Proc. Natl. Acad. Sci. USA
Vol. 78, No. 11, pp. 6932–6935, November 1981
Cell Biology

Nuclear DNA from uninfected or potato spindle tuber viroid-infected tomato plants contains no detectable sequences complementary to cloned double-stranded viroid cDNA

(restriction DNA fragments/recombinant DNA)

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Contributed by Theodor O. Diener, July 22, 1981

ABSTRACT **High molecular weight tomato nuclear DNA was isolated from uninfected and potato spindle tuber viroid (PSTV)-infected tomato leaves. Restriction digests were fractionated on agarose gels, denatured and transferred to diazobenzyloxymethyl-paper, and hybridized to ^{32}P-labeled cloned double-stranded PSTV cDNA. No hybridization to DNA from either uninfected or infected tissue could be detected under conditions that permitted detection of cloned double-stranded PSTV cDNA at a concentration equivalent to one-fifth copy of PSTV-related DNA per haploid tomato genome. Hybridization of tomato DNA to ^{32}P-labeled cloned soybean 18S and 28S ribosomal DNA sequences showed that the restricted nuclear DNA was suitable for hybridization to probes containing homologous sequences. Our results indicate that neither PSTV nor its complementary strand is transcribed from nuclear DNA but do not rule out the possibility of sequence homology between host DNA and a small portion of PSTV or its complement.**

In vitro ^{125}I-labeled citrus exocortis viroid (CEV) and potato spindle tuber viroid (PSTV) have been used previously as molecular probes to determine whether sequences complementary to CEV or PSTV occur either in the DNA of healthy susceptible plant species or in the DNA of infected cells (1–4). In the earliest hybridization studies, Semancik and Geelen (1) reported that ^{125}I-labeled CEV hybridized specifically with DNA-rich preparations from CEV-infected tomato or *Gynura aurantiaca*, but not with DNA-rich preparations from uninfected plants. In hybridization studies between ^{125}I-labeled PSTV and DNA from PSTV-infected or uninfected tomato plants, Hadidi *et al.* (2) found that infrequent, possibly single-copy, DNA sequences complementary to at least 60% of PSTV exist in both uninfected and infected cells and that no new DNA sequences related to PSTV appear as a consequence of infection. The authors suggested that PSTV may have originated from genes in normal solanaceous plants. In more recent studies, Grill and Semancik (5, 6) found that the CEV-related nucleic acid sequences described in the earlier study (1) were RNA, not DNA. Evidence has also been presented showing that DNA from uninfected or viroid-infected tomato plants does not contain regions complementary to ^{125}I-labeled PSTV (3, 4).

The cloning of double-stranded (ds) cDNA sequences of PSTV in plasmid pBR322 in *Escherichia coli* has been reported (7). These sequences were shown to hybridize to PSTV and to a RNA complementary to PSTV that is present in PSTV-infected tomato plants. The clone used in that study did not contain a complete cDNA copy of PSTV, but a full-length ds cDNA clone of PSTV has been constructed (unpublished data). A plasmid, pVH-3, was constructed by the insertion into pBR322 of a 365-base-pair (bp) fragment containing a full-length 359-bp ds PSTV cDNA attached to *Hin*dIII oligonucleotide linkers. Because this cloned ds cDNA contains the PSTV sequence as well as its complement, nick-translated pVH-3 DNA can function as a specific hybridization probe for viroid-related DNA sequences in host tissue.

To determine whether sequences related to PSTV occur in tomato nuclear DNA, ^{32}P-labeled pVH-3 DNA was hybridized to restriction fragments of nuclear DNA covalently bound to diazobenzyloxymethyl (DBM)-paper. We report that nuclear DNA from uninfected or PSTV-infected tomato plants does not contain detectable sequences related to PSTV.

MATERIALS AND METHODS

Preparation of High Molecular Weight Nuclear DNA from Tomato Plants. Ten-gram batches of chilled, freshly harvested leaves from PSTV-infected or uninfected tomato plants (*Lycopersicon esculentum* Mill, cv. Rutgers) were ground for 2 min with a mortar and pestle at 0°C in 50 ml of maceration buffer (500 mM sucrose/10 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/5 mM 2-mercaptoethanol/0.02% diethylpyrocarbonate). The homogenate was filtered through Miracloth (Calbiochem) and the nuclei were pelleted at 1000 × *g* for 10 min. The pellet, which contained nuclei, chloroplasts, and other cell components, was suspended at 0°C in maceration buffer containing 3% Triton X-100 for 10–15 min to solubilize contaminating chloroplasts. The nuclei were pelleted and resuspended in maceration buffer. Solubilization of contaminating chloroplasts and pelleting of nuclei was repeated several times until chloroplasts were completely removed. One to 2 ml of nuclear suspension was layered over a 9-ml cushion of 1.8 M sucrose containing maceration buffer and 3% Triton X-100, and the nuclei were pelleted at 5°C in a SW 41 rotor at 35,000 rpm for 30 min. The pellet containing nuclei and starch granules was resuspended in 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0 (NaCl/Cit). Contaminating starch was removed by resuspending the nuclei in 5 ml of NaCl/Cit and leaving the tube at 0°C for 10–15 min; under these conditions, nuclei sediment but starch granules stay in suspension.

After most of the starch granules had been removed, the sample was centrifuged at 1000 × *g* for 10 min, and pelleted nuclei were suspended in 40 ml of 50 mM Tris·HCl, pH 8.0/50 mM NaCl/50 mM EDTA containing ethidium bromide at 400 μg/ml. DNA was extracted from nuclei by a modification of the

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Abbreviations: PSTV, potato spindle tuber viroid; CEV, citrus exocortis viroid; DBM-paper, diazobenzyloxymethyl-paper; ds, double-stranded; bp, base pair(s); NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

procedure of Bendich *et al.* (8). Nuclei were lysed by the addition of 10 ml of 10% Sarkosyl. After occasional gentle mixing at 0°C for 10 min, 48 g of solid CsCl was added and dissolved at 0°C. The lysate was centrifuged at 28,000 × *g* for 30 min at 4°C to float the protein pellicle and to pellet starch. The supernatant was adjusted to a refractive index of 1.393 and centrifuged at 40,000 rpm in a Ti 50 rotor for 40 hr at 15°C. The fluorescent DNA band was collected and rebanded in a CsCl/ethidium bromide gradient at 40,000 rpm for 66 hr. The fluorescent band was again collected, ethidium bromide was removed by gentle extraction with CsCl-saturated 2-propanol, and the DNA was dialyzed extensively against 10 mM Tris·HCl/1 mM EDTA, pH 8.0.

Restriction Endonuclease Digestion and Gel Electrophoresis. DNA was digested with *Bam*HI, *Eco*RI, or *Hin*dIII according to conditions recommended by the supplier (New England BioLabs or Bethesda Research Laboratories). The extent of digestion was monitored by the appearance, upon gel electrophoresis, of limit bands containing highly repetitive DNA. Digested DNA was extracted with phenol, precipitated with ethanol, resuspended in 10 mM Tris·HCl/10 mM EDTA/8% (vol/vol) glycerol/0.02% bromophenol blue, and electrophoresed through a 1% agarose gel at 2 V/cm for 16–18 hr in Tris acetate buffer (9).

Isolation of Probe DNA. Plasmid pVH-3, which contains full-length 359-bp ds PSTV cDNA and 6 bp of oligonucleotide linkers, was purified (10) and used under P1 containment conditions in compliance with the National Institutes of Health guidelines for recombinant DNA research. For preparation of the hybridization probe, plasmid pVH-3 was labeled with [^{32}P]dCTP (Amersham; ≥400 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) by nick translation (11), digested with *Hin*dIII, and fractionated by polyacrylamide gel electrophoresis. The 365-bp band was identified against a background smear of radioactivity and eluted (12). The probe was thus enriched for the PSTV-specific insert but also contained pBR322 vector sequences which do not hybridize to tomato DNA or ds PSTV cDNA. The specific activity of the probe was 2 × 10^8 cpm/μg. A bacteriophage λ clone containing 18S and 28S ribosomal DNA sequences from soybean, *Glycine max* (Ch4A-RB115), was obtained from P. Jackson (13). Phage DNA was isolated according to standard methods, and nick-translated total phage DNA was used as hybridization probe. The specific activity of the probe was 6 × 10^7 cpm/μg.

DNA Transfer and Hybridization. DNA transfer and hybridization in the presence of 10% dextran sulfate were carried out on DBM-paper as described (14). Cleaved DNA fragments were transferred from agarose gels to DBM-paper and were then hybridized for 65 hr at 42°C to denatured probe DNA. After hybridization, DBM-papers were washed with double-strength NaCl/Cit/0.1% $NaDodSO_4$ at room temperature for 30 min and with 1/10th-strength NaCl/Cit/0.1% $NaDodSO_4$ at 50°C for 45 min. The DBM-papers were then autoradiographed with Kodak X-Omat S film and a Du Pont Cronex intensifying screen at −70°C.

RESULTS

Digestion of purified nuclear DNA from uninfected or PSTV-infected tomato plants showed that each restriction enzyme produced a different pattern of DNA fragments and that DNA from uninfected and PSTV-infected tomato plants gave identical patterns (Fig. 1*A*). To demonstrate that our nuclear DNA digests were suitable for hybridization and to identify ribosomal genes, DNA fragments were hybridized with ^{32}P-labeled cloned soybean 18S and 28S ribosomal DNA (Ch4A-RB115) after transfer to DBM-paper. Fig. 1*B* shows that the ribosomal probe hybridized well to DNA fragments from uninfected and infected

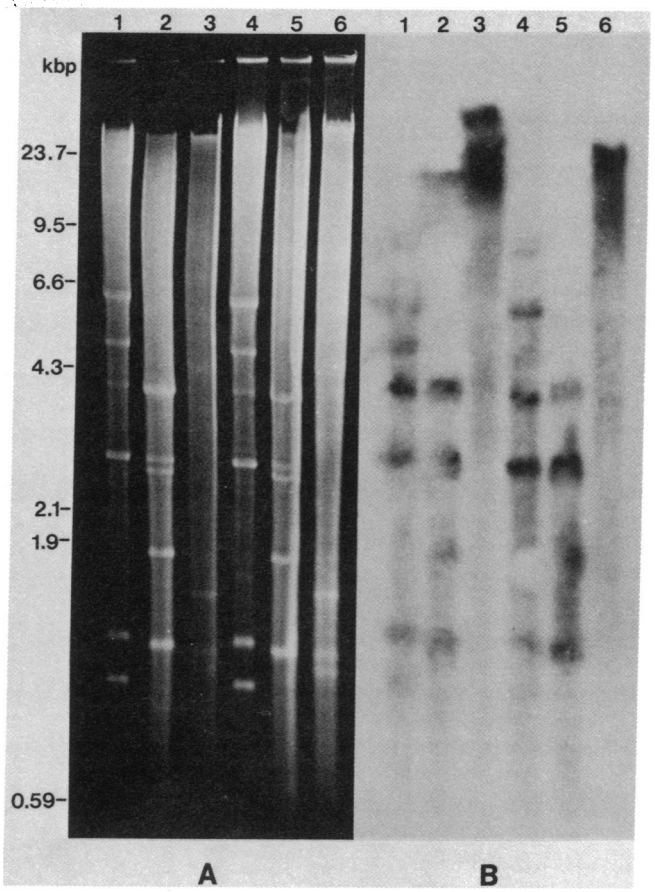

FIG. 1. Restriction fragments of nuclear DNA from uninfected and PSTV-infected tomato plants detected by ethidium bromide stain and by hybridization with nick-translated ^{32}P-labeled cloned 18S and 28S soybean ribosomal DNA. (*A*) Digested DNA samples (25 μg of DNA per sample) electrophoresed in a horizontal agarose gel at 2 V/cm for 16 hr, stained with ethidium bromide, and photographed. (*B*) DNA fragments transferred to DBM-paper and hybridized at 42°C for 65 hr with 10^7 cpm of ^{32}P-labeled cloned soybean ribosomal DNA in 15 ml of hybridization mixture (14). The DBM-paper was then washed and autoradiographed for 1 day. Lanes: 1–3, DNA from uninfected tomato plants; 4–6, DNA from PSTV-infected tomato plants. DNA was digested with *Bam*HI (lanes 1 and 4), *Eco*RI (lanes 2 and 5), and *Hin*dIII (lanes 3 and 6). Fragment sizes were determined from the mobilities of unlabeled fragments of a *Hin*dIII digest of λ phage DNA run in parallel.

tomato plants and that the hybridization patterns obtained varied according to the enzyme used. These results are consistent with published results (4, 15).

To determine whether PSTV-related sequences exist in host nuclear DNA or appear as a consequence of infection, ^{32}P-labeled cloned ds PSTV cDNA was hybridized to restriction fragments of nuclear DNA from uninfected and PSTV-infected tomato plants on DBM-paper. Fig. 2 (lanes 1–3) shows that no hybridization was detected between the labeled ds PSTV cDNA probe and nuclear DNA from uninfected plants digested with *Bam*HI, *Eco*RI, or *Hin*dIII. Identical results were obtained with DNA from infected plants (not shown).

To determine the sensitivity of this hybridization test, unlabeled plasmid pVH-3 was digested with *Hin*dIII to release the ds PSTV cDNA insert from the pBR322 plasmid vector. Different amounts of this DNA digest were then electrophoresed, transferred to the same DBM-paper, and then hybridized with the ^{32}P-labeled ds PSTV cDNA probe. In this experiment, 1 pg of unlabeled ds PSTV cDNA insert was detectable (Fig. 2, lanes 4–7).

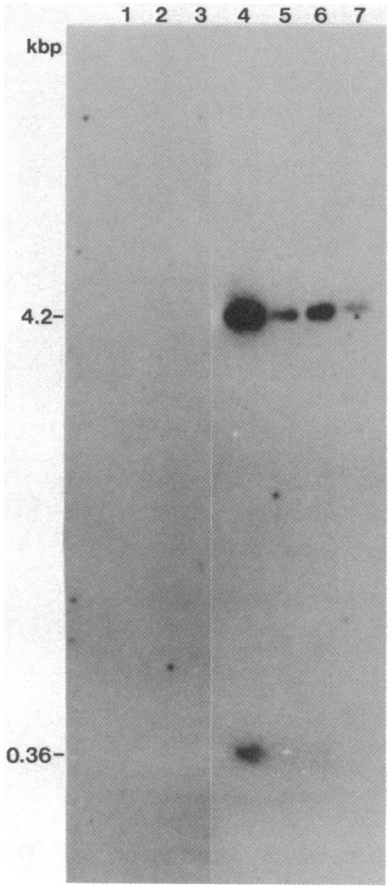

FIG. 2. Hybridization of ^{32}P-labeled cloned ds PSTV cDNA to restriction fragments of nuclear DNA from uninfected tomato plants and *Hin*dIII digests of cloned ds PSTV cDNA. Twenty-five micrograms of DNA restriction fragments (digested with *Bam*HI, *Eco*RI, or *Hin*dIII) and 0.1–10 pg of *Hin*dIII digests of pVH-3 DNA were fractionated by electrophoresis in a horizontal agarose gel at 2 V/cm for 16 hr. The DNA fragments were transferred to DBM-paper and hybridized with 3.5×10^8 cpm of ds PSTV cDNA insert at 42°C for 65 hr in 15 ml of hybridization mixture. The DBM-paper was then washed and autoradiographed for 8 days. Lanes 1–3, nuclear DNA from uninfected tomato plants; DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3). Lanes 4–7, *Hin*dIII digests of unlabeled pVH-3 plasmid containing ds PSTV cDNA insert and pBR322 plasmid; 10 pg of insert (lane 4), 1 pg of insert (lanes 5 and 6), and 0.10 pg of insert (lane 7). The positions of pBR322 DNA (4.2 kbp) and PSTV cDNA insert (0.36 kbp) are indicated.

DISCUSSION

Our results show that cloned ds PSTV cDNA does not detectably hybridize with restriction fragments of nuclear DNA from uninfected or PSTV-infected tomato plants. Under identical hybridization conditions, as little as 1 pg of unlabeled ds PSTV cDNA could be detected in reconstruction experiments. Tomato nuclear DNA has a calculated molecular weight of 1.175×10^{12} per haploid cell (2), and full-length ds PSTV cDNA has a molecular weight of 2.37×10^5. Thus, the fraction of the tomato genome corresponding to a single copy of PSTV-specific sequences is 2.02×10^{-7}; i.e., 25 μg of nuclear tomato DNA should contain 5 pg of PSTV-specific sequences. It follows that, under our experimental conditions, as little as one-fifth copy of a hypothetical DNA copy of PSTV could have been detected. Evidently, tomato nuclear DNA contains either less than one-fifth copy of PSTV-specific sequences per haploid genome or no such sequences at all.

The nucleotide sequence of PSTV (16) predicts that a complete 359-bp DNA copy of PSTV would contain one *Bam*HI site but no *Eco*RI or *Hin*dIII site. One should therefore expect that *Eco*RI or *Hin*dIII digestion would generate unique restriction fragments larger than 359 bp and containing the PSTV-specific sequence. However, we could not detect hybridization of specific DNA fragments with the ds PSTV cDNA probe, regardless of the restriction enzyme used. Transfer of the restriction fragments to DBM-paper should have permitted retention of small DNA fragments (17). Our DNA fragments were evidently suitable for hybridization as indicated by the patterns obtained after their hybridization with 18S and 28S soybean ribosomal DNA probes (4, 15).

Our results contradict those of an earlier report (2) in which the presence of infrequent, if not unique, PSTV-complementary sequences in tomato DNA was reported on the basis of solution hybridization of ^{125}I-labeled PSTV to total DNA fragments. Although thermal denaturation of the presumed viroid·DNA hybrids indicated that they were well-matched RNA·DNA duplexes, no competition experiments with unlabeled PSTV or host plant RNA were performed to rule out unambiguously hybridization of host DNA with cellular RNA contaminants in the ^{125}I-labeled PSTV preparations (2). In light of our present results, as well as of those of other recent studies (3, 4), it is evident that the hybrids obtained earlier were indeed complexes between DNA and contaminating host RNA and not between host DNA and the viroid. Contrary to statements made (3), however, the high $C_0t_{1/2}$ value (6×10^3 mol sec liter^{-1}) required for hybrid formation (2) makes it unlikely that the major contaminant could have been rRNA.

Although there are other possible explanations for our inability to detect viroid-related sequences in restricted DNA fragments (4), a complete DNA copy of PSTV clearly is not present in the nuclear genome of uninfected or PSTV-infected host plants. This conclusion is consistent with results of other studies (3, 4) as well as with evidence indicating that a complementary RNA strand is involved in viroid replication (5, 7, 18).

The possibility that chloroplast or mitochondrial DNA from uninfected or infected host plants contain PSTV-related sequences was not investigated, but it is unlikely in view of observations indicating that PSTV is synthesized in the nuclei of infected cells (19).

Our data do not rule out the possibility that short PSTV-specific regions occur in host DNA. If less than 20-30 bases long, such regions could not have been detected in our experiments (20). The nuclear location of PSTV and its apparent inability to code for viroid-specific proteins (19, 21) suggest that viroid-induced disease symptoms may be caused by direct interaction of the viroid with the host genome—that is, by interference with gene regulation in the infected cell. If so, it is possible that viroid-complementary recognition sites on the host DNA are involved.

We thank P. Jackson for the generous gift of cloned soybean ribosomal DNA and N. Grinberg and B. Smith for able technical assistance.

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