Correlation Between Leaf Epinasty Symptoms on Two Apple Cultivars and Results of cRNA Hybridization for Detection of Apple Scar Skin Viroid

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ABSTRACT

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Within 2 mo following double bud inoculation with apple scar skin viroid (ASSV), apple (*Malus domestica*) trees of the cultivars Stark's Earliest and Sugar Crab expressed pronounced leaf epinasty when maintained for 24-hr photoperiods in growth chambers at 18 or 28 C. Similar uninoculated trees and trees infected with the common apple latent viruses remained symptomless. Typical viroidlike symptoms were observed on fruit produced within 2 mo after inoculation of greenhouse-grown trees. Dot blot hybridization of leaf midrib and petiole extracts using a cRNA probe specific for ASSV indicated that ASSV accumulated in these tissues at 18 or 28 C but not at 38 C. The two apple cultivars not only were sensitive indicators for this viroid but also appeared to be useful amplification hosts for nucleic acid hybridizations.

Apple scar skin is a severe disease of apples (*Malus domestica* Borkh.) recognized by color dappling, cracking, and distortion of the fruit (1,5,6). The disease appears to be relatively rare in the United States but may be widespread in the Orient (1). Because the symptoms are apparent only on the fruit (1), trees propagated from infected source trees can be several years old before the disease is observed. Therefore, it is important that propagation source trees are free of the causal agent, apple scar skin viroid (ASSV). Unfortunately, biological tests for ASSV involve expression of fruit symptoms in the field, which requires several years to complete (1). Newly developed tests using nucleic acid probes, on the other hand, can be completed in just a few days (2,3). However, there are several reasons a rapid biological indicator for ASSV would be useful: 1) Testing facilities in many parts of the world are not equipped to handle nucleic acid probes; 2) indexing on woody indicators is still required to detect many other virus and viruslike disease agents of apple, and the availability of a quick woody indicator for ASSV would be expedient; and 3) such an indicator might also provide rapid biological confirmation of laboratory tests.

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ASSV-infected trees of the apple cultivars Red Delicious and Stark's Earliest have occasionally displayed downward leaf curvature (epinasty) when grown in our greenhouse. The objective of this study was to define the environmental conditions required to obtain reliable expression of foliage symptoms on ASSV-inoculated trees. Concurrently, the test trees were analyzed for viroid content by cRNA hybridization.

MATERIALS AND METHODS

Viroid isolates. Viroids that cause apple scar skin and a similar disease, dapple apple (2,5,10), have a high degree of homology (2,4). Recent reports demonstrate that [^{32}P]-cRNA probes prepared against ASSV can be used in diagnostic tests to detect both viroids (2). For purposes of this paper the causal agents of these two diseases will be referred to as isolates of ASSV. A third ASSV isolate was obtained from a tree of the Japanese apple cultivar Heiya grown at Prosser, Washington.

The scar skin and dapple apple isolates were maintained in Red Delicious apple trees grown in 7.6-L containers in the greenhouse. These trees were inoculated in 1988 with apple scar skin (SS) or dapple apple (DA) diseased bud and stem tissue obtained from G. Agrios (University of Massachusetts, Amherst). Inoculum of these two isolates was obtained from these greenhouse trees. When acquired, all three viroid sources were coinfected with three common apple latent viruses—apple stem grooving virus, apple stem pitting virus, and apple chlorotic leaf spot virus—as determined by assays on apple selections routinely used as indicators of these viruses, i.e., Virginia Crab, Radiant, and Russian Seedling R12740-7A, respectively. An ASSV-free tree labeled UA-1, which contained all three of these latent viruses, was included in all experiments as a virus-infected, viroid-free control; the ASSV-free status of the UA-1 tree had been determined by 4 yr of observations of fruit on four trees of the cultivar Red Delicious that had been graft-inoculated with tissue from the UA-1 tree.

Propagation, inoculation, and symptom observation. Single dormant buds of the indicator cultivars Stark's Earliest or Sugar Crab were chip-budded onto 1-yr-old apple seedlings grown in torpedo-shaped pots, which were 23 cm long and tapered gradually from a diameter of 6 cm at the top to 5 cm approximately 2 cm from the base, then sharply to 1.4 cm at the base (Deepot Container, McConkey Co., Sumner, WA), in a soil mixture of loam, sand, and peat moss (2:2:1). Two inoculum buds or bark chips were inserted below the indicator buds. After 1 wk at 26 C, these double-budded trees were pruned to just above the indicator bud to force growth.

Shortly after indicator bud emergence, trees budded with vegetative buds were placed into constant-temperature growth chambers and observed for symptom development. Inoculated plants were grown in one of nine environmental regimes consisting of three temperatures—18, 28, and 38 C—and three photoperiods—4, 14, and 24 hr of light (250 $\mu E \cdot m^{-2} \cdot s^{-1}$) provided by 12 40W cool-white fluorescent tubes. Leaf symptoms were recorded weekly.

Trees budded with flower buds were maintained in the greenhouse at 26 C. Emerging flowers were hand-pollinated with a commercial pollen mixture from four apple cultivars (Golden Delicious, Gravenstein, Winesap, and Jonathan). Vegetative shoots were subsequently cut back to encourage fruit set. Symptoms were recorded as the fruit matured.

ASSV probe preparation. An SP-6 generated [^{32}P]-cRNA probe was produced from PVAS14 plasmid (provided by A. Hadidi, USDA, Beltsville, MD) containing a 274-nucleotide-long cDNA copy of the Japanese isolate of ASSV (2). Transcription was done with a Riboprobe kit (Promega, Madison, WI) using [α-^{32}P] UTP (New England Nuclear, Burbank, CA). The specific activity of the probe was in the range of 10^9 cpm/μg of RNA.

Preparation of apple tissue extracts and application of samples. Samples (0.1–0.2 g) were prepared for blots on

membranes by extracting plant tissue 1/5 (w/v) in KT buffer (0.2 M potassium monobasic phosphate, 0.1% Triton X-100, 0.005 M dithiothreitol, 0.01 M 2-mercaptoethanol) in a sterile mortar and pestle. An equal volume of 0.18 M sodium citrate, 1.8 M sodium chloride, and 14.8% formaldehyde was added, and the sample was then denatured for 30 min at 60 C (9). The incubated samples were centrifuged at 16,000 *g* for 3 min or were allowed to settle overnight at 4 C. Supernatant (5 μl) was spotted on nylon membrane (Hybond N+, Amersham, Arlington Heights, IL) equilibrated in 20× SSC (1× SSC: 0.15 M NaCl, 0.15 M sodium citrate) using a sandwich blot procedure (9); the 5-μl sample had a tissue equivalent of about 500 μg. Test sensitivity was determined using 10-fold serial dilutions of these extracts. Blots of tissue equivalents as low as 1–2 μg were detectable.

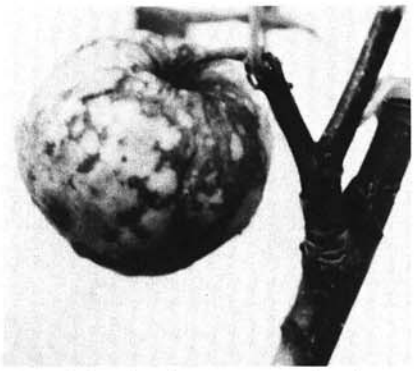

Fig. 1. Color dappling symptom of apple scar skin viroid (ASSV) on the fruit of a 4-mo-old tree of the apple cultivar Stark's Earliest. The tree was formed by chip-budding a seedling rootstock with a dormant flower bud. The tree was inoculated by grafting two chips of ASSV-infected apple bark tissue just below the flower bud. The subsequent flowers were hand-pollinated.

Fig. 2. (Right) Epinasty of leaves on a 2-mo-old Stark's Earliest apple tree inoculated with apple scar skin viroid; (left) uninoculated tree.

Hybridization and film development. Prehybridization and hybridization were according to Hadidi et al (2). Filters were autoradiographed 12–24 hr at −70 C using Kodak XAR5 film and intensifying screen.

RESULTS

Fruit symptoms. Fruit on ASSV-inoculated trees (SS or DA) of both Stark's Earliest and Sugar Crab developed dappling and cracking beginning at the calyx end about 2 mo after pollination (Fig. 1). No symptoms were observed on fruit from the virus-infected but viroid-free control trees or on uninoculated trees. However, many of the trees that were budded with buds presumed to contain flower initials did not produce flowers. Thus, only seven of more than 100 budded and inoculated trees developed fruit, and all seven trees displayed symptoms.

Foliar symptoms. Leaf epinasty was observed on ASSV-inoculated Stark's Earliest and Sugar Crab trees 6–8 wk after inoculation. Expanding leaves curled downward, with the leaf tip bending toward the petiole, creating a C-shaped curvature of the midrib (Fig. 2). Little, if any, lateral twisting was noted. With extended incubation periods, necrotic areas were noted on the lower midrib surface. No differences were detected in symptoms produced by the three ASSV isolates.

Effect of temperature and photoperiod on incidence of symptom development. Cool temperatures and long photoperiods enhanced the probability and intensity of leaf epinasty development on ASSV-inoculated trees. All Stark's Earliest and most Sugar Crab trees exhibited symptoms of leaf epinasty within 58 days when grown under constant light at 18 C (Table 1). When grown at 28 C, all similarly illuminated Stark's Earliest but only a few of the Sugar Crab expressed visual symptoms. Also, only a few trees of either cultivar showed epinasty at these temperatures under 14-hr photoperiods. No inoculated trees developed leaf epinasty symptoms when grown at 38 C under any light regime or at 4-hr photoperiods at any temperature (Table 1). Epinasty was not observed on uninoculated trees or on trees inoculated with the mixture of latent viruses.

Chlorotic spotting and unilateral deformation occurred on leaves of viroid-inoculated Sugar Crab trees maintained at 18 C with 4-hr photoperiods. However, the same symptoms were observed on latent virus-infected trees of this cultivar.

Effect of temperature and photoperiod on ASSV titer within apple trees. Positive hybridizations were obtained between the ASSV cRNA probe and midrib petiole extracts of apple trees grown at 18 and 28 C (Fig. 3). Estimates of relative viroid titer based on the intensities of the hybridization signals in comparison with internal standards suggest that day length had little or no effect on ASSV titer.

Viroid titer appeared reduced at higher temperatures. Strong reactions were observed in dot blot hybridization tests at all leaf positions at 18 C but only in the middle and lower leaves at 28 C (Fig. 3). No visible reactions were obtained with tissue from trees grown at 38 C. All ASSV isolates reacted similarly. No positive reactions were observed with petiole extracts from the latent virus-infected or uninfected control trees.

Effect of tissue type on ASSV titer. ASSV samples prepared from midribs and petioles appeared to be diagnostically more credible in dot blot hybridization tests than preparations from the leaf lamina because they produced much stronger reactions (Fig. 4). Extracts of ASSV-infected petioles that had been air-dried at room temperature reacted nearly as strongly in dot blot tests with the ASSV cRNA probe as extracts of freshly harvested petioles (Fig. 4).

DISCUSSION

Results indicate that ASSV isolates can be reliably detected in less than 2

Table 1. Leaf epinasty symptom expression on apple tree cultivars Stark's Earliest and Sugar Crab inoculated with isolates of apple scar skin viroid (obtained from Red Delicious trees with dapple apple and scar skin and from the Japanese apple cultivar Heiya) and grown at various light conditions and temperatures

Day length (hr)	Temperature (C)	No. of trees with leaf epinasty/total no. of trees					
		Stark's Earliest			Sugar Crab		
		Dapple apple	Heiya	Scar skin	Dapple apple	Heiya	Scar skin
24	38	0/5	0/5	0/5	0/5	0/5	0/5
	28	5/5	5/5	5/5	1/5	1/5	1/5
	18	5/5	5/5	5/5	5/5	4/5	5/5
14	38	0/5	0/5	0/5	0/5	0/5	0/5
	28	0/5	0/5	0/5	1/5	0/5	0/5
	18	0/5	0/5	3/5	2/5	2/5	0/5
4	38	0/5	0/5	0/5	0/5	0/5	0/5
	28	0/5	0/5	0/5	0/5	0/5	0/5
	18	0/5	0/5	0/5	0/5	0/5	0/5

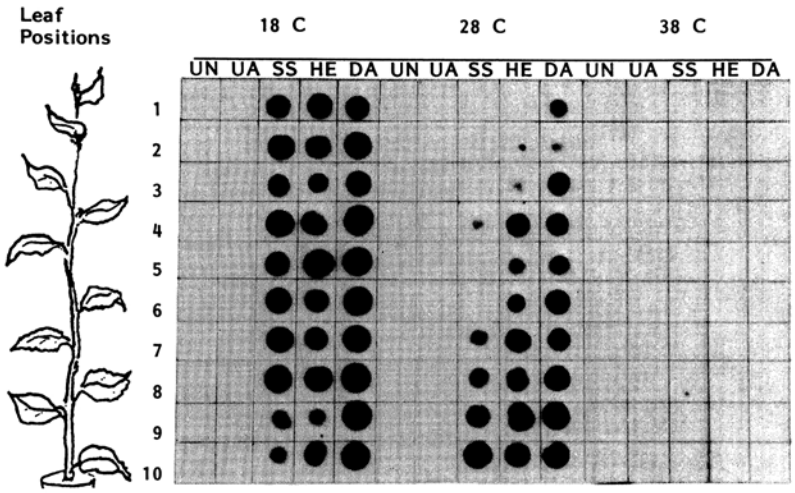

Fig. 3. Detection by cRNA probe of three isolates of apple scar skin viroid (ASSV) from Stark's Earliest apple trees grown at three temperatures and at constant illumination. Trees were inoculated with apple scar skin viroid isolates scar skin (SS), Heiya (HE), or dapple apple (DA) or with latent virus (UA); UN = uninoculated. Each spot contained denatured extract from 500 μg of petiole.

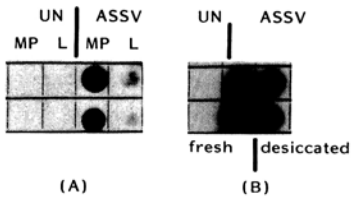

Fig. 4. Autoradiograph of crude tissue extracts: **(A)** Fresh midribs and petioles (MP) or leaf lamella (L) from Stark's Earliest apple trees inoculated with apple scar skin viroid (ASSV); UN = uninoculated. **(B)** Fresh and desiccated samples of ASSV-infected petioles and midribs.

mo by inoculated woody indicator plants kept under controlled growing conditions in contrast to the 2–3 yr required for present field testing methods (1). This method could be effectively used in certification schemes that already utilize woody plant indicators for virus detection. Results could be verified with cRNA hybridization tests of petiole tissue. Conversely, this rapid indicator assay could provide biological disease verification for nucleic acid hybridization detection techniques conducted in the laboratory.

Symptoms were reliably obtained within 2 mo on both fruit and vegetative tissues. However, the usefulness of the fruit test has been hindered by the low proportion of flower to vegetative buds on trees of Stark's Earliest and by inadequate techniques to discriminate in the dormant season between the few flower buds and the more common vegetative buds.

Although the ASSV indicators reported here have a function in the rapid procurement of diagnostic information, dot blot hybridization requires less time and allows for detection of viroid specific sequences in extracts of tissues that are difficult to test by woody indicators, such as fruit, seed, and foliage.

Visible symptoms and hybridization reactions of leaf petioles from the test trees indicated that ASSV titer appeared to decrease at elevated temperatures, especially near tree apexes. This observation suggests that ASSV-free trees might be obtained from infected ones by normal heat treatment, tip propagation methods. Preliminary results here suggest, in fact, that following standard heat therapy techniques, a small percentage of the resultant trees are viroid-free. Although viroids tend to be extremely heat-stable, apparently the viroid causing citrus exocortis disease was also removed by prolonged exposure to 38 C followed by shoot-tip propagation (8).

ASSV accumulation was greatest at 18 C, the lowest temperature tested. This apparent temperature preference is substantially lower than the optimum reported for the potato spindle tuber viroid in potato (7), suggesting that replication requirements for these two viroids may be quite different.

ACKNOWLEDGMENT

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