PLANT VIRUS SATELLITE AND DEFECTIVE INTERFERING RNAs: New Paradigms for a New Century

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Key Words subviral RNAs, virus-induced gene silencing, *Turnip crinkle virus*, *Cucumber mosaic virus*, *Tomato bushy stunt virus*

■ **Abstract**   Although many subviral RNAs reduce or intensify disease symptoms caused by the helper virus, only recently have clues concerning the mechanism of disease modulation been revealed. New models for DI RNA-mediated reduction in helper virus levels and symptom attenuation include DI RNA enhancement of post-transcriptional gene silencing (PTGS), which is an antiviral defense mechanism in plants. Symptom enhancement by the satRNA of *Cucumber mosaic virus* is caused by minus-strand induction of the programmed cell death pathway. In contrast, symptom enhancement by satC of *Turnip crinkle virus* is due to satC interference with virion formation, leading to increased levels of free coat protein, which is the viral suppressor of PTGS. Mutualism between satRNA and helper virus can be seen for the satRNA of *Groundnut rosette virus*, which contributes to the virus by allowing virion assembly. These novel findings are leading to re-evaluation of the relationships between subviral RNAs, helper viruses, and hosts.

INTRODUCTION

One feature that distinguishes plant viruses from animal viruses is their common association with a variety of subviral RNAs. Subviral RNAs can be distinguished from the viral genome by their dispensability for normal virus propagation. Subviral RNA replication is completely dependent on enzymes encoded by their helper virus and thus amplification is limited to coinfected cells. There are two subgroups

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of subviral RNAs based on their origin. Defective interfering (DI) RNAs (or just "defective" [D] RNAs if they do not interfere with the replication of their helper virus) are derived mainly from the genome of the helper virus by one or more premature termination and reinitiation events that infrequently result in a functional template capable of further amplification. Whereas DI RNAs also are commonly associated with animal viruses, the second subgroup, satellites, are nearly exclusively associated with plant viruses. Satellites are RNAs with sequence that is either mostly or completely unrelated to any large contiguous segment of the helper virus genome.

Subviral RNAs, many of which are noncoding, can cause profound alterations to the normal disease progression induced by their helper virus (15, 36, 77, 110) and thus have posed a fascinating enigma to virologists for over 40 years. Recent advances in understanding how the host responds to viral invasion have led to breakthroughs in elucidating how subviral RNAs cause, enhance or prevent disease symptoms. For example, symptom attenuation was once mainly attributed to simple competition between the subviral RNA and helper virus for limited quantities of replication factors. However, recent evidence from several systems suggests that subviral RNA-mediated enhancement of host resistance might be of equal or greater importance. Concepts delineating the evolutionary relationship between subviral RNA and helper virus are also evolving. Where once the relationship between subviral RNA and helper virus was viewed as purely parasitic, with the subviral RNA reaping benefits at the expense of the helper, recent results suggest that some pairs have more complex relationships, including ones that are mutualistic, benefiting both participants.

This review is not intended as a comprehensive examination of subviral RNAs, which can be found in other reviews (77, 103, 110). Rather, our goal is to present exciting recent findings on a few well-defined systems that are reshaping the way subviral RNAs are thought to interact with their helper viruses and hosts.

DEFECTIVE AND DEFECTIVE INTERFERING RNAs

DI RNAs are associated with many plant and nearly all animal RNA viruses. These truncated and often rearranged versions of viruses are generally missing some or all of the viral genes required for movement, replication, and encapsidation but contain all *cis*-acting elements necessary for replication by the RNA-dependent RNA polymerase (RdRp) of the parental virus. De novo generation of DI RNAs has been studied extensively in the past decade, and the RdRp-mediated copy choice model originally described for the generation of DI RNAs from animal viruses is also valid for plant virus DI RNAs (110). Following de novo generation, DI RNAs are likely under strong selective pressure for biological competitiveness. While most DI RNAs moderate the symptoms of their helper virus, DI RNAs of *Broad bean mottle virus* and *Turnip crinkle virus* (TCV) possess the unusual attribute of exacerbating symptom severity (49, 74).

DI RNAs of Tombusviruses

The first example of a plant virus DI RNA was described for the genus Tombusvirus (34). Tombusvirus DI RNAs represent important prototypes and are the most extensively studied of the plant virus DI RNA systems (79). Several DI RNAs have been described in tombusvirus infections and all possess common structural features that maintain noncontiguous elements of the parental genome corresponding to terminal regions and an internal segment while losing all coding capacity (109). De novo generation of DI RNAs resulted from high-multiplicity passages of plasmid transcript-derived and therefore presumably DI RNA-free tombusvirus inocula in *Nicotiana clevelandii* and *N. benthamiana* (43).

The presence of DI RNAs in virus-infected plants dramatically suppresses virus accumulation and leads to persistent attenuated symptoms (Figure 1). Infected plants containing DI RNAs are protected from lethal necrosis normally induced by their helper virus and in most cases are able to flower and set seeds. The biological role of DI RNAs is not clear because no DI RNAs have been found associated with over 50 field isolates of *Tomato bushy stunt virus* (TBSV) obtained from eggplant and tomato (12). These results suggest that DI RNAs may not play an important role in modulating natural virus-host interactions in these hosts. However, additional studies are required to fully examine the biological significance of DI RNAs under natural field condition.

To date, there is no single mechanism that is generally accepted to account for DI RNA interference with the accumulation of their helper virus and corresponding symptom attenuation. Mechanisms of DI RNA symptom modulation have been proposed to involve either (*a*) competition with the helper virus for trans-acting factors necessary for replication, (*b*) specific interaction with virus-encoded products, or (*c*) activation of posttranscriptional gene silencing (PTGS) (Figure 2).

DI RNA COMPETITION WITH THE HELPER VIRUS   One logical and widely used explanation for the reduction of helper virus genomic RNA levels when associated with DI RNAs is a competition between the DI RNA and the viral genome for limited factors required for replication; reduced virus levels in turn lead to attenuated symptoms (38). Supporting this hypothesis is the ability of DI RNAs to reduce helper virus accumulation at the single-cell level. Cotransfection of *Nicotiana benthamiana* protoplasts by equimolar amounts of TBSV (cherry strain) genomic and DI RNA resulted in a 65% suppression of genomic RNA accumulation at 9 h posttransfection compared to DI RNA-free transfection (38). Moreover, this inhibition of helper virus RNA accumulation was dose dependent, suggesting an efficient competition by the DI RNAs for limiting factors such as the RdRp.

Recently, however, results have been obtained that question a simple interference model for DI RNA-mediated reduction of helper virus levels leading to reduced symptoms. DI RNAs of Cucumber necrosis virus (CNV) suppress >50% of virus accumulation in cucumber protoplasts (13). However, one DI RNA species (C40) was only weakly interfering, resulting in virus reductions of only 30%
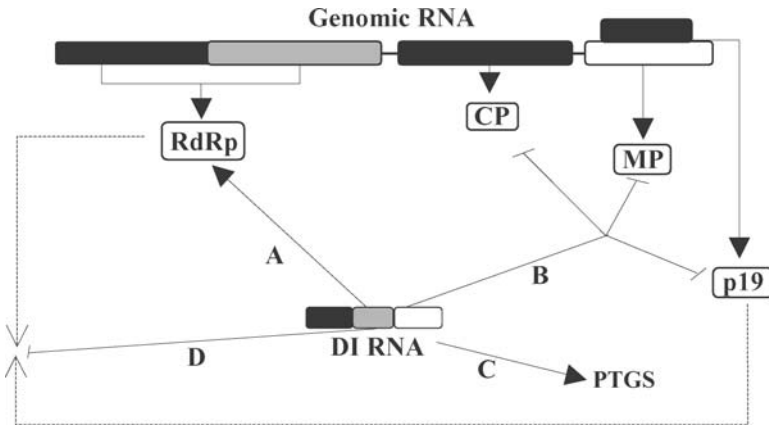
Figure 2 Proposed factors influencing symptom modulation of DI RNAs. Schematic representation of tombusvirus genomic and DI RNAs. RdRp, RNA-dependent RNA polymerase; CP, coat protein; MP, movement protein; p19, PTGS suppressor protein. Dotted lines indicate a presumed interaction of virus-derived proteins. *A*. The most widespread explanation for the interference mediated by DI RNAs is the ability of these subviral molecules to replicate efficiently competing for a limited supply of virus-encoded trans-acting factors such as the RdRp (38). *B*. Selective inhibition of sgRNAs and the corresponding proteins has been demonstrated in TBSV-infected plants. In this case, the genomic RNA and the RdRp (which is translated directly from the genomic RNA), accumulation was decreased less dramatically (84). *C*. The presence of DI RNAs can also activate PTGS, which may result in enhanced degradation and reduced spread of the helper virus (93; Z. Havelda, unpublished data). *D*. Protein-protein interactions play an important role in the development of severe symptoms and can be inhibited by DI RNAs. Some DI RNAs were not able to block this interaction and although they accumulate to high levels, cannot protect the infected plant (32).

compared with DI RNA-free virus. Despite such limited interference, C40 accumulation and symptom modulation in infected plants was similar to that of the prototypical DI RNAs. A DI RNA deletion mutant was also identified that did not efficiently interfere with the accumulation of the helper virus although the DI RNA accumulated to high levels (13). Investigation of *trans*-accumulation competence of RdRp-deficient TBSV deletion mutants resulted in the identification of a small region in the RdRp gene that acts as an inhibitor of *trans*-rescue (ITR) (65). Introduction of the ITR element into a DI RNA in the reverse orientation strongly suppressed DI RNA accumulation. More surprisingly, introduction of the ITR element in the positive orientation resulted in a DI RNA species that replicated efficiently but was not able to interfere significantly with either helper virus accumulation or the onset of lethal necrotic symptoms.

Replication of different tombusvirus DI RNAs was efficiently supported by closely related heterologous helper viruses (32, 78) but not distantly related or

unrelated viruses (78). For some DI RNA-helper virus combinations, accumulation of the genomic RNA was reduced and the heterologous DI RNAs accumulated to high levels. However, infected plants did not exhibit attenuated symptoms (32). Based on the nature of the symptom-modulating properties, protective (symptom attenuating) and nonprotective (no symptom-modulating) DI RNAs have been identified. Moreover, substantial variation has been observed in the ratios of protective DI RNAs to the helper virus genome with no particular ratio determining symptom development (32).

Interestingly, the accumulation and symptom-modulating ability of DI RNAs appears to be host specific. Inoculation of cucumber plants with sap containing CNV and DI RNAs reduced the normally high levels of DI RNA to undetectable levels (13). Since cucumber protoplasts support efficient replication of DI RNAs, the authors suggested that factors such as cell-to-cell and long-distance movement or specific interaction with host factors could contribute to the accumulation of DI RNAs in a given host.

These observations strongly suggest that competition between DI RNAs and the helper virus genome for limited replication factors cannot explain decreased accumulation of virus RNA or symptom attenuation by at least some DI RNAs.

SPECIFIC INTERACTIONS BETWEEN DI RNA AND VIRAL DERIVED PRODUCTS Alternative models have been proposed suggesting that a link between DI RNA accumulation and viral gene expression leads to symptom attenuation (85). One study found that the presence of DI RNAs in TBSV-infected plants caused a pronounced reduction in subgenomic (sg) RNAs and their encoded proteins while only mildly suppressing genomic RNA and RdRp levels. The 19-kDa protein (p19), translated from sgRNA2 (79), is both an important symptom determinant responsible for the induction of necrotic symptoms (6, 14, 82) and a posttranscriptional gene-silencing (PTGS; see below) suppressor (86). Moreover, sgRNA2 is also responsible for translation of a 22-kDa protein that is the cell-to-cell movement (MP) of the virus (18, 83). Since the presence of the DI RNA greatly reduced accumulation of p19 and MP, the authors suggested that, in addition to competition between DI RNAs and the helper virus, DI RNAs may also act via selective inhibition of virus-derived products responsible for development of severe symptoms or basic viral functions.

In contrast, no targeted inhibition of sgRNAs was detected using TBSV pepper isolate (TBSV-P) as helper virus and DI RNAs of *Carnation Italian ringspot virus* (CIRV) and TBSV-P. Although plants coinoculated with TBSV-P and CIRV DI RNA contained a high level of DI RNA and a low level of viral genomic RNA, they were still necrotized. These findings suggested that suppression of genomic RNA replication by the DI RNA was not directly responsible for symptom attenuation, unless the lowest accumulation of viral genomic RNA observed was below an unidentified threshold value that is sufficient for development of necrosis. Specific interaction of protective DI RNAs with viral-derived products was proposed to be important for the induction of attenuated symptoms (32). However, p19 accumulated to similar levels in infected plants in the presence of either protective or

nonprotective heterologous DI RNAs (32). The ability of heterologous DI RNAs to protect infected plants against systemic necrosis was mapped to the 5′-proximal region of the helper virus genome. Since direct or indirect interaction of p19 and p33 (the component of the RdRp encoded by the 5′-proximal region of the helper virus) was required for induction of necrotic symptoms (6), a model was proposed in which protective DI RNAs prevent, whereas nonprotective DI RNAs allow, the establishment of this interaction.

ACTIVATION OF PTGS   PTGS is an adaptive sequence-specific RNA degradation system that plays a role in control of transposon transposition, preservation of genome integrity, and defense against viruses (30, 104). According to recent models, PTGS is activated by double-stranded (ds) RNA and results in cleavage of the RNA into 21–26 nt long double-strand (ds) small interfering (si) RNAs by an RNase III-like enzyme called DICER. The generated siRNAs are associated with an enzyme complex (RNA-induced Silencing Complex; RISC) and guide the RISC to degrade any RNA having sequence homology with the inducer dsRNA. In addition to the cell-autonomous defense function, PTGS in plants generates a mobile signal that instructs target RNA degradation at a distance (systemic PTGS) (105). The sequence specificity of PTGS implies that the signal must consist of a nucleic acid component homologous with the target RNA. Recent findings have suggested that the 21-nt siRNAs are the systemic signaling molecules (35).

In plants, PTGS is triggered by dsRNA products and/or highly structured RNA elements of cytoplasmically replicating viruses (1, 93). Infection of nepo-, tobra-, and caulimoviruses, which are not able to efficiently cope with PTGS, often result in the development of a recovery phenotype displaying suppressed virus accumulation and symptom attenuation (69, 70). Consistent with the antiviral function of PTGS, many viruses have developed gene-silencing suppressor proteins such as p19 of tombusviruses (48, 106). These suppressor proteins act at different stages of the silencing pathway, targeting initiation, maintenance, or systemic signaling events (48). The p19 binds to and sequesters the majority of the 21-nt virus-specific siRNAs, thus blocking the spread of the 21 nt siRNAs ahead the infection front and preventing the onset of systemic silencing (86; L. Lakatos, unpublished data). In the absence of p19, virus accumulation is restricted to veins and adjacent tissues (31). Infection with a p19-defective mutant of *Cymbidium ringspot virus* (CymRSV) resulted in the development of a PTGS-associated recovery phenotype and symptoms that were surprisingly similar to those associated with DI RNA-containing infections (93). The role of PTGS in DI RNA-symptom modulation was further investigated by analyzing the composition of virus-derived siRNAs in CymRSV-infected plants. The siRNAs were essentially evenly distributed along the viral genome except in the 5′ region, which was significantly underrepresented (93). In contrast, the presence of DI RNAs caused dramatic changes to the siRNA distribution profile, leading to a large increase in the number of siRNAs corresponding to sequences encompassed by the DI RNAs. These observations demonstrated that siRNAs could be generated efficiently from DI RNAs, with a corresponding alteration of the accumulation pattern of siRNAs. More interestingly, the DI

RNAs were not efficiently targeted by the siRNAs, indicating that DI RNAs are effective inducers, but poor targets, of virus-induced PTGS (93). PTGS may thus play an important role in the evolution of DI RNAs by eliminating superfluous target sequences. Moreover, since DI RNAs are efficient inducers of PTGS, PTGS may contribute significantly to DI RNA-mediated symptom attenuation by degrading the helper virus genome. In situ analysis of tombusvirus-infected plants in the presence or absence of DI RNAs revealed that virus accumulation was now confined to the vicinity of vascular bundles, a phenotype similar to that caused by infection of p19-defective tombusviruses (Figure 1*C*) (Z. Havelda, unpublished data). Since the activity of p19 depends on its ability to bind to the 21-nt siRNAs, the accumulation of virus-derived siRNAs has been investigated in the presence and absence of DI RNAs. DI RNAs elevated the level of virus-specific siRNAs in the infection process, resulting in the saturation of p19 and consequently in the accumulation of unbound siRNAs. These data suggest that unbound siRNAs in DI RNA-containing infections are able to induce systemic PTGS ahead of the infection front similarly to p19-defective virus infections, resulting in the restricted spread of the virus. This finding was further supported by the observation that at low temperatures, which restrict the efficiency of PTGS (94), DI RNAs were unable to reduce the levels of TBSV-P genomic RNA and protect the plant from necrotic symptoms (Z. Havelda, unpublished data).

SATELLITE RNAs AND SATELLITE VIRUSES

The classical definition of a satellite is a linear or circular RNA that requires a helper virus to supply proteins for replication but shares little sequence relatedness with the helper virus and is not required for the accumulation of the helper virus (55). Satellites that encode their own CP, four of which have been described, were labeled as satellite viruses to distinguish them from satellite RNAs (satRNAs), which are encapsidated in particles assembled from helper virus CP. SatRNAs are roughly divided into two classes: large satRNAs that generally encode a single nonstructural protein and smaller satRNAs with no functional coding capacity.

While this classical description of satellites continues to be useful, an increasing number of exceptions have emerged requiring past definitions to become more flexible. For example, satC associated with TCV is a hybrid molecule composed of sequence from a second satRNA and two portions from the 3′ end of TCV genomic RNA (87). Also complicating previous definitions is the satRNA associated with the umbravirus GRV. This noncoding satRNA, while dispensable for virus movement within a host, is required for encapsidation of GRV in the CP of its luteovirus partner, *Groundnut rosette assistor virus* (GRAV), which is a prerequisite for aphid transmission (72).

SatRNAs of *Cucumber Mosaic Virus*

The satRNAs of *Cucumber mosaic virus* (CMV satRNAs) are linear and single stranded, and range from 330 to 405 nt. No functional open reading frames (ORFs)

have been documented for any CMV satRNAs. Although several small ORFs can be found in many CMV satRNAs, they are not well conserved among the more than 75 isolates that have been sequenced, and some have been shown to be nonfunctional by mutational analyses (reviewed in 23, 77). The helper virus, CMV, is a plus-stranded RNA virus, and by definition the packaged strand of satRNAs is also called the plus strand, even though it lacks protein coding functions.

STRUCTURAL AND SEQUENCE CONTRIBUTIONS TO PATHOLOGY   The majority of CMV satRNAs attenuate the symptoms induced by the helper virus. The pathogenic variants of CMV satRNAs also attenuate symptoms in most hosts, but induce either necrosis (26, 39, 41, 46, 68) or chlorosis (25, 62, 95) on tobacco, *N. benthamiana*, or tomato hosts. In most cases, the pathogenic phenotypes have been mapped to one or a few specific nucleotides (37, 89–91, 117); however, this information has not helped to elucidate the mechanism of pathogenicity. A study using a Potato virus X (PVX) vector to express D4 satRNA in tomato indicated that the minus strand, not the plus strand, was responsible for necrotic lesions on the infected leaves (100). In addition, in a separate study, the ratio of minus strands to plus strands increased during the onset of necrosis (112). In the in vivo secondary structure model for CMV satRNA, a stem and tetraloop was identified near the 3′ terminus of the plus strand of D4 satRNA that was conserved by covariation analysis in all necrogenic satRNAs, and which contained the three nucleotides previously mapped as responsible for necrosis (73). How this structure affects the plant cell is not clear, but given the proximity of the hairpin to the 3′ end, it is possible that it is involved in enhancing satRNA minus-strand synthesis.

MECHANISMS OF PATHOGENICITY   Detailed studies of tomato necrosis induced by CMV satRNA D4 demonstrated that programmed cell death (PCD) was involved (112). PCD was detected by the signature events of chromatin clumping, microscopic detection of DNA fragmentation, and the demonstration of discrete-sized DNA degradation fragments after the onset of cell death. PCD occurred in developing phloem cells in the stem just below the apical meristem. Subsequently, nearby vascular cells also underwent PCD, and within 24 to 48 h systemic necrosis was induced and the entire plant collapsed (Figure 3). The timing of the onset of PCD and necrosis was reproducible and could be altered only by inoculating plants at different ages. Although both plus- and minus-strand D4 satRNA were found in the cells of infected stems, satRNA was found in vascular bundles only where PCD was also detected. In addition, whereas levels of plus-strand satRNA rose rapidly and remained high throughout the infection, minus-strand levels rose dramatically just before the onset of PCD, further indicating involvement of the minus strand in satRNA pathogenesis (112).

CMV infection by itself does not elicit a detectable defense response in most infected plants, and no classical markers of defense are expressed at elevated levels. However, when the D4 satRNA was coinoculated with CMV in tomato a large number of defense-related genes, including PR1, PR5, and PR10, exhibited dramatic increases in expression prior to any visible onset of cell death. Expression
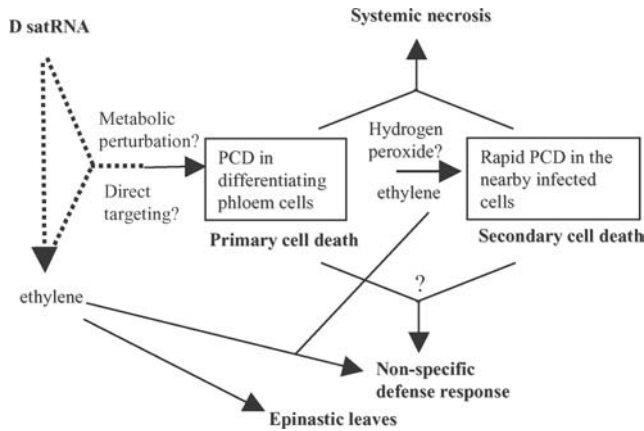
Figure 3   The complex pathway leading to CMV/satRNA systemic necrosis. The D4 satRNA induces PCD in developing phloem cells either by some metabolic perturbation or by a direct but unidentified effect. Secondary cell death then occurs via a signal such as hydrogen peroxide or ethylene in nearby infected cells. Finally, systemic necrosis develops, also via an undetermined signal. The involvement of ethylene in the process is suggested by leaf epinasty, which occurs just prior to collapse of the plant from necrosis. It is not clear when or how the multiple host-defense responses are activated, or if they are a cause or an effect of the PCD, but they appear before any visible symptoms or evidence of PCD is discernible.

of the putative antiapoptosis Bax inhibitor, tomBI-1, also increased in level and distribution. However, in spite of a "best effort" by the plant, it still dies (111).

The sequence determinants for chlorosis induction in tomato have been known for nearly a decade, but no further studies on the mechanisms of pathogenicity for the chlorotic satRNAs in tomato have been published. The phenotype in tomato induced by the WL2 satRNA is a white chlorosis (25), which is apparently a loss of both chlorophyll and carotenoid pigments such as xanthophyll. In these plants the number of chloroplasts is unchanged, but the size is reduced (M. Yassi, personal communication). With the B5 satRNA, a yellow chlorosis is induced in tomato, indicating a loss of chlorophyll only (24). In tobacco, yellow chlorosis induction by satRNAs has been linked to a single host gene, showing incomplete dominance (52). However, no further characterization of this phenotype has been performed.

EVOLUTIONARY CONSIDERATIONS OF CMV satRNAs   The origin of satRNAs, including CMV satRNAs, remains an intriguing mystery. Although commonly detected in experimental systems, CMV satRNAs are not as common in agricultural settings (42) or in nature. In wild hosts of CMV, satRNAs have been found frequently only in *N. glauca* in southern California (A. Dodds, personal communication) and southern Spain (F. García-Arenal, personal communication), and

occasionally have been described in weed species surrounding agricultural settings (88). In experimental studies, reduced aphid transmission occurred with CMV strains containing satRNA, which could account at least in part for differences seen between natural infections and experimental infections (22).

No significant sequence identity exists between the satRNA and the helper virus, except in very small segments. The genome of *Arabidopsis thaliana* also does not contain any intact sequence that is similar to CMV satRNA. However, there are significant stretches of similarity (Y. Bao, personal communication), and it is possible that the satRNAs are generated from the host plant under some unique conditions. Recent characterization of numerous species of small regulatory RNAs in plants (51 and references therein) are suggestive. During CMV infection, the PTGS pathway is activated, and it is possible that rare events bring together the right combination of small RNAs is able to generate a satRNA. In addition, multiple transcription termination and reinitiation events by the RdRp leading to satRNA generation cannot be ruled out. Strong selection for better replicating (i.e., more "fit") satRNA has likely resulted in substantial alternation of sequence from the original molecule, further obscuring progenitor RNA identification.

The CMV satRNAs have been used for a number of experimental evolution studies (47, 63, 64, 76). These studies demonstrated that a population of satRNA variants was rapidly generated in progeny of cDNA clones, and the population structure could be affected by the helper virus as well as by the sequence context of the satRNA. CMV satRNAs have been used to assess the evolution of virulence as well. A variant of the B5 satRNA causes chlorosis when the helper virus is a subgroup II strain, but with subgroup I strains the satRNA attenuates symptoms. However, the nonpathogenic variant reproducibly mutated to the pathogenic variant upon passage, even when the helper virus was a subgroup I strain. This demonstrates that the evolution of virulence in this system was unrelated to pathology per se (64). Clearly, the necrotic satRNAs could not have evolved in the hosts where they cause this disease. When a parasite kills its host it is also suicide for the parasite, unless it is able to rapidly escape to a new host. The necrotic phenotype in tomato develops very rapidly. These satRNAs most likely normally infect another plant, where they do not cause disease, and only when they accidentally infect tomato are they lethal.

Epidemiological studies of CMV and its satRNAs from fields in Spain indicated that the virus and satRNA do not always move together (2). This is a remarkable finding, because it is contrary to the notion that the satRNA must be packaged by the helper virus to be acquired by aphids for natural transmission. In order for the satRNA to spread independently, transmission must be more complex than what is currently known from laboratory experiments. This study also noted higher levels of sequence variation in the satRNA populations, as compared to the helper virus populations. Clearly, the evolutionary mechanisms and constraints for CMV and the satRNA are different. Phylogenetic analyses have been helpful in understanding the evolutionary history of the helper virus (75). A limited number of phylogenetic analyses have been done for CMV satRNAs (2, 5, 23). However,

an accurate estimation of a phylogeny, or the history of ancestry and descent of a group of taxa, depends on divergent evolution. Since experimental evolution studies have clearly shown convergent evolution in CMV satRNAs, the true phylogeny of satRNAs is probably obscured in the sequence data.

SatRNAs of *Turnip Crinkle Virus*

TCV is unique among members of the genus Carmovirus in being associated with several small satRNAs (3, 87). SatD, at 230 nt, shares many features of traditional satRNAs by having little contiguous sequence similarity with TCV beyond the 3′ terminal seven bases. As with other satRNAs, the origin of satD is obscure. However, a study on repair of 3′ terminal deletions suggests that satD may have evolved by repeated recombination events that linked short (8- to 10-nt) segments of TCV plus and minus strands to produce an RNA capable of being replicated and packaged (8, 9). SatD has no discernible effect on either replication or symptoms associated with TCV. However, laboratories working with satD are unable to keep TCV-infected plants free of satD, which can accumulate to levels equivalent to that of 5S rRNA within three weeks of inoculation with an inoculum that does not purposely contain satD (A.E. Simon, unpublished data). Although the reappearance of satD has been suggested to imply emergence from the plant's genome (4, 17), restricting the handling of satD in a new laboratory setting can eliminate its presence in TCV-infected plants (A.E. Simon, unpublished data). In addition, detection of satD sequences in the genome of turnip (4) could not be confirmed (A.E. Simon, unpublished data). These results and observations suggest that despite extraordinary efforts to keep TCV-infected plants free of satD in settings where satD is also being studied (17; A.E. Simon, unpublished data), TCV-infected plants exposed to minute traces of satD in the immediate environment rapidly accumulate the satRNA.

A second satRNA associated with TCV, satC, has a sequence consistent with its generation by a recombination event between satD and two regions of TCV. Recombination between satD and TCV is a common event, generating large populations of similar, but not identical recombinant molecules within 20 h posttransfection of turnip protoplasts with transcripts of satD and TCV (7). Junctions in recombinant molecules were not random, with crossovers in TCV located at the base of enhancers or RNA motifs similar to those found in promoter elements (7, 10, 11, 57, 113). These findings led to a model suggesting that the RdRp was responsible for generating recombinant molecules by reinitiating RNA synthesis at promoter-like elements during plus-strand synthesis of satD and TCV (11, 59).

Since the population of satD/TCV recombinants amplified and sequenced at various times postinoculation of plants and protoplasts were mainly unique species (7), and since randomly selected recombinants were incapable of efficient amplification in protoplasts (57), the ability of satC to accumulate efficiently must reflect additional parameters beyond the simple presence of satD and 3′ TCV sequences. In addition to a number of positional differences between satD and

TCV sequence and the corresponding segments in satC, the region joining satD and the two fragments from TCV results in the formation of a hairpin (M1H), which, in its minus-sense orientation, is an enhancer of satC replication in vivo and in vitro (57–59, 92, 116). The enhancer function of the M1H likely derives from the minus-sense TCV junction sequences, which contain promoter-like motifs (92, 116). This possibility was confirmed by replacing the 28-base MIH with randomized sequences and then subjecting the satC population to in vivo genetic selection. Following multiple rounds of competition between functional satC species, satC that were more fit to accumulate were determined to contain short (6- to 10-nt) motifs on minus strands that could replace the M1H, with most motifs having strong similarity to sequences found in TCV promoter elements (92, 116).

SatC REDUCES ACCUMULATION OF TCV   Levels of TCV accumulating in infected plants and protoplasts are reduced by 25% to 50% in the presence of satC (27, 28, 50, 92, 116). Since satC and TCV share 3' sequences and both are replicated by the viral-encoded RdRp, satC may be interfering with the replication (or stability) of TCV by competing for the RdRp. Despite the conservation of 3' sequences, replication of satC differs in some fundamental aspects from the replication of TCV. For example, satC accumulated to levels that were three- to fourfold greater in protoplasts when either the TCV CP was deleted or when the TCV CP was replaced with the CP of the related virus *Cardamine chlorotic fleck* (CCFV; 45). In contrast, elimination of CP expression by alteration of its wt and alternate initiation codons in TCV resulted in a modest reduction in TCV accumulation in protoplasts (107).

SatC-MEDIATED SYMPTOM MODULATION   SatC, being composed of both satD and TCV sequence, is an unusual satRNA with features of both DI RNAs and satRNAs. Unlike satD, satC strongly influences the moderate symptoms (stunting, slight leaf crinkling, and chlorosis) induced by TCV. On all hosts where TCV infection causes visible symptoms, satC amplifies these symptoms (50). Turnip and other hosts in the Brassicacea become severely stunted with strongly crinkled, dark-green leaves in the presence of satC. Using *A. thaliana*, inclusion of satC in the inoculum results in the death of the plant within two to three weeks postinoculation. Enhanced virulence occurs in these hosts despite the satC-specific reduction in virus levels. Hosts that are tolerant to TCV infection exhibit no additional symptoms in the presence of satC (50). These findings suggest that, unlike CMV satRNAs, satC is not directly contributing to symptom intensification but rather is directly or indirectly affecting the symptoms induced by the TCV genomic RNA.

Initial efforts to determine how satC contributes to TCV symptom expression focused on the TCV replicase. Single amino acid changes in the p88 (RdRp) ORF at positions 1025 or 1144 were sufficient to convert an isolate of TCV that produced mild symptoms in the presence of satC (TCV-B) to an isolate that induced severe symptoms in the presence of the satRNA (16, 60). Although at least one of these changes affected the extent of satC accumulation (16), these studies
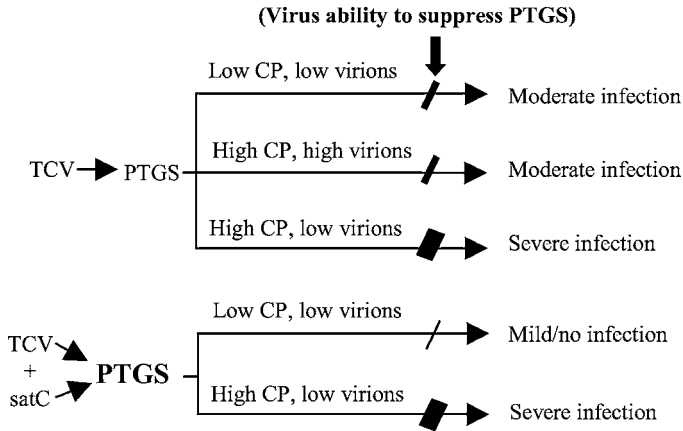
(Virus ability to suppress PTGS)

Figure 4 Model for symptom modification by satC. Both satC and TCV are thought to induce PTGS. The level of free CP (i.e., amount translated or increased amount as a result of virion suppression) directly correlates with the level of symptom expression since CP is the viral suppressor of silencing. High (wt) levels of CP combined with low levels of virions (due to N-terminal mutations or association with satC) lead to the most efficient suppression of PTGS and thus the most advantageous conditions for virus movement and symptom development.

did not address the mechanism by which satC modifies TCV symptoms. However, the variation in symptom intensity associated with early accumulation of satC suggested that satC influences an early step in the TCV life cycle. More recent reports suggest a direct role for satC in TCV symptom expression by enhancing the rate of viral cell-to-cell and long-distance movement. Alterations in TCV movement result from satC-mediated interference with the accumulation of stable TCV virions (Figure 4) (115). Reduced virion accumulation was also correlated with enhanced symptoms associated with TCV-CPm3, a TCV mutant with two additional amino acids at the N terminus of the CP (107, 115). Using a novel method to track the presence of virus within whole plants that involves cytoplasmic localization of a normally nuclear-targeted GFP fusion protein (114), both cell-to-cell and long-distance movement were observed to be enhanced when TCV infects hosts in a virion-free form (F. Zhang & A.E. Simon, unpublished data) (Figure 5).

SatC's ability to reduce the accumulation of virions may also enhance the ability of TCV to overcome PTGS. The TCV suppressor of silencing is the CP, which interferes with an early step in the silencing pathway (67, 101). The TCV CP is a weak silencing suppressor in the context of the intact virus yet is a very strong suppressor when expressed independently and delivered by agroinfiltration to plants containing a silenced reporter gene (67, 101). The lack of detectable suppression by the CP when expressed from the viral genome is likely due to a limited amount of free CP present during the early period in the viral life cycle when

the CP is required to suppress silencing. By interfering with virion formation, satC would increase the amount of free CP, thereby enhancing silencing suppression, which may result in more efficient movement of TCV (Figure 4).

The mechanism by which satC interferes with the accumulation of stable virions is unknown. However, the sequences flanking the M1H in satC plus strands have been implicated in the process. In vivo genetic selection of the M1H sequence using 10 or 28 random bases to replace the 28-base M1H resulted in three types of winning sequences: those that enhanced replication by containing promoter-like motifs in the minus-sense orientation (as described above); those that formed a stable hairpin in plus-strands; and those that had both attributes (92, 116). A model was proposed whereby a hairpin that brings together flanking single-stranded sequences is required to reduce accumulation of virions. This model was supported by finding that the satRNA most efficient at reducing virion levels contained a deletion of the M1H (92).

In addition to suppressing virion accumulation, it is likely that satC also induces the RNA silencing pathway similar to TBSV DI RNAs. Mutant TCV (TCV-CPm), which expresses only 10% of wild-type levels of a CP with two additional N-terminal amino acids, produces symptoms similar, but not identical, to wild-type TCV (107). The synthesized CP is identical to the CP of TCV-CPm3 and no virions are detected in TCV-CPm-infected protoplasts (107). Unlike the severe symptoms associated with TCV-CPm3, however, TCV-CPm produces mild symptoms, possibly due to less efficient silencing suppression by the reduced level of CP. Plants inoculated with TCV-CPm and satC display a variety of symptoms ranging from no effect to complete attenuation (44, 107). Current models suggest that attenuation of TCV-CPm symptoms mediated by satC is due to the additional silencing induction by satC without sufficient CP to suppress silencing (Figure 4) (115).

SatC CONTRIBUTION TO TCV FITNESS   The interrelationship between satC and TCV suggests that the satRNA is not a simple parasite of the virus. A purely parasitic nature for satC was originally proposed because of the satRNA's dependence on the helper virus (and host cell) for all life-cycle components. In addition, the presence of satC in turnip and *Arabidopsis* always resulted in a reduction of virus accumulation in whole plants and protoplasts. However, the recent finding of enhanced TCV movement in the presence of satC suggests that the relationship between satC and TCV is more mutualistic, where both participants gain fitness from the interaction. By interfering with virion accumulation, satC indirectly enhances the ability of TCV to move cell-to-cell and long distances (F. Zhang & A.E. Simon, unpublished data), permitting the virus to advance the timing of the infection by rapidly colonizing young host tissue. This would allow virus in the presence of satC to outcompete nonsatellite-containing virus for access to uninfected cells, thus promoting the amplification of virus associated with satC. While such mutualism is apparent within a manually inoculated host in a laboratory setting, it is unclear if the virus would derive an equal benefit from satC association in the field. For example, reduced levels of virions attributed to satC might impede vector- (flea beetle) mediated transfer of virus between hosts. Alternatively, more

rapid colonization of the plant might aid the beetle in transferring a limited, but still sufficient, amount of virions between plants.

The Satellite Virus of *Panicum Mosaic Virus*

Another example of a satellite that benefits its helper virus is the satellite virus associated with PMV (SPMV). SPMV (826 nt) has limited sequence similarity with PMV and requires the helper virus for components involved in replication. SPMV encodes its own 17.5-kDa CP, which packages both SPMV RNA and a satRNA of PMV into $T = 1$ icosahedral virions (20). The mild symptoms associated with PMV on natural host St. Augustinegrass are exacerbated into severe chlorotic mottling in the presence of SPMV, leading to the economically important disease St. Augustine decline. As with TCV satC, symptom intensification is associated with more rapid virus movement (84), which requires expression of the SPMV CP (66). However, unlike TCV satC, symptom intensification correlated with a two- to fourfold increase in PMV levels in infected plants (84). The authors suggested that SPMV might suppress or interfere with host defense pathways, or facilitate cell-to-cell movement without specific involvement of host defense, or allow more pervasive invasion of tissue types. To date, it is not known if the CP of SPMV is a suppressor of RNA silencing, which could explain the SPMV/PMV synergism.

SatRNAs of *Groundnut Rosette Virus*

A third example of a satellite beneficial to its helper virus is the satRNA associated with the umbravirus *Groundnut rosette virus* (GRV). Umbraviruses are small, positive-sense, single-stranded viruses with the unusual feature of not encoding any capsid proteins (98). GRV is capable of cell-to-cell and long-distance spread within a host in the absence of CP, and transfer of the GRV movement protein to CMV and PVX, which normally require their CP for cell-to-cell movement, allows these viruses to move locally in the absence of CP (80, 81). Transmission of GRV and other umbraviruses between hosts requires an assistor virus, usually a member of the family Luteoviridae, to provide the missing CP. In addition, unlike some umbraviruses, GRV is always associated with a satRNA. The GRV satRNA is between 895 and 903 nt and shares a requirement with other satRNAs for a helper virus to provide replication proteins (19). Different satRNA variants contain up to five possible ORFs; however, none of the ORFs are required for any known satRNA function (97).

SatRNA-MEDIATED SYMPTOM MODULATION   As with the severe CMV satRNAs, GRV satRNAs are primarily responsible for the symptoms of groundnut rosette disease. In the absence of satRNAs, groundnut plants infected with GRV are either symptomless or exhibit a transient mottling (56). Different GRV satRNAs are responsible for a variety of symptoms, including stunting, bright leaf chlorosis, or dark-green leaves. The brilliant yellow blotch mosaic symptoms associated with satRNA YB3b (GRV isolate YB) on *N. benthamiana* did not correlate with any

discernible differences in the accumulation or spread of GRV or the satRNA (97) and symptoms were not altered when the satRNA used *Pea enation mosaic virus* (PEMV) as helper (19). Two noncontiguous regions of the satRNA were identified as specifically contributing to the symptom phenotype. Since a pair of satRNAs, each deleted in one of the two regions produced symptoms when coinoculated with helper virus, these RNA segments appear to function in *trans*.

SatRNA NM3c, a mild satRNA variant, reduced the accumulation of GRV genomic and subgenomic RNAs by tenfold in inoculated leaves and by 64-fold in uninoculated leaves at 14 dpi (96). The 5′ 280 nt of the satRNA were determined to be involved in reducing virus accumulation (97). A similar effect was also found when either the full-length satRNA NM3c or its 5′ region was expressed in transgenic *N. benthamiana* plants inoculated with GRV (99). Although it was suggested that the satRNA could be competing with the helper virus for replication components, alternate possibilities such as translational repression or interference with silencing suppression cannot be ruled out.

SatRNA CONTRIBUTION TO GRV FITNESS   Unlike other satRNAs, the GRV satRNA is absolutely required for GRV transmission between hosts in nature. Both GRV genomic and satRNA are encapsidated by the CP of the assistor luteovirus GRAV (53). Studying the complex relationship among GRV, GRAV, and GRV satRNA is particularly problematic since no experimental hosts outside of groundnut can support all three constituents and virion yields in groundnut are very low. Using transgenic *N. benthamiana* plants expressing the GRAV CP, the satRNA was demonstrated to be essential for transcapsidation of GRV and satRNA in GRAV particles and thus for aphid transmissibility, although such transmission could not be confirmed using the experimental conditions (72). None of the possible satRNA ORFs were required for transcapsidation, suggesting that the RNA is directly involved in either promoting virion formation or stabilizing virions once formed. While some other umbraviruses are associated with satRNAs, such as PEMV, no other umbraviral satRNAs are required for transcapsidation (98).

The lack of required satRNAs associated with other umbraviruses suggests that the GRV satRNA has usurped a virion assembly/stability function that still exists in the remaining umbraviruses. Why GRV has established this relationship with a satRNA while other umbraviruses have not is not known. Nor is it clear whether seceding this function to a satRNA contributes to the fitness of GRV. Nonetheless, the GRV satRNA provides an extreme example of the evolution of mutualism beyond simple fitness enhancement to a required role for the satRNA in helper virus viability.

CONCLUSIONS

The past few years have witnessed a renaissance in our understanding of the complex interactions between subviral RNAs and their helper viruses. Where once subviral RNAs were viewed as selfish, parasitic hitchhikers that derived full

benefits from their relationship with a helper virus without contributing positively in return, current studies suggest that many of these relationships are far more complex. Repetitive elements in DNA genomes also were viewed as purely parasitic "selfish DNA" that detrimentally disrupted gene expression during transposition generating huge expanses of genomic "junk" (21, 61, 102). However, current views suggest that some selfish DNA can escape junk status and become "domesticated," i.e., molded over evolutionary time into functional genes, regulatory elements, or chromatin attachment sites that contribute positively to the host (40, 54). Similarly, subviral RNAs, which might begin existence as purely parasitic benign or detrimental entities, would be under strong selective pressure to evolve into "domesticated" agents that give the helper virus a competitive advantage. Thus satD, the original TCV satRNA, is apparently benign, whereas satC could be considered as the evolutionary outcome of satD domestication, having acquired additional sequences from TCV that now confer beneficial properties on the satRNA. Support for this idea also comes from finding subviral RNAs in various states between parasitic/detrimental and domesticated/essential. Unfortunately, the limited number of experimental hosts in which subviral RNA/helper virus combinations have been studied, coupled with different contributions of various hosts to the subviral RNA/virus combination, complicate determination of the evolutionary status of most subviral RNAs.

Complete understanding of the molecular mechanisms underlying the unexpectedly complex phenomenon of DI RNA-mediated symptom modulation is also lacking. In addition to the simplest explanation, the ability of DI RNAs to compete for the limited supply of *trans*-acting factors required for replication, several reports suggest the involvement of other factors such as selective inhibition of pathogenicity determinants, prevention of protein-protein interactions associated with symptom development, and induction of PTGS-based host defense. The sometimes contradictory results may be explained by a synergy among the different factors described in this review. Greater understanding of the biology of DI RNA- and satRNA-mediated symptom attenuation could provide information on the induction and development of virus-induced symptoms and the feasibility of the application of DI RNAs for disease moderation. Future studies that assess the contribution of subviral RNAs to viral fitness will continue to provide exciting new information on the relationship between viruses, subviral RNAs, and their hosts.

ACKNOWLEDGMENTS

The *Annual Review of Phytopathology* is online at http://phyto.annualreviews.org

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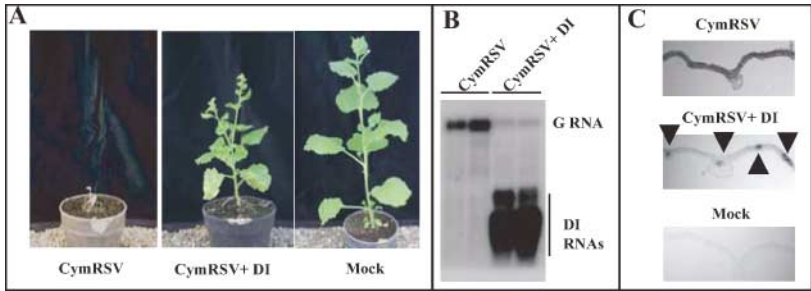
Figure 1 Effects of the presence of DI RNAs on tombusvirus infection. *A*. Symptoms caused by CymRSV infection in the absence and presence of DI RNAs at 28 days postinoculation (dpi). *B*. Total RNAs, prepared from systemically infected tissues of CymRSV and CymRSV+DI at 7 dpi, were separated under denaturing conditions, blotted, and hybridized with a CymRSV-derived probe. G, viral genomic RNA. *C*. In situ hybridization of 12 µm paraffin-embedded leaf cross sections probed with digoxigenin-11-UTP-labeled virus-specific RNA. Samples were taken from the first systemically infected leaves at 7 dpi. Black arrowheads indicate the sites of virus accumulation in panel displaying CymRSV+DI infection.
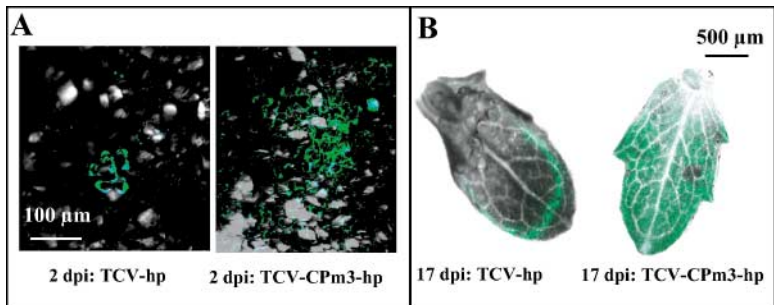
Figure 5 Cell-to-cell and long-distance movement of TCV is affected by the level of virions. Localization of TCV (contains wt levels of virion) and TCV-CPm3 (low virion levels) was achieved using a novel experimental approach whereby normally nuclear-localized GFP becomes cytoplasmic in the presence of TCV (114). Transgenic *A. thaliana* plants expressing a fusion protein consisting of GFP, a nuclear localization signal, and the CP of MS2 bacteriophage were inoculated with TCV-hp or TCV-CPm3-hp, which contain a 19-nt hairpin (hp) that is the binding sequence for the MS2 CP. *A*. Inoculated leaf at 2 dpi. *B*. Systemically infected (youngest expanded) leaf at 17 dpi. TCV-CPm-hp consistently showed more rapid and extensive spread in inoculated and uninoculated leaves compared to TCV-hp.

Ⓐ*Annual Review of Phytopathology*
Volume 42, 2004

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