeled ASSV cRNA did not hybridize to nucleic acids from uninfected tissues (Fig. 3A and B, lanes 1–3).

**Return gel electrophoresis and Northern blot hybridization analyses of DAV.** Total nucleic acids isolated as described in the Materials and Methods section from American DAV-uninfected and infected apple fruit skin and from potato spindle tuber viroid (PSTV)-infected tomato leaves were analyzed by return gel electrophoresis (Fig. 4). DAV-infected apple fruit skin contained a low-molecular-weight RNA which was absent from the uninfected control (Fig. 4, lanes 1 and 2). This RNA has an electrophoretic mobility that is slightly faster than that of PSTV (Fig. 4, lanes 2 and 3).



**Fig. 2.** Autoradiograph of Northern blot hybridization with <sup>32</sup>P-labeled apple scar skin viroid (ASSV) cRNA to LiCl soluble and insoluble fractions of nucleic acids from dapple apple viroid (DAV)-infected or uninfected apple tissue. LiCl-soluble nucleic acid fractions from: 1, uninfected leaves; 2, DAV-infected leaves; 3, uninfected bark; 4, DAV-infected bark. LiCl-insoluble nucleic acids from: 5, uninfected leaves; 6, DAV-infected leaves; 7, uninfected bark; 8, DAV-infected bark. Samples were electrophoresed in a 5% polyacrylamide gel under non-denaturing conditions. Approximately 20 µg of nucleic acids per sample was analyzed.



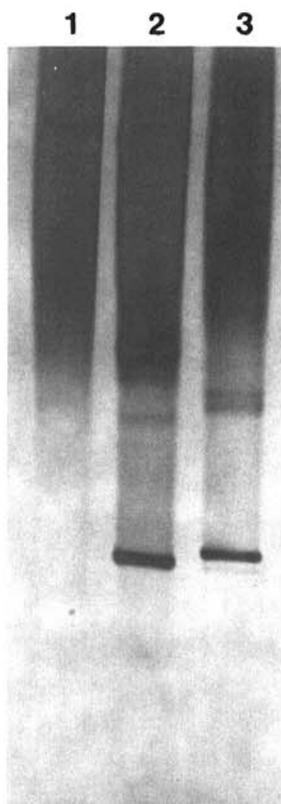
**Fig. 3.** Autoradiographs of Northern blot hybridization with <sup>32</sup>P-labeled apple scar skin viroid (ASSV) cRNA to total nucleic acids from: 1, uninfected apple roots; 2, uninfected apple bark; 3, uninfected apple leaves; 4, dapple apple viroid (DAV)-infected apple roots; 5, DAV-infected bark; and 6, DAV-infected leaves. **A**, Autoradiography was for 24 hr. **B**, Autoradiography was for 72 hr. Arrows indicate the position of a minor DAV-related RNA species. Samples were electrophoresed in a 5% polyacrylamide gel under non-denaturing conditions. Approximately 14, 17, and 36 µg of nucleic acids from root, bark, and leaf tissue, respectively, were analyzed.

Total nucleic acids from DAV-infected and uninfected apple leaves from Canada and fruit skin from the United States were separated by return gel electrophoresis, transferred by electrotransfer to Nytran membrane, and hybridized with  $^{32}\text{P}$ -labeled ASSV cRNA. A low-molecular-weight RNA species with very similar electrophoretic mobility hybridized with an equal intensity to the probe in both the infected leaf and fruit tissues (Fig. 5, lanes 2 and 4, respectively). Nucleic acids from uninfected tissue did not hybridize to the probe (Fig. 5, lanes 1 and 3).

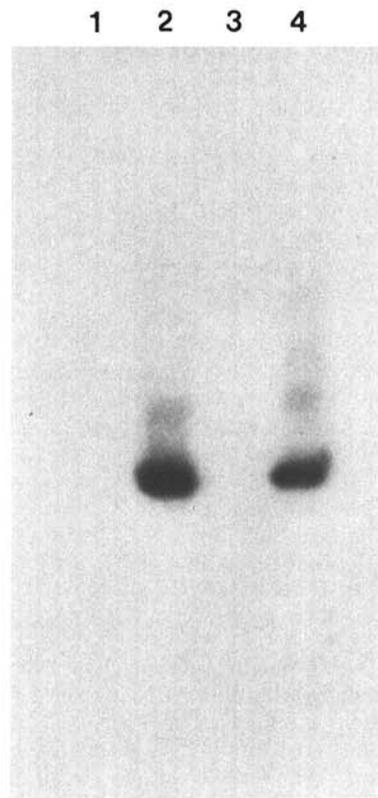
**Detection of DAV by dot blot and Northern blot hybridization assays.** A dot blot hybridization assay of  $^{32}\text{P}$ -labeled ASSV cRNA with total RNA from DAV-infected or uninfected apple fruit skin, seed, bark, or leaf tissue is shown in Figure 6. All RNAs extracted from DAV-infected tissues, including that of seeds, hybridized with the probe. The intensity of hybridization was correlated with sample RNA concentration (Fig. 6, lanes 2, 4, 6, and 8). The highest and lowest viroid concentration, as shown by the intensity of the hybridization signals, was found in infected fruit skin and leaves, respectively (Fig. 6, lanes 2 and 8). Dot blot hybridization assays with total nucleic acid preparations containing DNA were unreliable (data not shown). Digestion and removal of DNA from total nucleic acids of infected or uninfected apple tissues were required before blotting samples on membranes. Northern blot hybridization analysis of  $^{32}\text{P}$ -labeled ASSV cRNA with total nucleic acid samples from DAV-infected or uninfected apple fruit skin, seed, bark, or leaf tissue, however, was reliable. Results obtained with Northern blot hybridization analysis were very similar to those obtained with dot blot hybridization (results not shown).

**Application of molecular hybridization assays for detecting DAV or ASSV from recently imported apple cultivars.** Nucleic acid extracts from leaves of recently imported Asian apple cul-

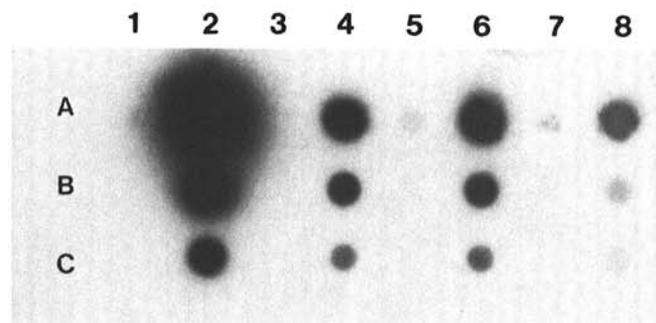
tivars were assayed for DAV or ASSV by dot and Northern blot hybridization analyses. The results of these assays and assays of nucleic acid samples from apple leaf, bark, or root tissue of DAV-infected, ASSV-infected, or uninfected controls are presented in Table 1. Viroid-infected and uninfected apple controls tested positive and negative, respectively, for the presence of the viroid (Table 1). One of 24 Asian apple cultivars tested positive for the presence of the viroid (Table 1).



**Fig. 4.** Purification of dapple apple viroid (DAV) from total nucleic acids by return-gel electrophoresis. Total nucleic acids from: 1, uninfected apple fruit skin; 2, DAV-infected fruit skin (American isolate); 3, potato spindle tuber viroid-infected tomato leaves. The first gel electrophoresis of the samples was in 5% polyacrylamide gel under nondenaturing conditions at 46 mA for 2.5 hr, and the second was under denaturing conditions at 46 mA for 2 hr. Approximately 40  $\mu\text{g}$  of nucleic acids per sample was analyzed and then visualized by silver staining.



**Fig. 5.** Autoradiograph of Northern blot hybridization of  $^{32}\text{P}$ -labeled apple scar skin viroid (ASSV) cRNA to total nucleic acids analyzed by return gel electrophoresis and then transferred electrophoretically to Nytran membrane. Total nucleic acids from: 1, uninfected apple leaves; 2, dapple apple viroid (DAV)-infected leaves (Canadian isolate); 3, uninfected apple fruit skin; and 4, DAV-infected fruit skin (American isolate). Approximately 140  $\mu\text{g}$  of nucleic acids of apple leaf tissue and 40  $\mu\text{g}$  of apple fruit skin tissue were analyzed.



**Fig. 6.** Autoradiograph of dot blot hybridization of  $^{32}\text{P}$ -labeled apple scar skin viroid (ASSV) cRNA with RNA (in 12 $\times$  standard saline citrate containing 6% formaldehyde) at increasing dilutions extracted from: 1, uninfected apple fruit skin; 2, dapple apple viroid (DAV)-infected apple fruit skin; 3, uninfected apple seeds; 4, DAV-infected apple seeds; 5, uninfected apple bark; 6, DAV-infected apple bark; 7, uninfected apple leaves; and 8, DAV-infected apple leaves. **A**, Undiluted RNA samples. **B**, RNA samples diluted 1:10. **C**, RNA samples diluted 1:100. Approximately 20, 17, 26, and 75  $\mu\text{g}$  of undiluted RNA samples and their 10- and 100-fold dilutions from uninfected or DAV-infected fruit skin, seed, bark, and leaf tissue, respectively, were analyzed.

## DISCUSSION

The present study demonstrates that the agent associated with the American or Canadian isolate of dapple apple disease is a viroid that displays close homology to apple scar skin viroid from Japan and that a molecular detection method for these viroids has been developed. Several lines of evidence substantiate that DAV is closely related to ASSV: 1) Under the hybridization conditions used in our study, <sup>32</sup>P-labeled ASSV cRNA hybridized with RNA from DAV-infected tissue but not with RNA from uninfected tissue; 2) strong hybridization signals were obtained with RNA from tissues infected with the American or Canadian dapple apple disease; 3) RNase A treatment of the hybrids as described in the Materials and Methods section removes mismatched hybrids; 4) hybridizing RNA is mainly distributed in LiCl soluble fraction, which is characteristic of viroids; and 5) comparison of the electrophoretic mobility of DAV with that of PSTV suggests that DAV consists of fewer than 359 nucleotides, a size that is compatible with that of ASSV (330 nucleotides). Analysis of nucleotide sequence of various isolates of ASSV and DAV should elucidate their relationship.

Although the nucleic acid extraction method of Koganezawa (11) was suitable for analysis of leaf nucleic acids by Northern blot hybridization, this method was not satisfactory for hybridization analysis of nucleic acids extracted from other tissues. By combining the procedure for isolating nucleic acids from apple tissue developed in this investigation with Northern blot and dot blot hybridization analyses, we demonstrated the systematic distribution of DAV or ASSV in infected apple trees (Figs. 2–6, Table 1). By combining the nucleic acid extraction procedure with the recently developed technique of return gel polyacrylamide electrophoresis, DAV molecules could be separated from other plant nucleic acids (Fig. 4). Analysis of DAV by return gel electrophoresis may be used for DAV or ASSV purification, detection, and/or other studies.

In view of these findings, it appears that strain(s) of ASSV are associated with the dapple apple disease in North America.

The advantages of the molecular hybridization detection method over the currently used biological method are as follows:

- 1) A few days are required for positive identification of the disease from infected tissue in contrast to an average of 3 yr that are needed to detect the pathogen by the biological method (2,5,24).
- 2) Leaf, bark, root, seed, and/or fruit tissue may be used for this assay. Only apple fruits are used in diagnosis in the biological assay.
- 3) The cRNA assay is accurate, specific, and very sensitive (6,8,12,20,22). In contrast, symptoms of ASSV or DAV on apple fruits vary considerably among cultivars (2,5,24, Hadidi et al, *unpublished*), and they depend on changes in fruit phenolic metabolism (10). In addition to these difficulties with the biological assays, the constant presence of certain apple viruses and agents causing fruit malformation may make the diagnosis of the disease based on fruit symptoms alone inaccurate.
- 4) The cRNA assay is relatively inexpensive and does not require a large space, whereas the biological assay is expensive, laborious, and requires more space.

The detection of DAV or ASSV in apple seed and vegetative tissues suggests that the viroid may be transmitted through these tissues. Seed and vegetative transmission could be a serious hazard because the viroid may spread from apple root stocks, grown from infected seeds or vegetative tissues, to grafted scions varieties. Because DAV or ASSV is systemically distributed in infected apple trees, it is likely to be transmitted in orchards by pruning equipment and natural root grafting.

Nucleic acid extracts of one Asian apple cultivar tested positive with the <sup>32</sup>P-labeled ASSV cRNA probe. ASSV and DAV can infect this cultivar as well as many Asian apple cultivars without causing visible symptoms in their fruits. These viroids, however, can cause scar skinned, dappled, and cracked fruits when they infect popular American apple varieties such as Red Delicious and McIntosh (2,5,24; Hadidi et al, *unpublished*). Thus, by using this assay, foreign apple cultivars can be screened more rapidly and introduced into the United States without threatening the American apple industry.

The availability of highly specific ASSV cRNA probes, together with the nucleic acid extraction procedure and hybridization assays reported here, will make monitoring ASSV and DAV worldwide possible.

## LITERATURE CITED

TABLE 1. Detection of dapple apple viroid (DAV) or apple scar skin viroid (ASSV) from imported Asian apple cultivars and from controls of domestic apple cultivars by molecular hybridization assays with SP6-generated <sup>32</sup>P-labeled ASSV cRNA probe<sup>a</sup>

Apple tree	Source of nucleic acids	Hybridization assay
Asian cultivar 1	Leaves	+
Asian cultivar 2 to 24	Leaves	—
DAV-infected control 1	Old leaves	+
	Young leaves	+
	Bark	+
DAV-infected control 2	Old leaves	+
	Young leaves	+
	Old bark	+
	Young leaves	+
	Roots	+
ASSV-infected control 3	Old leaves	+
	Young leaves	+
	Bark	+
Uninfected control 4	Old leaves	—
	Young leaves	—
	Bark	—
	Roots	—
Uninfected control 5	Leaves	—

<sup>a</sup> Nucleic acids extraction method and Northern and dot blot hybridization assays were performed as described in the Materials and Methods section. Total RNA (75–100 µg/sample) and total nucleic acids (100–150 µg/sample) were analyzed for dot blot hybridization and Northern blot hybridization, respectively. A plus sign indicates a positive hybridization reaction; a minus sign indicates no hybridization reaction. The experiments were repeated at least once.

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