Mutational analysis does not support the existence of a putative tertiary structural element in the left terminal domain of *Potato spindle tuber viroid*

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Comparative sequence analysis suggests that the left terminal domain of *Potato spindle tuber viroid* (PSTVd) and other large pospiviroids may assume a branched tertiary structure containing two pseudoknots. To search for evidence of such a structure *in vivo*, the nucleotide sequences proposed to interact were mutagenized, tomato seedlings were inoculated with mixtures of potentially infectious PSTVd RNA transcripts and the resulting progeny were screened for compensatory sequence changes. Positions 6–11 and 330–335 tolerated only limited sequence variation, and compensatory changes consistent with formation of an intact pseudoknot were observed in only two of the plants examined. No variation was detected at positions 14–16 or 29–31. Passage of selected variants in Rutgers tomato led to an increase in virulence only upon reversion to wild-type PSTVd Intermediate. The ability of the left terminal domain to assume a branched conformation containing pseudoknots does not appear to be an important determinant of PSTVd fitness.

Viroids are the smallest autonomously replicating pathogens yet described – they are small (246–399 nt), unencapsidated, circular RNAs that lack mRNA activity, yet are able to induce a wide range of disease symptoms in susceptible plant hosts (Hadidi *et al.*, 2003). The 29 viroid species described to date can be allocated to one of two families: *Potato spindle tuber viroid* (PSTVd) and other members of the family *Pospiviroidae* have an unbranched lowest free-energy structure with a central conserved region, do not exhibit ribozyme activity and replicate in the nucleus; members of the family *Avsunviroidae* lack a central conserved region, exhibit ribozyme activity and replicate in the chloroplast. Most avsunviroids have a branched structure.

In addition to the rod-like native conformation (Fig. 1a), PSTVd can also form a variety of other structures, at least some of which have been shown to have biological relevance [e.g. secondary hairpin II (Qu *et al.*, 1993) and a ‘tetraloop motif’ within the central conserved region that is recognized by a host nuclease activity during replication (Baumstark *et al.*, 1997)]. As discussed by Woese & Pace (1993), comparative sequence analysis is the most reliable method to establish higher-order RNA structure; this approach was used 20 years ago to identify five structural/functional domains within PSTVd and related viroids (Keese & Symons, 1985). Based upon a combination of chemical/enzymic probing and comparative sequence analysis, Gast and colleagues (Gast *et al.*, 1996; Gast, 2003) subsequently suggested that one of these domains (the left terminal domain) may have a branched structure. Characterization of the left terminal domain of PSTVd by nuclear magnetic resonance and thermodynamic analysis *in vitro*, however, has clearly shown the concentration of the branched conformation to be <1 % of that of the rod-like, elongated form (Dingley *et al.*, 2003). The mutational analysis described below was designed to search for evidence of a branched conformation *in vivo*.

As shown in Fig. 1(b), the ability of the left terminal domain of PSTVd and related viroids to fold into a branched conformation is due to the presence of a ‘terminal conserved repeat’, two pairs of complementary hexanucleotides that flank the left terminal loop (Stasys *et al.*, 1995). Nearby (and denoted by a combination of empty and filled symbols) are two additional pairs of shorter sequences that covariation analysis suggests may interact to form a double pseudoknot (Gast, 2003). If such interactions do, indeed, occur *in vivo*, the introduction of mutations into one sequence would be expected to result in the appearance of compensatory change(s) in its complement. Table 1 summarizes our mutagenesis strategy and the results from a series of four bioassays. Experiments 1–3 were screening assays in which groups of 20 Microtom seedlings were inoculated with mixtures of RNA transcripts containing 48–3072 potentially infectious PSTVd sequences; in experiment 4, groups of five Rutgers
seedlings were inoculated with selected variants recovered from experiment 2 to detect the possible appearance of compensatory mutations. Unlike Rutgers and other sensitive cultivars, Microtom tomatoes do not respond to PSTVd infection with the appearance of severe stunting and epinasty.

Wild-type PSTVd_Int(emediate) was eliminated from the inoculum in experiments 1–3 by replacing one or more residues with an equimolar mixture of the other three nucleotides (Owens et al., 2003). Infected plants were identified by dot-blot hybridization (Podleckis et al., 1993) and the progeny from individual plants were characterized by RT-PCR followed by automated sequence analysis of uncloned PCR products (Owens et al., 2003). Chromatograms were examined for signs of sequence heterogeneity, indicating the need to sequence collections of cloned cDNAs derived from individual infected plants. No evidence of significant heterogeneity was observed.

As shown in Fig. 1(b), pseudoknot 1 involves complementary GUG and CAC sequences located at PSTVd positions 14–16 and 29–31. The close proximity of these two sequences allowed them to be mutagenized by using a single pair of degenerate primers, resulting in a mixture of 3072 potentially infectious sequences, each lacking the A residue found at position 30 in wild-type PSTVd_Int. One-third of these transcripts contained the same A/U substitution at position 30 that was noted by Gast (2003) in his covariation analysis of large pospiviroids; nevertheless, none of the resulting progeny contained a compensatory G/A substitution at position 15. Fifteen of 16 infected plants contained wild-type PSTVd_Int, and the only variant contained a single, spontaneous A/G substitution at position 271 (results not shown).

Similar results were obtained when both sequences forming pseudoknot 2 were mutagenized simultaneously. In this case, covariation analysis of large pospiviroids; nevertheless, none of the resulting progeny contained a compensatory G/A substitution at position 15. Fifteen of 16 infected plants contained wild-type PSTVd_Int, and the only variant contained a single, spontaneous A/G substitution at position 271 (results not shown).
### Table 1. PSTVd variants recovered after mutagenesis of the left terminal domain

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<th>Progeny*</th>
<th>Targeted positions†</th>
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<tr>
<td>PSTVd-Int Mutant(s)</td>
<td>6 7 8 9 10 11 330 331 332 333 334 335</td>
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*Data are expressed as no. infected plants containing individual sequence variants. In experiments 1–3, each treatment contained 20 plants; in experiment 4, treatments contained either five (variant c) or 10 (variants a, b and d) plants.

†The sequence of PSTVd_Int is provided as reference. Positions targeted for mutagenesis are shown in bold (D = A + G + U; H = A + C + U; N = A + C + G + U). Sequences of progeny arising from individual inocula are boxed.

‡Variants a–d were isolated in experiment 2 and subsequently passaged in Rutgers tomato (experiment 4) to monitor the possible appearance of compensatory mutations (in square brackets).

§Variants containing compensatory mutations that restore Watson–Crick base pairing completely in pseudoknot 2.

334 and 7, is consistent with a possible tertiary interaction involving positions 330–335 (GUUUAG) and 6–11 (CUAAAC) (Gast, 2003). To reduce the complexity of the mutagenized inoculum, only three positions in each hexanucleotide were mutagenized, resulting in a mixture of 48 × 48 = 2304 potentially infectious variants. As shown in Table 1 (see experiment 1), sequence analysis of the resulting progeny revealed that all 10 infected plants contained wild-type PSTVd_Int.

The sequences possibly interacting to form pseudoknot 2 lie far enough from one another that two pairs of degenerate primers were required for mutagenesis. When simultaneous mutagenesis of positions 6–11 and 330–335 failed to yield informative sequence variants, we decided to examine the effect of mutagenizing just one sequence at a time. The resulting bioassay (experiment 2) yielded several informative sequence variants. Mutagenesis of positions 6, 8 and 10 yielded a single novel variant, a sequence in which the C residue at position 6 was replaced by a U. The resulting conversion of a G : C to a G : U base pair should have little effect on the stability of either the rod-like or branched structure of PSTVd, but the weakening of a single G : C pair could have a much greater destabilizing effect on pseudoknot 2, which contains only six base pairs. Mutagenesis of positions 331, 333 and 335 also resulted in several sequence changes (including a spontaneous A/G change at position 334) that could weaken pseudoknot 2.
Having demonstrated the possibility of individually mutating each partner in this potential tertiary interaction, we tried once again to find evidence for sequence covariation in pseudoknot 2. Starting with PSTVd_Int, we first introduced the U/A and G/U changes at positions 333 and 335 that were present in many of the progeny recovered from experiment 2, and then randomized positions 6–10 as before. Data presented in Table 1 (see experiment 3) revealed that 15 of 20 inoculated plants became infected – none with wild-type PSTVd. Each variant retained the U/A and G/U changes at positions 333 and 335 that were present in the inoculum; furthermore, changes consistent with the potential interaction of positions 330–335 and 6–11 were also detected at positions 6 and 8.

In order to completely restore Watson–Crick pairing with the modified GUAAU sequence at positions 330–335, new variants would be expected to contain an A/U substitution at position 8 and either a C/A or a C/G substitution at position 6. Both of these variants were recovered from single infected plants, but neither was the predominant sequence in the population. Note, however, that the variants recovered from a majority of the plants examined (eight of 15) contained a C/U substitution at position 8. One effect of this change is to recreate a pseudoknot 2 containing five rather than six contiguous base pairs. At best, the observed pattern of changes was only partially consistent with pseudoknot 2 formation.

The appearance of spontaneous sequence changes during passage of viroid mutants in vivo often signals a significant loss of fitness by the initial mutant (Góra-Sochacka et al., 1997). Although many such spontaneous changes appear to be neutral in nature (Matoušek et al., 2001), others restore important interactions disrupted by the initial mutagenesis [e.g. secondary hairpin II (Qu et al., 1993)]. Thus, if formation of pseudoknot 2 was essential for PSTVd replication, one would expect at least some spontaneous changes to restore base pairing between nt 6–11 and 330–335. In experiment 4, groups of five Rutgers tomato seedlings were inoculated with PSTVd variants recovered from our initial mutant screens and then monitored for changes in symptom expression consistent with an increase in viroid fitness. Fig. 2 contrasts the appearance of representative plants from this bioassay and sequence changes detected in the progeny are summarized in Table 1 (see experiment 4).

Two of the variants tested contained single nucleotide changes, a third contained two changes and the fourth variant contained three changes. Of the 25 seedlings that became infected after inoculation with preparations of total RNA recovered from experiment 2, only two plants inoculated exhibited symptoms as severe as those in the control plants inoculated with PSTVd_Int. As shown in Fig. 2, the vast majority (23 of 25) of infected plants showed only mild symptoms, and sequence analysis of uncloned full-length RT-PCR products revealed that both severely affected plants contained PSTVd_Int. Reversion to wild-type provides no information about the existence/non-existence of pseudoknot 2; thus, we also determined the sequences of progeny isolated from a number of

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**Fig. 2.** Effect of spontaneous sequence changes on PSTVd symptom expression. The two plants in the middle of the panel were inoculated with a variant of PSTVd_Int containing two targeted substitutions at positions 333 and 335 that are predicted to weaken pseudoknot 2, plus a spontaneous change at position 4 (see Table 1). Five weeks post-inoculation, sequence analysis revealed that the severely diseased plant on the right contained wild-type PSTVd_Int. The progeny isolated from the plant on the left showing milder symptoms, in contrast, had undergone a more complex series of changes that only partially repaired the pseudoknot. Far left, uninoculated control; far right, plant inoculated with wild-type PSTVd_Int.
other, randomly chosen plants. Data presented in Table 1 (see experiment 4) revealed all but the triple mutant to be reasonably stable. For this variant, reversion at position 333 restores one of the central base pairs in pseudoknot 2, whilst the appearance of a new A/G transition at position 334 weakens an adjacent A:U pair.

Although the Subviral RNA Database (http://subviral.med.uottawa.ca) now contains the sequences of nearly 100 different PSTVd variants, virtually all variability is located outside portions of the left terminal domain that are potentially involved in pseudoknot formation. Thus, the covariation analysis of Gast (2003) was based entirely on comparisons of PSTVd with related pospiviroid species. Our mutational analysis represents the first attempt to obtain direct experimental evidence for pseudoknot formation in PSTVd. As described above, we were able to generate sequence diversity affecting only pseudoknot 2, a putative Watson-Crick pairing between PSTVd positions 6–11 (CUAAC) and 330–335 (GUUUAG). Pseudoknot 2 is the least conserved of the two potential pseudoknots and comparable interactions in the other large pospiviroids involve as few as three and as many as nine base pairs (Gast, 2003).

Structural calculations (Lück et al., 1999) suggest that several of our mutagenesis-induced sequence changes could facilitate formation of pseudoknot 2 indirectly by destabilizing the rod-like structure of the left terminal domain (results not shown). Only two variants, however, contained all of the compensatory changes necessary to maintain Watson-Crick base pairing completely in pseudoknot 2. We note that these compensatory changes did not appear spontaneously during passage of the initial mutant, but required a second round of in vitro mutagenesis. Overall, the pattern of mutagenesis-induced and spontaneous sequence changes observed provides little support for the existence of pseudoknot 2.

Pseudoknot 1, a second, more highly conserved potential interaction between PSTVd positions 14–16 and 29–31, did not respond to mutagenesis. Several explanations for such a result are possible, but the failure to recover even a single variant is surprising. Several large pospiviroids, including the closely related Citrus exocortis viroid and Tomato apical stunt viroid, contain a central U:A rather than an A:U base pair, and the reappearance of wild-type PSTVd_Int in 15 of 16 infected plants indicates that there was ample opportunity for sequence evolution/seletion in vivo. A recent report (Wang et al., 2004) suggests that RNA silencing exerts considerable pressure on viroids and viral satellites to maintain a compact, highly base-paired structure. Our results, together with those from in vitro structural studies with model RNAs (Dingley et al., 2003), suggest strongly that the left terminal domain of PSTVd does not assume a branched or folded conformation containing pseudoknots in vivo.

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References


