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Identification of multiple structural domains regulating viroid pathogenicity

(infectious cDNA/chimeras)

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ABSTRACT **To investigate the role of individual structural domains in viroid pathogenicity and replication, a series of interspecific chimeras was constructed by exchanging the terminal left (T_L) and/or pathogenicity (P) domains between tomato apical stunt (TASVd) and citrus exocortis (CEVd) viroids. All six chimeras tested were replicated stably in tomato, and the symptoms exhibited by infected plants were intermediate between those induced by the parental viroids. Quantitative comparisons of symptom development and progeny accumulation revealed that: (*i*) the T_L domain of TASVd contains a determinant required for appearance of severe veinal necrosis in tomato, (*ii*) the severe epinasty and stunting characteristic of TASVd requires the presence of its T_L and P domains, and (*iii*) the variable (V) and terminal right (T_R) domains comprising the right side of the native structure also play an important role in viroid pathogenicity. Chimeras containing the right side of TASVd accumulated to higher levels early in infection, and infected plants developed more severe symptoms than those whose right halves were derived from CEVd. Although the individual contributions of the T_L and P domains to symptom induction could not be completely separated from that of viroid titer, the T_L domain appears to exert a greater effect upon symptom severity than does the P domain. The T_L, P, V, and T_R domains of TASVd and CEVd contain three discrete regions of sequence and/or structural variability that may correspond to the pathogenicity determinants uncovered by our genetic analysis.**

Viroids are the smallest known agents of infectious disease—small [246–375 nucleotides (nt)], highly structured, single-stranded RNA molecules that lack a protein capsid and detectable mRNA activity (1, 2). The apparent absence of pathogen-specified proteins implies that the disease process is initiated by the *direct interaction* of one or more structural determinants with as yet unidentified host cell constituents. Thus, viroid replication and pathogenesis provide an attractive model system in which to investigate the molecular mechanisms controlling host–pathogen interaction as well as gene expression in the uninfected host cell.

Based upon sequence similarities between potato spindle tuber viroid (PSTVd) and several other viroid species, Keese and Symons (3) have proposed that viroids contain five structural domains—i.e., a conserved central region (CCR), flanking pathogenicity (P) and variable (V) domains, and left (T_L) and right (T_R) terminal loops (Fig. 1). Sequence analysis of naturally occurring PSTVd and citrus exocortis viroid (CEVd) isolates (4, 5) as well as infectivity studies with chimeric cDNAs derived from portions of mild and severe CEVd isolates (6) have shown that sequence and/or structural elements within the P domain play an important role in viroid pathogenicity. Schnölzer *et al.* (4) have proposed that virulence is determined by the interaction of nucleotides within a portion of the PSTVd P domain known as the "virulence modulating" region with as yet unidentified host factor(s), but the possible role of structural elements outside the P domain in regulating viroid symptom expression is unclear. Many of the changes in host metabolism associated with viroid (or virus) disease are probably secondary consequences of infection, and symptom expression need not be directly related to replication. Indeed, symptom expression in tomatoes infected with naturally occurring isolates of CEVd and PSTVd has been reported to be independent of viroid concentration (4, 5).

Using chimeras constructed from mild and severe isolates of CEVd, Visvader and Symons (10) were able to demonstrate a clear correlation between pathogenicity (i.e., stunting and epinasty) and the source of the P domain. Furthermore, a recent study from our laboratory (11) has shown that a similar chimera containing the left half of CEVd and the right half of tomato apical stunt viroid (TASVd) is stably maintained in tomato. Here we describe the biological properties of five additional chimeras in which individual structural domains have been exchanged between TASVd and CEVd. By exchanging individual structural domains between these closely related viroid species, we have been able to identify the individual contributions of the T_L, P, and V/T_R domains to viroid pathogenicity and replication. Regulation of viroid pathogenicity appears to be more complex than previously believed, involving structural elements within at least three of their five proposed structural domains.

MATERIALS AND METHODS

Viroids and cDNA Clones. Construction of an infectious cDNA clone derived from the Ivory Coast strain of TASVd (360 nt) has been described (12), and similar methodologies were used to clone an infectious CEVd-J(c) cDNA (371 nt; see ref. 5) within the *Bam*HI site of plasmid pUC9. As shown in Fig. 1*B*, cDNA chimeras were constructed by reciprocal exchange of corresponding *Bam*HI–*Hin*dIII, *Msp* I–*Pst* I, or *Hin*dIII–*Msp* I plus *Pst* I–*Bam*HI fragments between these full-length TASVd and CEVd cDNAs. The infectivity of clone CEAS (previously designated pCEAS22 and containing the left side of CEVd joined to the right side of TASVd via

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Abbreviations: CEVd, citrus exocortis viroid; PSTVd, potato spindle tuber viroid; TASVd, tomato apical stunt viroid; nt, nucleotides; p.i., postinoculation; CCR, conserved central region; T_L and T_R, terminal left and terminal right; P, pathogenicity; V, variable.

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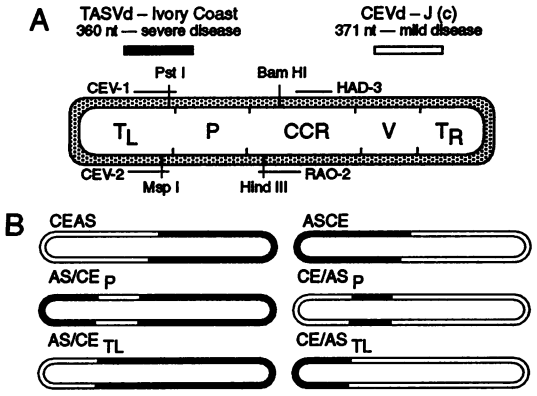

FIG. 1. Construction of TASVd-CEVd chimeras. (*A*) Locations of the five structural domains proposed by Keese and Symons (3) are shown within a schematic representation of the rod-like native structure: T_L loop, P domain, CCR, V domain, and T_R loop. Boundaries of these domains and the locations of restriction sites used for cDNA fragment exchanges (i.e., *Pst* I, *Bam*HI, *Hin*dIII, and *Msp* I) are indicated by tic marks and vertical lines, respectively. Binding sites for the oligonucleotide primers used for amplification of viroid cDNAs and nucleotide sequence analysis (i.e., HAD3, RAO2, CEV1, and CEV2) are also marked. (*B*) Structures of the six interspecific TASVd-CEVd chimeras. TASVd- and CEVd-derived sequences are indicated by filled and open lines, respectively.

a *Hin*dIII site in the lower portion of the CCR) has been described (10). Clones ASCE (an analogous construction containing the left side of TASVd joined to the right side of CEVd), AS/CE-T_L and AS/CE-P (TASVd whose T_L or P domains have been replaced by the corresponding portions of CEVd), and CE/AS-T_L and CE/AS-P (CEVd whose T_L or P domains have been replaced by corresponding portions of TASVd) were specially constructed for these studies.

Infectivity Assays and Symptom Quantitation. Infectivities of the parental (i.e., TASVd and CEVd) and chimeric cDNAs were assayed by inoculating young tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) seedlings with plasmid DNAs purified by alkaline lysis followed by RNase A treatment and polyethylene glycol precipitation (13). Three to 8 weeks postinoculation (p.i.), leaf tissue collected from the apex of each plant was assayed for the presence of viroids by molecular hybridization using RNA probes specific for TASVd and CEVd (14, 15).

Prior to bioassays comparing viroid pathogenicity, the concentration of viroid progeny in their respective low molecular weight RNA inocula was estimated by return-polyacrylamide gel electrophoresis (16) using known amounts of purified PSTVd as standards. One hundred microliters of appropriately diluted RNA inocula containing 4, 0.4, or 0.04 ng of viroid per 10 μl of 0.1 M Tris·HCl (pH 7.5) was used to inoculate groups of 10 tomato seedlings, and inoculated plants were maintained as described above. During the early-mid stages of infection (2–4 weeks p.i.), symptom severity was evaluated at weekly intervals using a specially developed numerical index.

Inoculated plants were assigned scores of 1–8 depending upon the leaf position where epinasty was first detectable—i.e., 1 = first true leaf, and 2–8 = second through eighth true leaves. Symptomless and mock-inoculated plants were given scores reflecting the number of true leaves present on each plant at the time of evaluation. For each individual treatment, scores from all 10 inoculated plants were then averaged to provide a composite score. With this scoring system, lower values indicate more severe disease. Later in infection, as symptoms intensified and spread to the older leaves, scores for several chimeras tended to decrease.

Quantitation of Viroid Accumulation. Viroid accumulation in individual leaves was monitored by quantitative dot blot hybridization. Two to 6 weeks p.i., 4-mm leaf discs were removed from each true leaf, and discs collected from the same leaf position were combined within treatments. After homogenization in 200 μl of AMES buffer (17) and extraction with 200 μl of phenol/chloroform, 1:1 (vol/vol), the resulting supernatants were diluted 10-fold with 10 mM Tris·HCl/1 mM EDTA, pH 8, and 2-μl samples (three spots per sample) were spotted onto 20× SSC-equilibrated nitrocellulose (1× SSC = 0.15 M NaCl/0.015 M sodium citrate). Aliquots from a series of 2-fold dilutions containing 100–0.39 pg of each viroid were applied to the same nitrocellulose membrane as standards. Hybridization using a 1:1 mixture of TASVd- and CEVd-specific RNA probes was performed as described (14), and the radioactivities of individual spots were quantitated using a Betascope image analyzer (Betagen, Waltham, MA). Mean values of the three spots were compared to those from the appropriate standards to estimate viroid titers.

Sequence Analysis of Viroid Progeny. Nucleotide sequences of the chimeric progeny were determined from enzymatically amplified viroid cDNAs as described (11). Binding sites for the primer pairs used in these analyses [i.e., Had3 (5′-CTCCAGGTTTCCCCGGG-3′) plus RAO2 (5′-GCGGATC-CGGTGGAAACAACTGAAGC-3′) and CEV1 (5′-TGCTC-TCCTGACCCTGCAG-3′) plus CEV2 (5′-CTTACAC-CCTAGCCCGGAG-3′)] are shown in Fig. 1*A*.

RESULTS

Recognition sites for restriction endonucleases *Bam*HI, *Hin*dIII, *Msp* I, and *Pst* I are located at similar positions within the native structures of TASVd and CEVd. In particular, Fig. 1*A* shows that the *Msp* I and *Pst* I sites are located very close to the border between the T_L and P domains. Although the T_L and CCR domains of these two viroids are highly homologous (i.e., 91% and 99%, respectively), their P domains exhibit only 54% sequence similarity (3). Thus, it was possible to construct six interspecific cDNA chimeras in which the T_L and/or P domains had been exchanged with only minimal disruption of viroid secondary structure (see Fig. 1*B*).

Infectivities of Chimeric Viroid cDNAs. Eleven to 40 days p.i., the stunting, leaf epinasty, and veinal necrosis typical of viroid infection began to appear among tomato plants inoculated with TASVd, CEVd, and each of the six chimeric cDNAs. Viroid replication in symptomatic plants was confirmed by dot hybridization analysis, and the strong signals observed with TASVd- and CEVd-specific RNA probes suggested the presence of chimeric progeny in plants inoculated with cDNA chimeras. Sequence analysis of enzymatically amplified cDNAs derived from each progeny preparation revealed that all viroids were replicated stably—i.e., the nucleotide sequence of each was precisely that predicted by their respective cDNA constructs (results not shown).

These progeny preparations were used as inocula in a series of three bioassays comparing the pathogenicities of these viroid chimeras with those of TASVd and CEVd. Each trial included three different inoculum concentrations (i.e., 4, 0.4, and 0.04 ng of viroid per plant), and virtually every plant inoculated with 4 or 0.4 ng viroid became infected. Progeny accumulation and symptom development in plants inoculated with 0.4 ng of viroid were carefully monitored over a 6-week period p.i., and results are summarized in Table 1. Representative data are presented in Figs. 2 and 3.

Viroid Replication/Accumulation Is Regulated by the V and T_R Domains. Sequences within the V domain of CEVd (i.e., the so-called "P_R" domain) appear to influence either infection or replication efficiency (10). As shown in Fig. 2, measurements of viroid titers during the middle to late stages of infection also point to an important role for the V and/or

Table 1. Biological properties of TASVd-CEVd chimeras

Construct	Replication accumulation		Epinasty*	Stunting†	Necrosis
	Early	Late			
TASVd	Rapid	Rapid	Severe	Severe	Severe
ASCE	Slow	Medium	Severe	↓	Severe
AS/CE$_P$	Rapid	Medium	Severe	↓	Severe
AS/CE$_{TL}$	Rapid	Slow	Severe	Medium	Weak
CEAS	Rapid	Rapid	Medium	Mild	Weak
CE/AS$_{TL}$	Slow	Slow	Weak	↓	Medium
CE/AS$_P$	Slow	Slow	Weak	↓	Weak
CEVd	Slow	Slow	Weak	Mildest	Weak

*See Fig. 3*A*.
†See Fig. 3*B*; the arrows indicate a gradual reduction in severity of stunting.

T_R domains in determining the titer of our CEVd-TASVd chimeras. Thus, only those chimeras containing the right side of TASVd reached high titers 4 weeks p.i., whereas those whose V plus T_R domains were derived from CEVd accumulated to much lower concentrations (see Fig. 2*A*). This correlation was still evident 6 weeks p.i., except that the titer of ASCE had increased to relatively high levels and that of AS/CE$_{TL}$ had decreased (Fig. 2*B*).

Contributions of the T_L and P Domains to Viroid Pathogenicity. Data summarized in Table 1 show that symptoms exhibited by tomato seedlings inoculated with CEVd were much milder than those induced by TASVd, even late in infection. Stunting, leaf curling, and veinal necrosis were visible on the TASVd-infected plants as early as 11 days p.i., but at least 21 days passed before mild stunting and leaf epinasty were visible on the CEVd-inoculated plants. All six TASVd-CEVd chimeras produced symptoms that were intermediate in severity between those of the parental viroids.

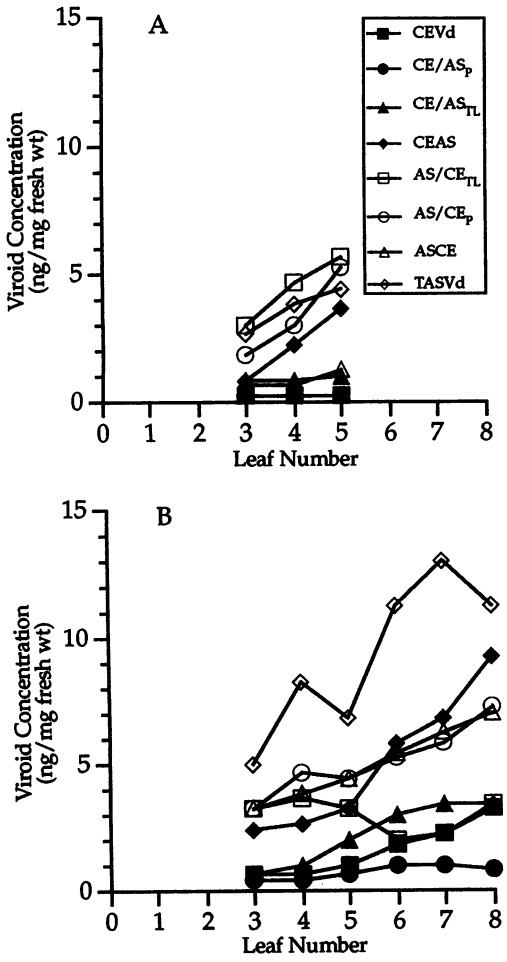

FIG. 2. Viroid accumulation in systemically infected tomato leaf tissue 4 weeks (*A*) and 6 weeks (*B*) p.i. Plants were inoculated with 0.4 ng of viroid per plant, and each point is the average of three measurements.

These differences in symptom expression could be a consequence of the different titers attained by the various viroids. Indeed, Fig. 2 shows that viroid concentrations in the severely affected TASVd-infected plants were much higher than those in mildly affected CEVd-infected plants. For the chimeras, however, differences in viroid titer alone cannot account for the symptom differences observed. Thus, ASCE, which contains the V plus T_R domains of CEVd and reached only low–medium titers, induced severe epinasty and stunting, whereas the reciprocal construction (i.e., CEAS) reached high titers in infected plants but induced only mild symptoms (see Table 1). These results emphasize the importance of sequences within the T_L plus P domains in determining symptom severity. When such a comparison is broadened to include chimeras ASCE, CE/AS$_{TL}$, and CE/AS$_P$, however, the induction of severe symptoms is seen to require the P and T_L domains of TASVd. Constructs containing only one of these domains, either P (CEAS$_P$) or T_L (CE/AS$_{TL}$), induced only mild symptoms.

That viroid titer does influence the severity of symptom expression is shown by comparing the stunting and epinasty observed with the three chimeras that reach only low titers (constructs containing the CEVd V plus T_R domains) with those of the three chimeras that reach high titers (those whose right sides are derived from TASVd). As discussed above, induction of severe symptoms by the low-titer chimeras requires the presence of *both* the T_L and P domains. With the high-titer chimeras, on the other hand, moderate–severe symptoms are induced when *either* the T_L or P domain of TASVd is present. Thus, a high viroid titer can substitute, at least partially, for the lack of either the TASVd P or T_L domain.

TASVd-CEVd chimeras also varied in their ability to induce veinal necrosis during the early to middle stages of infection. Data summarized in Table 1 show that the most important determinant of veinal necrosis resides within the T_L domain, with the P domain playing a subordinate role. Although plants infected with CE/AS$_{TL}$, which contains only the T_L domain from TASVd, exhibited moderate veinal necrosis, CE/AS$_P$ infections were accompanied by only a weak necrotic reaction. Chimera ASCE, which contains the TASVd T_L and P domains, induced severe veinal necrosis.

As with the induction of epinasty and stunting, viroid titer also appeared to influence the severity of veinal necrosis. Thus, plants infected with the low-titer chimera CE/AS$_{TL}$ exhibited only a mild necrotic reaction, despite the presence of the TASVd T_L domain, whereas in plants infected with the high-titer chimera AS/CE$_P$ veinal necrosis was severe—despite the absence of the TASVd P domain. Unlike the situation with epinasty and stunting, however, high viroid titer can substitute only for the absence of the subordinate (TASVd P domain) determinant, not for the lack of the primary T_L determinant.

Quantitative Comparisons of Pathogenicity. Attempts were also made to identify quantitative differences in viroid pathogenicity, using as criteria *leaf epinasty* (the first visible sign of infection) and *stunting* (a slower, more generalized response). In general, the results of these analyses confirmed the conclusions drawn from the experiments described above. Evaluation of epinasty using a specially developed numerical index (see *Materials and Methods*) revealed a definite correlation between the time at which disease symptoms first began to appear and the score assigned 2–4 weeks

p.i. that was independent of either inoculum concentration or time of year. Data shown in Fig. 3*A* reveal that the symptoms produced by chimeras AS/CE_{TL} and AS/CE_P, although slightly less severe than those of TASVd, were much more severe than those of CEVd. Symptoms produced by CE/AS_{TL} and CE/AS_P, on the other hand, were very similar to those of CEVd. As previously reported (11), the severity of CEAS symptoms was intermediate between those of TASVd and CEVd. Four weeks p.i., the severity of epinasty induced by ASCE, AS/CE_{TL}, and AS/CE_P infection was virtually identical (see also Table 1).

Later in infection, symptom expression was most clearly assessed by measurements of internode length (i.e., degree of stunting). Using this criterion, data presented in Fig. 3*B* show that (*i*) stunting induced by ASCE was almost as severe as that induced by TASVd, (*ii*) that induced by AS/CE_P was the next most severe, and (*iii*) the stunting induced by CE/AS_{TL} and CE/AS_P was not much different than that induced by CEVd. Also worthy of note (and denoted by arrows in Fig. 3*B*) is an apparent "recovery" in growth rate of plants infected with either AS/CE_{TL} or CEAS. In the case of AS/CE_{TL}, this recovery was associated with a decrease in replication/accumulation during the later stages of infection (see Fig. 2*B*).

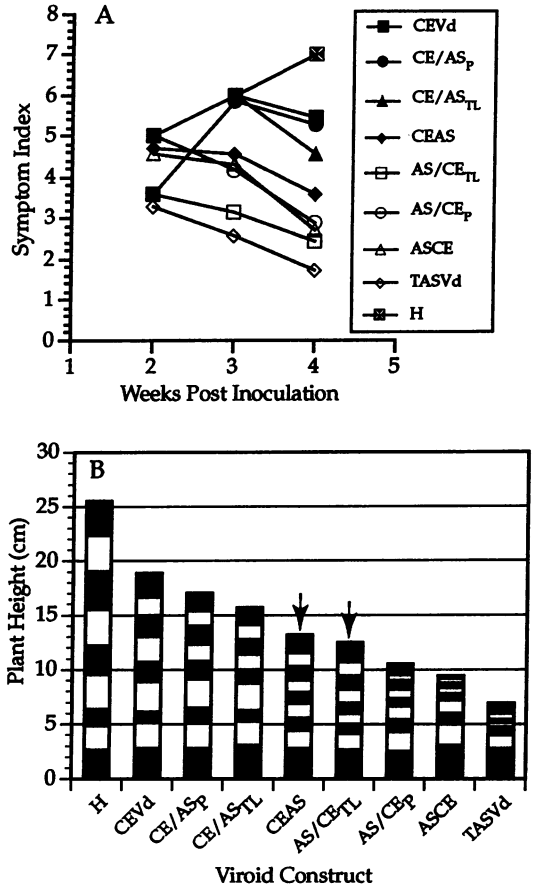

FIG. 3. Quantitative assessment of epinasty and stunting. (*A*) Severity of symptoms early in infection (2–4 weeks p.i.) was compared using an index based upon position of the first leaf exhibiting epinasty/curling. (*B*) Total plant height and length of individual internodes measured 6 weeks p.i. The values shown are averages computed after discarding the largest and smallest measurements for each chimera. Lengths of individual segments within each bar represent the distance between successive nodes. Arrows denote chimeras where the rate of growth/elongation increased significantly during the later stages of infection. Plants were inoculated with 0.4 ng of viroid.

DISCUSSION

Previous reports (18–22) have amply documented the ability of many single and clustered point mutations to abolish viroid infectivity. Thus, the ability to exchange entire structural domains without obvious ill effect may seem somewhat surprising. Two points are worthy of note: First, each of our chimeras is able to form secondary hairpins I and II, two alternative structural interactions believed to be essential for viroid replication (6, 23); second, our strategy for chimera construction was designed to minimize disruption of the native structure. The importance of the native structure is illustrated by the noninfectious nature of chimeras analogous to CE/AS_P or AS/CE_P in which only the upper portion of the P domain had been exchanged (results not shown). Sequence analysis of *Columnea* latent (7) and Australian grapevine viroids (8) has previously suggested that viroid evolution has involved the simultaneous exchange of top and bottom portions of individual structural domains. The viability of our TASVd-CEVd chimeras provides direct evidence that the structural domains proposed by Keese and Symons (3) are also biologically functional domains.

Previous attempts to understand symptom expression have focused upon sequence and/or structural variation within the P domain (4). With one notable exception (10), there has been little evidence that a more rapid rate of replication/accumulation leads to more pronounced disease. Our molecular genetic analysis has clearly shown, however, that at least three determinants are involved—one each within the T_L and P domains regulating symptom severity plus a third determinant that is located within the V and/or T_R domains and regulates viroid replication/accumulation. A high viroid titer can partially or wholly substitute for the lack of either the T_L or P determinant, but not for both.

As first suggested by Visvader and Symons (10), our data show that sequences within the V and/or T_R domains regulate viroid replication/accumulation. Only chimeras containing the V plus T_R domains of TASVd were able to reach high titers in systemically infected tissue by 4 weeks p.i., and, with only two exceptions, this correlation between the origin of the V plus T_R domains and viroid titer was maintained until at least 6 weeks p.i. These apparent anomalies (i.e., an increase in ASCE titers to levels similar to the high-titer chimera AS/CE_P and a decrease in the titer of AS/CE_{TL} to levels similar to those of CE/AS_{TL}) may be a result of complex interactions between viroids and their hosts late in infection.

Though confirming the importance of the P domain for symptom induction in tomato, our results clearly show that it is not the only domain that influences symptom severity. In addition to viroid titer, the T_L domain also plays a critical role in symptom induction. The T_L and P domains from TASVd are required for the induction of severe epinasty and stunting by chimeras that reach only relatively low titers, but either domain alone is sufficient for chimeras reaching higher concentrations (see Table 1). For these latter constructs (i.e., AS/CE_{TL} and AS/CE_P), stunting and epinasty were more severe with the chimera that contained the T_L domain of TASVd. Indeed, the T_L domain may have a greater effect upon the severity of epinasty and stunting than does the P domain.

Studies of veinal necrosis largely confirmed the conclusions drawn from the induction of epinasty and stunting. Here again, the T_L and P domains play important roles, and the titer reached by the various viroids in the infected plants also modulates symptom expression. For low-titer chimeras, severe necrosis was observed only when the T_L and P domains were derived from TASVd. The appearance of moderate necrosis in plants infected by CE/AS_{TL}, a low-titer chimera containing the TASVd T_L domain, but not in those infected by CE/AS_P provides a second piece of evidence for the predominance of the T_L domain in our experimental

FIG. 4. Sequence and structural variation between TASVd and CEVd. Comparison of minimal free energy secondary structures (9) for TASVd and CEVd reveals three main areas of variation—one each in the T_L and P domains plus a third area that spans the junction between the V and T_R domains (indicated by horizontal bars). The nucleotide sequence of TASVd-Ivory Coast is shown, and positions where this sequence differs from that of CEVd-J(c) are marked by solid dots. Many (but not all) of these sequence differences lead to changes in predicted secondary structure. Boundaries of individual structural domains (3) are indicated by vertical lines, and the locations of sequences forming the stems of secondary hairpins I and II are shaded. Locations of the *Bam*HI, *Hin*dIII, *Pst* I, and *Msp* I sites used to construct the various TASVd-CEVd cDNA chimeras have also been marked (arrows).

system, and it is reinforced by the results obtained with high-titer chimeras. AS/CE_P, a high-titer chimera that contains the T_L, but not the P, domain of TASVd induced severe veinal necrosis, whereas chimera AS/CE_{TL} induced only mild and scattered necrosis.

Comparison of the native (i.e., lowest free energy) structures of TASVd and CEVd-J(c) has identified several discrete regions of sequence and/or structural variation within the T_L, P, V, and T_R domains. Fig. 4 shows that variation within the T_L domain is quite localized, but that within the P domain begins in a region that has been termed the "virulence-modulating" region (4) and extends all the way to the border with the CCR. Variation within the V and T_R domains is also quite extensive. Although we are encouraged by the apparent correspondence between the number and location of these structural elements and the genetic determinants identified by our studies, we wish to point out that essentially nothing is known about viroid structure *in vivo*. Likewise, the molecular mechanisms by which such determinants might influence viroid replication/pathogenesis are unknown. Attempts are necessary, using small-scale PCR-mediated sequence exchanges, to identify the structural element(s) within these domains responsible for their individual contributions to viroid pathogenicity.

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