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Mutational Analysis of Viroid Pathogenicity: Tomato Apical Stunt Viroid

Robert A. Owens

Microbiology and Plant Pathology Laboratory, Plant Sciences Institute, USDA-ARS Beltsville, MD 20705 U.S.A.
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A series of nucleotide substitutions within the pathogenicity domain of tomato apical stunt viroid have been evaluated for their effects upon infectivity and symptom expression. None of the 12 A→G substitutions and one C→U substitution that were examined abolished infectivity in a whole plant bioassay, and the resulting progeny were characterized by nucleotide sequence analysis of cDNAs amplified by the polymerase chain reaction. Four of the 13 substitutions gave rise to altered progeny, but the patterns of sequence changes observed were unexpectedly complex. Mutations that did not rapidly revert to the wild-type sequence are located near the right border of the pathogenicity domain, a region which shows considerable natural sequence variability. None had a detectable effect upon symptom expression. The ability to observe viroid sequence evolution *in vivo* may provide insight into the molecular interactions responsible for viroid host range and symptom formation.

Additional keywords: infectious cDNA, pathogenesis.

Viroids are the smallest known agents of infectious disease; they are small (246 to 375 nucleotides), single-stranded, circular RNA molecules that are unencapsidated and lack detectable messenger activity. Although viroids replicate autonomously, they appear to be completely dependent upon host-encoded functions for replication. The combination of their small size and the existence of extensive nucleotide sequence data make viroids prime candidates for molecular studies of host-pathogen interaction. Thus far, viroids have only been found associated with higher plants (see Diener 1987 for a collection of recent reviews).

Comparative sequence analysis suggests that viroids contain five structural and functional domains (Keese and Symons 1985). Sequence changes within one of these domains, the pathogenicity domain in the left side of the rodlike native structure, appear to modulate symptom expression in potato spindle tuber viroid (PSTV) and citrus exocortis viroid (CEV) (Visvader and Symons 1985; Schnölzer *et al.* 1985). Although two naturally occurring strains of tomato apical stunt viroid (TASV), a third member of the "PSTV group," share only 91% sequence similarity, both induce very similar severe symptoms in tomato (Owens *et al.* 1990).

The infectivity of both longer-than-full-length viroid cDNAs (Cress *et al.* 1983) and their respective RNA transcripts (Ohno *et al.* 1983) allows viroid pathogenicity to be studied by a variety of site-directed mutagenesis techniques. This report describes the effect of intensive mutagenesis within the pathogenicity domain of TASV upon the viroid's ability to replicate and move systemically in tomato. None of the mutations tested was lethal, but only four were even partially stable *in vivo*. Changes that did not rapidly revert to the wild-type sequence are located in the right border of the pathogenicity domain, a region which shows considerable natural sequence variability.

MATERIALS AND METHODS

Viroid strains and infectious cDNA clones. Kiefer *et al.* (1983) have described the construction of a full-length (360-base pair), infectious cDNA derived from the Ivory Coast strain of TASV; the corresponding 363-base pair cDNA derived from the Indonesian strain of TASV was cloned and sequenced by Candresse *et al.* (1987). Both cDNAs have *Bam*HI termini derived from the upper portion of the central conserved region, and when cloned in the *Bam*HI site of pUC9 or related plasmids, both are flanked by the 11-nucleotide sequence duplication (GGATCCCCGGG) shown by Tabler and Sänger (1984) to be essential for infectivity. Although these two strains of TASV have quite distinct nucleotide sequences (91% sequence similarity), both elicit very similar severe symptoms in tomato (*Lycopersicon esculentum* Mill. cv. Rutgers).

Isolation and characterization of altered TASV cDNAs. A full-length cDNA from the Ivory Coast strain of TASV with *Bam*HI termini (clone pASB3; Owens *et al.* 1986) was transferred from its pUC9 vector to the phagemid vector pBS+ (Stratagene, La Jolla, CA) using standard techniques (Maniatis *et al.* 1982). The viroid-specific sequence in the resulting single-stranded pBS+ recombinant has the same polarity as TASV. Uracil-containing, single-stranded phage DNA was isolated from *Escherichia coli* CJ236 and used as the template for oligonucleotide-directed mutagenesis essentially as described by Kunkel (1987).

A 15-fold degenerate oligonucleotide primer complementary to TASV nucleotides 41–94 was used to introduce single A→G substitutions throughout the pathogenicity domain. During the synthesis of the oligonucleotide primer (5′-GATCCCTGAAGGACTTCTTCCTTCCCGCTCCTA

TCTTTTTTCTTGATGCCTGCA-3′), a mixture of 93% thymine and 7% cytosine was substituted for the wild-type monomer (T) at the boldfaced positions. The resulting pool of oligonucleotide primers favors the introduction of single A→G substitutions over either multiple or no mutations (Derbyshire *et al.* 1986) and was purified by denaturing polyacrylamide gel electrophoresis prior to use.

Altered TASV cDNAs were identified by temperature gradient gel electrophoresis and silver staining (Riesner *et al.* 1989). After digestion of recombinant plasmids with *Bam*HI to release their full-length TASV cDNA inserts, appropriate aliquots (approximately 0.5 μg) of each digest were mixed with an equal volume of 9 M urea, 0.2× TBE buffer (17.8 mM Tris, 17.8 mM boric acid, 0.48 mM EDTA) and applied to a 5% polyacrylamide gel containing 0.2× TBE buffer and 6 M urea. Electrophoresis in the presence of a 35–55° C gradient was conducted for 4.75 hr at 200 V. Where appropriate, approximately 0.3 μg of wild-type TASV cDNA was added to each putative mutant digest as an internal standard. The number and location(s) of nucleotide substitutions in individual TASV cDNA clones were determined by a dideoxy chain termination sequencing protocol (Korneluk *et al.* 1985) using a 5′-^{32}P-labeled primer complementary to TASV nucleotides 83–100 (that is, RAO1, 5′-CCCGGGGATCCCTGAAGG-3′).

Bioassay of altered TASV cDNAs. The infectivities of the various TASV cDNA constructions were determined as described elsewhere (Cress *et al.* 1983; Hammond *et al.* 1989), using either *Bam*HI-digested plasmid DNAs or (+) strand RNAs synthesized by T3 RNA polymerase as inocula. Aliquots of the inoculum (approximately 2–3 μg of DNA or 1–2 μg of RNA in 10 μl of 20 mM sodium phosphate, pH 7.0) were rubbed on the Carborundum-dusted cotyledons of 1-wk-old Rutgers tomato seedlings with a sterilized glass rod. Inoculated plants were maintained in a greenhouse under conditions suitable for viroid replication and symptom development. Samples collected from the uppermost leaves 3–4 wk later were assayed for the presence of progeny by molecular hybridization (Owens and Diener 1984) using a full-length RNA probe for TASV. Plants that initially tested negative were retested 2–3 wk later.

Characterization of viroid progeny. Viroid progeny were isolated from frozen leaf tissue by phenol-chloroform extraction and LiCl fractionation as previously described (Owens and Diener 1984). Protocols for ribonuclease mapping of viroid RNAs and sequence analysis of polymerase chain reaction (PCR)-amplified viroid cDNAs using *Taq* DNA polymerase have been described elsewhere (Owens *et al.* 1990; Carothers *et al.* 1989). Binding sites for RAO2 (5′-GCGGATCCGGTGGAAACAACTGAAGC-3′) and Had3 (5′-CTCCAGGTTTCCCCGGG-3′), the primers used for PCR amplification and sequence analysis of TASV cDNAs, are shown in Figure 1A.

Calculation of RNA secondary structures. RNA structures having minimal free energies were calculated as described by Zuker (1989) using a Digital AI VAX station (Digital Equipment Corporation, Maynard, MA). The folding model underlying the computer algorithm takes into account free-energy increments for single-base stacking at the ends of helices and for terminal mismatched pairs within interior and hairpin loops.

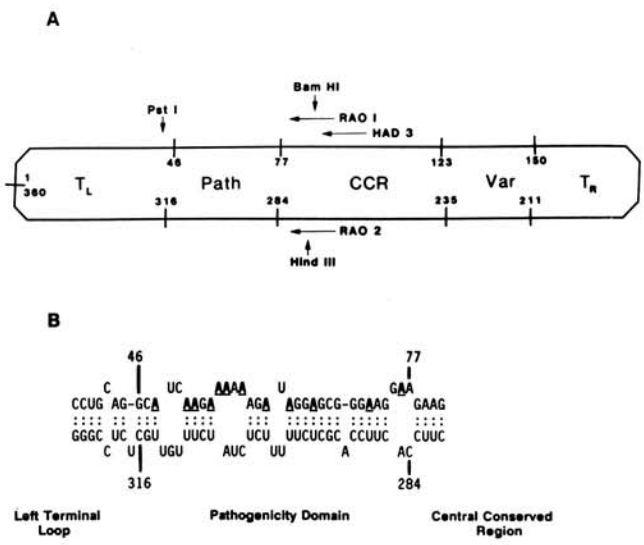

Fig. 1. Structural organization of the Ivory Coast strain of tomato apical stunt viroid (TASV). **A**, Boundaries of the left terminal loop (T_L), right terminal loop (T_R), central conserved region (CCR), pathogenicity (Path) domain, and variable (Var) domain are indicated within the rodlike native structure of TASV Ivory Coast. *Bam*HI, *Pst*I, and *Hind*III are restriction sites used to manipulate the various recombinant plasmid DNAs. HAD3, RAO1, and RAO2 are the oligonucleotide primers used for amplification of viroid cDNA and nucleotide sequence analysis. **B**, The lowest free-energy structure of the pathogenicity domain of TASV. Locations of the 12 individual A→G substitutions introduced by oligonucleotide-directed mutagenesis are underlined.

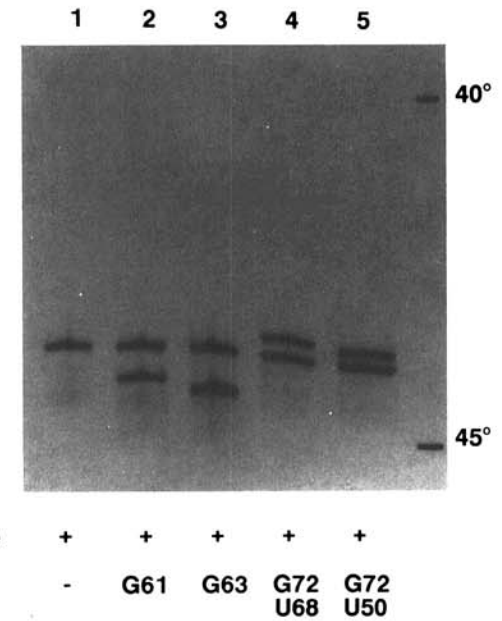

Fig. 2. Analysis of double-stranded tomato apical stunt viroid (TASV) cDNAs by temperature gradient gel electrophoresis. Appropriate aliquots of *Bam*HI-digested plasmid DNAs were adjusted to 4.5 M urea, 0.1× TBE buffer before electrophoresis in the presence of a parallel 35–55° C temperature gradient. Lane 1 contains only wild-type TASV Ivory Coast cDNA, while lanes 2–5 contain mixtures of wild-type TASV Ivory Coast and the various mutant TASV cDNAs: lane 2, A61→G; lane 3, A63→G; lane 4, A72→G and C68→U; and lane 5, A72→G and C50→U.

RESULTS

Mutagenesis within the pathogenicity domain of TASV. Figure 1A illustrates the spatial relationship between the pathogenicity domain of TASV and the adjacent left terminal loop and central conserved region; Figure 1B shows a secondary structure for the TASV pathogenicity domain that is compatible with those proposed for various isolates of PSTV by Schnölzer *et al.* (1985). This computer-generated lowest free-energy structure contains several short helical regions separated by internal or bulge loops.

Because rather subtle changes in the predicted structure of the corresponding regions in PSTV and CEV are associated with dramatic differences in symptom expression (Schnölzer *et al.* 1985; Visvader and Symons 1985), introduction of single A→G substitutions throughout the upper portion of the TASV pathogenicity domain seemed likely to produce a variety of interesting biological effects. Therefore, a collection of TASV cDNAs containing the desired substitutions was prepared using a mutagenesis strategy based on that of Derbyshire *et al.* (1986). A comparatively new technique, temperature gradient gel electrophoresis, was used to identify altered TASV cDNAs prior to nucleotide sequence analysis and bioassay.

The ability of temperature gradient gel electrophoresis to resolve full-length double-stranded TASV cDNAs that differ by only a single base pair exchange is documented in Figure 2. Compared to the wild-type TASV cDNA, mutant cDNAs containing A→G substitutions at positions 61 or 63 are more stable and migrated further into the gel before denaturation began to slow their migration (compare lanes 2 and 3 with lane 1 of Fig. 2). In addition to an A→G substitution at position 72, the two TASV cDNAs analyzed in lanes 4 and 5 of Figure 2 each contain single C→U substitutions elsewhere in the pathogenicity domain. In both cases, the overall effect of the double substitution was a slight destabilization of the mutant cDNA.

Infectivity studies with altered TASV cDNAs. Infectivities of the 12 altered TASV cDNAs were evaluated in a series of five tomato bioassays using both intact plasmid DNAs and the corresponding "plus" sense *in vitro* RNA transcripts as inocula. In contrast to previous studies with point mutations in PSTV (Hammond and Owens 1987; R. Hammond, personal communication), not one of the mutations tested could be classified as lethal. Each TASV mutant was infectious in at least one bioassay, and RNA transcripts were more consistently infectious than intact plasmid DNAs. Thus, only three of the five mutants that rapidly and reproducibly produced progeny when inoculated as RNA transcripts were infectious when intact plasmid DNA was used as inoculum (that is those containing substitutions at positions 61, 63, or 66). For the remaining seven mutants, progeny appearance was either delayed or erratic. In all experiments, the mutant cDNA containing an A→G substitution at position 72 also contained a C→U substitution at position 68.

Characterization of TASV progeny. Initial attempts to characterize the progeny derived from the mutant TASV cDNAs used a ribonuclease-mapping protocol that had been developed to characterize novel TASV-TASV and CEV-TASV chimeras (Owens *et al.* 1990). A series of 12 ^{32}P-labeled RNA probes complementary to the various mutants was prepared for these analyses by transcription of the mutant TASV cDNAs with T7 polymerase, but unambiguous results could not be obtained (data not shown).

Fortunately, however, the same PCR primers used to amplify and sequence single-stranded viroid cDNAs derived from the left side of a novel CEV-TASV chimera are also well suited for sequence analysis within the pathogenicity domain of TASV (that is primers HAD3 and RAO2; see Fig. 1A). These primers were used to examine each preparation of TASV progeny for the presence or absence of the expected nucleotide substitutions, and the results of these analyses are summarized in Table 1. Because the pattern of sequence changes detected was unexpectedly complex, relevant portions of selected sequencing gels are shown in Figure 3.

Variants were recovered from the upper leaves of plants inoculated with three of the 12 different TASV mutants tested. Progeny derived from inocula containing single substitutions at positions 61 or 63 appeared rapidly (3–4 wk after inoculation with RNA transcripts), while those originating from inocula containing substitutions at positions 68 + 72 appeared more slowly.

Introduction of an A→G substitution at position 61 led to sequence changes in the flanking positions (that is a complete U→A substitution at position 62 and deletion of the G residue at position 60 in a portion of the progeny). A second infectivity trial with this mutant produced a similar mixture of progeny plus a small amount of wild-type TASV (results not shown). Inoculation of tomato seedlings with RNA transcripts containing an A→G substitution at position 63 also produced a complex mixture of variants. In this case, position 63 contained both G and A residues, while positions 64 and 65 contained a mixture of all four ribonucleotides.

Examination of the progeny arising from two independent infectivity trials with inocula containing nucleotide substitutions at positions 68 and 72 provided yet more

Table 1. Sequence alterations detected after mutagenesis within the pathogenicity domain of tomato apical stunt viroid

Inoculum	Progeny[a]	
	First passage	Second passage
G48[b]	Wild-type	Analysis not done
G51	Wild-type	Analysis not done
G52	Wild-type	Analysis not done
G53	Wild-type	Analysis not done
G55	Wild-type	Analysis not done
G56	Wild-type	Analysis not done
G58	Wild-type	Analysis not done
G61	[G60], G61, A62	[G60], G61, A62
G63	R63, N64, N65	Wild-type
G66	Wild-type	Analysis not done
U68, G72	A62, Y68, R72	A62, U68
G76	Wild-type	Analysis not done

[a]R, adenine plus guanine; Y, cytosine plus uridine; N, a mixture of all four ribonucleotides; and [ ], deleted nucleotide.

[b]The nature and location of each alteration are denoted by a one-letter, two-digit code (that is, G48 indicates an A→G substitution at position 48).

evidence for sequence instability and/or evolution *in vivo*. In one trial, the A→G substitution at position 72 was retained, but position 68 contained a mixture of C and U residues. Progeny from the second trial contained an A residue at position 72, a U residue at position 68, and an A residue at position 62 (see Fig. 3). The presence of this U→A substitution at position 62 was completely unexpected.

The complex and variable composition of these progeny populations highlighted the need to monitor their long-term stability. Therefore, after estimating the concentration of circular TASV in selected low molecular weight RNA preparations by polyacrylamide gel electrophoresis, a set of suitably diluted RNAs was used to inoculate a second group of tomato seedlings. Symptom expression in the inoculated plants was monitored for 5 wk before low molecular weight RNA was isolated from systemically infected leaf tissue, and the resulting TASV progeny were characterized by sequence analysis of PCR-amplified TASV cDNAs. Results of these analyses are presented in Table 1, and the predicted structural effects of certain sequence alterations are shown in greater detail in Figure 4.

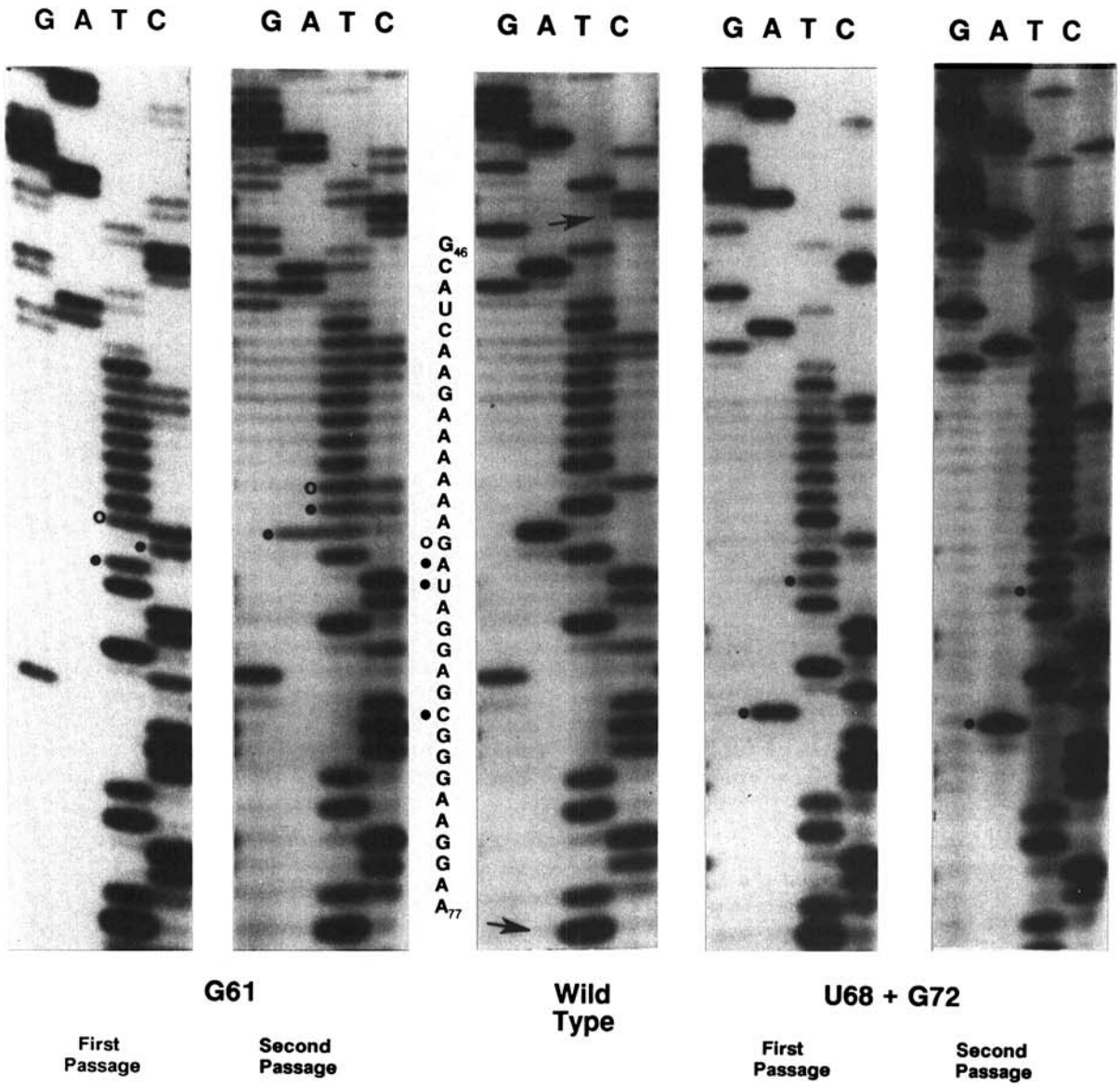

Fig. 3. Sequence analysis of selected tomato apical stunt viroid variants. As described in the text, single-stranded cDNA templates derived from the left side of the native structure were generated by successive symmetric and asymmetric polymerase chain reactions containing primers HAD3 + RAO2 or RAO2 alone. Because the 5′-^{32}P-labeled HAD3 primer used for sequence analysis anneals at positions 111–95 of the tomato apical stunt viroid "plus" strand, the sequences determined are complementary to that shown in Figure 1. The locations of sequence alterations in variants derived from initial mutations at positions 61 (left) and 68 + 72 (right) are marked: ●, nucleotide substitutions; ○, nucleotide deletion. The presence of a deletion at position 60 in a portion of the progeny arising from inoculum containing an A→G substitution at position 61 is clearly shown by the sequence heterogeneity apparent at each succeeding position (left two panels). For purposes of comparison, the complete sequence of the wild-type pathogenicity domain between positions 46 and 77 is shown beside its autoradiograph (center).

Both variants derived from inocula that contained an initial A→G substitution at position 61 were still detectable among the progeny from the second passage (that is a variant containing a double nucleotide substitution at positions 61 and 62 as well as one with an additional deletion of the G residue at position 60). The proportion of wild-type TASV present in this preparation was greater than after the first passage, however. The variants originating from an A→G substitution at position 63 showed an even greater tendency to be replaced by wild-type TASV; all traces of variants containing nucleotide substitutions at positions 61–63 had disappeared after the second passage in tomato (see Fig. 3).

The most stable TASV variant was derived from a cDNA containing mutations at positions 68 and 72. After a second passage in tomato, this variant still contained a C→U substitution at position 68 plus the unexplained U→A substitution at position 62. All TASV variants were also examined for the presence of sequence changes in the lower portion of the pathogenicity domain (3′ to position 288), but no evidence for any such compensatory changes was found (results not shown).

The secondary structures for the different TASV variants presented in Figure 4 are those predicted to have the lowest free energies. The net effect of sequence alterations between positions 60 and 62 appears to be the removal of a small, uracil-containing internal loop present in the wild-type Ivory Coast strain of TASV. Structural calculations indicated that this internal loop should not be affected by simultaneous nucleotide substitutions at positions 68 and 72, but such changes may convert a nearby bulged A residue into a two-nucleotide interior loop. None of these alterations, however, had any detectable effect upon symptom expression in tomato (results not shown).

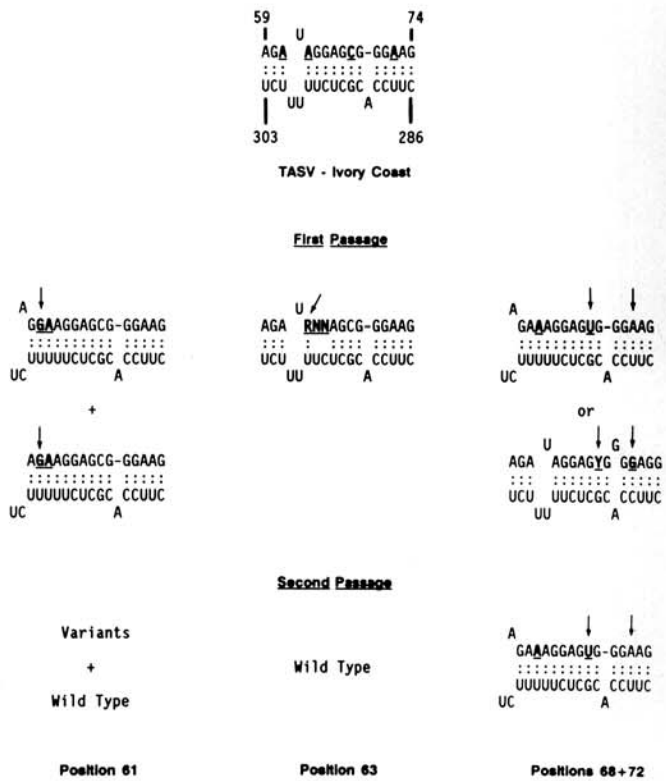

Fig. 4. Mutation-induced rearrangements within the pathogenicity domain of tomato apical stunt viroid (TASV). Only that portion of the pathogenicity domain that adjoins the central conserved region of TASV and contains the initial nucleotide substitutions at positions 61, 63, 68, and 72 is shown. Structures for variants detected in the first and second tomato passages (below) are compared to that of the parental strain, TASV Ivory Coast (above). Nucleotide substitutions are underlined, and locations of the initial substitutions are indicated by arrows. **R**, adenine plus guanine; **Y**, cytosine plus uridine; and **N**, an approximately equimolar mixture of all four ribonucleotides. Two independent preparations of progeny derived from inocula containing an A→G substitution at position 61 were analyzed, and both contained a mixture of two different variants plus variable amounts of wild-type TASV. In the case of the inocula containing substitutions at positions 68 and 72, only one of two progeny preparations contained a variant with an unexpected C→U substitution at position 62.

DISCUSSION

Previous studies have shown that nucleotides within the pathogenicity domain control symptom expression by PSTV and CEV, two members of the "PSTV group." Schnölzer *et al.* (1985) have proposed that the thermal stability of a "virulence-modulating" region within the PSTV pathogenicity domain controls symptom expression by modulating the viroid's ability to interact with unidentified host factor(s). Visvader and Symons (1985) were unable to find a similar correlation for CEV, but the variation in symptom expression observed by these investigators in studies with novel CEV chimeras confirmed the important role of the pathogenicity domain in symptom expression (Visvader and Symons 1986). The existence of extensive sequence similarities among TASV, PSTV, and CEV (Keese and Symons 1985) strongly suggests (but does not prove) that symptom expression by TASV is controlled by the nucleotides which form its pathogenicity domain.

As shown in Figure 5, the pathogenicity domain of TASV shows considerable natural sequence variation. Differences between the previously described Ivory Coast and Indonesian isolates are concentrated within a predominantly helical region adjacent to the central conserved region, and many of the changes appear to be compensatory. Although the position of a small internal loop containing three U residues appears to be somewhat variable, both naturally occurring TASV isolates induce severe symptoms in tomato (Owens *et al.* 1990). Interestingly, the only *in vitro* mutations in TASV Ivory Coast that led to the appearance of variant progeny were also located in this portion of the pathogenicity domain.

Mutations expected to increase the size of either the uracil-containing internal loop (a single A→G substitution at position 63) or a nearby bulge loop containing a single A residue (simultaneous substitutions at positions 68 and 72) were unstable and rapidly reverted to the wild type. Variants in which the uracil-containing internal loop may be absent, however, were much more stable. The symptoms produced by one such variant (one which contained substitutions at positions 62 and 68) were indistinguishable from those of wild-type TASV Ivory Coast.

Four naturally occurring viroids have been reported to produce severe symptoms in tomato (that is, TASV, CEV, PSTV, and tomato planta macho viroid), but structural

calculations indicate that only TASV and tomato planta macho viroid contain such an internal loop. Furthermore, CEV variants that induce severe symptoms lack this loop, while variants that induce mild symptoms contain a smaller, two nucleotide loop. The presence or absence of a small, uracil-containing internal loop within the pathogenicity domain thus seems to have little or no relationship to symptom severity.

The precise series of events responsible for the appearance of the TASV variants detected in this study is unclear. Although the amount of inoculum applied to each plant was 100- to 1,000-fold greater than that required to infect 50% of the inoculated plants (Tabler and Sänger 1984, 1985), probability calculations argue against nucleotide misincorporation *in vitro* as an explanation for the appearance of variant progeny. In this respect, it should be noted that the presence of single nucleotide substitutions at positions 61 and 63 both led to the appearance of variants containing multiple, clustered alterations. Furthermore, the unpaired U residue at position 62 in the Ivory Coast strain of TASV was replaced by an A residue in two completely independent progeny preparations (see Fig. 5). Thus, the variants present in systemically infected leaf tissue probably represent the products of a multistep *in vivo* selection for molecules able to replicate and move from cell-to-cell at near wild-type rates. Whether this sequence evolution results from a need to conserve the viroid's native structure per se or its ability to interact with certain host components remains to be determined. Similar sequence changes have previously been observed during infectivity trials with cucumber mosaic virus satellite RNAs derived from infectious RNA transcripts (Collmer and Kaper 1988; Kurath and Palukaitis 1990).

Although the symptoms induced by these TASV variants in tomato were indistinguishable from those of the wild-type viroid, it remains to be determined whether these or other changes in nucleotide sequence and/or secondary structure within the pathogenicity domain will affect symptom expression in other host species. Walter (1987) has reported that symptoms of TASV infection vary widely among different *Nicotiana* species. It will also be important to determine whether or not inoculation of several different hosts with a given TASV mutant is followed by the appearance of the same mixture of variants. Reproducible sequence differences between the progeny isolated from different hosts may provide insight into the structural determinants of viroid host range. Such host-specific variants could be very useful in biochemical studies of possible interaction between the pathogenicity domain and host-encoded polypeptides.

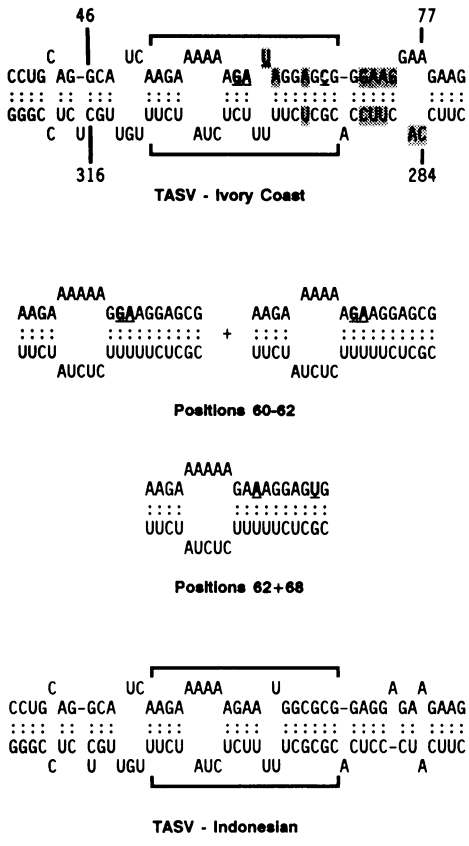

Fig. 5. Comparison of the pathogenicity domains of naturally occurring and laboratory-derived tomato apical stunt viroid (TASV) variants. Sequence variation between the Ivory Coast and Indonesian isolates of TASV is denoted by shading, while that induced by oligonucleotide-directed mutagenesis has been underlined. For the laboratory-derived variants, only a portion of the pathogenicity domain is shown; corresponding regions within the Ivory Coast and Indonesian isolates are indicated by the horizontal brackets.

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