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Genetics

Potato spindle tuber and citrus exocortis viroids undergo no major sequence changes during replication in two different hosts

(RNA fingerprinting/iodine-125/viroid diseases)

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ABSTRACT **Potato spindle tuber viroid and citrus exocortis viroid, each purified from tomato (*Lycopersicon esculentum*) and from *Gynura aurantiaca,* were iodinated *in vitro* with ^{125}I, digested with ribonuclease T1, and subjected to two-dimensional RNA fingerprinting analysis. With the exception of minor variations, each viroid retained its distinctive fingerprint pattern irrespective of the host species from which it was isolated. We conclude that the nucleotide sequences of these viroids are principally determined by the infecting viroid and not by the host.**

Viroids are subviral plant pathogens composed exclusively of low molecular weight RNA (1) of low complexity (2–4) that cause a number of severe disease symptoms (usually including stunted growth) in a variety of plants (5). Viroids have single-stranded genomes with highly developed secondary structures (6). Various proportions of the infectious RNA (20%–99%) may be isolated as covalently closed circles (7–9). Viroids have genomes about one-tenth the size of the smallest known bacteriophage genomes (10) (about 350 nucleotides; ref. 9). Viroids appear to exist without the protection of a capsid (11, 12) and lack detectable messenger RNA activity (13, 14). Demonstration that viroid replication occurs in host nuclei (15) and is inhibited by actinomycin D (16) makes plausible the idea that viroid replication could be the result of transcription of a previously existing region within the host genome (1, 17, 18). This view gains further support from hybridization studies showing that regions of complementarity exist between viroid RNA and host DNA prior to viroid infection (19). If viroid replication is mediated by host transcription, then it is formally possible that the viroid genome could undergo major alterations upon replication in different hosts.

The observation that potato spindle tuber viroid (PSTV) and citrus exocortis viroid (CEV) produce similar disease symptoms across a common host range led to suggestions that they might be independent isolates of the same pathogen (20–22). Analysis of two-dimensional RNA fingerprints of PSTV isolated from tomato plants and CEV isolated from Gynura plants has shown that these two RNA species do not resemble each other (4, 23). However, this does not rule out the possibility that viroids are modified during replication in different hosts. For example, it is formally possible that, when infecting tomato plants, CEV isolated from Gynura plants could trigger the synthesis of RNA with a fingerprint like that of PSTV from tomato plants. It has been shown previously that RNA species can be uniquely and reproducibly characterized by two-dimensional RNA fingerprinting after *in vitro* labeling with ^{125}I (4, 24). This approach has been used in the studies reported here to determine whether the genomes of PSTV and CEV undergo major sequence alterations after replication in tomato and Gynura plants.

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MATERIALS AND METHODS

Viroid RNA. *(i) Multiple plant extraction.* PSTV from tomato plants (*Lycopersicon esculentum* Mill. cv. Rutgers), PSTV from *Gynura aurantiaca* DC, and RNA with similar size from unfected Gynura plants were prepared by standard methods (25). CEV isolated from Gynura plants was the kind of gift of J. S. Semancik, University of California, Riverside, CA. *(ii) Single plant extraction.* Low molecular weight RNA was prepared from 6 g (fresh weight) of individual uninfected, PSTV-infected, and CEV-infected tomato plants by the extraction procedure of Morris and Smith (26). All RNA samples were prepared for gel electrophoresis by the addition of one-quarter volume of 50% sucrose containing 0.01 M Tris·HCl (pH 7.4) and bromphenol blue.

Polyacrylamide Gel Electrophoresis. All RNA species were subjected to a final fractionation on eight-slot slab gels 20 × 20 × 0.3 cm containing 5% acrylamide (Canalco) and 0.25% bisacrylamide (Canalco). Gels were made up and samples were electrophoresed in 40 mM Tris·HCl/20 mM sodium acetate/1 mM EDTA at pH 7.2 (26). After 5 min of electrophoresis, samples were electrophoresed at 4° at 160 V (75 mA) for 5 hr, by which time the bromphenol blue marker had migrated 10 cm. The gels were then stained for 15 min in 20 μg of ethidium bromide per ml/1 mM EDTA, destained in 1 mM EDTA for an additional 15 min, and photographed. Gel bands of interest were excised and stored at −70° until extraction; the gels were rephotographed to record accuracy of excision.

Extraction of RNA from Gel Bands. RNA was extracted by homogenization of the gel bands in phenol and 50 mM Tris·HCl/0.1 M sodium chloride/1 mM EDTA at pH 7.0, freed of acrylamide by hydroxylapatite chromatography (27), and concentrated by CF11 cellulose chromatography (28) and ethanol precipitation. When the total amount of RNA present in the gel band was in the range of 0.05–1.0 μg, this procedure was able to produce intact RNA suitable for subsequent *in vitro* iodination to specific activities in the range of 40–160 × 10^6 dpm/μg.

***In Vitro* Iodination of RNA.** Commerford's method for *in vitro* labeling of nucleic acids with ^{125}I (29) as described by Prensky (30) was carried out in a glovebox with built-in activated charcoal filter system. Reactions were carried out at 64° for 3 min. These conditions of iodination yielded intact RNA with more than 80% of the iodinated material appearing as a single band in 5% polyacrylamide gels.

Abbreviations: PSTV, potato spindle tuber viroid; CEV, citrus exocortis viroid.

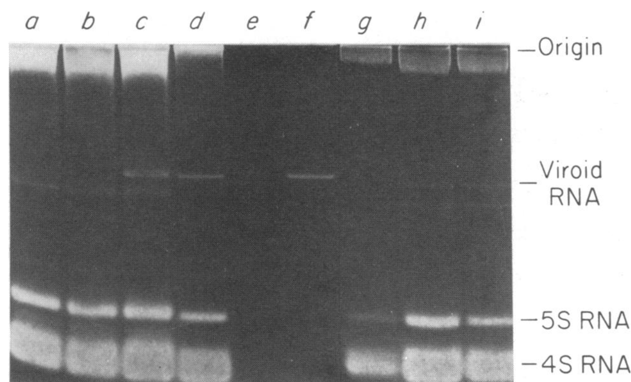

FIG. 1. Polyacrylamide gel electrophoretic fractionation of RNA from individual tomato plants. The photograph represents a composition of regions from two gels stained with ethidium bromide (slots a–f from one gel and slots g–i from a second gel). Eight-slot 5% acrylamide slab gels (20 × 20 × 0.3 cm) and low molecular weight RNA samples were prepared as outlined in *Materials and Methods*. Slots e and f contain known amounts of highly purified viroid RNA isolated in multiple plant preparations: (e) CEV, 0.2 μg, isolated from CEV-infected Gynura; (f) PSTV, 1.0 μg, isolated from PSTV-infected tomato plants. The remaining slots each contain the total low molecular weight RNA from one tomato plant of the following descriptions: (a, b, and g) uninfected; (c and d) PSTV-infected; (h and i) CEV-infected. Viroid-specific RNA and the equivalent regions from the RNA of uninfected plants were excised from the gel and the RNA was extracted and iodinated (see *Materials and Methods* and Table 1).

Digestion and Fingerprinting of RNA. Iodinated RNA samples were digested with ribonuclease T1 (Sankyo Co., Ltd., Japan) in the presence of 10 μg of *Escherichia coli* tRNA and were fingerprinted by standard techniques (24, 31). Radioactive oligonucleotides were detected by autoradiography on Dupont Cronex 2 x-ray film.

RESULTS

PSTV was originally discovered as a disease of potatoes (32) and CEV as a disease of lemon trees (33). Attempts to find good indicator plants capable of reacting more rapidly to these pathogens with unambiguous symptoms led to the practice of propagating PSTV in tomato (34) and CEV in Gynura plants (35). The PSTV and CEV inocula used in the studies reported here were isolated from these two indicator plants. Each of these two viroids has been grown in the indicator host of the other to find out whether any major alterations in sequence would occur. In particular, PSTV isolated originally from tomato plants was grown in Gynura plants and CEV isolated originally from Gynura was grown in tomato plants. The viroid-specific RNA was isolated in each case, labeled *in vitro* with ^{125}I, and, after digestion by ribonuclease T1, subjected to two-dimensional RNA fingerprinting analysis.

Fig. 1 shows a representative example of 5% polyacrylamide slab gel fractionation of low molecular weight RNA from tomato plants. Each of slots a–d and g–i contains the total low molecular weight RNA from a single plant. A comparison of slots a, b, and g (RNA from uninfected plants) with c, d, h, and i (RNA from viroid-infected plants) permits the identification of a band that stains with ethidium bromide and is present only in RNA preparations from viroid-infected plants. Furthermore, this new material has a mobility similar to that of highly purified CEV from Gynura (slot e) and PSTV from tomato (slot f) and therefore coincides in mobility with the position of maximal concentration of infectivity, as shown previously (36, 37). Gel regions containing the viroid-specific material and equivalent regions from slots containing RNA from uninfected plants were excised and the RNA was extracted and iodinated. Amounts of RNA extracted from the gel bands and specific activities of ^{125}I labeling are summarized in Table 1.

One-quarter of each iodinated sample was digested with ribonclease T1 and subjected to two-dimensional fingerprinting analysis as shown in Figs. 2 and 3. Because the amount of RNA present in samples varied over a wide range (0.01–0.1 μg: see Table 1), the time of exposure of each fingerprint to x-ray film was varied to obtain uniform intensities of the spots constituting the major patterns.

Fingerprinting analysis of viroid-specific RNA isolated from PSTV-infected Gynura plants (Fig. 2*E*) revealed only minor changes in the pattern characteristic of the PSTV from tomato used as inoculum (Fig. 2*B*). Likewise, fingerprinting analysis of viroid-specific RNA isolated from CEV-infected tomato plants (Fig. 2*C*) revealed only minor changes in the pattern characteristic of the CEV from Gynura used as inoculum (Fig. 2*F*). Thus, no major alteration of either of these two viroids occurs upon replication in a second plant host.

The arrows positioned within the fingerprint patterns of Fig. 2 point to detectable minor differences. In PSTV, the pair of arrows points to two spots whose relative intensities are greatly reduced in PSTV isolated from Gynura as compared to PSTV isolated from tomato. In CEV, a pair of arrows indicates a vertical pair of spots, the upper of which has a greater relative

Table 1. Extraction of viroid RNA from gels and its subsequent *in vitro* iodination

Plant extraction	Fig. 1 (slot)	Fingerprint (Fig.)	Amount on gel, μg	Amount iodinated, μg	^{125}I specific activity, dpm/μg
Single plant					
Uninfected tomato	g	2*A*	Not visible	≤0.05	≥160 × 10^6
PSTV-tomato	c	3*A*	1.0*	0.40	90 × 10^6
PSTV-tomato	d	3*B*	1.0*	0.30	100 × 10^6
CEV-tomato	h	2*C*	0.7*	0.2	60 × 10^6
CEV-tomato	i	3*C*	0.7*	0.15	67 × 10^6
CEV-tomato	Not shown	3*D*	0.7*	0.10	40 × 10^6
Multiple plant					
CEV-Gynura	e	2*F*	0.2	0.07	114 × 10^6
PSTV-tomato	f	2*B*	1.0	0.2	150 × 10^6
Uninfected Gynura	Not shown	2*D*	Not visible	≤0.05	≥40 × 10^6
PSTV-Gynura	Not shown	2*E*	0.05*	0.05	40 × 10^6

* These amounts are approximate; they were estimated by visual comparison of the intensity of ethidium bromide staining in the gel band as compared to the intensities of gel bands containing known amounts of RNA.

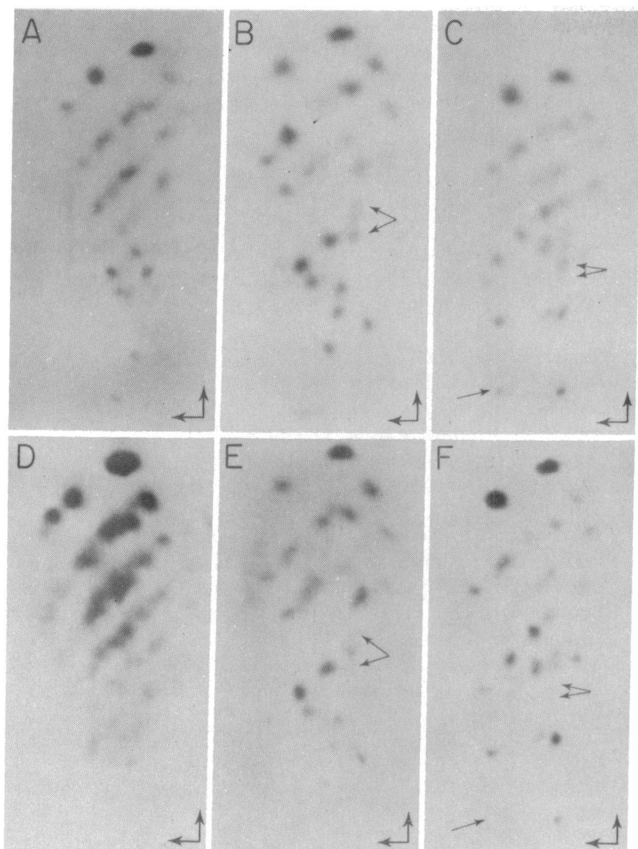

FIG. 2. Ribonuclease T1 fingerprints of ^{125}I-labeled viroid RNAs from tomato and Gynura plants. Amounts of RNA ranging from less than 0.05 to 0.4 μg were subjected to iodination, digestion by ribonuclease T1, and two-dimensional fingerprinting analysis as described in *Materials and Methods* and as summarized in Table 1. (*A*) RNA from a single uninfected tomato plant migrating in gels at the position of viroid RNA; (*B*) PSTV from bulk extraction of tomato plants infected by PSTV; (*C*) CEV from a single CEV-infected tomato plant; (*D*) viroid-sized RNA from a multiple-plant extract of uninfected Gynura plants; (*E*) PSTV from bulk extraction of PSTV-infected Gynura plants; and (*F*) CEV from bulk extraction of CEV-infected Gynura plants. For comparative purposes, attention should be focused on the lower portions of fingerprints, where the longer oligonucleotides that characterize each RNA species are located. The origin of each autoradiograph is at the lower right. The first dimension (right to left) consists of high-voltage electrophoresis on cellulose acetate at pH 3.5, while the second consists of ascending RNA homochromatography on thin layers of DEAE-cellulose (28, 33). Exposure time for the autoradiographs was adjusted to give equal spot intensities in these photographs. Thus, the autoradiograph in *A*, resulting from the low amount of RNA recovered from viroid-sized RNA of uninfected plants [no band visible in the gel (Fig. 1g); see also Table 1], was exposed 12 times longer than fingerprints of RNA species isolated in higher amounts (e.g., *B*). Possible minor sequence variations are indicated by arrows. The horizontal and vertical arrows at the lower right-hand corner of each fingerprint show the directions of first and second dimension separation, respectively.

intensity in CEV isolated from tomato plants, and the lower of which may be altogether absent from this RNA species. Furthermore, a spot indicated by an arrow in the lower left corner of the fingerprint is absent from the CEV isolated from Gynura but present in CEV isolated from tomato.

It is unlikely that these minor differences are the result of contamination by variable amounts of host-specific RNA. Fig. 2 *A* and *D* shows fingerprints of viroid-sized RNA prepared from uninfected tomato and Gynura plants, respectively. These patterns cannot be seen in the fingerprints of viroid RNA prepared from these plants (Fig. 2 *B* and *C*, and *E* and *F*, respectively) unless the time of exposure to x-ray film is increased about 10-fold (data not shown). Furthermore, positions of increased intensity within the viroid fingerprints do not coincide with major spots of the host-specific RNA fingerprints.

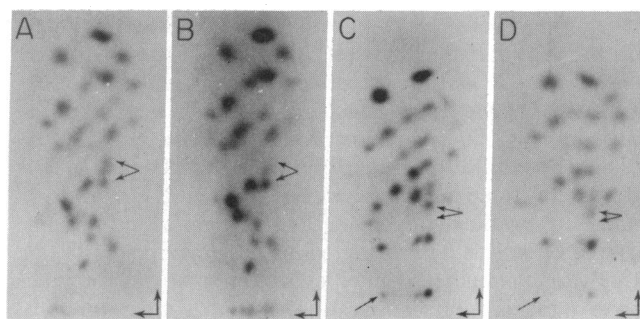

FIG. 3. Additional ribonuclease T1 fingerprints of ^{125}I-labeled viroids from individual tomato plants. (*A* and *B*) PSTV from single plant extraction of PSTV-infected tomato plants; (*C* and *D*) CEV from single plant extractions of CEV-infected tomato plants. RNA fingerprinting and autoradiography were as described in *Materials and Methods* and the legend to Fig. 2.

One way to determine whether the minor differences observed are a result of reproducible host-specific alteration of the viroid genome is to analyze viroid RNA extracted separately from a number of viroid-infected plants. Fig. 3 illustrates the results of such a study. Two tomato plants were inoculated with the PSTV whose fingerprint is shown in Fig. 2*B*. The RNA was extracted separately from each plant and fractionated as shown in Fig., 1 slots c and d, and the viroid-specific RNA was iodinated and fingerprinted (Fig. 3 *A* and *B*). While the fingerprint in Fig. 3*A* is just like that in Fig. 2*B*, the spots indicated by the pair of arrows in Fig. 3*B* have different relative intensities from those in Figs. 2*B* and 3*A*. Thus, it is possible to observe variation in the intensities of these spots without growing PSTV in a second host. Similarly, in two additional examples of CEV isolated from tomato plants (Fig. 3 *C* and *D*), the spot indicated in the lower left corner can either be present (Fig. 3*C*) or absent (Fig. 3*D*) from the fingerprint of CEV grown in this host. On the other hand, the pair of spots indicated in these fingerprints retains the relative intensities observed in Fig. 2*C*, making these oligonucleotides good candidates for further study in future attempts to find host-specific modification of viroids.

DISCUSSION

Comparative RNA fingerprinting studies have demonstrated that PSTV isolated from tomato plants (*Lycopersicon esculentum*) remains largely unchanged by replication in a second host, *Gynura aurantiaca*. Similarly, CEV isolated from Gynura plants remains largely unchanged by replication in tomato plants.

As has been shown by previous work, RNA fingerprinting is an ideal way to detect limited differences among RNA species. In particular, Robertson and Jeppesen (38) studied the extent of sequence variation in the RNAs of three closely related RNA bacteriophages. They were able to show by sequence analysis that the f2, MS2, and R17 RNA species each were 96%–97% homologous with the others, revealing an extent of variation of 3%–4%. The ribonuclease T1 fingerprints of these three RNA species reflected this limited extent of variation by the presence of large and unmistakable differences in their patterns involving the positions of many spots within the lower regions of their fingerprints. Since such major changes in pattern are not observed in the comparative fingerprinting studies

of the products of viroid replication in second hosts reported here, then any variations in nucleotide sequence must be limited to fewer than 3% of the nucleotides. Thus, it is estimated that such minor changes could affect, at most, 10–11 residues in the approximately 350-nucleotide genomes of these viroids.

The detectability of minor differences depends upon the nature of the sequence alteration. Nearly identical fingerprints could differ by the disappearance of a spot, reflecting a change in sequence, or could show altered intensity of a spot, reflecting either variation in the extent of iodination or the disappearance of one of two or more comigrating oligonucleotides. Identical fingerprint patterns could contain three types of hidden differences: (*i*) spots in the upper portion of fingerprints containing several comigrating oligonucleotides could lose or gain an oligonucleotide as a result of sequence alteration within a short ribonuclease T1-resistant oligonucleotide without producing a noticeable intensity difference; (*ii*) oligonucleotides lacking cytidylate residues [and therefore not labeled *in vitro* by ^{125}I (24)] would not be detected; and (*iii*) specific chemical modification (for example, methylation) of a base would pass unnoticed if the modified oligonucleotide had the same mobility as the unmodified one. In order to evaluate possible sources for the minor variations observed in the viroid fingerprints presented here, it will be necessary to apply the methods of RNA sequence analysis to the oligonucleotides concerned.

An extension of the approach described here, combining viroid isolation from individual plants with radioiodination and RNA fingerprinting, should help to define the generality of our findings. For example, Diener and Smith have shown that PSTV from tomato can infect chrysanthemum plants and produce a disease indistinguishable from that caused by chrysanthemum stunt viroid (preliminary study cited in ref. 5), but yielding an infectious RNA with a mobility in gels like that of PSTV from tomato rather than like that of chrysanthemum stunt viroid (unpublished experiments). A testable prediction based on these preliminary observations and the results reported here is that the fingerprint of PSTV would not be significantly changed by replication in chrysanthemum plants. Also, further studies along these lines should allow us to determine the nature of any limited and specific differences that may arise as viroids replicate in different hosts.

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