Inhibition of Cell Growth and Shoot Development by a Specific Nucleotide Sequence in a Noncoding Viroid RNA

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Viroids are small noncoding and infectious RNAs that replicate autonomously and move systemically throughout an infected plant. The RNAs of the family Pospiviroidae contain a central conserved region (CCR) that has long been thought to be involved in replication. Here, we report that the CCR of Potato spindle tuber viroid (PSTVd) also plays a role in pathogenicity. A U257A change in the CCR converted the intermediate strain PSTVdInt to a lethal strain that caused severe growth stunting and premature death of infected plants. PSTVd with nucleotide U257 changed to C or G did not cause such symptoms. The pathogenic effect of the U257A substitution was abolished by a C259U substitution in the same RNA. Analyses of the pathogenic effects of the U257A substitution in three other PSTVd variants established A257 as a new pathogenicity determinant that functions independently and synergistically with the classic pathogenicity domain. The U257A substitution did not alter PSTVd secondary structure, replication levels, or tissue tropism. The stunted growth of PSTVdIntU257A-infected tomato plants resulted from restricted cell expansion but not cell division or differentiation. This was correlated positively with the downregulated expression of an expansin gene, LeExp2. Our results demonstrate that specific nucleotides in a noncoding, pathogenic RNA have a profound effect in altering distinct cellular responses, which then lead to well-defined alterations in plant growth and developmental patterns. The feasibility of correlating viroid RNA sequence/structure with the altered expression of specific host genes, cellular processes, and developmental patterns makes viroid infection a valuable system in which to investigate host factors for symptom expression and perhaps also to characterize the mechanisms of RNA regulation of gene expression in plants.

INTRODUCTION

Development of disease symptoms in a host plant when infected by a particular pathogen is the result of complex pathogen–host interactions. The pathogen usually has pathogenicity determinants that elicit the types and degrees of severity of host symptoms. In the host, altered cellular functions lead to changes in physiology and/or development that exhibit as disease symptoms. Thus, understanding the cellular and molecular mechanisms of disease formation will not only provide the basis for the development of rational approaches to combat pathogen infection but also will provide insights about the basic cellular processes that underlie normal plant growth and development (Kasschau et al., 2003).

Viroid infection provides a simple experimental system in which to study direct interactions between a pathogenic RNA genome and the host. A viroid genome consists of a single-stranded, covalently closed circular, noncoding, and noncapsidated RNA. Without encoding capacity, the viroid RNA genome and its replication intermediate interact directly with host components for nearly all aspects of the infection process, including replication, intracellular movement, intercellular movement, systemic movement, and pathogenicity (Hull, 2002).

The pleiotropic functions of the viroid RNA genome are remarkable given its small size, structural simplicity, and noncoding capacity. Viroid RNAs range from 246 to 399 nucleotides in size (Flores et al., 1997). Viroids in the Pospiviroidae family assume a rod-shaped secondary structure in their native state, and some viroids in the Avsunviroidae family have branched structures (Flores et al., 2000). A rod-shaped viroid RNA, as shown in Figure 1, contains five broad structural domains: (1) a central conserved region (CCR) involved in replication; (2) a pathogenicity domain implicated in symptom expression; (3) a variable domain; and (4) right- and (5) left-terminal domains (Keese andSymons, 1985). During replication, metastable structures such as hairpins may be formed through structural rearrangement (Loss et al., 1991; Owens et al., 1991; Qu et al., 1993; Schröder and Riesner, 2002). Local tertiary structures also may exist (Branch et al., 1985; Gast et al., 1996). A viroid is essentially an RNA mosaic with tightly packed and perhaps overlapping structural and functional motifs.

Viroid-infected plants can be symptomless or develop symptoms that range from mild to lethal, depending on viroid strains and host species (Schnöller et al., 1985; Owens, 1990; Owens et al., 1991, 1995, 1996; Hammond, 1992; de la Peña et al., 1999; Škorić et al., 2001). Because viroid pathogenesis is a consequence of direct RNA–cellular factor interactions, viroids are considered to belong to a group of noncoding RNAs that regulate cellular functions through means other than by encoding proteins for specific functions (Storz, 2002). In this context, elucidating the molecular mechanisms of viroid disease may help us understand the mechanisms of the RNA regulation of cellular processes.
Potato spindle tuber viroid (PSTVd) is the type species of the Pospiviroidae family (Flores et al., 2000). Sequence variations that contribute to different degrees of symptom severity have been mapped to the pathogenicity domain (Schnölzer et al., 1985; Owens et al., 1991, 1995, 1996; Hammond, 1992). Most studies have been limited to analyses of viroid structure and general plant symptoms (Schnölzer et al., 1985; Owens, 1990; Owens et al., 1991, 1995, 1996; Hammond, 1992; Schmitz and Riesner, 1998). Changes in the global gene expression patterns of infected plants also have been described (Itaya et al., 2002). In general, however, we have little knowledge of how a specific PSTVd sequence or structure can evoke distinct changes in host gene expression that lead to alterations in specific cellular processes and the development of particular symptoms.

We have taken a comprehensive approach that includes molecular, cellular, biophysical, and whole-plant analyses to investigate the mechanisms of viroid pathogenicity using PSTVd infection of tomato as an experimental system. Here, we report that a specific nucleotide change in CCR, a region conserved in all viroids of the family Pospiviroidae and long thought to be involved in replication (Keese and Symons, 1985) and host range determination (Wassenegger et al., 1996), confers a novel lethal symptom on the infected tomato plants. Underlying this symptom is inhibited cell growth and shoot development, marked by the repressed expression of a tomato expansin gene implicated in cell growth. The biological implications of these results are discussed.

RESULTS

The Nucleotide Substitution U257A Converted PSTVd<sup>Int</sup> to a Lethal Strain in Tomato

The predicted secondary structure of the intermediate PSTVd strain (PSTVd<sup>Int</sup>) with nucleotide sequences (Gross et al., 1978) is shown in Figure 1A. We showed previously that two independent mutations in loop E of CCR, C259U and U257A, converted PSTVd<sup>Int</sup> to tobacco infectious variants PSTVd<sup>Int</sup>C259U and PSTVd<sup>Int</sup>U257A, respectively (Figure 1B) (Qi and Ding, 2002; Zhu et al., 2002). We were interested in knowing whether these mutations would alter infectivity in tomato, a convenient experimental host for the study of viroid symptoms. We inoculated tomato seedlings at the cotyledon stage (6 days old, before the first leaf was visible) with in vitro transcripts of PSTVd<sup>Int</sup> and its two variants. Water was used as the inoculum in mock inoculation. As shown in Figure 2A, PSTVd<sup>Int</sup>C259U infection caused symptoms similar to those caused by PSTVd<sup>Int</sup> and its two variants. Water was used as the inoculum in mock inoculation. As shown in Figure 2A, PSTVd<sup>Int</sup>C259U infection caused symptoms similar to those caused by PSTVd<sup>Int</sup>. Strikingly, plants infected with PSTVd<sup>Int</sup>U257A showed severe growth stunting and relatively small leaves (Figure 2B). These plants displayed a characteristic flat appearance on the top of the shoot, with all lateral organs (leaves) at similar vertical levels (Figure 2B). We designate this phenotype the “flat-top” symptom. Furthermore, the leaves showed yellowing and necrosis. Sequence analyses indicated that PSTVd progeny maintained the mutated sequences in the infected plants (data not shown).
Figure 2. Symptoms Caused by Infection of Rutgers Tomato with PSTVd Variants.

Cotyledons of 6-day-old seedlings were inoculated with 100 ng/μL PSTVd transcripts. Photographs were taken and lengths of internodes were measured at 6 weeks after inoculation.

(A) PSTVd\textsuperscript{int}C259U and PSTVd\textsuperscript{int}U257C cause symptoms similar to those caused by PSTVd\textsuperscript{int}. PSTVd\textsuperscript{int}U257A causes severe growth stunting, flat top, and premature plant death. PSTVd\textsuperscript{int}U257G causes growth stunting intermediate between that caused by PSTVd\textsuperscript{int}C259U and PSTVd\textsuperscript{int}U257A.

(B) Closer view of the PSTVd\textsuperscript{int}U257A-infected plant showing leaf necrosis and the flat-top appearance of the shoot tip.

(C) Quantitative analyses of the stunted growth of PSTVd-infected tomato plants at 6 weeks after inoculation. The lengths of individual color bars represent the lengths of successive internodes, with the first internode represented at bottom. Each value is the average of measurements from six plants infected with each PSTVd variant.

(D) The double mutant PSTVd\textsuperscript{int}C259U/U257A causes a symptom similar to that caused by PSTVd\textsuperscript{int}C259U or PSTVd\textsuperscript{int}.

(E) to (G) The U257A substitution in parental PSTVd\textsuperscript{Mild} (E), PSTVd\textsuperscript{KF440-2} (F), and PSTVd\textsuperscript{RG1} (G) causes the flat-top symptom in each case. Note the normal “pyramid-shaped” appearance of shoot tips of plants infected with each of the parental viroids.
The heights of the plants and the lengths of successive internodes were measured from six plants infected with these PSTVd variants at 6 weeks after inoculation. The internodes were numbered sequentially, with the first one being immediately above the cotyledons. As shown in Figure 2C, the lengths of the first and second internodes were similar among plants infected with PSTVd<sup>int</sup>, PSTVd<sup>int</sup>C259U, or PSTVd<sup>int</sup>U257A. Starting from the third internode, the PSTVd<sup>int</sup>U257A-infected plants showed shortened length compared with plants infected with PSTVd<sup>int</sup> or PSTVd<sup>int</sup>C259U. The magnitude of shortening increased with subsequent internodes (Figure 2C). Thus, PSTVd<sup>int</sup>U257A-infected plants developed the flat-top symptom as a result of increased shortening or stunted growth of successive internodes.

At 7 weeks after inoculation, the leaves of PSTVd<sup>int</sup>U257A-infected tomato plants started to die. These plants did not produce flowers. At the same time, plants infected with PSTVd<sup>int</sup>C259U or PSTVd<sup>int</sup> had produced flowers, although they were delayed compared with flower production in mock-inoculated plants.

The U257A Substitution in PSTVd<sup>int</sup> Was Required Specifically for the Development of the Flat-Top Symptom

To determine whether the absence of U or the presence of A at position 257 was critical for PSTVd<sup>int</sup>U257A to cause the flat-top symptom in tomato, we inoculated tomato plants with PSTVd<sup>int</sup>-derived variants that have U257 replaced with nucleotide C or G by site-directed mutagenesis (Qi and Ding, 2002). As shown in Figure 2A, PSTVd<sup>int</sup>U257C caused a symptom similar to that caused by PSTVd<sup>int</sup>. On the other hand, PSTVd<sup>int</sup>U257G caused growth stunting intermediate between that caused by PSTVd<sup>int</sup>U257A and PSTVd<sup>int</sup>C259U (Figures 2A and 2C). These results indicate that nucleotide identity at position 257 plays a role in symptom expression and that A257 is required specifically for the development of the severe stunting and flat-top symptoms.

The U257A substitution could have caused changes in the secondary or higher structure of PSTVd to evoke new interactions with host factors to produce the flat-top symptom. Alternatively, the modified CCR could confer the pathogenic effect at the nucleotide sequence level. We showed previously that the computed PSTVd secondary structure was not altered by the U257A substitution (Qi and Ding, 2002; Zhu et al., 2002). To verify the computed data, we used temperature gradient gel electrophoresis (TGGE) (Figure 3A) to analyze the denaturation profiles of PSTVd<sup>int</sup>U257A and PSTVd<sup>int</sup>. A comparison of the denaturation profiles of different PSTVd RNAs on the same TGGE gel would reveal their structural similarities or differences (Rosenbaum and Riesner, 1987; Riesner, 1991). Our results showed that PSTVd<sup>int</sup> and PSTVd<sup>int</sup>U257A exhibited indistinguishable denaturation profiles in TGGE (Figure 3B). As a control, PSTVd<sup>int</sup> and variant PSTVd<sup>int</sup>A135G exhibited the expected differences in thermal stability (Figure 3C) (Owens et al., 1996). Similar differences were observed between PSTVd<sup>int</sup>U257A and PSTVd<sup>int</sup>A135G (Figure 3D), further confirming that PSTVd<sup>int</sup> and PSTVd<sup>int</sup>U257A share the same thermal stability.

These structural analyses indicate that the U257A substitution does not cause significant structural changes in PSTVd in vitro. Whether the same holds true in vivo remains to be determined. Nonetheless, our data suggest that a local change in the CCR sequence most likely was responsible for the induction of the flat-top symptom. The specificity of the pathogenic effect of A257 in the CCR was further established in the experiments described below.

The Symptoms Caused by the U257A Substitution Were Abolished by the C259U Substitution in a Double PSTVd Mutant

Given that the U257A substitution caused severe growth-stunting and flat-top symptoms and the C259U substitution did not, we tested the effect of the presence of both of these mutations on symptom expression. We created a double mutant, PSTVd<sup>int</sup>C259U/U257A, that contains both mutations in the same viroid RNA (Qi and Ding, 2002). Surprisingly, the double mutant caused symptoms similar to those caused by PSTVd<sup>int</sup>C259U (Figures 2C and 2D). Thus, host interactions mediated by U259 appear to dominate interactions mediated by A257. Alternatively, the presence of U259 may abolish the interactions of A257 with host factors. Regardless of the specific mechanisms, these data provide additional evidence for
the specificity of A257-conferred host interactions for the expression of the flat-top symptom. Furthermore, these data demonstrate that the effect of A257 can be modulated by other specific nucleotides in the CCR.

The CCR and the Pathogenicity Domain Function Independently and Synergistically in Symptom Expression

To test the general importance of A257 in pathogenicity, we replaced nucleotide U257 with A in three other PSTVd variants, PSTVdMild, PSTVdKF440-2, and PSTVdRG1, by site-directed mutagenesis. These variants differ in nucleotide sequences in the virulence-modulating region of the classic pathogenicity domain (Figure 4). PSTVdMild causes mild symptoms (Schnölzer et al., 1985), whereas PSTVdKF440-2 (Schnölzer et al., 1985) and PSTVdRG1 (Gruner et al., 1996) cause lethal symptoms in infected tomato.

Tomato plants infected with the new PSTVd variants (PSTVdMildU257A, PSTVdKF440-2U257A, and PSTVdRG1U257A) also developed the characteristic flat-top symptom in addition to further growth stunting in each case (Figures 2C to 2G). These data indicate that A257 plays a specific role in conferring the flat-top symptom, regardless of the parental PSTVd background. Furthermore, the classic pathogenicity domain can modulate the pathogenic effects of A257. Sequence variations in this domain allow infected plants to develop different severity levels of the final symptoms. Therefore, the classic pathogenicity domain and the CCR appear to function independently and synergistically in symptom expression.

The U257A Substitution Did Not Enhance PSTVd Accumulation in Infected Tomato

Increased severity of a plant disease could be the result of enhanced accumulation of a pathogen. Both the U257A and C259U substitutions in PSTVdInt enhance replication in tobacco cells (Qi and Ding, 2002), likely accounting for the increased infectivity in tobacco plants (Zhu et al., 2002). Therefore, we asked whether these nucleotide substitutions also could enhance PSTVd accumulation in tomato and whether U257A particularly would enhance accumulation in tomato to account for the development of the flat-top symptom.

To analyze the replication capacity of PSTVdIntC259U and PSTVdIntU257A compared with that of PSTVdInt at the whole-plant and cellular levels, we inoculated tomato plants as well as protoplasts with in vitro transcripts of these PSTVd variants. Total RNAs were extracted from infected plants at 1-week intervals for up to 6 weeks and from infected protoplasts at 3 days after inoculation. Accumulation levels of PSTVd were determined by RNA gel blot analysis. Such analyses showed that these PSTVd variants accumulated to similar levels in infected whole plants (Figures 5A and 5B) and infected protoplasts (Figure 5C). Thus, in contrast to the situation in tobacco, the U257A and C259U substitutions did not enhance PSTVd accumulation at either the cellular or the whole-plant level in tomato. Therefore, the flat-top symptom caused by PSTVdIntU257A infection was not correlated with viroid accumulation levels.

The U257A Substitution Did Not Alter Tissue Tropism of PSTVd in Infected Tomato

It was possible that PSTVdIntU257A invaded tomato cells that were not invaded normally by PSTVdInt, causing the flat-top symptom in the infected plants. Given the central role of the shoot apical meristem (SAM) in development (Sussex, 1989) and the observation that PSTVdInt did not invade the tomato SAM (Zhu et al., 2001), we asked whether PSTVdIntU257A would invade the SAM to interfere with its normal function, leading to perturbed development. To address this question, we performed in situ hybridization experiments using riboprobes specific for the plus-strand PSTVd. We examined SAMs of tomato plants infected with PSTVdInt and PSTVdIntU257A. Consistent with previous observations (Zhu et al., 2001), PSTVdInt was absent from the SAM (Figures 6A and 6B). PSTVdIntU257A also was absent from the SAM (Figures 6C and 6D).

We also extended the analyses of PSTVd localization to leaves and stems of infected plants. The two PSTVd variants were present in all tissues of leaves (Figures 7A to 7D) and stems (Figures 7E to 7H). Thus, the U257A substitution did not alter the cellular localization patterns of PSTVd in tomato.

Because the U257A substitution did not alter the accumulation levels or tissue tropism of PSTVd, we attributed its pathogenic effect to specific molecular interactions with a host factor(s). Such interactions resulted in inhibited shoot development to account for the flat-top symptom. To elucidate the basis of the
molecular interactions that led to the inhibited shoot development, we performed experiments to identify key cellular processes that were altered in the PSTVd<sup>IntU257A</sup>-infected tomato plants.

**Cell Expansion Was Inhibited in Stems and Leaves of PSTVd<sup>IntU257A</sup>-Infected Plants**

Plant development results from cell division to increase cell numbers, from growth to increase cell size, and from differentiation to form specialized cells/tissues (Steeves and Sussex, 1989). The restricted shoot development in PSTVd<sup>IntU257A</sup>-infected tomato could be attributable to inhibited cell division leading to the production of fewer cells, inhibited growth, abnormal differentiation of tissues, or a combination of any of these processes. Distinguishing between these possibilities should help us understand the cellular processes that were affected specifically to cause restricted shoot development and the appearance of the flat-top symptom.
We examined the number, size, and type of cells in the stems of mock-inoculated and PSTVd\textsuperscript{int}, and PSTVd\textsuperscript{int}U257A-infected tomato plants at 3 weeks after inoculation. To ensure that the analyses were conducted on organs at comparable developmental stages in tomato plants, we focused on the fifth internodes from the bottom (i.e., the first internode being the one immediately above the cotyledons). This internode was chosen because it was the one closest to the shoot apex that still could be identified reliably in the PSTVd\textsuperscript{int}U257A-infected plants. As shown in Figure 8, the length of the fifth internodes from PSTVd\textsuperscript{int}U257A-infected tomato plants was approximately one-third to one-half of that from the mock-inoculated and PSTVd\textsuperscript{int}U257A-infected plants, even though these plants should be at similar developmental stages. The fifth leaf also was much smaller in the PSTVd\textsuperscript{int}U257A-infected plants.

Cytological analyses revealed that all tissue types, including epidermis, cortex, vasculature, and pith, were present in the fifth internodes of PSTVd\textsuperscript{int}U257A-infected plants, as in the other plants (Figure 9). The numbers of cells in a transverse section were similar in PSTVd\textsuperscript{int} and PSTVd\textsuperscript{int}U257A-infected and mock-inoculated plants (Table 1). However, the cells in tomato plants infected with PSTVd\textsuperscript{int}U257A were approximately half the size of those in the other plants (Table 1, Figure 9). In the fifth leaf of PSTVd\textsuperscript{int}U257A-infected plants, all cell types were present and the cell size was smaller than that in the other plants (Figure 10).

The cytological data suggest that a major cause of the reduced internode length and leaf size was restricted cell growth. Cell division and differentiation were not affected visibly. To gain further evidence at the molecular level for the inhibited cell growth, we analyzed the expression levels of LeExp2 in the fifth internodes and leaves of mock-inoculated, PSTVd\textsuperscript{int}-infected, and PSTVd\textsuperscript{int}U257A-infected tomato plants. LeExp2 encodes an expansin that has been suggested to play a critical role in cell expansion in rapidly growing organs, based on its expression patterns in tomato (Reinhardt et al., 1998; Caderas et al., 2000; Catalá et al., 2000). We cloned a 248-bp fragment (nucleotides 855 to 1102) corresponding to the 3' untranslated region of LeExp2 from tomato using primers synthesized based on the published LeExp2 sequences (Caderas et al., 2000). The cloned LeExp2 fragment was used as a template for the in vitro synthesis of radiolabeled riboprobes. RNA gel blot analysis showed that LeExp2 expression was repressed severely in leaves and young stems of tomato infected with PSTVd\textsuperscript{int}U257A but less so in tomato infected with PSTVd\textsuperscript{int} (Figure 11). As an internal control, the expression of glyceraldehyde-3-phosphate dehydrogenase (\textit{GAPDH}) was analyzed and found to be unaltered in any organs (Figure 11). Thus, suppressed LeExp2 expression

Figure 6. Localization of PSTVd in Shoot Apices of Infected Tomato Plants by in Situ Hybridization. (A) and (B) Serial sections showing the absence of PSTVd\textsuperscript{int} from the SAM. Viroid signal is present in the vascular tissues (arrows) as well as in other cells in the subapical region. (C) and (D) Serial sections showing the absence of PSTVd\textsuperscript{int}U257A from the SAM. Viroid signal is present in the vascular tissues as well as in other cells in the subapical region.
Figure 7. Localization of PSTVd in Leaf and Stem Tissues of Infected Tomato Plants by in Situ Hybridization.

(A) and (B) Transverse (A) and paradermal (B) leaf sections showing the presence of PSTVd\textsuperscript{Int} in all tissue types. Cx, cortex; Ep, epidermis; Ms, mesophyll; Vas, vascular tissue.

(C) and (D) Transverse (C) and paradermal (D) leaf sections showing the presence of PSTVd\textsuperscript{IntU257A} in all tissue types.

(E) and (F) Transverse (E) and longitudinal (F) stem sections showing the presence of PSTVd\textsuperscript{Int} in all tissue types. Pi, pith.

(G) and (H) Transverse (G) and longitudinal (H) stem sections showing the presence of PSTVd\textsuperscript{IntU257A} in all tissue types. Arrows point to PSTVd localization signals.
was specific and was correlated positively with restricted cell expansion in the internodes and leaves of PSTVdIntU257A-infected tomato plants.

**DISCUSSION**

A Viroid Can Evolve Multiple Pathogenicity Determinants Spanning the RNA Genome

All nucleotide substitutions in PSTVd that influence symptom expression have been mapped to the classic pathogenicity domain (Schnölzer et al., 1985; Owens et al., 1991, 1995, 1996; Hammond, 1992; Gruner et al., 1996). The CCR of PSTVd has long been thought to be involved mainly in replication (Keese and Symons, 1985) and host range determination (Wassenegger et al., 1996). Thus, our finding that a single U257A substitution in the PSTVd CCR confers symptoms such as severe growth stunting, flat top, and premature death is quite unexpected. Considering the dramatic symptoms that the U257A substitution caused in tomato, it is surprising that this nucleotide substitution was not reported in previous studies. One explanation might be that this mutation has adverse effects on viroid replication, so that viroid progeny carrying this mutation disappear rapidly from a host cell. However, our studies showed that this mutation does not affect viroid accumulation in tomato and can be maintained stably in the infected plants.

Importantly, the U257A substitution did not alter PSTVd accumulation levels in single cells or in a whole plant. This finding is consistent with previous work showing no close correlation between accumulation levels and the severity of pathogenicity of some other PSTVd isolates (Schnölzer et al., 1985; Owens et al., 1991; Hammond, 1992). Our results further showed that the U257A substitution does not alter cellular localization patterns of PSTVd. Based on these findings, we conclude that the U257A substitution alters molecular interactions between the viroid and host factors to cause the flat-top symptom. Because the U257A substitution does not appear to alter the PSTVd structure, we postulate that A257 in the CCR functions independently of the classic pathogenicity domain as a separate pathogenicity determinant.

In testing the role of individual viroid structural domains in pathogenicity, Sano et al. (1992) constructed interspecific chimeras by exchanging domains between *Tomato apical stunt viroid* and *Citrus exocortis viroid*, both in the family Pospiviroidae. Infection studies showed that except for the CCR, all other viroid domains contribute to symptom expression. Here, we demonstrated that the nucleotide sequence of PSTVd CCR also plays a role in pathogenicity. Together, these data suggest that there is no definitive viroid pathogenicity domain. Spontaneous mutations in a viroid genome create diverse sequence and structural variants. As long as a sequence or structural change does not compromise the ability of the viroid variant to replicate and move systemically, its interaction with a host factor(s) to perturb cellular functions can lead to symptom expression. In this regard, a viroid can serve as a powerful source material for the study of how an RNA can evolve particular sequences/structures through nucleotide changes to achieve distinct biological functions.

**Figure 8.** Size Comparison of Fifth Internodes and Fifth Leaves of Mock-Inoculated and PSTVd-Infected Tomato Plants.

**A** The fifth internode from PSTVdU257A-infected tomato is one-third to one-half the size of those from mock-inoculated and PSTVdInt-infected plants.

**B** The fifth leaf of PSTVdU257A-infected plant is much smaller than those of mock-inoculated and PSTVdInt-infected plants.

A Viroid Motif Can Have Multiple Functions

This study, like previous studies, reveals the remarkable capacity of a viroid RNA motif to possess multiple and diverse functions. Subtle nucleotide changes can occur to introduce new functions without altering RNA structure or compromising other functions. Loop E of PSTVd CCR is involved directly in processing of longer-than-unit-length linear plus strands of PSTVd into unit-length circular molecules (Baumstark et al., 1997). It also is involved in host adaptation (Wassenegger et al., 1996). We showed that the role of loop E in host adaptation could be attributed at least partially to its regulation of transcription levels (Qi and Ding, 2002). Specifically, the U257A or C259U substitution in this motif, although not altering the PSTVd structure or processing capacity, enhances transcription levels ~5- to 10-fold in tobacco cells (Qi and Ding, 2002). The present study shows that the U257A substitution can specifically cause the flat-top and growth-stunting symptoms in tomato. Thus, loop E of PSTVd functions in processing, transcription, and pathoge-
nicity. PSTVd loop E’s role in pathogenicity is similar to the role of a tetraloop of *Chrysanthemum chlorotic mottle viroid* (CCMVd) in pathogenicity. A UUUC82-85 → GAAA substitution in the tetraloop converted the symptomatic strain CCMVd-S to a non-symptomatic strain, CCMVd-NS (de la Peña et al., 1999).

Loop E is present in a wide range of RNAs (Branch et al., 1985; Wimberly et al., 1993; Szewczak and Moore, 1995; Leontis and Westhof, 1998a) and functions as an important motif in RNA–RNA and RNA–protein interactions (Correll et al., 1997; Leontis and Westhof, 1998a, 1998b; Gongadze et al., 1999; Hampel and Burke, 2001). This loop may contain submotifs (Leontis and Westhof, 1998c). Based on these observations, two alternative mechanisms can be speculated to account for the pleiotropic functions of PSTVd loop E. First, submotifs of loop E mediate interactions with different host factors to accomplish separate functions. Second, different metastable structures are formed via interactions of loop E submotifs with other viroid regions, and these metastable structures then interact with different host factors to perform distinct functions. Elucidating how loop E performs multiple functions should provide valuable insights about RNA structure-function relationships.

**Roles of RNA Sequences in Viroid and Viral Pathogenicity**

Viroid variants with slight nucleotide sequence differences often cause different degrees of symptom severity (Schnölzer et al., 1985; Owens, 1990; Owens et al., 1991, 1995, 1996; Hammond, 1992; Škorić et al., 2001). Such differences may result in differences in viroid structure. For instance, nucleotide differences in the virulence-modulating domain within the pathogenicity domain of PSTVd*\(_{\text{Mild}}\)*, PSTVd*\(_{\text{Int}}\)*, and PSTVd*\(_{\text{RG1}}\)* lead to different degrees of bending of the virulence-modulating region.

**Figure 9.** Structure of the Fifth Internodes of Mock-Inoculated and PSTVd-Infected Tomato Plants.

(A) and (B) Transverse (A) and longitudinal (B) sections of the fifth internodes from mock-inoculated plants. Cx, cortex; Ep, epidermis; Pi, pith; Vas, vascular tissue.

(C) and (D) Transverse (C) and longitudinal (D) sections of the fifth internodes from PSTVd*\(_{\text{Int}}\)*-infected plants.

(E) and (F) Transverse (E) and longitudinal (F) sections of the fifth internodes from PSTVd*\(_{\text{IntU257A}}\)*-infected plants. All tissue types are present. The cell size is one-third to one-half of that from the other plants.
The nucleotide at position 257 must be A, and not U, G, or C, in the PSTVd\textsuperscript{Int} background to confer the flat-top symptom. This represents an example of how subtle nucleotide sequence differences in the viroid genome can confer distinct interactions with host factors. Thus, there are numerous possibilities for a viroid RNA to generate sequence variants, which may or may not assume higher level structures, that can interact with and perturb host functions and lead to disease formation. There is already an example of how subtle nucleotide differences without obvious effect on the PSTVd secondary structure have significant impact on interactions with host factors. Diener et al. (1993) showed that PSTVd\textsuperscript{Mild} and PSTVd\textsuperscript{Severe}, which differ by two nucleotides in the lower portion of the classic pathogenicity domain, have a 10-fold difference in activating the interferon-induced, double-stranded RNA-activated protein kinase (P68). Whether such differential activation of P68 is directly responsible for the development of some viroid symptoms remains to be investigated.

Direct RNA–host factor interactions for symptom expression may not be limited to viroid infection. Although most studies of viral pathogenesis have revealed roles of specific viral proteins (reviewed by Hull, 2002; Maule et al., 2002), a few studies demonstrate that noncoding elements of a viral genomic RNA (Rodríguez-Cerezo et al., 1991; Fernandez et al., 1999) or a viral satellite RNA (Taliany and Robinson, 1997; Taliany et al., 1998) can control symptom development in an infected host, potentially via direct interactions between the RNA sequences/structures and host factors. Thus, direct RNA–host factor interactions may be a fundamental mechanism of pathogenicity shared by viroids, viruses, and virus-associated RNAs such as satellite RNAs.

### The Possibility of Linking Viroid Structure, Host Gene Expression, Cellular Response, and Symptom Expression

Studies of plant viral and viroid diseases have produced much information about the pathogen determinants of symptom expression (Hull, 2002). The global gene expression pattern in an infected host has been examined by microarray analysis during viral infection (Whitham et al., 2003) and by macroarray analysis during viroid infection (Itaya et al., 2002). In general, there is a lack of understanding of how the altered expression of a partic-

| Table 1. Cell Number and Size in the Fifth Internodes of Mock-Inoculated and PSTVd-Infected Tomato Plants |
|-----------------|-----------------|-----------------|
| Inoculum       | Cortex Cell No. | Pith Cell No.   | Pith Cell Size (μm × μm) |
| Water          | 1486 ± 173      | 1735 ± 185      | 78.2 ± 10.3 × 89.1 ± 12.6 |
| PSTVd\textsuperscript{Int} | 1432 ± 145      | 1676 ± 203      | 60.0 ± 5.4 × 75.2 ± 6.7 |
| PSTVd\textsuperscript{IntU257A} | 1551 ± 218      | 1786 ± 156      | 33.5 ± 5.6 × 55.8 ± 8.1 |

\(a\) The number of cells represents the number on a transverse section. The number in each case is the mean ± SE calculated from cell counts obtained from four complete transverse sections of the internode.

\(b\) The size was measured as length (along the length of the internode) and width (across the width of the internode) of a cell in a longitudinal section in each case. For each longitudinal section, the cell length was calculated by dividing the entire length of the section by the total number of cells along the length. Similarly, the cell width was calculated by dividing the entire width of the section by the total number of cells along the width. Four sections were used to measure the cell size in each case.

![Figure 10. Structure of the Fifth Leaves of Mock-Inoculated and PSTVd-Infected Tomato Plants.](image_url)

(A) Transverse section of a leaf from a mock-inoculated plant.
(B) Transverse section of a leaf from a PSTVd\textsuperscript{Int}-infected plant.
(C) Transverse section of a leaf from a PSTVd\textsuperscript{IntU257A}-infected plant. All tissue types, including cortex (Cx), epidermis (Ep), mesophyll (Ms), and vascular tissue (Vas), are present in all leaves. The size of cells from the PSTVd\textsuperscript{IntU257A}-infected plant is one-third to one-half of that from the other plants.
Viroid Infection: A Useful Model in Which to Study Direct RNA Regulation of Cellular Processes

Without encoding proteins, a viroid RNA interacts directly with cellular factors to alter cellular functions that lead to changes in growth and development. Thus, a viroid is a de facto noncoding and regulatory RNA. Cellular noncoding and regulatory RNAs occur widely in organisms ranging from animals (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) to plants (Llave et al., 2002a; Reinhart et al., 2002). Recent studies have underscored the potential importance of such RNAs in the regulation of plant growth and development (Llave et al., 2002b; Rhoades et al., 2002).

Disruption of microRNA-controlled developmental processes is at least partly responsible for the formation of viral symptoms (Kasschau et al., 2003). Thus, viroid pathogenicity in all cases and viral pathogenicity in at least some cases share a common ground with the regulation of gene expression by cellular noncoding and regulatory RNAs: the direct effect of RNA on cellular functions. Studying the mechanisms of viroid and viral pathogenicity should provide fundamental insights into the mechanisms of RNA-regulated gene expression. Well-understood viroid structure/function relationships also may permit the engineering of RNA motifs with specific functions as research tools to study various biological processes.

METHODS

Plant Material and Growth Conditions

Tomato plants (Lycopersicon esculentum cv Rutgers) were grown in a growth chamber with 27/22°C day/night temperatures and a 14-h/10-h light/dark cycle.

PSTVd Variants and cDNA Cloning

Plasmids pRZ6-2, pRZ:Mild, and pRZ:RG1, which have cDNAs of PSTVdHd (Gross et al., 1978), PSTVdMo (Schnölzer et al., 1985), and PSTVdRg1 (Zimmatt et al., 1990), respectively, flanked by double ribozymes, were constructed by Hu et al. (1997) and kindly provided to us by Robert Owens (U.S. Department of Agriculture/Agricultural Research Service, Beltsville, MD). Construction of plasmids pRZ:Ut257A and pRZ:Ut257A was described by Zhu et al. (2001). Plasmids pRZ:Ut257A and pRZ:Ut257A with the U257A substitution and pRZ:Ut257A and pRZ:Ut257A with the A135G substitution were generated by site-directed mutagenesis using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The reactions were performed with plasmids pRZ:Mild, pRZ:RG1, or pRZ6-2 and complementary primers with sequences corresponding or complementary to PSTVd sequences with the desired mutation. The introduced mutations were verified by sequencing.

cDNA Cloning of Tomato LeExp2 and Glyceraldehyde-3-Phosphate Dehydrogenase Gene Fragments

A 248-bp fragment corresponding to the 3′ untranslated region of LeExp2 (corresponding to nucleotides 855 to 1102) (Caderas et al., 2000; Catalá et al., 2000) and that with other proteins to regulate cell wall expansion (Caderas et al., 2000). We recognize that LeExp2 likely functions in concert (Reinhardt et al., 1998; Caderas et al., 2000; Catalá et al., 2000). This finding is fully consistent with the observations that LeExp2 is expressed in rapidly growing parts of hypocotyls, stems, and leaves and with the hypothesis that the product of this gene functions to loosen cell walls to permit cell expansion (Reinhardt et al., 1998; Caderas et al., 2000; Catalá et al., 2000). We recognize that LeExp2 likely functions in concert with other proteins to regulate cell wall expansion (Caderas et al., 2000; Catalá et al., 2000) and that LeExp2 is likely one of the many genes whose expression is altered in PSTVdU257A-infected plants. Furthermore, altered expression of LeExp2 may occur downstream of the cascade of altered gene expression that leads to the flat-top symptom. Nonetheless, our data provide a concrete example of how a pathogen-elicited symptom at the whole-plant level can be linked to the disturbance of a specific cellular process, which in turn is correlated positively with the repressed expression of a cellular gene implicated explicitly in this process. Further studies now are possible to investigate the upstream genes that regulate LeExp2 expression and the primary targets of PSTVdU257A.
(2002) was amplified by reverse transcriptase–mediated PCR and then cloned into pGEM-T vector (Promega, Madison, WI) in the antisense orientation. This gave rise to plasmid pLeExp2(−), which was used as the template to produce antisense LeExp2 riboprobes. Plasmid pGAPDH(−) was provided by Asuka Itaya (Department of Plant Biology and Plant Biotechnology Center, Ohio State University, Columbus) and used to produce antisense glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobes. pGAPDH(−) has a 500-bp fragment of tomato GAPDH (corresponding to nucleotides 276 to 775), which was inserted into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA).

In Vitro Transcription

To prepare in vitro transcripts of the PSTVd variants for inoculation, the plasmids described above containing PSTVd cDNAs flanked by double ribozymes were linearized with HindIII and used as templates for in vitro transcription using the T7 MEGAscript Kit (Ambion, Austin, TX) according to the manufacturer’s directions. To obtain 32P-labeled PSTVd transcripts for temperature gradient gel electrophoresis (TGGE) analysis, UTP was replaced by α-32P-UTP in in vitro transcription reactions. Spliced-linear pInter(+) was used as a template to synthesize marker RNAs of 414 nucleotides that contain monomeric, linear (+)-PSTVd sequences and 55 nucleotides from the vector.

To prepare riboprobes for RNA gel blot analysis or in situ hybridization, α-32P- or digoxigenin-labeled antisense riboprobes were prepared in vitro transcription using the T7 Maxiscript Kit (Ambion) according to the methods recommended by the manufacturer using Spliced-linear pInter(−) pLeExp2(−), and pGAPDH(−) as templates.

After transcription, DNA templates were removed by digestion with RNase-free DNase I, and RNA transcripts were purified using the MEGAclear kit (Ambion). Nonradioactive RNA transcripts were quantified by UV spectrometry.

Plant and Protoplast Inoculation

Water and in vitro transcripts of PSTVd variants were used as inocula. Ten microfilters of water or PSTVd transcripts (100 ng/μL) was rubbed onto each carborundum-dusted cotyledon of each 6-day-old tomato seedling with a Pasteur pipette. Six plants were inoculated with water or transcripts of each PSTVd variant in one set of experiments. At least four independent sets of experiments were conducted to ensure the reproducibility of symptom expression caused by each PSTVd variant.

Protoplasts were prepared from young leaves of 3-week-old tomato plants as described by Mühlbach and Sänger (1977) with the following modifications: solution I [0.5 M mannitol and 3.6 mM 2-(N-morpholino)-ethanesulfonic acid, pH 5.9] was used instead of 0.78 M mannitol and 0.5% potassium dextran sulfate; protoplasts were resuspended in solution II (solution I plus 0.1 mM CaCl2) to a density of 2 × 10⁸ protoplasts/mL and kept on ice for 1 h before inoculation. The electroporation method used to inoculate protoplasts with PSTVd transcripts was essentially as described by Qi and Ding (2002). After inoculation, protoplasts were cultured in solution III (Schenk and Hildebrandt medium [Sigma, St. Louis, MO] plus 0.45 M mannitol). At 3 days after inoculation, the protoplasts were harvested for RNA extraction.

RNA Extraction and RNA Gel Blot Analysis

Total RNA was isolated from plants or protoplasts using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and quantified by UV spectrometry. For detection of PSTVd accumulation, RNA aliquots (10 μg each) were fractionated by electrophoresis at 55°C on 5% polyacrylamide gels containing 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0) and 8 M urea. To determine the expression levels of LeExp2 and GAPDH, RNA aliquots were subjected to 3-(N-morpholino)-propanesulfonic acid-formaldehyde agarose gel electrophoresis (Sambrook et al., 1989). After electrophoresis, the gels were stained with ethidium bromide and examined under UV light to determine the integrity and loading of the same quantity of RNA samples. The RNAs were transferred to HybondXL nylon membranes (Amersham Biosciences, Piscataway, NJ) using a vacuum-blotting system (Amersham). Hybridizations were performed at 65°C with ULTRAhyb reagent (Ambion) and in vitro transcribed α-32P-UTP–labeled antisense riboprobes. The membranes were washed at 68°C and exposed to a Storage Phosphor Screen (Kodak, Rochester, NY). Quantification of radioactivity was performed with Molecular Imager FX using Quantity One-4.1.1 software (Bio-Rad, Hercules, CA).

Sequencing of RNA Progeny

The protocols for preparing cDNAs of the PSTVd progeny isolated from infected plants or protoplasts were essentially as described by Qi and Ding (2002). cDNAs of PSTVd RNA were amplified by reverse transcriptase–mediated PCR and sequenced in both directions using the ABI377 DNA sequencer (Perkin-Elmer, Boston, MA) at the DNA Sequencing Facility at Ohio State University.

Tissue Processing

Tissue processing was performed as described previously (Zhu et al., 2001). Tomato samples were fixed in 10% formaldehyde, 50% ethanol, and 5% acetic acid, dehydrated, and embedded in paraffin (Electron Microscopy Sciences, Fort Washington, PA). Sections (10 μm) were obtained with a rotary microtome (model HM 340 E; Microm International, Walldorf, Germany).

Histology

Tissue sections were dewaxed in xylene, treated sequentially with anhydrous isopropanol and 70% isopropanol, stained with 0.5% (w/v) safranin in 95% isopropanol, and rinsed with 50% isopropanol. Stained sections were mounted and examined with a Nikon Eclipse 600 light microscope (Nikon, Tokyo, Japan). Images were captured with a SPOT 2 Slide charge-coupled device camera and the associated software (Diagnostics Instruments, Sterling Heights, MI). Image processing and figure preparation were performed using Adobe Photoshop (Mountain View, CA).

In Situ Hybridization

In situ hybridization was performed as described previously (Zhu et al., 2001) using digoxigenin-labeled antisense PSTVd riboprobes. After hybridization, the sections were examined and photographed with the Nikon Eclipse 600 light microscope as described above.

Analysis of PSTVd RNAs by Perpendicular TGGE

Preparation of PSTVd RNAs for TGGE was performed essentially as described by Owens et al. (1996). 32P-labeled linear unit-length PSTVd RNAs were purified by electrophoresis at 55°C on 5% polyacrylamide gels containing 1× TBE buffer and 8 M urea and circularized by incubation with wheat germ extract (Promega). Perpendicular TGGE analysis of 32P-labeled circular and linear PSTVd RNAs was performed using a TGGE system (Biometra, Göttingen, Germany) according to the instructions of the manufacturer using 5% polyacrylamide gels (acrylamide: bisacrylamide [30:1], 0.2× TBE, and 5 mM NaCl) and running buffer (0.2× TBE and 5 mM NaCl). The gel electrophoresis procedure was as follows: (1) prerunning at 25°C and 200 V for 5 min; (2) loading the mixture of
The GenBank accession number for tomato available in a timely manner for noncommercial research purposes.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

**Accession Number**
The GenBank accession number for tomato GAPDH is U97257.

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Inhibition of Cell Growth and Shoot Development by a Specific Nucleotide Sequence in a Noncoding Viroid RNA
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