Destabilization of potato spindle tuber viroid by mutations in the left terminal loop

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Infectivity studies with highly infectious RNA inocula generated by ribozyme cleavage were used to compare the biological properties of three apparently nonviable mutants of potato spindle tuber viroid (PSTVd). One of these mutants (PSTVd-P) contains three nucleotide substitutions in the left terminal loop, and mechanical inoculation of tomato seedlings with RNA transcripts at levels equivalent to 10^3–10^5 times the ID50 for PSTVd-Intermediate failed to result in systemic infection. Viable progeny containing a spontaneous C → G change at position 4 could, however, be recovered from transgenic Nicotiana benthamiana plants that constitutively expressed PSTVd-P RNA. The initial mutations in PSTVd-P led to an overall weakening of its native structure in vitro, and the precisely-full-length molecule released by ribozyme cleavage in vivo was also unstable. Even RT–PCR analysis failed to reveal detectable amounts of circularized PSTVd-P among the RNAs isolated from uninfected plants. Predicted stabilizing effects of a spontaneous mutation at position 4 suggest that the appearance of viable progeny was dependent on a combination of events: errors by host RNA polymerase II during transcription of the mutant transgene coupled with a strong selective pressure against alterations in the native structure of PSTVd.

Introduction

Viroids, the smallest known agents of infectious disease, are small (246–463 nt), highly structured single-stranded RNA molecules which lack both a protein capsid and detectable mRNA activity (reviewed by Diener, 1987; Semancik, 1987). Despite their exceedingly small size and lack of mRNA activity, viroids replicate autonomously and are able to induce a wide variety of disease symptoms in susceptible plant species. Physical studies (reviewed by Riesner, 1990; Riesner & Steger, 1990) have shown that potato spindle tuber viroid (PSTVd) and related viroids share several unusual structural features: (i) a rod-like native structure composed of an alternating series of short double-helical regions and small internal loops; (ii) three comparatively unstable ‘premelting (PM) regions’ present at conserved positions within the native structure; and (iii) formation of a series of alternative structural interactions during thermal denaturation in vitro. Pairwise sequence comparisons suggest that the rod-like native structure of viroids contains five structural domains whose boundaries are defined by sharp changes in sequence similarity (Keese & Symons, 1985), i.e. a conserved central domain believed to contain the site where multimeric viroid RNAs are cleaved and ligated to form circular progeny (Baumstark & Riesner, 1995), flanking pathogenicity and variable domains, and two terminal domains.

A variety of molecular approaches has been used in efforts to identify structural features which allow viroids to replicate, move from cell-to-cell and induce disease. For example, Sano et al. (1992) constructed a series of interspecific chimaeras between tomato apical stunt and citrus exocortis (CEVd) viroids in order to investigate the role of individual structural domains in viroid pathogenicity and replication. At the biochemical level, D. Riesner and co-workers have examined the effects of nucleotide substitutions within the central conserved region on the cleavage and ligation of longer-than-unit-length PSTVd RNAs in vitro in an effort to define the
structural requirements for the ‘processing’ needed to convert multimeric replicative intermediates to monomeric viroid progeny (Steiger et al., 1992; Baumstark & Riesner, 1995).

The ability of many point mutations and small insertions/deletions to abolish viroid infectivity has been well-documented (e.g. Ishikawa et al., 1985; Visvader et al., 1985; Hammond & Owens, 1987; Owens et al., 1991; Lakshman & Tavantzis, 1992). Introduction of such a mutation may also lead to the appearance of one or more ‘compensatory’ mutations in the progeny, and the nature of these secondary mutations has provided valuable insight into the role of certain structural elements in PSTVd replication and movement. For example, the time-course and pattern of sequence reversion associated with several unstable mutations affecting secondary hairpin II suggest that formation of a metastable structure containing secondary hairpin II controls the ability of (+)-PSTVd RNAs to serve as templates for the synthesis of (+) strand progeny (Qu et al., 1993).

The ability of a ‘disabled’ viroid RNA to evolve in vivo has certain practical consequences. Constitutive expression of antisense RNAs targeting the (+) strand of CEVd can result in a moderate reduction in viroid accumulation in transgenic tomatoes after challenge inoculation (Atkins et al., 1995). Although RNAs containing rather large (i.e. 50 nt) deletions showed no tendency to revert, Wassenegger et al. (1994) have reported that a PSTVd mutant containing a smaller deletion was able to resume replication after undergoing a second, spontaneous deletion event that restored its rod-like secondary structure. The frequency and molecular mechanism(s) responsible for such in vivo ‘repairs’ are not yet clear. Here, we describe the effects of certain mutations in the left terminal loop on the stability of PSTVd both in vitro and in vivo.

**Methods**

**Construction of PSTVd cDNAs.** Construction of a full-length (359 nt) PSTVd cDNA clone with BamH I termini derived from the upper portion of the central conserved region has been described elsewhere (Cress et al., 1983). Transfer of this cDNA, derived from the Intermediate strain of PSTVd (PSTVd-Int) to a derivative of pTZ18R (US Biochemical), resulted in plasmid pRZ6-2, a molecule in which the full-length PSTVd cDNA is flanked by specially modified versions of the hammerhead and paperclip (or hairpin) ribozymes derived from satellite tobacco ringspot virus (stbRSV) RNA (P. A. Feldstein, unpublished data). Transcription of this plasmid DNA by T7 RNA polymerase produces an RNA molecule which spontaneously self-cleaves in vitro to release a precisely full-length PSTVd-Int RNA whose 5'-hydroxyl and 2',3'- cyclic phosphate termini are derived from positions 86 and 87, respectively (see below).

Isolation of several apparently non-infectious cDNAs, containing an A → G substitution at position 135 via random chemical mutagenesis, was reported by Owens et al. (1991). Partial characterization of additional mutants containing multiple substitutions in either the left terminal loop (i.e. PSTVd-P; Hammond, 1994) or the pathogenicity domain (i.e. 43G + 310–311U; Owens et al., 1995) has also been described. These mutations were transferred into the double ribozyme expression cassette, described above, by replacing the 294 bp Eagl–Eco47III fragment of PSTVd-Int cDNA (positions 145–359/1–79) with the corresponding fragment of mutant cDNA. Recombinant plasmids were propagated in *Escherichia coli* strains JM83 or DH5α using 1 × yeast–tryptone medium supplemented with 50–100 µg/ml ampicillin (Sambrook et al., 1989).

**Synthesis and bioassay of PSTVd RNA transcripts.** For synthesis of PSTVd RNAs containing an 11 nt sequence duplication (i.e. GGATCCCGCGG), plasmid pST64-B5 (Owens et al., 1986), or homologous plasmids containing mutant cDNAs, were linearized with EcoRI and transcribed with SP6 RNA polymerase as suggested by the manufacturer (Promega). Ribozyme-containing plasmid templates were linearized by digestion with *Hind* III before transcription with T7 RNA polymerase, and reactions were incubated for 3–4 hrs at 37 °C as described by the manufacturer (Promega) except for an increase in the MgCl₂ concentration to 15 mM. After digestion of the DNA template with RNase-free DNase, unlabelled PSTVd RNA transcripts intended for bioassays were recovered by phenol-chloroform extraction and ethanol precipitation. RNA pellets were dissolved in sterile water and stored at −20 °C prior to use.

PSTVd RNAs were diluted to a final concentration of 0.1–100 ng/µl (+)-PSTVd RNA using 20 mM sodium phosphate buffer (pH 7.0), and aliquots (10 µl) were rubbed on the carrot-dusted cotyledons of 1-week-old tomato seedlings using a sterilized glass rod. Inoculated plants were maintained in a greenhouse for 6–7 weeks under conditions suitable for viroid replication. Samples of tissue collected from the uppermost leaves of individual plants were periodically assayed for the presence of viroid progeny by dot-blot hybridization using a full-length, digoxigenin-labelled RNA probe specific for (+)-PSTVd (Podleckis et al., 1993).

**Expression of PSTVd mutants in transgenic Nicotiana benthamiana.** To construct transgenic *N. benthamiana* plants which constitutively expressed (+)-PSTVd RNA, ribozyme cassettes containing either PSTVd-Int or PSTVd-P cDNA were excised from pRZ6-2 (or an analogous plasmid) by digestion with EcoRI and *Hind* III and recloned in plasmid pGEM-7Zf (+) (Promega). After removal of the unique *Hind* III site from the resulting plasmid by cleavage with *Hind* III and Klno-sequence-filled-in, the PSTVd-ribozyme cassette was excised by digestion with *Nhe* I and *Xba* I. After partial fill-in of its termini with dCTP and dTTP, the resulting fragment was transferred to the partially filled *Hind* III site of pGA643 (An et al., 1988). The resulting recombinant plasmid, from which transcription driven by the cauliflower mosaic virus (CaMV) 35S promoter will produce a (+)-PSTVd-P RNA flanked by active ribozyme sequences in planta, was directly transformed into competent *Agrobacterium tumefaciens* (strain 5922) containing the helper plasmid pC2760 (Gallie et al., 1984). Protocols for the *Agrobacterium*-mediated transformation of *N. benthamiana* leaf tissue and identification of transformed plants by PCR have been described elsewhere (Hammond & Kamo, 1995; McFarley & Kaper, 1991).

**Analysis of PSTVd RNAs synthesized in vitro.** Temperature gradient gel electrophoresis (TGGE) analysis of 32P-labelled circular and linear PSTVd RNAs was carried out as previously described (Owens et al., 1995). The 5% w/v polyacrylamide gel and buffer reservoirs contained 0.2 × TBE, 5 mM NaCl. Approximately equimolar mixtures of wild-type and mutant PSTVd RNAs (ca. 40–60 000 c.p.m. each) were applied to the single 12 cm sample slot. Following electrophoresis, the gel was fixed in 10% ethanol, 1% acetic acid and dried before analysis in a GS-363 Molecular Imager (Bio-Rad).

**Analysis of PSTVd-related RNAs synthesized in vivo.** Total RNA was extracted from leaf tissue samples (0.1 g) collected from individual transgenic *N. benthamiana* plants using the TRI reagent (Molecular Research Center) and quantified by UV spectrophotometry. Concentrations of PSTVd-related RNAs in individual RNA preparations
were estimated by dot-blot hybridization as described above. For Northern blot analysis, aliquots (15 µl) containing 40–60 pg (+)PSTVd RNA were mixed with an equal volume of loading buffer (90% formamide, 10 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol), heated for 3 min at 94 °C, and chilled on ice before electrophoresis on 5% (w/v) polyacrylamide gels containing 1× TBE buffer, 8 M urea. After electrophoresis, the RNAs were electrotransferred to Nytran Plus membranes (Schleicher & Schuell), UV-crosslinked, and hybridized with digoxigenin-labelled, strand-specific RNA probes.

As shown in Table 2, six pairs of primers were used to amplify sequences derived from different portions of PSTVd-related nucleic acids by PCR. Binding sites for primers HH-1 (5’ GGGAATTCTGCAG- GGGGATCTGATGAGTCCGTGA 3’) and PC-2 (5’ GTACCAAGTG- AATATACCAAACGTGTGTTTCTCTG 3’) are located in the ribozymes flanking the PSTVd cDNA. For RNA analyses, total cellular RNA was treated with RNase-free DNase to remove contaminating plant enzymes. For RNA analyses, total cellular RNA was treated with RNase-free DNase to remove contaminating plant enzymes flanking the PSTVd cDNA. For RNA analyses, total cellular RNA was treated with RNase-free DNase to remove contaminating plant enzymes flanking the PSTVd cDNA. For RNA analyses, total cellular RNA was treated with RNase-free DNase to remove contaminating plant enzymes flanking the PSTVd cDNA. For RNA analyses, total cellular RNA was treated with RNase-free DNase to remove contaminating plant enzymes.

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Expression of PSTVd-related RNAs in transgenic *N. benthamiana*.

(A) Dot-blot analysis of leaf sap samples prepared from groups of ten independently transformed plants (columns 1–10) which constitutively express one of four PSTVd-specific RNAs [precisely-full-length (+)PSTVd-Int (row a), (−)PSTVd-Int (row b) or (+)PSTVd-P (row c) RNAs, or a truncated RNA containing only 16 nt of PSTVd-specific sequence derived from the upper portion of the central conserved domain (row d)]. Sap samples were prepared 2 weeks after transfer of the plants to soil and hybridized with an RNA probe specific for (+)PSTVd. PC, total RNA isolated from PSTVd-infected tomato; NC, comparable sample from mock-inoculated tomato. (B) Dot-blot analysis of leaf sap from five independent (+)PSTVd-P transformants (columns 1–5) collected 2 weeks (row a) and 8 weeks (row b) after transfer to soil. All samples contained equal volumes of clarified leaf sap.

Fig. 2. Expression of PSTVd-related RNAs in transgenic *N. benthamiana*. (A) Dot-blot analysis of leaf sap samples prepared from groups of ten independently transformed plants (columns 1–10) which constitutively express one of four PSTVd-specific RNAs [precisely-full-length (+)PSTVd-Int (row a), (−)PSTVd-Int (row b) or (+)PSTVd-P (row c) RNAs, or a truncated RNA containing only 16 nt of PSTVd-specific sequence derived from the upper portion of the central conserved domain (row d)]. Sap samples were prepared 2 weeks after transfer of the plants to soil and hybridized with an RNA probe specific for (+)PSTVd. PC, total RNA isolated from PSTVd-infected tomato; NC, comparable sample from mock-inoculated tomato. (B) Dot-blot analysis of leaf sap from five independent (+)PSTVd-P transformants (columns 1–5) collected 2 weeks (row a) and 8 weeks (row b) after transfer to soil. All samples contained equal volumes of clarified leaf sap.

Expression of PSTVd-P RNA in transgenic plants

As part of efforts to measure the frequency of viroid RNA recombination *in vivo*, we constructed a number of transgenic *N. benthamiana* lines designed to constitutively express precisely-full-length (+)PSTVd-P RNA. As shown in Fig. 2(A), initial dot-blot analysis of the original (+)PSTVd-P transformants yielded only a weak hybridization signal, much weaker than those from plants designed to express comparable (+)PSTVd-Int or (−)PSTVd-Int RNAs (compare row c with rows a and b). When, however, these (+)PSTVd-P plants were retested 4–5 weeks later, the amounts of PSTVd-related RNA present in two plants had increased dramatically. As shown in Fig. 2(B), the signals produced by those two plants appeared very similar in intensity to those from plants which constitutively express wild-type PSTVd RNA of either polarity.

To determine the nature of the viroid-related RNAs present in these plants, total cellular RNA extracted from two plants giving strong hybridization signals 8 weeks post-infection and three plants where the signal intensity remained low was subjected to Northern blot analysis. As shown in Fig. 3, the amounts of circular and linear PSTVd RNA in the two plants producing strong hybridization signals were very similar to those in plants which constitutively express (+)PSTVd-Int RNA (compare lanes 1 and 2 with lane 6). In plants where the signal intensity remained low (i.e. lanes 3–5), the only PSTVd-related RNAs detected were partially processed molecules containing either (+)PSTVd-P plus hammerhead ribozyme...
Table 2. PCR-mediated analysis of PSTVd-related RNAs isolated from (+)PSTVd-P transgenic plants

<table>
<thead>
<tr>
<th>Primers</th>
<th>(+)PSTVd-P transgene*</th>
<th>PCR products†</th>
<th>(+)PSTVd-int</th>
<th>(+)PSTVd-P</th>
<th>genomic DNA</th>
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<tr>
<td>a</td>
<td>HH (88) 160 232 304 17 87 PC</td>
<td>†</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>b</td>
<td>RAO-2 (96-116)</td>
<td>(263-282) RAO-2</td>
<td>(212 bp)</td>
<td>+</td>
<td>+</td>
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<td>RAO-2 (263-282)</td>
<td>RAO-2 (279 bp)</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>d</td>
<td>RAO-14 (88-109)</td>
<td>RAO-34 (278 bp)</td>
<td>(530-552)</td>
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<tr>
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<td>RAO-34 (530-552)</td>
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<td>−</td>
<td>+</td>
</tr>
<tr>
<td>f</td>
<td>HH-1 (435 bp)</td>
<td>PC-2</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
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</table>

* Numbers above the (+)PSTVd-P transgene with its flanking hammerhead (HH) and paperclip (PC) ribozymes refer to nucleotide positions in PSTVd RNA. Numbers above or below each primer indicate the location of their specific binding site.
† PCR products were generated from either genomic DNA isolated from a (+)PSTVd-P transgenic plant or cDNA synthesized from total RNA extracted from one (+)PSTVd-Int and four (+)PSTVd-P transgenic plants. As shown in Figs 2 and 3, two of these (+)PSTVd-P plants showed a strong positive reaction (+++ during dot-blot hybridization; the others were only weakly positive (+/−).
‡ The presence (+) or absence (−) of a PCR product of the expected size is indicated for each combination of template and primers. Note that products were obtained from PCR reactions containing (CD-1 + RAO-2) or (RAO-2 + RAO-33) only when the cDNA synthesis reaction contained a circular PSTVd RNA template.

The nature of the PSTVd-related RNA sequences present in the transgenic plants was further examined by RT–PCR. Binding sites for the six primer pairs used in these analyses are shown in the central portion of Table 2, and the results obtained for each template (i.e. five cDNAs plus one preparation of PSTVd-P genomic DNA) are summarized in the columns to the right. Primers HH-1 and PC-2 are complementary to sequences in the ribozymes flanking the PSTVd-P cDNA and were used to verify that the PstI site originally present in the PSTVd-P cDNA had been stably maintained in the genomic DNA of our transgenic plants. Amplification of genomic DNA isolated from (+)PSTVd-P transgenic plants using primers HH-1 and PC-2 led to formation of a product of the expected size (i.e. 435 bp; see Table 2 row f), and the two fragments released by subsequent digestion with PstI were indistinguishable from those released by digestion of PCR products derived from the Agrobacterium strain used for plant transformation (results not shown). The absence of genomic DNA from reactions where randomly primed cDNAs were used as templates confirmed the ability of DNase treatment to destroy any genomic DNA contaminating our preparations of total cellular RNA.

The termini of the full-length PSTVd RNA released by ribozyme cleavage are located between the binding sites for primers CD-1 + RAO-2 (Table 2, row a) and RAO-2 + RAO-33 (Table 2, row b). Failure of RT–PCR reactions containing these primer pairs to give products of the predicted size thus confirmed the absence of circular (+)PSTVd-P RNA in plants containing only low levels of PSTVd-related RNA. Finally, results presented in Table 2, row c–f also showed that these plants contained few, if any, unprocessed (+)PSTVd-P RNA transcripts. While reactions containing primers RAO-2 + PC-2 (Table 2, row c) or HH-1 + RAO-34 (Table 2, row e) yielded products derived from partially processed (+)PSTVd-P transcripts, a product was obtained with primers HH-1 + PC-2 only when genomic DNA was used as template (Table 2, row f).
Potential structural effects of spontaneous mutations associated with PSTVd-P. Portions of the (+) strand (A) and (-) strand structures (B) surrounding the initial mutations at positions 2, 4 and 6 are shown. For (+)PSTVd-P, both the rod-like native structure (Gross et al., 1978) and an alternative branched structure (Gast et al., 1996) are shown. The structure of (-)PSTVd-P is based on that proposed by Hecker et al. (1988).

Stability of individual mutations in vivo

Several of the PCR products derived from (+)PSTVd-P RNAs were subjected to a combination of restriction enzyme digestion and sequence analysis in order to monitor the stability of the individual mutations. Products obtained with primers RAO-2 + PC-2 (see Table 2, row c) were cleaved by PstI, thereby indicating that all three changes were stably maintained in transcripts from plants which contained only limited amounts of PSTVd-related RNA. PCR products derived from plants containing high levels of PSTVd-related RNAs (i.e. Table 2, rows a and b), in contrast, were not cleaved by PstI. As shown in Fig. 4(A), sequence analysis revealed that the A → C substitution at position 4 in the initial RNA transcript had been replaced by a G residue in the progeny. Progeny from two infected plants were analysed, and the results were consistent.

Progeny recovered after mechanical inoculation of tomato seedlings with mutants 135G and 43G-310–311UU were also subjected to PCR-mediated sequence analysis. As shown in Table 1, only one plant became systematically infected after inoculation with mutant 43G-310–311UU, even when the inoculum pressure was extremely high. Sequence analysis indicated that the progeny isolated from that plant were wild-type (i.e. PSTVd-Int). Mutant 135G contains only a single A → G substitution, yet it was & 1000-fold less infectious than PSTVd-Int. Somewhat surprisingly, sequence analysis indicated that this mutation was stably maintained in progeny isolated from at least one infected plant. These molecules did contain what appears to be a ‘compensatory’ G → A change at position 134, however.

Discussion

Previous mutational analyses of PSTVd have yielded a number of weakly infectious sequence variants. While certain mutations appear to exert their phenotypic effects by disrupting alternative structural interactions (e.g. Qu et al., 1993; Baumstark & Riesner, 1995), most appear to act within the context of the rod-like native structure. Examples include (i) mutations within the pathogenicity domain which affect symptom expression (Owens et al., 1996), (ii) changes in the right terminal loop that inhibit replication and/or cell-to-cell...
Destabilization of PSTVd

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References


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