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The Coat Protein of Turnip Crinkle Virus Suppresses Posttranscriptional Gene Silencing at an Early Initiation Step

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Posttranscriptional gene silencing (PTGS), or RNA silencing, is a sequence-specific RNA degradation process that targets foreign RNA, including viral and transposon RNA for destruction. Several RNA plant viruses have been shown to encode suppressors of PTGS in order to survive this host defense. We report here that the coat protein (CP) of Turnip crinkle virus (TCV) strongly suppresses PTGS. The Agrobacterium infiltration system was used to demonstrate that TCV CP suppressed the local PTGS as strongly as several previously reported virus-coded suppressors and that the action of TCV CP eliminated the small interfering RNAs associated with PTGS. We have also shown that the TCV CP must be present at the time of silencing initiation to be an effective suppressor. TCV CP was able to suppress PTGS induced by sense, antisense, and double-stranded RNAs, and it prevented systemic silencing. These data suggest that TCV CP functions to suppress RNA silencing at an early initiation step, likely by interfering the function of the Dicer-like RNase in plants.

Posttranscriptional gene silencing (PTGS) is a sequence-specific RNA degradation process that leads to the elimination of the targeted RNA and loss of the function(s) encoded by the targeted RNA (1, 6, 62, 69). This phenomenon was first observed and intensively studied in plant systems (see reference 64 for a review), where it has been associated with several processes, including cosuppression (44), repeat induced gene silencing (70), RNA-mediated resistance (35, 58), or homology-dependent gene silencing (43). Similar mechanisms were later discovered in other organisms, including quelling in filamentous fungus Neurospora crassa (9) and RNA interference (RNAi) in Caenorhabditis elegans (18) and Drosophila melanogaster (30). Recent research has revealed that all of these different phenomena have many common features and are now considered to be manifestations of an RNA-targeting pathway, whose natural functions include protecting hosts from invading viral RNAs and transposons (see references 45, 54, and 72 for reviews). RNA silencing has been proposed as a more general term to describe these related processes (1).

Initiation and maintenance stages have been identified as distinct phases of the PTGS or RNA silencing process (10, 50, 65). In the initiation stage, the invading RNA triggers a pathway that results in its being degraded into a small RNA species of discrete size (21 to 25 nucleotides [nt]) called small interfering RNAs (siRNAs) that function as a guide for further degradation in the maintenance stage (22, 71). The most potent initiator of PTGS is thought to be double-stranded RNA (dsRNA) (8, 18, 30, 68), although single-stranded RNA (ssRNA), both sense and antisense orientations, or even DNA trigger RNA silencing (15, 65, 66). ssRNA is most likely converted to a double-stranded form with the help of a host RNA-dependent RNA polymerase (RdRP) in order to be effective (9, 11, 42, 52, 57). The dsRNA initiators are then degraded by an RNase III-like RNase (e.g., Dicer in Drosophila [3]) into siRNAs. The initiation stage of PTGS is dependent on the presence of the initiator RNAs and seemingly independent of mRNA of homologous host genes.

In the maintenance stage, siRNAs are brought to a multi-component nuclease called the RNA-induced silencing complex (RISC) to guide the sequence-specific cleavage of homologous host-encoded mRNA (23, 71). PTGS is independent of trigger RNAs at this stage. RNA silencing at the maintenance stage also involves an RdRP activity that amplifies the siRNAs and hence the efficiency of silencing (36, 55, 61). Another important feature of PTGS in plants is the systemic spread of the silencing signal between cells and in the vascular system, even if the initiator molecule remains localized or is removed. Hence, a system to amplify the silencing signal must also exist. It has been demonstrated that an invading viral RNA genome or a replication product in the form of a dsRNA intermediate, is both the initiator in the initiation stage and subsequently the target of RISC in the maintenance stage (reference 59 and references therein).

It has been suggested that a natural function of the RNAi pathway is to defend the host against invading viruses and transposons (1, 45). It is not unexpected, therefore, that such pathogens would be driven to evolve mechanisms to counter this potent defense system. Numerous reports have now appeared describing plant viral genes encoding proteins that suppress PTGS (4, 29, 66, 67). Most recently, a suppressor of PTGS was identified in the genome of Flock house virus, an RNA virus that infects insect cells (33). An interesting feature of these virus-encoded suppressors is that they have been found to target different stages of PTGS. In the case of Potato virus X (PVX), the p25 movement protein has been shown to interfere with an initiation step, possibly preventing ssRNA from being converted to dsRNA (67). In contrast, the helper component protease (HC-Pro) of Tobacco etch virus (TEV) appears to interfere with the maintenance stage (37, 39), whereas the 2b protein of Cucumber mosaic virus (CMV) inhibits the systemic transport of the silencing signal (19). These
results suggest that further characterization of additional silencing suppressors will certainly be of value in further dissecting the RNA silencing pathway.

_Turnip crinkle virus_ (TCV) is a small icosahedral virus with a single-stranded, positive-sense RNA genome of 4 kb. It infects a wide range of plant species, including the model plant _Arabidopsis thaliana_. The compact 4-kb RNA genome encodes only five open reading frames (ORFs) (see reference 46 for a review). The most 5′ proximal ORF (p28) and its readthrough product (p88) are both needed for viral RNA replication. Two small internal ORFs (p8 and p9) function as movement proteins and are translated from a subgenomic RNA (sgRNA) that gets expressed during viral replication. A second smaller sgRNA, derived from the 3′ half of the genome, serves as the mRNA for the viral coat protein (CP). Although the primary function of CP is structural, several studies have shown that it also has a vital role in virus-host interactions. This is supported from studies showing that certain TCV CP mutants cause altered symptom phenotypes (25) and that CP is responsible for modifying symptoms caused by satellite RNAs (31, 32). In addition, it is well established that TCV CP is needed for systemic movement in most hosts, and cell-to-cell movement in _Nicotiana benthamiana_ (20, 34). TCV CP has also recently been shown to be the elicitor of gene-for-gene resistance in _Arabidopsis_ (27). Our lab recently demonstrated that it specifically interacts with a transcription factor in _Arabidopsis_ that appears to be associated with this resistance response (49). Here we demonstrate that TCV CP also functions as a strong suppressor of RNA silencing. Our data suggest that it functions at a very early initiation step of RNA silencing and may represent a unique type of virus-encoded suppressor.

**MATERIALS AND METHODS**

Constructs. All constructs used in this work are listed in Fig. 1. The insert in PZP-green fluorescent protein (GFP) is the cDNA of mGFP4 (24), which was obtained by PCR amplification by using the genomic DNA of GFP 16c plants as a template. A _BspHI_ site and an _XbaI_ site was introduced into the 5′ and 3′ primers, respectively. The PCR product was then digested with _BspHI_ and _XbaI_ and ligated into pRTL2* (5) between _NcoI_ and _XbaI_ sites. The plasmid pRTL2 has a Cauliflower mosaic virus (CaMV) 35S promoter and a TEV translational enhancer. The resulting fragment containing 2b cDNA was ligated into pRTL2 (5) between _NcoI_ and _XbaI_ sites. The plasmid pRTL2 then digested with _NcoI_, _PstI_, and _XbaI_ sites. The plasmid pRTL2 was cut out of pRTL2 with _PstI_ and ligated into pPZP212, a binary vector, to produce PZP-GFP. PZP-TEVCp, PZP-TBSVp19, and PZP-TBSVCP were produced similarly. All inserts were sequenced to make sure no sequence errors were introduced by PCR. The CMV2b cDNA was obtained from plasmid pSK2b, which was kindly provided by Shou-Wei Ding (14). pSK2b was first digested with _NcoI_ and _XbaI_, the resulting fragment containing 2b cDNA was ligated into pRTL2 to produce pRTL-CMV2b, which was then subjected to digestion with _BamHI_ and relaxation to remove the redundant restriction sites (EcoRI, _PstI_, and _SmaI_, among others) between the 3′ end of 2b and _XbaI_ site. This modified pRTL-CMV2b* was then cut with _PstI_ to release the expression cassette. The TEV HC-Pro expression cassette was obtained by _PstI_ digestion of the plasmid pRTL2-0027S provided by Jim Carrington (28).

PZP-asGFP contains the GFP insert in a reverted orientation. PZP-CPΔ is a mutant of PZP-TEVCp that contains two consecutive stop codons after the first five amino acids of the CP. PZP-TCV has a full-length cDNA of TCV RNA as an insert. PZP-asGFP is a mutant of PZP-TEVCp that has 92 nt deletion within the movement protein coding region (34). PZP-CpStop is another PZP-TCV mutant that contains the same mutation in the CP coding region as PZP-CPΔ.

**Agrobacterium infiltration.** Agrobacterium suspensions carrying the various binary constructs were pelletted and resuspended in a solution containing 10 mM morpholinepropanesulfonic acid (pH 5.5), 10 mM MgCl₂, and 100 μM acetylsyringone to an optical density of 600 nm of 1.0. In coinoculations, equal volumes of each suspension were mixed prior to infiltration. Three-week-old GFP 16c plants (provided by D. C. Baulcombe) were infiltrated on the first two true leaves with a 3 ml, needleless syringe. The infiltrated plants were kept in growth chambers with a 12-h day length, a daytime temperature of 24°C, and a night temperature of 18°C.

**Analysis of RNAs.** Total RNAs were extracted from infiltrated leaves by grinding the individual leaves in the presence of 600 μl of phenol-chloroform-isomyl alcohol (25:24:1) and 300 μl of a solution containing 100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 2 mM EDTA, and 1% sodium dodecyl sulfate (SDS). The resulting supernatants were reextracted with 450 μl of phenol-chloroform-isomyl alcohol (25:24:1), and the second supernatants were precipitated with ethanol and washed twice with 70% ethanol. The concentration of RNAs was estimated by agarose gel and a UV spectrophotometer. The RNAs were then...
RESULTS

We recently published a study on the role of viral CP and a second viral gene (p19) on the systemic movement of a distant relative of TCV within *Tomobusviridae*, *Tomato bushy stunt virus* (TBSV) (48). Previous work by others (7, 66) suggested that the TBSV p19 likely functions as a suppressor of PTGS. We extended on these studies by showing that p19 is essential for maintaining a high level of TBSV RNA (48). More relevant to the current study, we showed that TCV CP was able to complement the functions of both p19 and CP of TBSV in a chimeric TBSV genome in which the p19 gene was deleted and the CP gene had been replaced with TCV CP gene. This result prompted us to speculate that TCV CP might well function as a suppressor of PTGS during TCV infection. To directly test this possibility, we utilized the *Agrobacterium* infiltration system developed by Baulcombe and coworkers (65, 67). The constructs we used in the current study are diagrammed in Fig. 1. The cDNAs of various proteins and their mutants were first cloned into pRTL2 (5), a plasmid containing the 35S promoter (P35S) of CaMV and the translational enhancer (TE) of TEV (5, 47) positioned upstream of multiple cloning sites, and the CaMV 35S terminator (T35S) downstream. The full expression cassettes, which included the P35S, TE, the genes to be tested, and T35S, were then subcloned into the binary vector pPZP212 (21) between the right and left borders of the T-DNA. These constructs were then transformed into *Agrobacterium* sp. strain C58C1, suspensions of which were used to infiltrate *Nicotiana benthamiana* plants carrying a GFP transgene (line GFP 16c [50]). The level of silencing suppression was monitored visually by assessing GFP fluorescence with a hand-held long-wavelength UV lamp and verified by evaluating GFP RNA accumulation in infiltrated leaves. A detailed description of the constructs and procedures used is provided in Materials and Methods.

**TCV CP is a strong suppressor of PTGS.** To investigate the function of TCV CP as a suppressor of PTGS, we infiltrated leaves of GFP 16c plants with mixtures of agrobacteria carrying the GFP and TCV CP constructs. Figure 2 shows that control leaves of the GFP 16c plants expressing GFP transgenically were fluorescent compared to nontransgenic leaves (Fig. 2A, compare panels 1 and 2). Leaves infiltrated with the GFP construct alone showed a marked increase in GFP fluorescence 2 days after infiltration (dpi; due to transient GFP expression) but then started to decrease at 4 dpi and had almost disappeared from leaf areas between the veins by 7 dpi (Fig. 2A, panel 3). This result is consistent with reports by Voinnet et al. (66, 67) demonstrating that transient expression of GFP mRNA at high levels rapidly triggered PTGS. When leaves were infiltrated with a mixture of suspensions carrying GFP and TCV CP constructs, fluorescence was initially much stronger than in leaves infiltrated with GFP alone. Moreover, fluorescence continued to increase to a very high level by 5 dpi and remained at this peak level for at least 2 weeks (Fig. 2A, panel 4 at 7 dpi). This result provides confirmatory evidence that the TCV CP is indeed a potent suppressor of PTGS initiated by transient expression of GFP in the GFP transgenic plants. We also tested the two movement proteins (p8 and p9) of TCV in a similar manner (data not shown), as well as the CP of TBSV (Fig. 2A, panel 8). None of these proteins showed any indica-

![FIG. 2. Suppression of PTGS by TCV CP.](image)
tion of suppressor activity in these assays. The result with the TBSV CP control also suggested that the increase of GFP fluorescence in the GFP+TCV CP-infiltrated leaves was not likely the result of protection of the GFP mRNA due to non-specific packaging.

We next compared the suppressor activity of TCV CP with other established viral PTGS suppressors by using the same assay. The level of enhanced GFP fluorescence in leaves infiltrated with a mixture of GFP and TBSV p19 (Fig. 2A, panel 5) was very marked and approached that observed for the TCV CP suppression. This is consistent with the previously published results showing it to be a potent suppressor of PTGS (7, 48, 56, 66). As shown by Guo and Ding (19), the 2b protein of CMV, which suppresses systemic PTGS by interfering with the systemic delivery of the silencing signal (2, 19), was much less effective in preventing GFP silencing in these assays. The HC-Pro of TEV, which functions at a maintenance step of PTGS (4, 52, 66), was almost as effective as TCV CP and TBSV p19, as evidenced by loss of the mRNA signal (lanes 6, 7, and 8). Their GFP mRNA levels were significantly lower than the buffer-infiltrated control (Fig. 3A, top panel, lane 1) and were comparable to the levels of TBSV p19, as judged from the levels of fluorescence. These results suggest that the TCV CP and TBSV p19 likely interfere with a step in the initiation or local maintenance stage of RNA silencing, as opposed to the suppression of systemic silencing as observed for CMV2b (19).

RNA blot analysis verified that increased GFP fluorescence resulted from elevated levels of the GFP mRNA (Fig. 2B, top panel). The results show a marked reduction in the level of GFP mRNA in leaves where silencing has been induced by infiltration with GFP (top panel, lane 3). Most evident is the very high level of GFP mRNA accumulation in leaves infiltrated with GFP+TCV CP, GFP+TBSV p19, and GFP+TEV HC-Pro. Consistent with the fluorescence assay results, GFP mRNA accumulation was below the limits of detection in leaves infiltrated with either GFP+CMV2b or GFP+TBSV CP.

In addition to GFP mRNA levels, we also assessed the levels of the GFP-specific siRNAs as a way of confirming that the reduced levels of mRNA were indeed the result of PTGS (22). In the GFP-infiltrated control (Fig. 2B, middle panel, lane 3), evidence for silencing is confirmed by loss of the mRNA signal in the top panel and the appearance of a strong 21- to 25-nt siRNAs signal in the middle panel. The GFP-specific siRNAs were also detected in leaves in which silencing suppression was not effective (CMV2b and TBSV CP, lanes 6 and 8). In the case of TEV HC-Pro, siRNAs were detected at a significant level despite the strong silencing suppression evident from the high-level GFP mRNA. In marked contrast, siRNAs were not detected when silencing was suppressed by the TCV CP, even after prolonged exposure to the X-ray film (data not shown). The TBSV p19 behaved similarly except that siRNAs were visible after prolonged exposure. This is consistent with a recent report describing the function of the p19 protein encoded by a related virus (56). The absence of siRNAs in the case of TCV CP demonstrates that the silencing suppression mechanism of TCV CP is different from that of TEV HC-Pro and suggests that TCV CP functions to suppress PTGS at an initiation step prior to the production of siRNAs.

**Suppression of RNA silencing by TCV CP requires its presence at the time of PTGS initiation.** To further assess the mechanism of silencing suppression by TCV CP and to determine the step at which CP interferes, we introduced the entire TCV genome into GFP 16c leaves by using *Agrobacterium* infiltration. This permitted us to compare the timing of expression of CP generated from a viral genome with that translated directly from the 35S promoter-driven mRNA. Three TCV genome constructs were tested: PZP-TCV, which encompasses the full-length cDNA of TCV; PZP-TCVΔ92, which contains a 92-nt deletion in the coding region of the two movement proteins that makes it defective in cell-to-cell movement (34); and PZP-TCVCpstop, which has two consecutive stop codons in the CP coding region after first five amino acid residues that prevent CP translation (Fig. 1, last three constructs). All three constructs were competent for viral replication. The construct PZP-CPΔ, which carried the corresponding CP cDNA of the Cpstop mutant, was used as an additional negative control.

GFP fluorescence was monitored daily after the leaves of GFP 16c plants were infiltrated with mixtures of *Agrobacterium* suspensions carrying the GFP construct and each of the genome constructs. While leaves infiltrated with the GFP+CP mixture showed strong GFP fluorescence as described previously, leaves infiltrated with the mixtures of GFP+TCV and GFP+Δ92 showed very weak fluorescence, and they were indistinguishable from those infiltrated with GFP+Cpstop. This was surprising because both TCV and Δ92 would be expected to produce large amounts of CP from their replicating genomes, whereas CPstop could not produce functional CP. Analysis of RNAs isolated from infiltrated leaves at 7 dpi confirmed that all three viral constructs replicated efficiently, as evidenced by high levels of accumulated progeny viral RNA in the leaves (Fig. 3A, bottom panel, a full-length viral RNA band is visible above the rRNA band in lanes 6, 7, and 8). Additionally, in plants infiltrated with GFP+TCV, systemic vein-clearing symptoms typical of TCV infection became evident starting at 7 dpi, indicating that CP was translated normally.

When RNA blot analysis was performed with a GFP-specific probe (Fig. 3A, top panel), only very low levels of GFP mRNA could be detected in leaves infiltrated with each of these three combinations, whether or not the CP gene could be translated (lanes 6, 7, and 8). Their GFP mRNA levels were significantly lower than the buffer-infiltrated control (Fig. 3A, top panel, compare lane 2 with lanes 6, 7, and 8), indicating that the silencing of transient, as well as transgenic, GFP mRNA had occurred. These results mean that TCV CP, when expressed as a product of the replicating viral genome, failed to suppress RNA silencing. This is in dramatic contrast to our results in the previous section (also shown on lane 4 of Fig. 3A, top panel), which demonstrated that CP is a strong silencing suppressor when expressed directly from the 35S promoter-driven mRNA.

Given these unexpected results, we examined the levels of CP mRNA in the infiltrated leaves by performing RNA blot analysis with a CP-specific probe (Fig. 3B). The accumulation levels of TCV genomic and subgenomic RNAs in leaves infiltrated with GFP+TCV, GFP+Δ92, and GFP+Cpstop were almost 100-fold higher (lanes 5, 6, and 7; note here that samples in these lanes were diluted 1:100 prior to loading onto the gel) than the level of CP mRNA transcribed from the transient CP construct (lane 3). Accordingly, we would expect that CP expressed from the transient CP construct to be much less abundant than in the viral infections, and yet it was considerably more effective at suppressing silencing of the coinfiltrated
GFP mRNA. Two possible explanations might account for these results. First, if the CP indeed suppresses silencing at a very early initiation step, then production of CP from the transient construct could be earlier than from the infectious genomes, which could account for the difference. Alternatively, it is possible that CP produced from infectious RNA is very quickly directed into virus assembly process and is therefore not available for suppressing the silencing of nonviral mRNA.

To test the first possibility, we infiltrated GFP 16c leaves with buffer, CP, CPΔ, TCV, Δ92, and CPstop constructs and
analyzed the infiltrated leaves for TCV RNAs at 1 and 2 dpi by RNA blot hybridization with a CP-specific probe. CP mRNA was detectable as early as 1 dpi in CP- and CPα-infiltrated leaves (Fig. 3C, top panel, lanes 2 and 3). In comparison, CP mRNA could not be detected in extracts of the three infectious constructs at this early time point (even with a fivefold longer exposure; Fig. 3C, top panel, lanes 4, 5, and 6 and data not shown). The results were very different at 2 dpi. CP mRNA accumulated to an ∼10-fold higher level in the CP-infiltrated leaves (bottom panel, lane 2). As might be expected, the CP mRNA had almost completely disappeared in the CPα-infiltrated leaves, likely the result of silencing due to absence of silencing suppression by CP (lane 3, bottom panel). At 2 dpi, both TCV genomic and subgenomic RNAs were detectable in leaves infiltrated with the infectious constructs (bottom panel, lanes 4, 5, and 6). These results suggest that CP is indeed expressed earlier from the transient CP construct than from the infectious constructs. Moreover, the result showing disappearance of CP mRNA from the CPΔ construct at 2 dpi provides compelling evidence that PTGS was very effective at eliminating the foreign RNA and that it was induced prior to the time of appearance of CP mRNA produced from the infectious constructs. This experiment supports the notion that CP acts to suppress RNA silencing at a very early initiation step.

CP expressed from infectious viral RNA was, however, able to suppress silencing targeting viral RNA. This is demonstrated by comparing the accumulation levels of viral RNAs at 7 and 13 dpi in leaves infiltrated with the three constructs carrying TCV replicons (TCV, ∆92, or CPstop). The levels of accumulation of viral RNAs in each case were comparable at 7 dpi (Fig. 3D, top panel). In the CPstop infections, however, the viral RNA was almost completely cleared from infected leaves at 13 dpi, demonstrating the need for CP to sustain vigorous replication of viral RNA. This experiment shows that CP produced during viral infection is indispensable for suppressing silencing that would otherwise target TCV viral RNA.

These experiments suggested that failure of TCV and ∆92 to suppress GFP silencing is likely due to a lag in the production of CP. To directly prove that CP produced in the process of TCV infection can indeed suppress silencing of an unrelated RNA, we inoculated GFP 16c plants with TCV and permitted systemic symptoms to develop. Leaves with systemic symptoms were then infiltrated with the GFP construct. We observed that GFP fluorescence began fading continuously on the control leaves (GFP-infiltrated leaves of healthy GFP16c plants) at 3 dpi. In contrast, the fluorescence in the TCV-infected leaves strengthened and peaked at 5 dpi (Fig. 3E) and remained at this level for at least 2 weeks. This result clearly demonstrates that, if present at the time of the introduction of an unrelated RNA, TCV CP produced by infectious virus is capable of suppressing silencing targeting the unrelated RNA. The results collectively support the conclusion that the silencing suppression function of CP requires it to be present at the time of silencing initiation, and it is ineffective when introduced after even a short delay.

**TCV CP suppresses PTGS induced by both antisense and dsRNA.** The evidence presented so far suggests that TCV CP functions as a suppressor of silencing very early in the initiation of the PTGS pathway. In the previous experiments, the GFP construct used to induce silencing in the transgenic GFP plants was always of positive-sense polarity. In order to further dissect where the TCV CP might operate in the PTGS pathway, we wanted to determine whether silencing induced by either antisense RNA or dsRNA would be able to circumvent suppression by CP. This is important to know also because both negative-stranded RNA and dsRNA are produced during TCV replication. To test this, we made an antisense construct in which the GFP coding sequence was incorporated into the PZP vector in the opposite orientation (PZP-asGFP). GFP 16c leaves infiltrated with asGFP in the absence of functional CP (asGFP + CPΔ) turned completely red, indicative of silencing of the transgenic GFP RNA. When the same construct was infiltrated with CP, no enhancement of GFP fluorescence was observed because no exogenous GFP mRNA was available for translation. However, the GFP fluorescence present prior to infiltration remained for as long as the leaves were alive, suggesting that asGFP-induced silencing was prevented by CP.

When a triple infiltration (GFP + asGFP + CPΔ) was performed, a moderate enhancement of GFP fluorescence was visible at 2 dpi. This fluorescence started to fade at 4 dpi, and only scattered fluorescent dots were visible by 7 dpi. In contrast, enhanced fluorescence was evident in leaves infiltrated with a mixture of GFP, asGFP, and TCV CP, and the high level was sustained for at least 2 weeks. In both triple infiltrations, the initial enhancement of GFP expression is an indication that a significant amount of GFP mRNA was not in the double-stranded form. However, the fluorescence was not as strong as that of GFP + CPΔ and GFP + TCVCP, respectively, suggesting to us that some GFP mRNA did form dsRNA with asGFP RNA. It has been shown that dsRNA is a much more efficient inducer of RNA silencing than either sense or antisense RNA (18). The fact that the silencing suppression capability of TCV CP was not compromised by coinfiltrating GFP and asGFP constructs suggests that TCV CP also suppresses PTGS triggered by dsRNA. This observation is further supported by the detection of RNA complementary to GFP mRNA in the GFP+TCVCP-coinfiltrated leaves (see below).

Total RNA was extracted from these infiltrated leaves and analyzed by RNA blot hybridization with a probe labeled by random priming to ensure detection of both sense and antisense GFP RNAs (Fig. 4A). A high level of GFP-specific RNA accumulation is evident in extracts from leaves of each of the GFP constructs protected by TCV CP (top panel, lanes 2, 4, and 6) and absent in those infiltrated with the CPΔ construct (lanes 3, 5, and 7). These results verify that TCV CP was equally effective at suppressing silencing regardless of the form of GFP RNA used to induce PTGS.

We also enriched for siRNAs from total RNA preparations. RNA blot analysis showed that siRNAs could be detected in all cases where silencing was induced (Fig. 4A, lower panel, lanes 3, 5, and 7) and not in cases where silencing was suppressed (lanes 2, 4, and 6). Note here that the siRNAs migrated as two distinct bands under these gel conditions (the gel was run at a higher temperature than in Fig. 2B). Other researchers observed similar results and found that the siRNAs were differentially suppressed by TEV HC-Pro (work carried out in D. C. Baulcombe’s lab [described as meeting reviews in references 16 and 41]). In our results, TCV CP prevented production of both size classes of siRNAs, providing one additional
A high abundance of GFP-specific RNA heterogeneous in size is noticeable in these siRNA blots in all samples lacking the siRNAs (Fig. 4A, bottom panel, lanes 2, 4, and 6). These RNAs produced a long smear in the 15% polyacrylamide–8 M urea gel, suggesting that they were no larger than 300 nt. We next examined the polarity of these RNAs. Additional gels were run and subjected to hybridizations with strand-specific probes (Fig. 4B). Surprisingly, RNAs of both polarities were detected in fairly high concentrations in both the GFP/H11001CP- and asGFP/H11001CP-infiltrated leaves (Fig. 4B, lane 1 of the bottom panel and lane 3 of the middle panel). Even if all of the positive-stranded RNAs in the asGFP+CP-extracts were derived from the transgenic GFP mRNA, the presence of even a small amount of negative-strand RNAs in the GFP+CP samples suggests that dsRNA must have been made. This result supports our conclusion that CP suppresses silencing induced by dsRNA and suggests that it functions to suppress the host enzyme that specifically digests dsRNA into siRNAs but not other nonspecific RNases that degrade the dsRNA into the heterogeneously sized RNA. The candidate host enzyme that best fits these properties would be an N. benthamiana analogue of the Dicer enzyme (3).

FIG. 4. TCV CP suppresses PTGS induced by mRNA, antisense RNA and dsRNA of GFP. (A) RNA blot analysis showing the accumulation of GFP-specific RNAs and siRNAs in leaves infiltrated with Agrobacterium suspensions carrying the constructs listed above the panels. Note that the probe used here was generated by random priming in order to detect both sense and antisense RNAs. The position of a 28-nt DNA oligonucleotide is shown to the right of the bottom panel, serving as the size marker for siRNAs. (B) Detection of heterogeneously sized RNAs of both polarities in the leaves subjected to treatments described in panel A. Probes used are indicated to the right of respective panels.

The action of TCV CP prevents systemic silencing. The fact that TCV CP suppresses the initiation of PTGS suggests that it should also prevent systemic silencing. We infiltrated the first two true leaves of GFP 16c plants with GFP+CP and then examined them for systemic spread of GFP silencing. GFP 16c plants infiltrated either with GFP alone (20 plants) or GFP+CPΔ (20 plants) served as controls. Signs of systemic silencing were seen in almost all of the control plants by 8 dpi and in some by as early as 5 dpi. When viewed under long-wavelength UV light, systemic silencing initially appeared as red minor veins on the leaf edges in young, green-fluorescing, sink leaves and gradually expand into major veins and the leaf mesophyll. Eventually, the entire plant showed signs of being silenced, with GFP fluorescence only seen on major veins of
older leaves. In our experiments, systemic silencing was observed on all GFP-infiltrated plants and 19 of 20 GFP+CPΔ-infiltrated plants. As anticipated, none of the GFP+CP-infiltrated plants showed any signs of systemic silencing. We also checked a total of 8 plants infiltrated with asGFP+CPΔ and asGFP+CP for systemic silencing. As expected, all asGFP+CPΔ-infiltrated plants developed systemic silencing, whereas none of the asGFP+CP-infiltrated plants did. Of four plants infiltrated with GFP+asGFP+CP, two developed delayed, sporadic systemic silencing, whereas all four plants infiltrated with GFP+asGFP+CPΔ developed systemic silencing. These data, summarized in Table 1, show that the action of TCV CP prevented systemic silencing.

**TCV CP does not reverse established silencing but is effective at blocking renewed initiation of silencing.** In an effort to further rule out the possibility that TCV CP might play a role in interfering with PTGS maintenance, we infiltrated leaves of systemically silenced GFP 16c plants with TCV CP. The CPΔ, TBSV p19, and TEV HC-Pro constructs were also infiltrated into the same leaves as controls. One representative leaf from this experiment at 9 dpi is shown in Fig. 5A. The region of Agrobacterium infiltration can be discerned as a faint boundary in all cases. Neither the negative control (CPΔ) nor areas expressing any of the three suppressor proteins (TCV CP, TBSV p19, or TEV HC-Pro) showed any sign of increased fluorescence indicative of a reversal of active silencing. This experiment was somewhat inconclusive because we were unable to confirm previous reports demonstrating that TEV HC-Pro was able to reverse PTGS in other systems (29, 66).

We next assessed whether viral infections by either TCV or TBSV might reverse PTGS in systemically silenced GFP 16c plants. A total of four plants were infected with infectious transcripts of each viral genome, and the plants were incubated under conditions optimal for symptom development (24°C day temperature, 22°C night temperature, 12 h of daylight). Typical necrotic symptoms characteristic of a TBSV infection led to the death of all of the infected plants by 14 dpi. No signs of increased GFP fluorescence were seen throughout this period. Similarly, TCV symptoms developed starting at 7 dpi with all infected plants showing typical vein clearing and leaf curling. Again, there was no indication of an increase in GFP fluorescence in any of the symptomatic leaves. We conclude from these results that infections by either TBSV or TCV were unable to reverse the process of established silencing of the GFP transgene.

The inability for TCV and TBSV to reverse PTGS provides additional proof that their silencing suppressors function differently than those characterized for both TEV and CMV. This also suggests that TCV CP does not interrupt the maintenance stage of PTGS, at which TEV HC-Pro acts (37, 39). To strengthen this conclusion, we designed the following experiment to show that the initiation step of newly induced silencing could still be interrupted by TCV CP in an already-silenced plant. To demonstrate this, we infiltrated GFP 16c leaves that were fully silenced with each of the following combinations: GFP+TCV CP, GFP+CPΔ, GFP+HC-Pro, and GFP+TBSV p19. Numerous studies have shown that these silenced plants are in the maintenance stage of PTGS, where transcription of
the GFP transgene occurs but the mRNA is degraded post-
transcriptionally and is independent of inducers of PTGS (50). 
Hence, it is expected that newly introduced homologous GFP 
RNA would be degraded in such silenced plants as long as 
the maintenance machinery is not interrupted. This expected re-
sult is readily demonstrated when a fully silenced leaf was 
infiltrated with a mixture of GFP and CPΔ (Fig. 5B). Note as 
well that the level of GFP fluorescence was also not detectably 
increased in the region infiltrated with GFP+HC-Pro. The 
important and more interesting results are shown in the lower 
half of the leaf in Fig. 5B. Both TCV CP and TBSV p19 were 
able to prevent silencing of newly introduced GFP mRNA as 
evidenced by the enhanced fluorescence in the infiltrated re-
gion. This result supports the conclusion that a new round of 
silencing initiation is needed to counter the introduction of a 
high level of homologous RNA even if an active maintenance 
level of silencing is operative. TCV CP (and TBSV p19) also 
suppresses this new round of silencing initiation.

**DISCUSSION**

RNA silencing is an ancient cellular defense mechanism 
conserved among different kingdoms of organisms (45) that 
likely appeared before animals and plants diverged (72). It 
is therefore understandable that viral parasites utilizing RNA 
during replication and gene expression would need to develop 
strategies to evade this defense system. It is now becoming 
evident that many RNA viruses have evolved an active mech-
anism to counteract silencing by encoding suppressor proteins 
that interfere with the process (for reviews, see references 6, 
33, and 62).

We have characterized a novel silencing suppressing func-
tion associated with the CP of the small RNA plant virus, TCV. 
This was interesting because it adds to an extensive list of 
important functions associated with this well-studied viral CP 
and suggests an explanation for numerous observations that 
have associated TCV CP with symptom modulation and patho-
genesis functions. In this report, we show that TCV CP is a very 
strong suppressor of RNA silencing, suppressing both local 
and systemic silencing. The illustration that CP suppresses 
silencing of unrelated RNA suggests that caution should be 
exercised in developing virus-resistant plants by using CP-me-
diated resistance, as the suppressor activity associated with 
viral CPs such as TCV might act synergistically to promote 
infection with other viruses.

TCV CP represents a novel class of virus-encoded suppres-
sors in that it is a structural protein. In natural infection, CP is 
expected to be available even before the viral multiplication 
cycle starts. Consistent with this, we demonstrate that the TCV 
CP suppressor works at an early step in the initiation of si-
encing prior to the production of siRNAs. Our data also suggest 
that it prevents the dsRNAs from being specifically cleaved 
into siRNAs. Bernstein et al. (3) have observed that multiple 

dsRNA-specific nucleases are present in *Drosophila*, but only 
Dicer, a bidentate RNase, was able to digest dsRNA to pro-
duce siRNAs. Our results suggest, therefore, that TCV CP 
functions to suppress a Dicer-like RNase in plants.

A simplified model of the silencing pathway patterned after 
Plasterk (45) is presented in Fig. 6. This updated model incor-
porates recent evidence that a host RdRP step is also necessary 
for the maintenance of RNA silencing (36, 55, 61). In addition, 
we have tentatively assigned locations where several known 
plant virus-encoded suppressors might act. For example, PVX 
p25 was reported to suppress silencing induced by ssRNA but 
not by RNA carried in the PVX vector and thus likely inter-
feres at a step that requires host RdRP (67). In earlier studies, 
TEV HC-Pro was reported to function during the maintenance 
step where it also blocked production of siRNAs (37, 39).

Subsequent studies showed that it only prevented the produc-
tion of siRNAs of a certain size class (16, 26, 41). This discrep-
ancy is probably explained by the fact that the earlier studies 
utilized transgenic plants expressing HC-Pro crossed to plants 
with a silenced reporter gene to assess whether the mainte-
nance of siRNAs could be reversed. They showed that 
HC-Pro suppressed the silencing of the reporter gene and 
prevented siRNA production in the maintenance stage. The 
later reports used the *Agrobacterium* infiltration system and 
primarily involved studies on the initiation of silencing. We 
speculate from these data that TEV HC-Pro is unable to abol-
ish siRNAs produced in the initiation stage. In the case of the 
TCV suppressor, our data convincingly demonstrate that CP, 
which suppresses silencing induced by both ssRNAs and 
dsRNAs, most likely interferes with the Dicer-like nuclease, 
making it unique among the plant virus suppressors character-
ized to date.

We provide evidence that a second viral suppressor, TBSV 
p19, likely functions at the initiation stage as well. The activity
of TBSV p19 differs somewhat from TCV CP in that it permits accumulation of a small amount of siRNAs (Fig. 2B). This is consistent with the recent report showing that the action of p19 of Cymbidium ringspot virus (CymRSV), a virus closely related to TBSV, did not completely eliminate the siRNAs (56). Silhavy et al. further demonstrated that CymRSV p19 specifically binds to the double-stranded form of the siRNAs, thus depleting the specificity determinants of the PTGS effector complex and preventing further amplification. This suggests that p19 functions at a step downstream of TCV CP.

TCV CP and TBSV p19 are similar in their inability to reverse the maintenance phase of the silencing process of a transgene such as GFP. Interestingly, both of these suppressors were able to block the silencing of a high level of transiently expressed GFP mRNA when introduced together into GFP-silenced leaves. We propose that the maintenance of silencing in the GFP-silenced plants is tightly regulated at a level sufficient to degrade the GFP RNA transcribed from the transgene. This is probably at a much lower level than the transient to degrade the GFP RNA transcribed from the transgene.

The guard hypothesis (13) proposed to explain R gene-mediated resistance in plants. One well-studied example supporting the guard hypothesis is the avrPro-Pto-Prf system conferring resistance to Pseudomonas syringae in the tomato plant (40, 51, 53, 60). The credibility of this hypothesis has been enhanced recently with the characterization of the RIN4 protein that interacts with both the avirulence protein avr-Rpm1 of P. syringae, and the corresponding resistance gene product RPM1 in Arabidopsis (38).

The guard hypothesis has also been invoked to explain the resistance-related interactions between TCV and Arabidopsis proteins in the expanded version of the model described by Dangl and Jones (13). We showed previously that the TCV-encoded elicitor, TCV CP, interacts with an NAC family transcription factor which we called TIP (49). The TCV CP-TIP interaction was highly correlated with the resistance response in Di-17 line carrying the NBS-LRR resistance gene, HRT. Although no direct interaction has been demonstrated between TIP and HRT, it was speculated that a TIP-CP complex might be the trigger that activates the resistance cascade through HRT, hence functioning in a manner consistent with the guard hypothesis. It will be very interesting to see whether we can connect the function of TIP with certain steps of RNA silencing, possibly as a transcription factor involved in activating the transcription of certain inducible factors in the RNA silencing pathway. Such demonstration would link the RNA silencing with R gene-mediated resistance.

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