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Site-specific mutagenesis of potato spindle tuber viroid cDNA:
Alterations within premelting region 2 that abolish infectivity

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Summary

The infectivity of cloned viroid cDNAs permits investigation of structure/function relationships in these unusual pathogenic RNAs by systematic site-specific mutagenesis of the cDNAs and subsequent bioassay. We have used three different strategies to create nucleotide substitutions within premelting region 2, a region of potato spindle tuber viroid (PSTV) believed to be important in viroid replication: sodium bisulfite-catalyzed deamination of deoxycytosine residues, oligonucleotide-directed mutagenesis, and construction of chimeric viroid cDNAs from fragments of infectious PSTV and tomato apical stunt viroid cDNAs. Although their effects upon the rod-like native structure of PSTV should be minimal, C → U transitions at positions 92 or 284 appeared to be lethal. When inoculation with PSTV cDNA containing a single nucleotide substitution was mediated by the Ti plasmid of *Agrobacterium tumefaciens*, PSTV progeny with an unaltered 'wild type' sequence was obtained. Two factors, the high error frequency characteristic of RNA synthesis and the use of a systemic bioassay for PSTV replication, may explain such sequence reversion and emphasize the importance of an appropriate bioassay system for screening mutant viroid cDNAs.

Viroids, the smallest autonomously replicating pathogenic agents known, cause transmissible diseases in several economically important crop plants (reviewed in 8). All known viroids are small, unencapsidated, and covalently closed circular single-stranded RNA molecules with extensive secondary structure. A combination of comparative sequence analyses and investigations of their physical-chemical properties have provided a detailed model of viroid structure *in vitro* (reviewed in 35 and 37). Knowledge of the mechanisms of viroid replication and symptom induction has accumulated more slowly.

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Evidence that viroids do not code for proteins (reviewed in 8 and 37) suggests that their biological properties are the consequence of direct viroid/host interactions. Diener (7) has postulated that disease induction might result from viroid interference with host gene expression, and a hypothetical mechanism for such interference (disruption of host pre-mRNA processing) has been proposed (reviewed in 9 and 37). Viroids thus appear to constitute a minimal genetic and biological system ideally suited to detailed structure/function analyses.

One genetic approach to the analysis of viroid-host interaction involves comparative sequence analyses of either different viroids (22) or viroid strains differing in symptom severity (38, 44). Regions such as the central conserved region (see

Fig. 5) that are strongly conserved among several different viroids may be essential for replication (37). On the other hand, strain-specific sequence differences in potato spindle tuber viroid (PSTV) and citrus exocortis viroid (CEV) define one (38) or two (44) pathogenesis-related domains within their native structures.

The recent demonstration that certain cloned viroid cDNAs and their corresponding *in vitro* RNA transcripts are infectious (6, 20, 36, 42) permits the use of an alternative approach to the systematic study of viroid-host interaction. This approach, often termed 'reverse genetics', involves introduction of desired mutations into an infectious viroid cDNA and subsequent bioassay to detect phenotypic variation. It has been used to analyze biological or structural functions encoded by a variety of DNA genomes [e.g. cauliflower mosaic virus (13)] but is equally applicable to RNA genomes that can be copied into biologically active cDNAs. In this communication we present results from ongoing site-specific mutagenesis studies of infectious PSTV cDNAs that suggest that the relationship between viroid structure and function may be more complex than previously believed. Preliminary reports of our findings have been published (16, 18).

Materials and methods

Construction and chemical mutagenesis of recombinant plasmids

The source of double-stranded PSTV cDNA fragments used in our constructions was plasmid pVB-6, an infectious pBR322 recombinant which contains a tandem dimer of full-length PSTV cDNA inserted into the BamHI site (6). All PSTV cDNAs have termini derived from the unique BamHI site (positions 87–92), and their orientations within the plasmid vectors are such that initiation of RNA synthesis at the adjacent promoter sequence will produce RNA transcripts with the same polarity as PSTV. All constructions in plasmid pBR322 contain a 6 nucleotide (GGATCC) sequence duplication (42).

C → U transitions in the upper portion of the central conserved portion of PSTV were created by directed chemical mutagenesis of pVB-6 DNA with sodium bisulfite (41). Plasmid DNAs containing the desired mutations contain only two of the three overlapping BamHI-SmaI sites present in pVB-6. The nucleotide sequence near the altered BamHI-SmaI site of pVB-6(HSO_3 34) was determined by the method of Maxam and Gilbert (24).

Standard methods (23) were used to transfer PSTV cDNA monomers and tandem dimers with BamHI termini from pVB-6 to plasmids pUC9 (43) or pSP64 (Promega Biotec). Plasmid pAS-B3, which contains a full-length tomato apical stunt (TASV) cDNA with BamHI termini, was constructed from two contiguous BamHI-HindIII restriction fragments of TASV cDNA (22). All PSTV and TASV cDNA constructions cloned in pUC9 or pSP64 vectors contain an 11 nucleotide (GGATCCCCGGG) sequence duplication as a result of the fortuitous identity of the overlapping viroid BamHI-SmaI site and the vectors' multiple cloning site (42).

Plasmid DNAs were isolated from chloramphenicol-amplified cultures of *Escherichia coli* strain JM83 by a 'boiling lysis' procedure and purified through two successive ethidium bromide-CsCl equilibrium centrifugations using a Beckman VTi80 vertical rotor (32). Restriction digestions to determine viroid cDNA orientation within recombinant plasmids or phage were performed according to protocols provided by the enzyme suppliers (New England Biolabs or Bethesda Research Laboratories).

Oligonucleotide-directed mutagenesis of recombinant phage DNAs

An M13mp9 recombinant containing a PSTV cDNA monomer in the PSTV orientation was constructed by insertion of a 359 base pair (bp) BamHI fragment of PSTV cDNA into BamHI-digested, phosphatase-treated M13mp9 replicative form. Single-stranded M13mp9 phage DNA and replicative form were prepared as described (27). A 14-nucleotide mutagenesis primer (dTTCTCGGAAGCTTC), the 17-nucleotide Had3 primer (dCTCCAGGTTTCCCCGGG) and the 19-nucleotide RF2 primer (dGGCTTCAGTTGTTTCCACC) were synthesized by the solid phase phosphoramidite method using an automated DNA synthesizer (Applied Biosystems, Foster City, CA). Descriptions of the syn-

thesis cycle and subsequent oligodeoxynucleotide purification by reverse phase HPLC using a μ-Bondapak C18 column (Waters Associates) have been published (4).

A 'two primer' method for oligonucleotide-directed mutagenesis (30) was used to introduce a C → T transition at position 284 of PSTV cDNA, thereby creating a unique recognition site for HindIII (positions 279–284). After recombinant single-stranded phage DNA (0.5 pmole) was annealed with a mixture of M13 sequencing primer (20 pmoles, 15-nucleotides) and the mutagenesis primer (20 pmoles, 14-nucleotides), PSTV cDNA containing the desired nucleotide change was synthesized by brief incubations with DNA polymerase I (Klenow fragment) and T4 DNA ligase. The PSTV cDNA was removed from the partially double-stranded phage DNA by digestion with EcoRI plus PstI and recloned in pUC9. Screening by HindIII digestion revealed three mutants among the twelve clones obtained. One of these mutants was purified and used for subsequent constructions after sequence analysis by the method of Maxam and Gilbert (24) to verify the nature of the introduced mutation.

Construction of recombinant Agrobacterium tumefaciens *Ti plasmids*

Construction of recombinant pTiA6 plasmids from pUC9 recombinants pST-B14 and pST-B2 and the octopine tumor-inducing *A. tumefaciens* plasmid pTiA6 will be described in detail elsewhere (15). Briefly, PSTV cDNAs with BamHI termini were inserted into the BglII site of pCGN149a, a shuttle vector which contains a region of T-DNA homology and a functional chimeric gene constructed from the 35S promoter of cauliflower mosaic virus (CaMV, 31) and coding sequences for bacterial (Tn5) kanamycin resistance. Homologous recombination with the *A. tumefaciens* octopine plasmid pTiA6 produced Ti plasmids in which *in vivo* transcription of PSTV RNA is controlled by the CaMV 35S promoter (Fig. 2).

Transcription of PSTV cDNAs by SP6 RNA polymerase

Plasmid DNA templates (1 μg, linearized by EcoRI digestion) were incubated for 2 hrs at 40 °C in 25 μl assays containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 500 μM ribonucleotide triphosphates, 30 units RNasin (Promega Biotec), and 4 units SP6 RNA polymerase (26). The DNA template was removed by addition of 1 μg pancreatic DNase (electrophoretically purified, Worthington or Fluka) and incubation at 37 °C for 10 min before dilution with 20 mM Na phoshate (pH 7.0) and bioassay. Transcript yields, estimated by polyacrylamide gel electrophoresis and ethidium bromide staining, averaged 8–10 RNA transcripts/plasmid template.

Bioassay of recombinant DNAs and RNA transcripts

Bioassays of intact pUC9 recombinant plasmid DNAs, electrophoretically purified PSTV cDNA restriction fragments, and PSTV-related RNAs synthesized by SP6 RNA polymerase were performed as described by Cress *et al.* (6). Plants were monitored visually for the appearance of the characteristic symptoms of PSTV infection and by nucleic acid spot hybridization of leaf sap extracts (32). [^{32}P]-labeled hybridization probes (sp. act. $0.5-1\times10^8$ cpm/μg) were prepared by nick-translation of pST-B5 and pAS-B3 DNAs in the presence of [α-^{32}P]dATP using a commercial reagent kit (Bethesda Research Laboratories).

Crown gall induction on tomato (*Lycopersicon esculentum* Miller cv. Rutgers) by recombinant *A. tumefaciens* pTiA6 plasmids containing PSTV cDNA inserts followed a published protocol (40) and is described in greater detail elsewhere (15). As required by the NIH Guidelines for Research Involving Recombinant DNA Molecules, Institutional Biosafety Committees at both Calgene and Beltsville reviewed proposed experimental protocols before work was begun. Plants were kept in dedicated growth chambers, and appropriate precautions were taken to prevent accidental release of *Agrobacterium* strains containing recombinant Ti plasmids into the environment. All pots, soil, and plant material were autoclaved before disposal.

Hybridization analysis with [5′-^{32}P]oligonucleotide probes

Low molecular weight RNA isolated from infected tomato leaf tissue was denatured by a 15 min in-

cubation at 60 °C in the presence of 4 × SSC-7.5% HCHO (47) and diluted with 16 × SSC-7.5% HCHO before application to nitrocellulose membranes (equilibrated with 20 × SSC and air dried before use). Membranes were baked for 2 hr at 80 °C *in vacuo* and prehybridized in 6 × SSC−5 × Denhardt's reagent-0.1% SDS (21) for approximately 60 min at 50 °C before addition of [5′-^{32}P]RF2 or mutagenesis primer (1 × 10^6 cpm/ml, sp. act. 2−4 × 10^8 cpm/μg). After overnight incubation at T_m−5 °C the membranes were washed in 6 × SSC (4−5 times at room temperature followed by a final 20 min wash at T_m−2 °C and prepared for autoradiography. T_m values for hybrids containing RF2 or the mutagenesis primer were calculated to be 54 °C and 42 °C respectively (25).

Southern analysis of bacterial and plant DNAs

Total cellular DNA was prepared from overnight cultures of *A. tumefaciens* and lyophilized tomato tumor tissue as previously described (40, 28). Standard procedures (23, 25) were used for electrophoresis of HindIII-digested DNAs on 1% agarose gels and unidirectional transfer to nitrocellulose. Conditions for hybridization with [^{32}P]PSTV cDNA and subsequent washing of the filters have been described (32). [^{32}P]PSTV cDNA was prepared as described (1) using the universal sequencing primer and a single-stranded recombinant phage DNA template.

Synthesis and restriction endonuclease mapping of PSTV cDNA

PSTV was purified by two-dimensional gel electrophoresis (39) from low molecular weight RNA that had been isolated from infected tomato leaf tissue as previously described (32). One μg (98 pmoles) PSTV plus 1.25 μg (250 pmoles) Had3 primer were dissolved in 15 μl 10 mM Tris-HCl (pH 7.5)-10 mM $MgCl_2$-50 mM NaCl, heated for 2 min at 90 °C, quenched in an ice-water bath for 1 min, and annealed at 37 °C for 2 hr. First strand cDNA synthesis was carried out at 42 °C in a 50 μl reaction containing 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 140 mM KCl, 10 mM DTT, 0.5 mM dCTP, dGTP, and dTTP, 0.05 mM dATP, 0.6 μM [α-^{32}P]dATP (800 Ci/mmole, NEN) p, and 40 units of avian myeloblastosis virus reverse transcriptase (Molecular Genetic Resources). After 90 min the synthesis was terminated by addition of 2 μl 500 mM EDTA, and the PSTV cDNA was recovered by phenol/chloroform extraction and ethanol precipitation.

The resulting RNA-DNA hybrid was dissolved in 25 μl water and denatured by heating (100 °C, 2 min) and quenching in an ice-water bath. Second strand cDNA synthesis catalyzed by DNA polymerase I (Klenow fragment) has been described elsewhere (22). Aliquots of the resulting double-stranded PSTV cDNA (12 ng) were digested with BamHI, BamHI plus AluI, or BamHI plus HindIII in 10 μl reactions and analyzed by electrophoresis in 5% acrylamide-0.125% N,N′-methylene-bis-acrylamide gels containing 1 × Tris-borate-EDTA buffer (23). Gels were dried prior to autoradiography at −70 °C using Kodak XAR X-ray film and Dupont Cronex Lightning-Plus intensifying screens.

Results

Inoculation of tomato seedlings with intact pBR322 recombinant DNAs containing tandem dimers of PSTV cDNA is followed by the appearance of PSTV progeny with the RNA sequence predicted by the sequence of the cloned PSTV cDNA (6). Because RNA transcribed from a recombinant plasmid containing a PSTV cDNA monomer with a 4 nucleotide sequence duplication also appeared to be weakly infectious, we decided to begin our systematic site-specific mutagenesis studies of PSTV by assessing the effect of sequence duplication on PSTV cDNA infectivity.

Infectivity of PSTV cDNAs containing nucleotide substitutions

Directed chemical mutagenesis of deoxycytosine residues within the three overlapping BamHI-SmaI sites of pVB-6 produced three mutant plasmids which could be easily distinguished from pVB-6 by the simultaneous loss of one BamHI plus one SmaI site. Because DNA sequence analysis revealed that pVB-6(HSO_3 34) contains C → T transitions at positions 92 and 103 within the central BamHI-SmaI site of its PSTV cDNA insert, its longest PSTV cDNA contains only 365 contiguous PSTV-specific nucleotides (see Table 1).

This double mutant was significantly less infec-

Table 1. Effect of selected C → T transitions on infectivity of PSTV cDNA.

Clone	No. Nucleotides	Structure	Infectivity[a]
pVB-6		87 359-1 87 88 359-1 92 PSTV PSTV	+ (6/6)
pVB-6 (HSO_3 34)	365,349[b]	87 91 104 92 x x	+ (1/12)
pVB-6 (HSO_3 30)		93 92 x	+ (7/12)
pVB-6 (HSO_3 54)		87 91 x	+ (6/6)
pST-B2		87 284 284 97 x x	- (0/10)

[a] Values represent number of infected plants/total number of plants inoculated with 10 μg intact plasmid DNA.
[b] There are 365 contiguous PSTV-specific nucleotides preceding the first C → T transition in pVB-6 (HSO_3 34) DNA, but only 349 PSTV-specific nucleotides following the second C → T transition.
The numbers above the lines refer to nucleotide positions within the PSTV cDNA sequences.
x: C → T transitions within individual PSTV cDNAs.

tious than either the parental pVB-6 or the two mutant plasmids which contain C → T transitions in the outer BamHI-SmaI sites. RNA sequence analysis indicated that the progeny possessed an unaltered intermediate strain sequence (data not shown). The simplest explanation for this observation would require that a greater than unit-length PSTV RNA transcribed *in vivo* from pVB-6(HSO_3 34) could undergo cleavage/ligation between positions 86 and 92 to excise both nucleotide changes present in its DNA template.

A 'two primer' method for oligonucleotide-directed mutagenesis of single-stranded M13mp9 recombinant DNAs (30) was also used to introduce a single C → T transition at position 284 of PSTV cDNA. This single nucleotide substitution creates a unique recognition site for HindIII in the mutant cDNA, thereby allowing the construction of chimeric PSTV-TASV cDNAs (see below). We were somewhat surprised to find that PSTV cDNAs containing this mutation were noninfectious (clone pST-B2, Table 1). Bioassays were conducted with a tandem dimer of the mutant PSTV cDNA (clone pST-B2) because preliminary experiments had shown that the specific infectivity of *in vitro* RNA transcripts from a wild-type tandem dimer was 100-fold greater than those from the corresponding monomer. RNA transcripts from the mutant cDNA templates were noninfectious (results not shown).

Infectivity of viroid cDNA chimeras

The extensive sequence homologies shared by PSTV and TASV which include the central conserved region (22) containing the unique BamHI recognition site make construction of mixed tandem dimers from PSTV and TASV cDNAs with BamHI termini straightforward. Inoculation of tomato seedlings with the resulting plasmid DNAs (pSTAS-B4 and pASST-B2, see Table 2) was fol-

Table 2. Infectivity of chimeric viroid cDNAs.

Clone	No. Nucleotides	Structure	Infectivity[a]
pST-B5[b]	359	87 359-1 97 ¦----------¦ PSTV	+ (pool)[c]
pAS-B3[b]	360	89 282 99 ¦- - -(x)- - ¦ TASV	+ (5/5)
pSTAS-B4[b]		87 87 90 99 ¦----------¦- - -(x)- - ¦	+ (2/35 PSTV) (3/35 TASV)
pASST-B2[b]		89 88 88 97 ¦- - -(x)- - ¦----------¦	+ (3/35 PSTV) (17/35 TASV)
pSTAS-B5[d]	365	87 279-278 99 ¦-----(x) - -¦	- (0/20)
pASST-B6[d]	354	89 277-280 97 ¦- - -(x)----¦	- (0/20)

[a] Except where noted values represent number of infected plants/total number of plants inoculated.
[b] Individual plants were inoculated with 10 μg intact plasmid DNA.
[c] Because 9 of 10 inoculated plants exhibited typical symptoms of PSTV infection, tissue samples were pooled before hybridization analysis.
[d] Individual plants were inoculated viroid cDNA fragments prepared by BamHI digestion of 7.5 μg plasmid DNA and electrophoresis in 5% acrylamide gels.
The numbers above the lines refer to nucleotide positions within the PSTV or TASV cDNA sequences.
¦: BamHI cleavage site; (x): HindIII cleavage site.

lowed by the appearance of the typical symptoms of viroid infection-stunting, epinasty, and leaf rugosity. Nucleic acid spot hybridization analysis using nick-translated PSTV- or TASV-specific probes indicated that individual infected plants contained either TASV or PSTV (Table 2). No evidence for either the replication of chimeric TASV-PSTV RNAs or the simultaneous replication of TASV and PSTV in a single plant was obtained (Fig. 1).

Two different chimeric cDNA 'monomers' can be constructed from BamHI-HindIII fragments of wild type TASV cDNA (clone pAS-B3) and the mutant PSTV cDNA which contains a single C → T transition at position 284 (pST-B2). Digestion of either mutant PSTV or TASV cDNA with BamHI plus HindIII produces 2 fragments, one derived from the more stable right side of the native structure and another derived from the less stable left side (see Fig. 6). The chimeric viroid cDNAs present in clones pSTAS-B5 and pASST-B6 (Table 2) were constructed by successive incubations of the appropriate mixtures of electrophoretically purified BamHI-HindIII fragments with T4 DNA ligase and BamHI. Even though the chimeric cDNA 'monomers' were excised with BamHI before bioassay in order to maximize their specific infectivity (42), neither cDNA was infectious.

A single nucleotide substitution abolishes PSTV cDNA infectivity

The only difference between the noninfectious PSTV cDNA clone pST-B2 and the corresponding infectious wild-type clone is the presence of a single

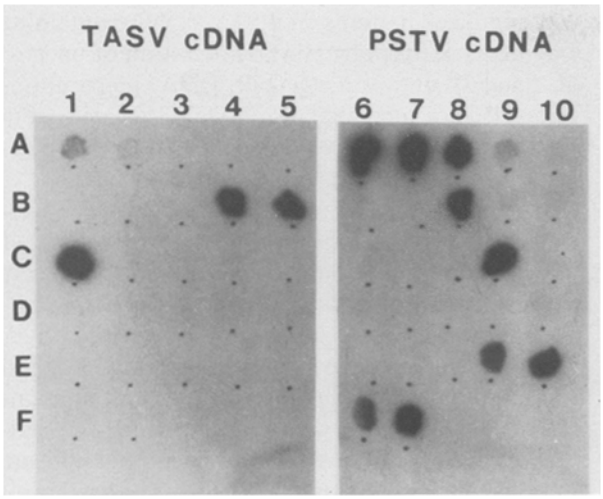


Fig. 1. Characterization of viroid progeny which appears following inoculation with chimeric viroid cDNAs. Aliquots (3 μl) of crude leaf sap prepared from tomato plants inoculated with intact recombinant plasmid DNAs containing mixed tandem dimers of PSTV and TASV cDNAs (10 μg/plant) were spotted on duplicate nitrocellulose membranes, incubated with [^{32}P]pST-B5 (left) or pAS-B3 (right) DNAs, and washed as described in Materials and methods. Row A contains serial tenfold dilutions of electrophoretically purified PSTV (3000 pg → 0.3 pg, left to right) in sap prepared from uninoculated plants. Rows B-D contain samples prepared from individual plants inoculated with either pASST-B2 or pSTAS-B4 (see Table 2). Rows E and F contain samples prepared from plants inoculated with a pUC9 recombinant whose tandem PSTV cDNA dimer contains a 24 nucleotide insertion in the central BamHI site. Samples from healthy or mock inoculated plants are found in row B (column 2), row C (column 2), and row D (column 3). The signal intensities shown required a 65 h exposure in the presence of a single intensifying screen.

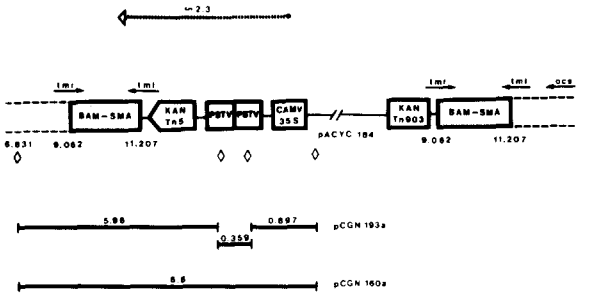


Fig. 2. Structure of the T-DNA region of *A. tumefaciens* pCGN193a. The relative positions of the various functionally important DNA sequences are shown as boxed areas in the middle of the figure; numbers refer to nucleotide positions within the T-DNA (2). The horizontal arrows above the DNA sequence indicate the location of certain T-DNA transcripts (solid arrows) and a potential 2300 nucleotide chimeric transcript which initiates at the CaMV 35S promoter and terminates within the *tml* region (dashed arrow). *In vivo* transcripts from similar recombinant Ti plasmids containing the CaMV 35S promotor have been characterized by Shewmaker *et al.* (40). Expected differences in the lengths of PSTV-specific fragments released by HindIII digestion of plasmids pCGN193a and pCGN160a are indicated below the T-DNA. ◇, HindIII cleavage sites.

C → T transition at position 284, a change which seemed unlikely to significantly destabilize the native structure of PSTV (see Fig. 5). Therefore, we decided to confirm its effects in a bioassay system where PSTV infection is mediated by recombinant *A. tumefaciens* tumor inducing (Ti) plasmids containing full-length PSTV cDNAs (15).

Figure 2 shows the structure of the relevant portions of pCGN193a, a pTiA6 recombinant containing a tandem dimer of mutant PSTV cDNA; a second plasmid, pCGN160a, contains an unaltered PSTV cDNA monomer. Details of their construction and characterization of their biological properties are reported elsewhere (15), but these two plasmids can be readily distinguished by Southern blot hybridization analysis using a PSTV-specific probe (Fig. 3).

When the stems of tomato seedlings were inoculated with either pCGN160a or pCGN193a to induce crown gall formation, typical symptoms of PSTV infection began to appear in the foliage 14–21 days later. In four independent experiments both pCGN160a and pCGN193a were consistently infectious. Nucleic acid spot hybridization analysis of leaf sap extracts from one experiment revealed that 17/20 and 20/20 pairs of seedlings became systemically infected with PSTV following inoculation with pCGN160a and pCGN193a respectively. Because these results were in apparent conflict with those obtained with a pUC9 recombinant containing the same C → T transition (Table 2), several aspects of PSTV infection mediated by the *A. tumefaciens* Ti plasmid were examined in greater detail.

Southern hybridization analysis of total cellular DNA preparations from the *A. tumefaciens* clones and the corresponding crown gall tumors was used to verify the structure of the PSTV cDNAs present in the T-DNA region. Figure 3 shows that digestion with HindIII releases 3 fragments containing PSTV specific sequences from both *A. tumefaciens* pCGN193a and the corresponding galls (lanes 3

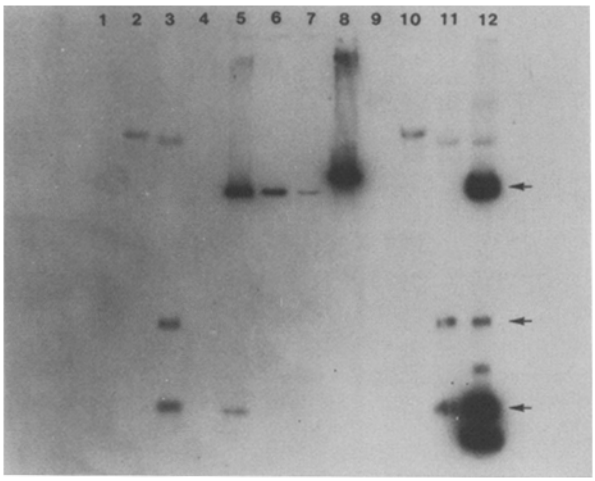


Fig. 3. Southern analysis of *A. tumefaciens* and tomato crown gall total DNA preparations. DNAs (25 μg) were incubated for 16 hr at 37 °C in 70 μl reactions containing 25 units HindIII; additional enzyme (15 units) was added after 6 hr. DNA fragments were recovered and concentrated by successive precipitations from 2.5 M NH_4 acetate and 0.3 M Na acetate before electrophoresis and Southern analysis with [^{32}P]PSTV cDNA as described in Materials and methods. Lanes 1–3, total bacterial DNAs (14 μg): lane 1, *A. tumefaciens* A722; lane 2, pCGN160a; and lane 3, pCGN193a. Lane 4, 0.7 μg lambda DNA. Lanes 5–7, pgST64-B2 DNA: lane 5, 500 pg; lane 6, 50 pg; and lane 7, 5 pg. Lane 8, 5000 pg pST-B14. Lanes 9–12, total DNA from tomato galls (14 μg): lane 9, *A. tumefaciens* A722; lane 10, pCGN160a; lane 11, pCGN193a; and lane 12, pCGN193a plus 1400 pST64-B2.

and 11) but only a single fragment from *A. tumefaciens* pCGN160a and its corresponding galls (lanes 2 and 10). The smallest fragment in the pCGN193a digests comigrates with the 359 bp fragment produced by HindIII digestion of pST-B2, the pUC9 recombinant used in its construction (compare lanes 3, 5, and 11). This 359 bp fragment is not present in HindIII digests of either pCGN160a, its corresponding galls, or pST-B14 (lanes 2, 10, and 8 respectively) where only a single, much larger fragment hybridized with [^{32}P]PSTV cDNA. As predicted the PSTV cDNA-containing fragment in pCGN160a digests was slightly larger than the largest fragment in the pCGN193a digests (compare lanes 2 with 3 and 10 with 11). *A. tumefaciens* A722, which does not contain PSTV cDNA, yielded no PSTV-specific fragments among the HindIII digestion products of either its total cellular DNA or the corresponding tumor tissue (lanes 1 and 9). The identical patterns of PSTV cDNA-containing fragments released by HindIII digestion of bacterial (lane 3) and tomato gall DNA preparations (lane 11) indicated that the T-DNA isolated from galls incited by pCGN193a had not undergone detectable sequence reversion at position 284.

Having established the integrity of the PSTV cDNA dimer within the T-DNA region of pCGN193a, we characterized the PSTV progeny (Fig. 4). Low molecular weight RNA was isolated from the leaves of tomatoes inoculated with either *A. tumefaciens* (strains pCGN193a and pCGN160a) or the infectious pBR322 recombinant pVB-6 and denatured with formaldehyde (47). Serial three-fold dilutions were applied to duplicate nitrocellulose membranes and hybridized with either [5′-^{32}P]-labeled RF2 or mutagenesis primer. Hybridization with RF2 was used to estimate the relative concentration of PSTV in each sample, while the ability of mutant PSTV containing a C → U transition at position 284 to form stable hybrids with the mutagenesis primer was used to differentiate the putative mutant PSTV from the unaltered intermediate strain.

Comparison of columns 7 and 8 demonstrates that hybridization of RF2 with *in vitro* RNA transcripts from the intermediate strain (pST64-B14) and mutant (pST64-B2) produced equivalent signals (upper panel). The mutagenesis primer, however, hybridized >9-fold less efficiently to pST64-B14 transcripts which lack the C → U transition than to mutant transcripts (lower panel). When this assay was used to compare the PSTV present in leaf tissue from tomatoes inoculated with pCGN193a (columns 4–6) with that from plants inoculated with unaltered cDNAs (pVB-6 and pCGN160a, columns 2 and 3), no evidence for the replication of PSTV containing a C → U transition at position 284 was apparent. The PSTV present in plants inoculated with mutant cDNAs showed no evidence of preferential hybridization with the mutagenesis primer.

Restriction endonuclease mapping of cDNAs synthesized using PSTV template purified from these low molecular weight RNA preparations confirmed the absence of mutant PSTV to tomatoes inoculated with pCGN193a (results not shown). A 17-nucleotide primer (Had3) which is perfectly complementary to nucleotides 92–108 of PSTV and nucleotides 95–111 of TASV was used to prime

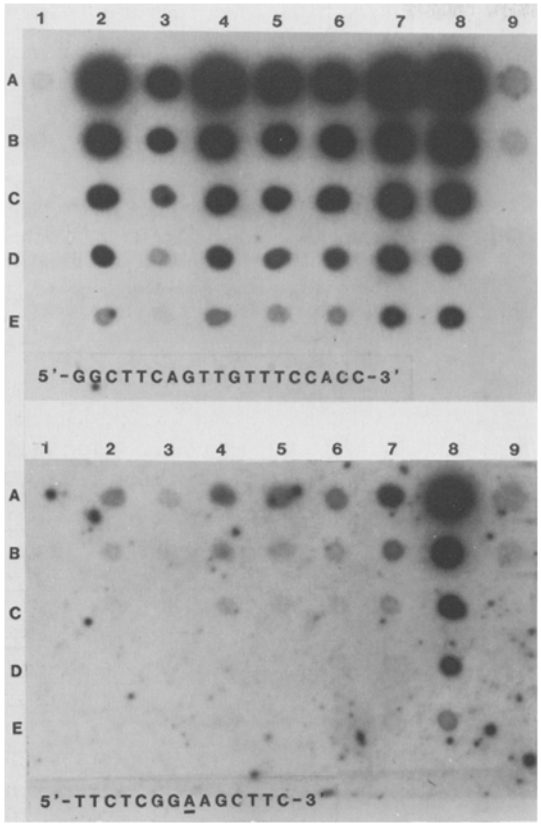


Fig. 4. Hybridization analysis of PSTV isolated from tomatoes inoculated with *A. tumefaciens* recombinants. Serial three-fold dilutions (rows A–E) of 8 different RNA preparations were spotted on duplicate nitrocellulose membranes in columns 1–8 and incubated with one of two different [5′-^{32}P]oligonucleotide hybridization probes (see Materials and methods). The 19-nucleotide RF2 primer (upper panel) is perfectly complementary to positions 265–282 of both intermediate strain PSTV and the putative mutant PSTV. The 14-nucleotide mutagenesis primer (lower panel) is perfectly complementary to positions 278–291 of PSTV RNA containing a C → U transition at position 284. Columns 1–6, 5 μg → 62 ng low molecular weight RNA from tomato leaf tissue: column 1, uninoculated control plants; column 2, plants inoculated with plasmid pVB-6; column 3, plants inoculated with *A. tumefaciens* pCGN160a; columns 4–6, plants inoculated with *A. tumefaciens* pCGN193a in three independent experiments. Columns 7 and 8, 20 ng → 247 pg *in vitro* RNA transcripts from pST64-B14 (column 7) and pST64-B2 (column 8). Samples in rows A and B of column 9 are identical to that in row A of column 6 except that the RNA preparations in column 9 were incubated with 2 μg pancreatic RNase [45 min at 37 °C in the presence of 10 mM Tris-HCl (pH 8.0)-1 mM EDTA] before denaturation. The autoradiograph in the upper panel was produced by a 5 hr exposure with a single intensifying screen, while the autoradiograph in the lower panel required a 69 hr exposure.

cDNA synthesis just upstream from the unique BamHI site present in both viroid cDNAs. Both unaltered and mutant PSTV cDNAs contain unique AluI recognition sites (positions 280–283), but only the mutant PSTV cDNA will contain a HindIII recognition site (positions 279–284). TASV cDNA contains a unique HindIII recognition site (positions 277–282), and one of its three AluI recognition sites occurs between positions 278–281.

Digestion of both double-stranded PSTV cDNA preparations with BamHI plus AluI released a discrete fragment corresponding to the left half of the PSTV native structure, and digestion of double-stranded TASV cDNA with BamHI plus HindIII released a slightly larger fragment. There was no evidence, however, for the presence of a HindIII recognition site in double-stranded PSTV cDNA synthesized from viroid RNA template isolated from plants inoculated with pCGN193a. The pattern of fragments produced by digestion with BamHI plus HindIII was identical to that produced by BamHI alone, and this cDNA preparation did not inhibit the cleavage of TASV cDNA by BamHI plus HindIII. We concluded, therefore, that the PSTV template for cDNA synthesis did not contain the C → U (T) transition at position 284 present in the Ti plasmid of *A. tumefaciens* pCGN193a.

Discussion

As the focus of plant viroid research has begun to shift from *in vitro* structural studies to the relationship between viroid structure and function *in vivo*, genetic approaches to the study of viroid/host interaction have become increasingly important. Structure/function assignment suggested by comparative sequence analysis (21, 38, 44) can be refined by a two step procedure, i.e. introduction of appropriate sequence changes by site-specific mutagenesis of infectious viroid cDNAs followed by subsequent bioassay to determine their phenotypic effect *in vivo*. The conventional tomato bioassay (8) provides a valuable screening tool for the characterization of PSTV mutants.

Comparative sequence analysis of naturally occurring viroids has identified regions that are strongly conserved among different viroids [e.g. the central conserved region (37), sequences homolo-

gous to the 'box' sequences of Group I introns (11, 12)], and regions containing pathogenesis-related sequence variations (38, 44). It should be noted that two of these regions lie within less stable portions of the viroid secondary structure, the premelting regions recently identified by Schnolzer *et al.* (38). Portions of the central conserved region are found within premelting region 2, while pathogenesis-related sequence variations in PSTV and CEV occur within premelting regions 1. Fig. 6 shows the locations of several potential functional regions within the familar native structure of PSTV.

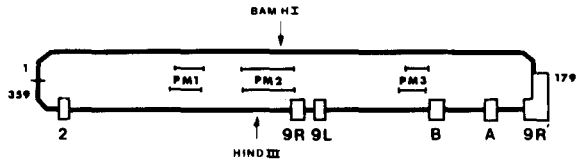


Fig. 6. Potential functional regions within the native structure of PSTV. The position of nucleotides comprising premelting regions 1–3 (38) are indicated by bars inside the native structure, while the open boxes indicate sequences homologous to the 'box' sequences of Group I introns (11, 12). The unique cleavage sites in PSTV cDNA for BamHI and HindIII (mutant cDNA only) are shown by the vertical arrows.

Biological effects of specific nucleotide changes

Our initial efforts to introduce defined nucleotide changes into infectious PSTV cDNAs have employed C → T transitions because the resulting C → U transitions in PSTV should have minimal effects upon its native structure (Fig. 5). Two of the 3 substitutions are located within premelting region 2 (see Fig. 6) and appear to abolish viroid replication.

The apparently lethal effect of simultaneous C → U transitions at positions 92 and 103 within the upper portion of the central conserved region was not completely unexpected in light of the suggested importance of this region for viroid replication (37). Although assignment of precise roles to the individual mutations awaits construction of the corresponding revertants, the data presented in Table 1 support a model recently proposed by Diener (10) to account for the processing of oligomeric viroid replication intermediates into monomeric circular progeny. This model postulates that partially or completely dimeric PSTV RNAs can form a thermodynamically extremely stable base-paired configuration involving PSTV nucleotide 79–110 that is essential for precise RNA cleavage and ligation. The C → U transition at position 92 in the central BamHI-SmaI site of pVB-6(HSO_3 34) lies within the proposed cleavage/ligation site.

The unexpected abolition of PSTV cDNA infectivity by a single C → T transition at position 284 is intriguing. CEV, CSV, and TASV cDNAs each contain a unique recognition site for HindIII, and the equivalent of U_{284} in their native structures is base-paired with an A rather than the G found at position 78 in PSTV. The presence of a G-U base-pair in the PSTV mutant may impair replication by destabilizing premelting region 2. Position 284 lies just outside the lower portion of the central conserved region but just inside the left border of premelting region 2 (Fig. 5). It is not involved in either secondary hairpins I-III (35, 37) or the sequences homologous to the Group I intron 'boxes' (Fig. 6). This hypothesis is being tested by con-

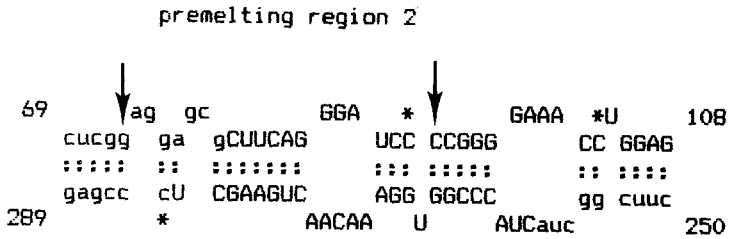


Fig. 5. Location of inactivating C → U transitions with respect to premelting region 2 and the central conserved region of PSTV. A portion of the PSTV native structure has been redrawn from Schnolzer *et al.* (38) using lower case lettering to indicate nucleotides outside the perfectly conserved central conserved region shared by PSTV and TASV. *: locations of C → T transitions in plasmids pVB-6(HSO_3 34) [positions 92 and 103] and pST-B2 [position 284]. Boundaries of premelting region 2 are indicated by vertical arrows.

struction of appropriate pseudorevertants in which the G at position 78 will be replaced by an A.

Infectivity of chimeric viroids

Construction of chimeric viroid cDNAs provides a second, conceptually different approach to site-specific mutagenesis of infectious viroid cDNAs. Visvader and Symons (44) have discussed the possible use of this technique to determine the relative contribution of the two pathogenesis-related regions of CEV to symptom expression in tomato. Our goal in constructing several different types of PSTV-TASV cDNA chimeras was somewhat different.

Mixed tandem dimers of viroid cDNA can theoretically give rise to a number of potentially viable full-length RNA transcripts, and individual plants might contain viroid clones whose sequence would vary from plant to plant. Characterization of such clones would identify nucleotide substitutions that do not destroy viability. If successful, this approach would be less laborious than the construction and testing of a number of individual point mutations in a single viroid cDNA.

Mixed tandem dimers constructed from full-length PSTV and TASV cDNAs were indeed infectious, but nucleic acid hybridization analyses using full-length cDNA probes provided no evidence for either mixed infections or the desired chimeric viroid RNAs (Table 2). In retrospect, three observations predict such a result: the infectivity of the individual PSTV and TASV cDNAs (Table 2), the probable location of the site for processing of oligomeric viroid replication intermediates within the overlapping BamHI-SmaI site which joins the two full-length cDNAs (10), and the well known phenomenon of viroid 'cross protection' (29). Suitably designed mixed tandem cDNA dimers can provide a means to study the molecular basis of cross protection in a system where every inoculated cell simultaneously receives a copy of both infectious cDNAs.

A second type of cDNA chimera, 'monomers' containing the thermodynamically less stable left half of one viroid and the more stable right half of a second viroid, was noninfectious (Table 2). Although the secondary structure of the central conserved region and the ability to form a Group I intron 'core' structure appear unaltered, sequence divergence just outside the central conserved region prevents formation of two of the 9 base-pairs in the stem of secondary hairpin I (Table 3). The construction of appropriate pseudorevertants will allow assessment of the relative importance of secondary hairpin I and the Group I intron 'core' structure to normal viroid function. Introduction of additional restriction sites into individual viroid cDNAs will permit the construction of chimeric cDNAs which contain less extensive sequence substitutions.

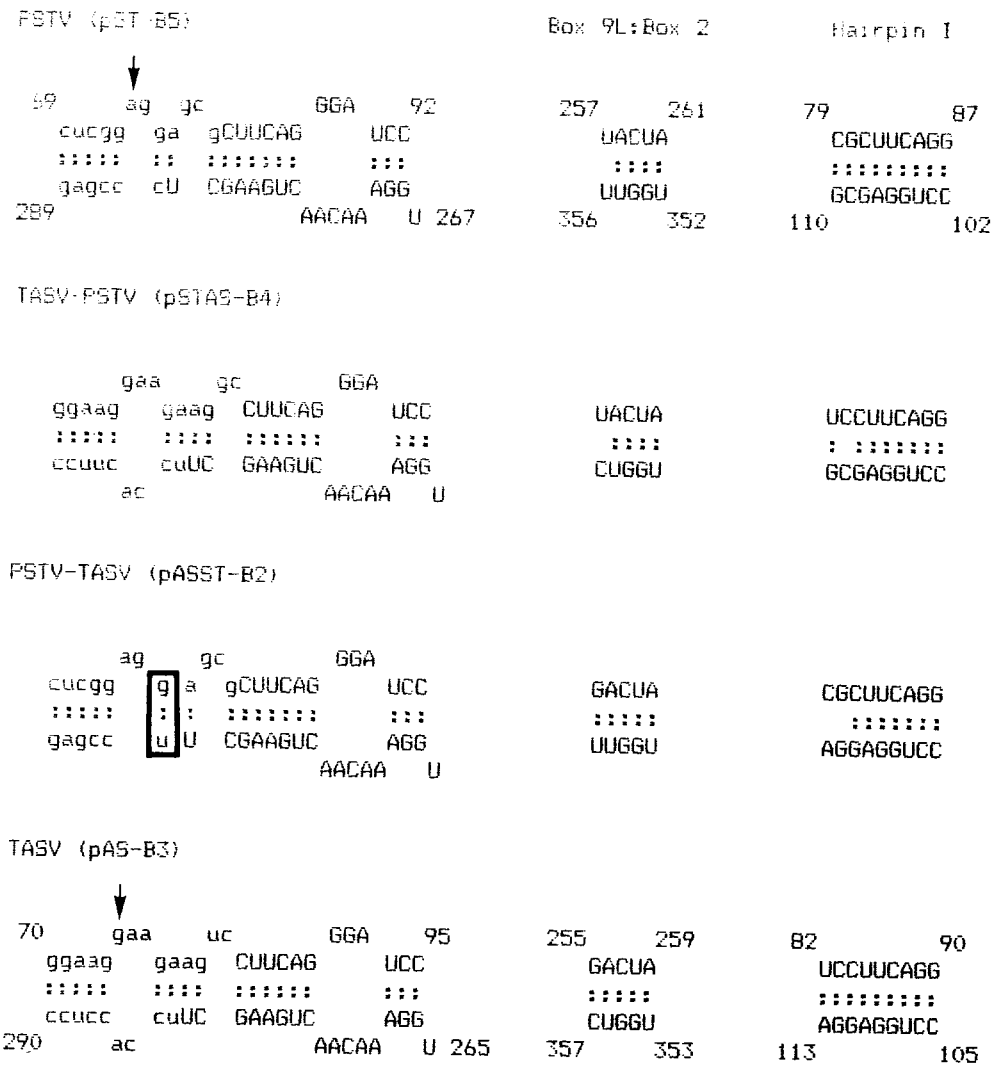
Characterization of viroid mutants

An ideal bioassay system must be able to differentiate mutations that are actually lethal from those that severely inhibit PSTV replication. In an effort to develop such a system we have placed potentially infectious PSTV cDNAs under the control of a functional promoter for RNA polymerase II and used the Ti plasmid of *A. tumefaciens* to introduce them into the host cell. This system has been used to confirm the lethal effect of a single C → U transition at PSTV position 284, but its full potential has not yet been realized.

Although Ti plasmid-mediated PSTV infections require only T-DNA transfer but not integration (17), transformed callus cultures or even intact plants can be readily obtained. Such experimental systems maximize the opportunity for PSTV infection and can provide large amounts of tissue in which viroid replication need not be systemic to be detectable. We have discovered, however, that increases in the theoretical sensitivity of a bioassay may be accompanied by unexpected experimental complications.

The appearance of wild type PSTV progeny after *A. tumefaciens*-mediated inoculation with an altered PSTV cDNA (Fig. 4) may well be a consequence of the high error frequency characteristic of RNA synthesis (45). We do not yet know whether or not reversion at the RNA level will be a serious problem when recombinant plasmid or phage DNAs are used as inocula in bioassays to detect systemic PSTV replication, but identification of mutations which severely impair PSTV replication may depend upon the use of mutations which cannot be 'rescued' by simple nucleotide substitutions. Because viroid cDNAs appear not to replicate in the infected plant (42), there is no obvious selection

Table 3. Potential structural interactions within PSTV-TASV viroid chimeras.



Nucleotides outside the perfectly homologous central conserved regions shared by PSTV and TASV are shown as lower case letters in the proposed viroid native structures (left). The left border of the premelting region 2 is indicated by a vertical arrow. The boxed G-U basepair in the PSTV-TASV chimera would also be present in PSTV containing a C → U transition at position 284. The Box 9L:Box 2 interactions for PSTV and TASV are those proposed by Diener and Hadidi (11).

pressure for sequence reversion at the DNA level.

Site-specific mutagenesis studies of the *Tetrahymena* rRNA intron (33, 46) have shown the importance of *in vitro* as well as *in vivo* assays to systematic studies of RNA structure/function relationships. Hall and his collaborators (14) have used the ability of mutant brome mosaic virus RNAs synthesized *in vitro* to act as substrates for tyrosyl-tRNA synthetase and the viral replicase to study structure/function relationships in the 3′-terminal region of the viral RNA. This approach permits the generation of targeted mutations without regard to their viability *in vivo*. The recent demonstration of spontaneous *in vitro* cleavage of multimeric viroid RNAs (36) may be the first step toward the development of similar *in vitro* as-

say systems for individual steps in viroid replication.

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Note added in proof

Based upon site-specific mutagenesis of infectious CEV cDNAs Visvader *et al.* (Nuc Acids Res 13:5843 – 5856, 1985) have also concluded that *in vivo* processing of longer-than-unit-length viroid plus strands occurs within the upper portion of the central conserved region.

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