Integrated Molecular and Biological Assays for Rapid Detection of Apple Scar Skin Viroid in Pear

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ABSTRACT

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A viroid in oriental pears (*Pyrus* hybrids) was associated with dappling and cracking fruit symptoms in graft-inoculated *Malus pumila* cv. Lord Lambourne. The viroid hybridized with a cRNA probe to apple scar skin viroid (ASSVd). Detection of the viroid by hybridization assays of tissue blots gave erratic results when used with some pear cultivars. Two methods for improving detection of the pear viroid were compared. In the first, pear bark chips were graft-inoculated into a viroid "amplification host," which was then tested by tissue blot hybridization. Digoxigenin-labeled cRNA probe and an antidigoxigenin antibody conjugate were used with a chemiluminescent substrate for detection of viroid in the tissue blots. Greenhouse-grown *Pyrus communis* cv. Nouveau Poiteau and *Malus pumila* cv. Virginia Crab amplified ASSVd to levels that were detectable in tissue blots of shoots and leaf petioles, even though the leaves and shoots were symptomless. In the second method, pears were indexed on the ASSVd indicator plants Stark's Earliest and Sugar Crab apple grown in growth chambers under a 24-h photoperiod. ASSVd-infected apple and the infected pears induced similar symptoms of leaf epinasty in the indicators in 5 to 8 weeks. Although infection in pear was rapidly and reliably detected by both methods, tissue blot hybridization was faster. Inoculation of an amplification host was useful when numerous cultivars or genetically diverse plants were tested and genetically similar positive and negative control plants were unavailable.

Apple scar skin viroid (ASSVd) (7) was the first viroid reported to infect pome fruit trees (*Malus*, *Pyrus*, and *Cydonia* spp.). This viroid is one of the most important pathogens in apple producing areas of China and Japan (15). The symptoms of ASSVd are restricted to the fruit. They include fruit dappling, scarring, and cracking. Most commercial pear cultivars do not develop symptoms when infected with ASSVd.

Two other diseases, dapple apple (5) and pear rusty skin (1), have been attributed to viroids that are closely related to ASSVd. Dapple apple viroid (DAVd) and pear rusty skin viroid (PRSVd) hybridize with and can be detected by a cRNA probe complementary to the full-length sequence of ASSVd (5,6). Analysis of the nucleotide sequences has shown that DAVd and PRSVd differ from ASSVd in only nine and 25 positions, respectively (16). Therefore, in this paper we will refer to ASSVd, DAVd, and PRSVd, collectively, as ASSVd. We also will assume that the viroid(s) we are detecting is one of these three entities and refer to it as ASSVd.

ASSVd is the prototype for a subgroup of phylogenetically related viroids designated apscaviroids (2). Pear blister canker viroid (PBCVd) (3,8) is the only other apscaviroid reported to infect pome fruit trees. Hop stunt viroid (HSVd) (14) and apple fruit crinkle viroid (AFCVd) (11,12) have been found in pome fruit trees in Japan but are unrelated to ASSVd.

Although these six viroids are prevalent in apples and/or pears in China and Japan, their incidence in pome fruits in the United States is rare. In the United States, quarantine regulations exist to prevent importation and distribution of viroid-infected trees. Vegetative propagations of apple and pear must be tested in an approved program before general distribution of the germ plasm is allowed. Since the viroids cause only fruit or bark blemishes in apple and pear cultivars of commercial importance, infection is not detected visually until infected trees mature.

Conventional biological detection of ASSVd is accomplished by growing graft-inoculated, red-fruited apple indicator hosts for 3 to 5 years in an orchard and observing them for the appearance of fruit dapple or scar skin symptoms. In 1992, Howell and Mink (9) described a bioassay for ASSVd-infected apples in which the graft-inoculated apple (*Malus* hybrids) cultivars Stark's Earliest and Sugar Crab were induced to express leaf curling and epinasty in 8 weeks by growth under constant light. At the same time, Hurtt et al. (10) reported the detection of an agent that caused dapple apple symptoms on the fruit of Lord Lambourne apple graft-inoculated with bark chips from imported pear trees. The pears originated in Liaoning, China, where pears were reported to be latent hosts for ASSVd (15). Tissue blots of the symptomatic Lord Lambourne trees hybridized with ASSVd cRNA probe (13).

We undertook this study to determine whether the viroid in the Chinese pear germ plasm would induce symptoms in the ASSVd indicator hosts, Stark's Earliest and Sugar Crab. We also compared the sensitivity and reliability of this bioassay to detection by a tissue blot, nonisotopic molecular hybridization-based assay of greenhouse-grown, symptomless, viroid "amplification hosts." We use the term "amplification host" to refer to plants that give rise to high titers of viroid, with or without symptoms of infection. We propose a protocol to rapidly detect ASSVd in a pome fruit indexing program where many genetically diverse plants within a genus are tested, e.g., a quarantine program.

MATERIALS AND METHODS

Plant materials. One-year-old apple seedlings (*Malus domestica* Borkh.) or pear seedlings (from seeds of *Pyrus communis* L. cv. Bartlett) were used as healthy rootstocks. Healthy budwood of Stark's Earliest, Sugar Crab, and Virginia Crab apples and *P. communis* cv. Nouveau Poiteau were from the NRSP5/IR2 (National Research Support Project No. 5/Interre-

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gional Project No. 2) Fruit Tree Collection, Irrigated Agriculture Research and Extension Center, Prosser, WA, or the United States Department of Agriculture, Agriculture Research Service orchards at Glenn Dale, MD.

Pears were propagated in orchards at Glenn Dale from budwood donated by the Research Institute of Pomology, Chinese Academy of Agricultural Science, at Xing Cheng, Liaoning, China. Thirty-five pear accessions were rated as viroid-infected or -uninfected based on the results of field bioassays in Lord Lambourne and/or Red Delicious. Three indicator trees were graft-inoculated with two bark chips from a pear tree, and then observed for fruit dappling symptoms (Fig. 1) over 4 years of fruiting. Budwood of healthy and ASSVd-infected apple was supplied by C. L. Parish, USDA, ARS, Wenatchee, WA.

Graft inoculations in the greenhouse were made according to a modification of the double-budding method described by Fridlund (4) and modified by Howell and Mink (9). One-year-old healthy rootstocks were removed from dormant storage, potted in torpedo-shaped (10 × 23 cm) deepots (J. M. McConkey & Co., Summer, WA), and grown in the greenhouse from January through February. One or two healthy buds of the indicator or amplification species were chip budded into the stem of the sprouting rootstock. Two bark chips from the inoculum source were grafted onto the rootstock directly below the bud graft. Seven to 10 days later, the rootstock was cut off 2 cm above the upper bud graft to force the indicator bud into growth.

Inoculated plants of Nouveau Poiteau pear and Virginia Crab apple were maintained at about 20 and 25°C, respectively, under ambient greenhouse light conditions from February through May at Beltsville, MD. Inoculated Stark's Earliest and Sugar Crab apples were maintained in an environmental chamber at 18°C under 24 h of incandescent and fluorescent light (9).

The reactions of Stark's Earliest and Sugar Crab when graft-inoculated with ASSVd-infected apple or viroid-infected pear were compared in duplicate tests at the IR-2/NRSP5) and USDA locations. Three viroid-infected pears and an ASSVd-infected apple were selected as sources of inoculum for the assays. Each of the viroid isolates or bark chips from healthy apple and pear trees was grafted into three plants of each of the two indicators. The indicator plants then were grown as described above and observed 8 weeks for symptom expression.

Tissue blots. Leaf and stem tissues were sampled beginning 4 to 6 weeks after rootstocks were cut off. Tissue blots were made by firmly pressing the freshly cut surface of young, succulent shoots or leaf petioles against positively charged nylon membranes (Boehringer Mannheim, Indianapolis, IN) prewetted with 20× SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7). The treated membranes were irradiated with ultraviolet light (UV) at 1.2×10^5 µJ/30 s in a UV crosslinker (Stratalinker, Stratagene, LaJolla, CA) and held in a desiccator at room temperature (RT) until hybridization tests were done.

Probe preparation and hybridization. Preparation of cRNA probe and hybridization assays were performed as previously described (13). Briefly, plasmid pSP64 transcription vector (Promega, Madison, WI) containing an insert complementary to ASSVd (5) was linearized using *Eco*RI restriction endonuclease. Nonisotopically labeled riboprobes were prepared using digoxigenin-11-UTP and SP6 RNA polymerase according to the manufacturer's directions (Boehringer Mannheim).

Membranes with tissue blots were soaked for 1 h at 55°C in 20 mM sodium cacodylate buffer, pH 7.0, containing 40% formamide, 10% dextran sulfate, 0.18 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and yeast tRNA at 400 µg/ml. The prehybridization buffer was replaced with fresh solution, and digoxigenin-labeled probe was added to a final concentration of 100 ng/ml. Membranes were incubated in hybridization solution for 16 to 20 h at 55°C, then washed twice with 2× SSC for 15 min at 55°C, once in 2× SSC with RNase A at 1 µg/ml for 20 min at RT, and once in 0.1× SSC with 0.1% SDS for 30 min at 65°C. Membranes were washed in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 3 min and blocked in maleate buffer containing 1% blocking reagent (Boehringer Mannheim) for 0.5 to 3.0 h. After the blocking step, membranes were incubated 30 min with diluted polyclonal antidigoxigenin alkaline phosphatase enzyme conjugate (1/5,000 in blocking buffer). Unbound conjugate was removed with two washes, 15 min each, in maleate buffer and then equilibrated for 2 min in 100 mM tris-hydrochloride, 100 mM NaCl, and 50 mM magnesium chloride, pH 9.5. Membranes were coated with the enzyme substrate 4-methoxy-4-(3-phosphatephenyl-spiro[1,2-digoxetane-3,2'adamantane] (Lumi-Phos 530, Boehringer Mannheim) at about 1 ml/100 cm^2 and immediately exposed to photographic film (Hyperfilm ECL, Amersham, Arlington Heights, IL) for 1 to 3 h. Alternatively, the substrate-coated membrane was held overnight, then exposed to film for 10 to 60 min.

RESULTS AND DISCUSSION

Of the 35 oriental pear germ plasm introductions tested, 13 (37%) gave dapple apple symptoms on Lord Lambourne and/or Red Delicious apples. When these 6- to 8-year-old orchard-grown pear trees were assayed by molecular hybridization with cRNA probe, tissue blots of the new shoots and leaf petioles gave inconsistent results (Table 1).

Tissue blot hybridization of Virginia Crab apple and Nouveau Poiteau pear graft-inoculated with bark chips from the Chinese pears consistently detected the infected introductions in the collection (Table 1). Although tests of tissue blots from graft-inoculated Nouveau Poiteau and Virginia Crab gave similar results, tissue blots of Virginia Crab produced more intense spots on the detection film. Positive hybridization reactions were obtained 4 to 6 weeks after the amplification host bud broke dormancy. Viroid-inoculated, greenhouse-grown Virginia Crab and Nouveau Poiteau were symptomless.

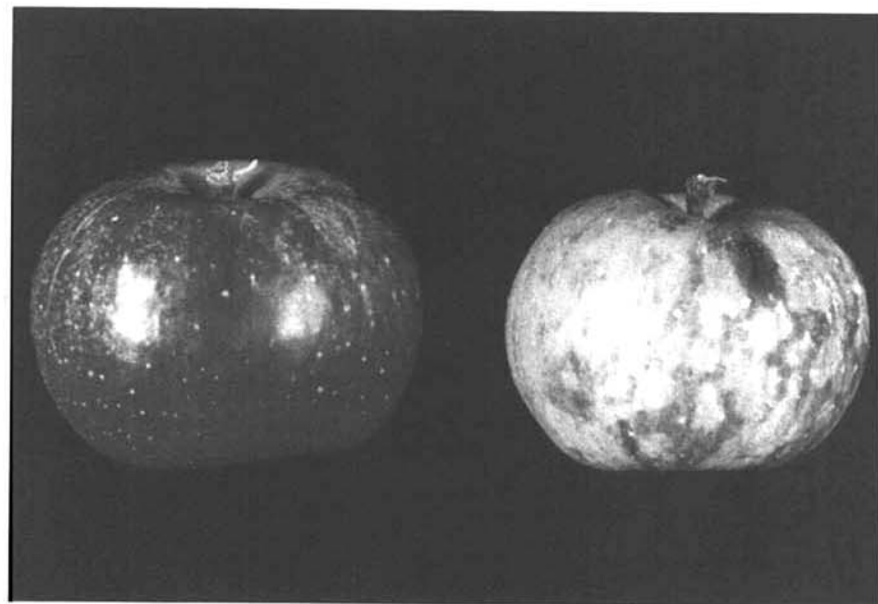

Fig. 1. Healthy (left) and dappled (right) fruit from 5-year-old Lord Lambourne apple tree graft-inoculated with bark chips from viroid-infected oriental pear.

Table 1. Comparison of detection of apple scar skin viroid by bioassay on field-grown Lord Lambourne (*Malus pumila*) and tissue blot hybridization assays of oriental pears and pear-inoculated Virginia Crab apple or Nouveau Poiteau pear

Pear accession[a]	Bioassay	Tissue blot hybridization		
		Pear	Nouveau Poiteau	Virginia Crab
An Li	3/3[b]	1/1[c]	4/4	1/1
Ba Li Shian	3/3	1/1	5/5	1/1
Guan Hong Shieo	2/2	2/2	5/5	6/6
Hong Li	3/3	2/5	0/0	3/3
Hua Gua	3/3	0/0	2/2	0/0
Liu Yue Shian	3/3	0/1	5/5	4/4
Mian Suan	1/2	1/3	4/4	5/5
Pingo Li	3/3	0/1	2/2	1/1
Ta Shian Sui Li	3/3	1/1	5/5	0/0
Tsu Li #1	3/3	1/4	5/5	2/2
Tsu Li #2	2/2	0/2	5/5	0/0
Tu Li	0/3	0/1	0/5	0/2
Ya Guang Li	3/3	1/1	2/2	0/0
Yang Nai Shian	3/3	0/0	0/0	0/0

[a] Designations are those provided with the germ plasm by the donating institute or the English transliteration thereof.
[b] Number positive/number Red Delicious or Lord Lambourne indicator trees inoculated and surviving to bear fruit.
[c] Number positive/number tested.

Fig. 2. Healthy (left) and ASSVd-infected (right) Stark's Earliest apple. Infected apple shoot shows symptoms of leaf epinasty 6 weeks after graft inoculation and incubation under a 24-h photoperiod at 18°C.

Table 2. Comparison of apple scar skin viroid (ASSVd) detection by bioassay and tissue blot hybridization at 4 and 6 weeks after inoculation of Stark's Earliest apple with viroid

Inoculum	4 Weeks		6 Weeks	
	Bioassay	Tissue blot	Bioassay	Tissue blot
Apple ASSVd	1/3[a]	3/3[b]	3/3	3/3
Pear ASSVd	0/3	3/3	3/3	3/3
Healthy apple	0/3	0/3	0/3	0/3
Healthy pear	0/3	0/3	0/3	0/3

[a] Number of plants with symptoms/number of plants inoculated.
[b] Number positive plants/number of plants inoculated.

Stark's Earliest and Sugar Crab apples developed leaf epinasty symptoms (Fig. 2) when graft-inoculated at the NRSP5/IR2 and ARS locations with bark chips from three viroid-infected oriental pear introductions and ASSVd positive control from apple. Symptoms appeared as early as 4 weeks after inoculation but sometimes required 6 to 8 weeks to develop. While the symptoms were similar, indicator plants inoculated with the apple ASSVd inoculum showed symptoms 1 week or more before those inoculated with the infected pear bark chips. Indicator plants inoculated with uninfected apple or pear bark chips were symptomless. Tissue blots of the Stark's Earliest and Sugar Crab apples also hybridized with the digoxigenin-labeled ASSVd probe. Symptomatic leaves and the adjoining stem segments gave strong positive reactions similar to those of Virginia Crab apple. In addition, viroid was detected in Stark's Earliest before symptoms appeared (Table 2).

Symptom expression in the two indicator hosts is photoperiod and temperature dependent (9). In one experiment, after 8 weeks in the environmental growth chamber, symptomatic plants were moved to a greenhouse. The new leaves that developed under greenhouse conditions of 18 to 25°C and ambient light were asymptomatic. After six to eight new leaves had emerged, petiole and stem sections at every internode were cut and blotted onto a membrane. The cRNA probe detected viroid in all internodes, including symptomless new shoot segments.

Based on these results, we adopted the procedures illustrated (Fig. 3) to rapidly and reliably index imported apple and pear accessions for ASSVd-related viroid. Two bark chips from the candidate budstick and a bud from the amplification host are double budded onto a healthy seedling rootstock. Four to 6 weeks after the bud breaks dormancy, the new shoot is cut for tissue blot hybridization with nonisotopically labeled cRNA probe for ASSVd. If bioassay results on Stark's Earliest or Sugar Crab are desired, plants must be grown under conditions described by Howell and Mink (9), and tissue sampling is delayed until symptom expression is rated at 8 weeks. The tissue blot hybridization assay can be repeated (to increase confidence in the test results) by sampling the second flush of growth induced in all of the bioamplification or assay hosts when the shoots are excised at the first sampling.

Of those hosts tested, Virginia Crab and Stark's Earliest are the preferred amplification hosts. Stark's Earliest is preferred to Sugar Crab because the former grows erect and requires less space in the environmental chamber. The apples were preferred to the Nouveau Poiteau pear because tissue blots of apple usually produced larger and darker positive spots on the detection film

than comparable imprints of pear. Pear tissue imprints often developed a brown color on the membranes. This pigmented material interfered with ASSVd detection, and its presence on the membrane produced corresponding clear areas on the detection film.

In developing this protocol for a quarantine program, several considerations were taken into account. First, since each accession is genetically unique, healthy and infected control plants for every species or clone cannot be included in each test. In the absence of such controls, there is no way for the clinician to know if an introduction will or will not give consistent tissue blot hybridization results if tested directly. Our data show that tissue blot detection of ASSVd directly from some pear trees is unreliable. Secondly, at the time of importation, the amount of plant sample available for testing may be limited and may consist of only a few small, dormant budsticks. These would not be expected to test well by tissue blotting because their sap content is low. This problem is overcome when a common amplification species is inoculated with bark chips from the candidate budsticks and the succulent, bioamplification host is tested using a nucleic acid probe to detect viroid. Pome fruits are rapidly indexed for common pome viruses on greenhouse-grown indicator hosts such as Virginia Crab apple and Nouveau Poiteau. If these hosts serve as the viroid amplification host, labor and space costs for viroid detection are minimized. Total detection time by biological amplification and tissue blotting hybridization is approximately 8 to 10 weeks, which is quicker by 2 to 3 years than the conventional field indexing protocol. Where hybridization analysis is not feasible, Stark's Earliest may be used as a rapid bioassay for apples and pears.

We cannot be sure of the identity of the viroid(s) infecting each of the 13 pears that gave positive viroid test results without isolating and sequencing the viroid(s). Several apscaviroids, including apple scar skin, dapple apple, pear rusty skin, and pear blister canker (2), have been reported to infect pear. All of these viroids share varying degrees of sequence homology (2,5,16). Pear blister canker is not known to infect apple and reacts only weakly with cDNA to ASSVd (3). Under our conditions of stringency for hybridization, we would expect to detect ASSVd (including DAVd and PRSVd) but not PBCVd, HSVd, or AFCVd.

Stark's Earliest indicator host inoculated with the apple isolate of ASSVd developed symptoms sooner than did those inoculated with the pear inoculum. The difference in the rate of symptom expression may reflect a difference in titer in the source inoculum, an impediment to transmission from pear tissue, or biological differences between apple isolate of ASSVd and the pear viroids.

Fruit symptoms on Lord Lambourne and Red Delicious inoculated with pear bark chips initially consisted of fruit dappling. Later, Lord Lambourne fruit also showed cracking and deformation. Red Delicious trees, inoculated several years after the Lord Lambourne indicator trees, are still under observation for changes in fruit symptoms. A comparison of symptoms caused by a number of isolates of dapple apple, scar skin, and pear rusty mottle viroids in a variety of apple cultivars grown in similar environments over a period of time has not been reported. Therefore, the taxonomic relationship of these viroids to each other and to the viroid that we detect remains uncertain, but available data indicate a relationship among them characteristic of strains of a pathogen.

In summary, we described a rapid indexing protocol for detecting apple scar skin-like viroid in pear germ plasm by a combination of graft-inoculation bioassay and a chemiluminescent molecular hybridization assay. Use of amplification hosts allows viroid detection by direct tissue blotting, thereby reducing the hazardous waste and safety problems associated with the alternate phenol–chloroform extraction procedure for dot blot nucleic acid hybridization assays. Similarly, the use of a nonisotopic, chemiluminescent detection method is safer, more convenient, and less expensive than methods that use radioactive isotopes. The host plants used in this research and others will be tested as amplification hosts for HSVd, PBCVd, and AFCVd. If a common amplification host can be found, detection of all six viroids could be performed by using the same graft-inoculated host for tissue blot hybridization assays with several different cRNA probes.

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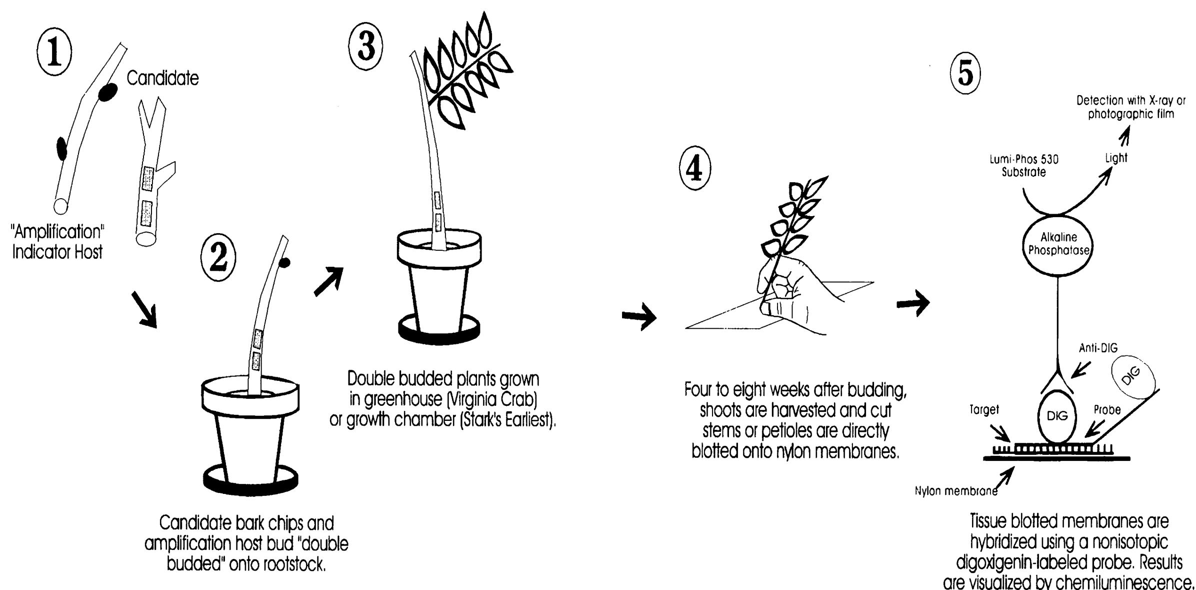

Fig. 3. Schematic summary of a protocol integrating biological and molecular assays for the rapid detection of apscaviroid subgroup in pome fruit trees: (1) budwood of the healthy indictor or amplification host and the pome to be tested (candidate) are gathered; (2) bark chips of the candidate pome and a bud of the amplification/indicator host are grafted into the trunk of a 1-year-old healthy pome rootstock which is cut off just above the host bud; (3) the host shoot is grown for 4 to 8 weeks in the greenhouse (amplification host) or in a controlled environment (bioassay host) and observed for symptoms; (4) stems are cut and tissue blotted to membranes; (5) blots are tested with digoxigenin-labeled cRNA probe to ASSVd, and hybridization is detected with antidigoxigenin alkaline phosphatase (AP) antibody conjugate and luminescent substrate.

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