Transmission of a Citrus Viroid to Avocado by Heterologous Grafting

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ABSTRACT

Hadas, R., Ashulin, L., and Bar-Joseph, M. 1992. Transmission of a citrus viroid to avocado by heterologous grafting. Plant Dis. 76:357-359.

A citrus viroid previously designated as CV-Ib was graft-transmitted from alimau (*Citrus macrophylla*) to seedlings of a West Indian avocado (*Persea americana*). It was found by subsequent graft transmission to have a host range limited to only a few avocado cultivars. Extracts from CV-Ib–infected avocado were infective on Etrog citron and induced a slight leaf kinking and fruit deformation. The possibilities of extending viroid host ranges as a result of heterologous grafting among noncompatible hosts are discussed.

Five viroidlike RNAs with estimated molecular sizes of 275, 295, 300, 330 and 371 bases were found in extracts of Etrog citron (*Citrus medica* L.) plants inoculated with a graft-transmissible dwarfing (GTD) agent 225-T (7). Molecular hybridization identified the 371-base citrus viroid (CV) as a strain of citrus exocortis viroid (CEV) (7). Sequencing revealed that the 300-base CV was actually 299 bases long and shares 98% sequence homology with hop stunt viroid (HSVd) (10). Previous host range studies (4,5,14) have shown that the CEV present in CV complexes could be separated by inoculating *Gynura aurantiaca* (Blume) DC. Both the 275-base CV, for which recent sequencing indicated a molecular size of 284 bases (11), and the 299-base CV could be transmitted and separated by serial transfer to cucumber (4,5,16) and *Benincasa hispida* (Thunb.) Cogn. (10). The host range of the 330-base CV, classified as CV-Ib and representative of the group I citrus viroids (5,14), was considered to be restricted to *Citrus* spp. This paper reports on the separation of CV-Ib by graft transmission from citrus to avocado plants.

Accepted for publication 11 June 1991 (submitted for electronic processing).

MATERIALS AND METHODS

Inoculation procedures. For graft transmission, groups of four West Indian (WI) avocado seedlings were chip-grafted with six to eight buds collected from alimau (*C. macrophylla* P. J. Wester) seedlings inoculated with two CV sources, obtained from subinoculations of GTD 225-T and designated as 225-S and 225-M (7). After 4 wk the plants were topped, and new growth was observed continuously for disease symptoms and tested for the presence of CVs and ASBV by the sequential PAGE (sPAGE) analysis (12).

For mechanical transmission, extracts prepared from the CV-Ib–infected avocado leaves were slash-inoculated on the stems of Etrog citron seedlings with a razor blade dipped in the CV-Ib extract,

and the wounded stem was wrapped with parafilm membrane.

Viroid extraction and PAGE analysis. Viroids were extracted from avocado and citrus leaf samples (5 g) using a minor modification of the method of Semancik et al (15). The extraction procedure included tissue pulverization in liquid nitrogen, followed by vigorous mechanical shaking for 20 min in 3 vol of TE (10 mM Tris-HCl and 1 mM EDTA)-saturated phenol (pH 8.0) and 1 vol of extraction buffer (0.4 M Tris-HCl [pH 8.9], 1% sodium dodecyl sulfate [SDS], 5 mM EDTA [pH 7.0], and 3% of 2-mercaptoethanol). After centrifugation at 10,000 *g* for 15 min, the aqueous phase, to which ethanol was added to 35%, was mixed with Whatman CF-11 cellulose powder (1 g/5 g of tissue). The slurry was shaken for 20 min or overnight at room temperature, packed in a column, and washed four times with STE (0.05 M Tris-HCl, 0.1 M sodium chloride, and 0.001 M EDTA [pH 7.0]) 30% ethanol. The viroid fraction was eluted with STE buffer, and LiCl was added to the solution. The fraction soluble in 2 M LiCl was ethanol-precipitated; redissolved in 10 mM Tris, 10 mM KCl, and 0.1 mM $MgCl_2$ (pH 7.4); and run in sPAGE. The viroid bands were detected by silver staining (8).

Hybridization assays. The nucleic acids from healthy and viroid-infected citrus and avocado plants were fractionated by sPAGE in the presence of 8 M urea using a Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, CA). The gel was treated with 7% formaldehyde for 20 min, electrotransferred to a nylon membrane, and fixed to the membrane by a 3-min exposure to UV light. The filters were incubated for 2 hr at 42 C in prehybridization solution consisting of 50% formamide, 5× concentrated Denhardt solution, 6× concentrated SSPE (1× SSPE is 0.18 M NaCl, 10mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.1% SDS, and 10 $\mu g/\mu l$ herring sperm DNA. A ^{32}P-labeled CV-Ib cDNA probe or an oligonucleotide probe (2) was used for overnight hybridization at 42 C. The filters were washed twice with a solution of 2× SSPE and 0.1% SDS for 10 min at room temperature, twice with 1× SSPE and 0.1% SDS for 20 min at 42 C, and once with 0.1× SSPE and 0.1% SDS at 42 C. They were then dried and autoradiographed.

RESULTS

After 1 yr, 16 of 40 citrus buds grafted on eight WI avocado seedlings remained viable. Occasionally the citrus buds sprouted and developed some leaves. Only one of the WI seedlings showed temporary epinasty symptoms. However, sPAGE analysis of extracts from young avocado leaves 6 mo after grafting revealed the presence of CV-Ib (Fig. 1, lane 4) in six of the eight grafted WI seedlings. Extracts and grafts of WI avocado carrying CV-Ib were infective on Etrog citron, and only the CV-Ib could be recovered (Fig. 1, lane 3). The Etrog citron seedlings that were infected with CV-Ib showed a slight leaf kinking (not shown) and fruit deformation (Fig. 2). Hybridization assay with a ^{32}P-labeled CV-Ib cDNA probe revealed the presence of CV-Ib in the infected avocado plant (Fig. 3, lane 2) and in GTD 225-S–infected Etrog citron (Fig. 3, lane 4). The CV-Ib from the symptomless WI seedlings and from the infected plant were further graft-transmitted to eight WI seedlings and to a range of commercial avocado cultivars including Nabal, Hass, Wourz, Reed, Ettinger, and Horshim. None of the graft-inoculated avocado plants showed symptoms for 18 mo after infection. Analysis by sPAGE re-

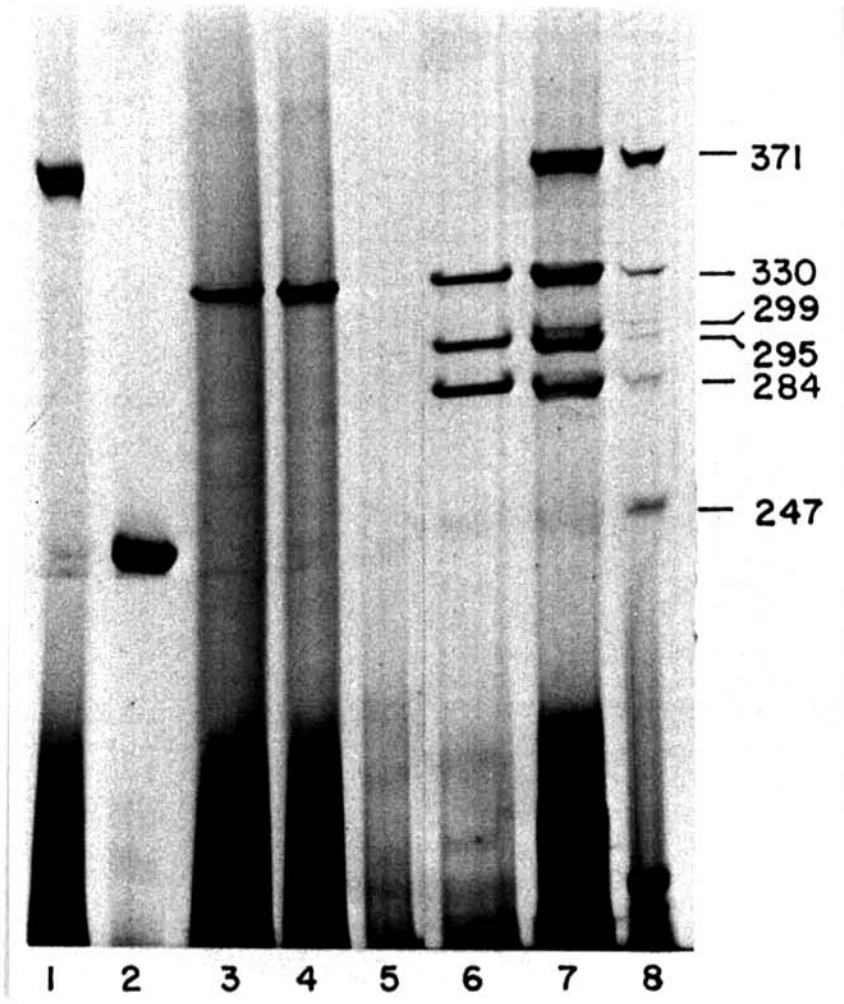

Fig. 1. Polyacrylamide (5%) gel containing 8 M urea, stained with silver after processing by sequential PAGE. Nucleic acid samples (2 M, LiCl soluble) were prepared from *Gynura aurantiaca* leaves infected with citrus exocortis viroid (lane 1), avocado leaves infected with avocado sunblotch viroid (lane 2), citrus leaves inoculated with extract from CV-Ib–infected avocado leaves (lane 3), avocado leaves from a plant graft inoculated with citrus buds infected with graft-transmissible dwarfing (GTD) agent 225-M (lane 4), healthy Etrog citron leaves (lane 5), Etrog citron infected by GTD 225-M and 225-S (lanes 6 and 7, respectively), and a mixture of citron leaves infected by GTD 225-S and avocado leaves infected by avocado sunblotch viroid (lane 8). Estimated molecular lengths in bases are indicated at right.

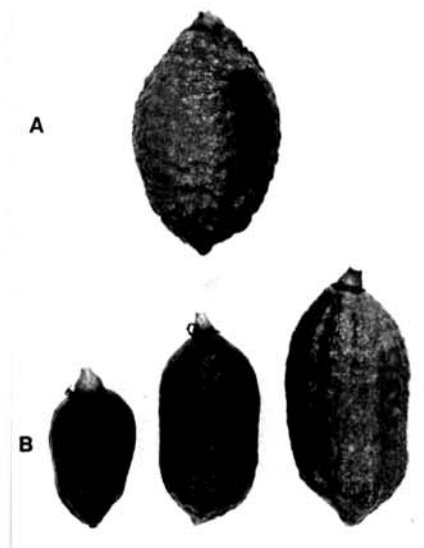

Fig. 2. (A) Normal, healthy citron fruit; **(B)** Etrog fruit malformations expressed as a result of the presence of the CV-Ib citrus viroid.

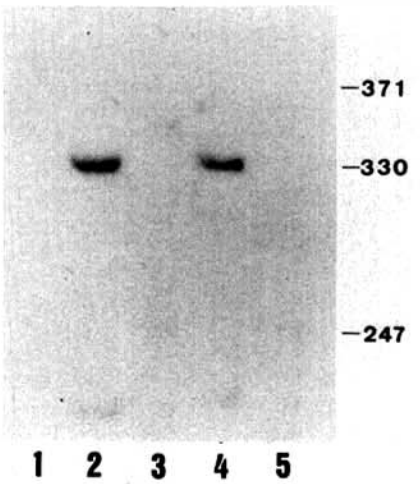

Fig. 3. Autoradiograph after electrotransfer onto nylon membrane and hybridization with a ^{32}P-labeled CV-Ib cDNA probe with extracts from healthy avocado leaves (lane 1); CV-Ib infected avocado (lane 2); avocado sunblotch viroid- and citrus exocortis viroid-infected avocado and *Gynura aurantiaca* leaves, respectively (lane 3); Etrog citron infected by graft-transmissible dwarfing agent 225-S (lane 4); and healthy Etrog citron leaves (lane 5). RNA patterns were as in Fig. 1.

vealed the CV-Ib viroid only in the WI plants and at a low concentration in the Hass cultivar.

DISCUSSION

A 332-base CV from a single citrus clone in Australia was recently described by Gillings et al (6). This CV, classified as CV-Ib (5), induced mild symptoms in Etrog but did not replicate in *Chrysanthemum* and *Gynura*. A similar-size CV has been previously considered as the agent of citrus variable viroid disease (CVaV) (13). Recently, however, Semancik et al (16) reported that the CVaV symptoms appear as a result of coinfection by four CVs (Ib, IIa, IIb, and IIIb). The recovery of single viroid isolates, including CV-Ia, after shoot-tip grafting from citron infected with complex sources of CVs has recently been reported by Juarez et al (9). Our study is believed to be the first report of transmission of the CV-Ib to a host other than citrus. Nucleic acid extracts and tissue grafts of WI avocado infected by CV-Ib were infective on Etrog citron. Only CV-Ib was revealed by sPAGE analysis of Etrog plants, which were either mechanically inoculated or tissue-grafted with CV-Ib–infected avocado. This suggests that the avocado plants did not become erratically (1) or subliminally infected by the other CVs present in *C. macrophylla* inoculated with GTD 225-S. The concentrations of the CV-Ib in avocado leaves were usually five to 10 times higher than in citrons (not shown), making avocado a better source for future characterization of this viroid. Bitters, Duran-Vila, and Semancik (3) were the first to record the association of Etrog fruit symptoms with CEV infection. The present study indicates that Etrog fruit deformation may also be expressed as a result of infection by CV-Ib. The long-term effects of CV-Ib on avocado and its possible natural distribution in mixed citrus and avocado plantings are still unknown.

Heterologous grafting between graft-incompatible hosts have been previously utilized for viroid transmission to indicator plants (17). The present study extends the host range of CV-Ib to a new economically important crop plant and indicates the advantage of using heterologous graft transmission assays for defining the potential host range of known viroids to different economically important fruit trees.

ACKNOWLEDGMENTS

This research was supported by the United States–Israel Binational Agricultural Research and Development Fund (BARD). We thank J. Ben-Shalom for technical assistance.

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