Tombusvirus P19-Mediated Suppression of Virus-Induced Gene Silencing Is Controlled by Genetic and Dosage Features That Influence Pathogenicity

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The p19 protein (P19) of *Tomato bushy stunt virus* (TBSV) is a pathogenicity determinant with host-dependent effects on virus spread and symptom induction. In addition, results in this study confirm that *Potato virus X*-mediated delivery of P19 suppresses posttranscriptional gene silencing (PTGS). To study the relevance of this activity for TBSV biology, we evaluated whether TBSV activates virus-induced gene silencing (VIGS) and if this process is suppressed by P19. TBSV vectors with the green fluorescent protein (GFP) gene, either active or inactive for P19 expression, were inoculated onto GFP-transgenic *Nicotiana benthamiana* plants. In the absence of P19 expression, VIGS was activated, as evidenced by the disappearance of GFP mRNA and green fluorescence. Coexpression of GFP and P19 from the TBSV vector suppressed VIGS, except in the newly emerging leaves. The suppressor activity required a central P19 region that is also known to be essential for host-dependent virus spread and symptom induction. Defective interfering RNAs (DIs) that contained the 3' end of the GFP gene induced silencing very effectively. The concomitant DI-instigated reduction in P19 accumulation failed to suppress this process, analogous to the known P19 dosage effects for other biological activities. In conclusion, (i) TBSV and its DIs are very effective inducers of VIGS, (ii) P19 is a strong suppressor of PTGS, (iii) P19 is a moderate suppressor of VIGS, and (iv) the suppressor activity is influenced by genetic and dosage features that are also important for P19-associated pathogenesis.

Virus-induced gene silencing (VIGS) appears to reflect an induced defense response to protect plants from virus infection. The mechanism of VIGS is similar to posttranscriptional gene silencing (PTGS), whereby RNA molecules are degraded in a sequence-specific manner (Baulcombe 2000). These phenomena may reflect an evolutionary relic, because PTGS (or RNA interference) has been described for di- and monocotyledonous plants (Dalmay et al. 2000; Ingelbrecht et al. 1999; Voinnet and Baulcombe 1997) and for other organisms (Bahramian and Zarbl

1999; Cogoni and Macino 1999; Fire et al. 1998; Hammond et al. 2000; Wu-Sharf et al. 2000). The precise mechanisms responsible for PTGS and VIGS have not yet been fully resolved, but it is generally agreed that VIGS proceeds in stepwise phases (Baulcombe 2000; Fagard and Vaucheret 2000; Sijen and Kooter 2000). Ultimately, a systemic silencing condition is acquired, which can result in resistance against, or recovery from, viral infection (Covey et al. 1997; Lindbo et al. 1993; Palauqui and Vaucheret 1998; Ratcliff et al. 1997; Ruiz et al. 1998; Waterhouse et al. 1999).

PTGS or VIGS can be counteracted by suppressors that are encoded by some plant viruses. This was first shown for the helper component-proteinase (HC-Pro) of potyviruses (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998) and, subsequently, for proteins of other viruses (Brigneti et al. 1998; Voinnet et al. 1999). In general, viral suppressors represent pathogenicity factors, including the p19 protein (P19) of *Tomato bushy stunt virus* (TBSV) (Voinnet et al. 1999). This protein has various biological activities: it is required in a host-dependent manner for viral invasion (Scholthof et al. 1995b), it is the elicitor of a hypersensitive response (HR) on *Nicotiana tabacum*, and it induces a systemic necrosis in *N. benthamiana* (Scholthof et al. 1995a). P19 activity depends on high levels of expression (Scholthof et al. 1999), and a centrally located cluster of amino acids on P19 is crucial for all biological activities, whereas N-proximal residues exert host-dependent effects (Chu et al. 2000).

In comparison to the HC-Pro suppressor, P19 differs in a variety of ways. Initially, P19 was reported to suppress PTGS mainly along the vein tissue and in newly emerging leaves, whereas HC-Pro reversed PTGS in a non-tissue-specific manner (Voinnet et al. 1999). No information was available on the effect of P19 on VIGS, but this might be important in the context of recent models that suggest that VIGS and PTGS may be controlled by separate, albeit overlapping, pathways (Carrington et al. 2001; Mallory et al. 2001; Voinnet et al. 2000). Furthermore, HC-Pro is able to increase levels of viral replication (Kasschau and Carrington 1998; Pruss et al. 1997), but such an effect is not observed for TBSV P19 in *N. benthamiana* (Chu et al. 2000; Qiu and Scholthof 2001; Scholthof et al. 1995b, 1999). These aspects indicated that studies on TBSV P19-mediated suppression could potentially contribute valuable new information to our understanding of suppression and its relevance for virus biology.

The goals of the current study were to investigate (i) whether TBSV and its defective interfering RNAs (DIs) induce VIGS, (ii) if P19 is able to suppress VIGS, and (iii) if suppressor ac-

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tivity correlates with features of P19 that are associated with its biological properties (Chu et al. 2000). We used transgenic *N. benthamiana* plants expressing a green fluorescent protein (GFP) (Voinnet and Baulcombe 1997) as an indicator host for silencing, i.e., disappearance of GFP expression. TBSV coat protein (CP) and P19 are both dispensable for high levels of TBSV accumulation in *N. benthamiana* (Scholthof et al. 1993, 1995b). This permitted the use of TBSV CP replacement vectors (Scholthof 1999; Scholthof et al. 1996) containing the GFP gene (silencer) that were either active or inactive for P19 (suppressor) expression. Although this is an artificial strategy, it does mimic a natural viral infection whereby the silencer and suppressor are introduced simultaneously by the virus in the same cells. The available TBSV P19 amino acid substitution mutants (Chu et al. 2000) permitted an analysis of the potential correlation between the P19-mediated suppression in *N. benthamiana* versus its other genetically defined biological activities on various hosts. To study whether the suppression activity of P19 was dosage dependent, as was shown for its other biological activities (Scholthof et al. 1999), we substantially increased the ratio of silencer versus suppressor produced in the same cell. This was achieved by using a well-characterized, abundantly accumulating, DI vector to express a portion of the GFP gene (silencer), while simultaneously exploiting the feature that DIs down-regulate the levels of P19 (suppressor) produced by the helper virus (Scholthof et al. 1995c).

The results show that TBSV- and DI-mediated delivery of GFP sequences are efficient systems to investigate VIGS. Simultaneous delivery of P19 and GFP from the TBSV vector prevented the early onset of gene silencing. The suppression depended on a central region of P19 that is also essential for its previously defined biological activities. In addition, the high accumulation of silencer by a DI vector, which simultaneously reduced P19 expression from the helper, prohibited suppression, illustrating the importance of the dosage of silencer and suppressor. These features of P19-mediated suppression correlate with those involved in pathogenesis (e.g., virus spread and symptom induction), supporting the model that the ability of a virus to suppress VIGS is a biologically important feature.

RESULTS

Separate delivery of silencer and suppressor.

Agrobacterium-mediated gene silencing. To permit comparison of our results with those in the literature, and to confirm that TBSV P19 acts as a suppressor of PTGS, we first employed an existing experimental silencing system (Voinnet et al. 1998, 1999), generously supplied by D. Baulcombe (Sainsbury Laboratories, John Innes Centre, Norwich, U.K.). In this system, *Agrobacterium tumefaciens* was used to deliver the silencer GFP by agroinfiltration of GFP-transgenic *N. benthamiana* line 16C (NbGFP). This treatment abolished GFP expression, as evidenced by the absence of green fluorescence at the site of infiltration upon UV illumination of NbGFP plants (data not shown). This initial effect was followed by a systemic fading of green fluorescence by 3 weeks postagroinfiltration (Fig. 1A), indicating that the GFP transgene was systemically silenced (Voinnet and Baulcombe 1997; Voinnet et al. 1998). The visible red fluorescence is emitted by chlorophyll upon UV illumination.

Potato virus X (PVX)-mediated delivery of P19. The upper leaves of silenced NbGFP plants that manifested uniform red fluorescence were inoculated with RNA transcripts of PVX/p19(+) (i.e., pHS142), which expressed TBSV P19 (Scholthof et al. 1995a), or of PVX/p19(-), carrying the TBSV p19 gene in the antisense orientation (H. Scholthof, unpublished data). GFP-associated fluorescence was monitored fre-

quently by UV illumination during a 3-week period postinoculation. The results showed that green fluorescence gradually replaced red fluorescence when the plants were infected with PVX/p19(+) (Fig. 1A). By contrast, red fluorescence persisted on the silenced plants inoculated with PVX/p19(-), similar to that observed for noninoculated silenced plants (Fig. 1A). These observations confirmed that TBSV P19 is a viral suppressor of *Agrobacterium*-mediated PTGS (Voinnet et al. 1999). A few days after the green fluorescence was reestablished by infection with PVX/p19(+), the plants died as a result of the lethal effects of P19 on *N. benthamiana* (Scholthof et al. 1995a).

Suppression of gene silencing was also conspicuous on the stems (Fig. 1A), and cross sections showed that the suppression occurred throughout these stems (Fig. 1B). Therefore, visual monitoring of plants often included inspection of the stem, as it was shown to be a good indicator for monitoring gene silencing and suppression.

Concurrent delivery of silencer and suppressor.

The above procedure of *Agrobacterium*-activated PTGS followed by virus vector-mediated expression of a suppressor has been very useful in demonstrating the concept of silencing and suppression. However, it might not be the most appropriate method to test the biological relevance of these processes for viral infections, especially since PTGS and VIGS might be influenced by different pathways (Matzke et al. 2001; Voinnet et al. 2000). During natural viral infections, silencing inducers and suppressors are introduced simultaneously in the same cells. To mimic such an event, we used the TBSV vector to simultaneously supply silencer (GFP) and suppressor (P19).

TBSV vector-mediated gene silencing. The first step was to determine the ability of TBSV to induce VIGS. For this purpose, a TBSV CP gene-replacement vector was used, in which expression of P19 was abolished (Scholthof 1999; Scholthof et al. 1996). The same GFP gene used to create the transgenic NbGFP plants was inserted into the TBSV vector to generate pHST3-4, further referred to as pTG (Fig. 2A). When *N. benthamiana* plants were inoculated with RNA transcripts of pTG, bright green fluorescent spots were visible with the confocal microscope (Fig. 2B). This confirmed that the GFP gene was expressed at high levels at the site of inoculation. However, as previously reported (Scholthof 1999; Scholthof et al. 1993), foreign inserts are rapidly deleted from the TBSV vector, precluding systemic spread and expression of the GFP gene (data not shown).

To examine if the localized GFP expression from pTG RNA would induce silencing, transcripts were inoculated onto NbGFP plants. The progressive transition of green to red fluorescence, reflecting the advancement of GFP silencing, was monitored under UV illumination throughout the infection. GFP silencing on the inoculated leaves was marked by vague reddish areas on the leaf, but visible levels of green fluorescence were often maintained. The onset of systemic silencing was frequently noted in the stems above the inoculated leaves, followed by systemic gene silencing in the upper leaves (Fig. 3A). In these upper leaves, red fluorescence was visible by 10 to 15 days postinoculation (dpi) predominantly along the class III veins and gradually expanded to class II veins by 20 to 24 dpi (Fig. 3A). Silencing throughout the entire plant was observed at 30 dpi. As expected, the disappearance of green fluorescence coincided with reduced levels of GFP mRNA (Fig. 3A). Upper, noninoculated NbGFP leaves showed chlorotic mottle symptoms and contained viral RNA, suggesting that infections with pTG RNA were not effectively blocked by the onset of gene silencing. When the TBSV vector was used to deliver a modified GFP gene with substantial nucleotide changes compared with the original version present in NbGFP plants (Chiu et al. 1996), silencing did not occur (data not shown).

Fig. 1. Suppression of green fluorescent protein (GFP) gene silencing upon *Potato virus X* (PVX)-mediated delivery of P19 on previously silenced plants. **A, 1**, Bright green fluorescence of GFP-transgenic *Nicotiana benthamiana* (NbGFP); **2**, the typical red autofluorescence of NbGFP systemically silenced by *Agrobacterium*-mediated delivery of a T-DNA binary plasmid carrying the GFP gene (Sil-NbGFP); **3**, Sil-NbGFP inoculated with PVX/p19(-); and **4**, Sil-NbGFP inoculated with PVX/p19(+). Plants were inoculated 4 weeks after agroinfiltration and pictures of UV-illuminated plants were taken 10 or 12 days after inoculation. **B**, Fluorescent microscope image of stem sections of plants treated as described for **1**, **2**, and **4** in **A**.

TBSV vector-mediated codelivery of silencer and suppressor. The above results showed that the TBSV vector provides an effective and convenient delivery vehicle for the induction of systemic gene silencing of homologous genes in plants. To test how codelivery of the suppressor (P19) would influence the silencing, the defect of pTG to express P19 was restored to yield pTG-p19/wt (Fig. 2A).

RNA of pTG to express the wild-type (wt) p19 gene was inoculated onto NbGFP plants, and the progressive transition of green to red fluorescence on these plants was compared with that on control plants inoculated with pTG transcripts (Fig. 3B). At 10 dpi, the plants infected with pTG-p19/wt RNA remained green fluorescent, while plants infected with RNA from pTG exhibited the first typical signs of vein-associated silencing (i.e., red fluorescence). At 24 dpi, silencing-associated red fluorescence was clearly visible on plants infected with pTG, but upper leaves of the plants infected with pTG-p19/wt RNA still maintained a green fluorescence (Fig. 3B). The influence of P19 on gene silencing is not due to indirect effects on RNA replication and cell-to-cell movement, because local GFP expression (Fig. 2B) and viral RNA accumulation in NbGFP (Fig. 3C) occurred efficiently and independently of P19. This is in agreement with previous results (Chu et al. 2000; Qiu and Scholthof 2001; Scholthof et al. 1995b) on nontransgenic *N. benthamiana*.

NbGFP plants inoculated with pTG-p19/wt RNA frequently showed severe systemic symptoms at time points beyond 24 dpi because of the expression of P19. This protein is responsible for induction of a lethal necrosis on *N. benthamiana* plants, as shown for several members of the *Tombusvirus* genus (Burgan et al. 2000; Chu et al. 2000; Rochon 1991; Scholthof et al. 1995a). However, three out of five NbGFP plants infected with pTG-p19/wt recovered from the effects of the initial severe necrosis, and the newly emerging leaves of these plants exhibited red fluorescence along the minor veins (Table 1). The disappearance of severe P19-mediated symptoms and the concurrent onset of gene silencing (i.e., disappearance of suppression) showed that the suppression was not complete.

These results show that the simultaneous expression of the suppressor with the silencer from the TBSV vector delayed the onset of silencing. Therefore, in addition to the ability of P19 to reverse PTGS (Fig. 1), it can also suppress the effective onset of VIGS.

Identification of P19 amino acid residues involved in suppression.

Five representative P19 amino acid substitution mutants (Table 1) with known biological effects (Chu et al. 2000) were selected to identify their ability to suppress gene silencing. Infectious transcripts of these P19 mutants, with the GFP gene substituted for the CP gene, were inoculated onto NbGFP plants. Immunoblot assays were performed to confirm the accumulation of P19 (Fig. 4A). It is important to discuss P19 accumulation, because in a separate study, we demonstrated that P19 dosage contributed to its biological activity (Scholthof et al. 1999). Previously, we showed that mutant and wild-type P19 accumulated to similar levels at early time points (Chu et al. 2000), but at later time points, reduced P19 levels were observed for P19/71-72 and P19/85 (H. Scholthof, unpublished data). This same effect was noticed in this study for the TBSV vector expressing GFP (Fig. 4A). However, the phenotype associated with pTG-p19/71-72 (discussed below) was comparable to that of pTG-p19/72^s, for which no reduction in P19 levels was observed (Fig. 4A). Furthermore, the accumulation of P19 was similar for pTG-p19/43 and pTG-p19/72^s (Fig. 4A), but their suppressor activities were different (Fig. 4B, Table 1). This indicated that, although dosage effects do probably play a contributing role for suppression, the qualitative differences observed for the mutants pTG-p19/71-72 and pTG-p19/72^s were directly influenced by the substitution of the arginine at amino acid position 72.

Reproducible results of five independent experiments showed that the P19-mediated suppression of silencing by the various mutant P19 proteins could be grouped into three classes (Table 1). The first is represented by pTG-p19/101 and pTG-p19/43 (Fig. 4B), which showed a phenotype similar to pTG-p19/wt. This was manifested by suppression of the onset of silencing compared with that in the absence of suppressor for pTG (no P19 expression). However, the suppression was not complete, because silencing eventually occurred on the new leaves at a later stage of infection (Table 1). The second class is represented by pTG-p19/85, which, like pTG (Fig. 3), failed to suppress gene silencing. The third phenotype was intermediate and observed for mutants p19/72^s and p19/71-72. This response was typified by a pattern of non-, partial-, and full-silencing on different branches of a single infected plant (Fig. 4B and C). Throughout the experiments, the appearance of red fluorescence on plants inoculated with the different mutants (Table 1) was consistently correlated with the disappearance of GFP mRNA accumulation. The GFP mRNA accumulation in plants in which silencing was suppressed by inoculation with pTG-p19/wt (data not shown) was similar as for the mock-inoculated plant in Figure 4D. Analyses of total RNA extracted from leaves on different branches of the same plant inoculated with RNA from pTG-p19/71-72 (Fig. 4C) showed that in the nonsilenced leaves, or in leaves in which silencing was barely initiated, GFP mRNA accumulated to levels similar to those in mock-inoculated plants (Fig. 4D). However, in partially silenced leaves, GFP mRNA was far less abundant than in the nonsilenced leaves. In the fully silenced leaves, GFP mRNA was not detected (Fig. 4D).

Effect of GFP dosage on silencing and P19-mediated suppression.

DI-mediated silencing. The results from the previous sections indicated that high levels of GFP expression from the TBSV vector induced silencing generally at least 1 week faster than the agroinfiltration method. This suggested that a high GFP RNA dosage produced by a replicating virus vector increased the efficiency of silencing. To further investigate this aspect, we used the feature of TBSV that it serves as a helper virus for the prolific accumulation of DIs (White and Morris 1999) or DI-like molecules containing short inserts (Qiu et al. 2001). We also applied the observation that gene silencing can be triggered by truncated GFP fragments (Ruiz et al. 1998). In combination, this strategy yielded pDI-P, with a 3'-proximal GFP segment 'P' (nucleotides [nt] 489 to 716) inserted into a biologically active DI clone (Fig. 5A).

NbGFP plants were inoculated with a mix of transcripts of pDI-P and those of pTΔp19 (i.e., pHs157), which is a TBSV derivative that expresses CP (instead of GFP) but is incapable of expressing P19 (Scholthof et al. 1995b). Compared with inoculation with pTG, inoculations with DI-P gave a very strong induction of local silencing, as inferred by the appearance of red spots on inoculated leaves upon UV illumination (data not shown). At 8 to 10 dpi, red fluorescence emerged along veins of upper leaves, indicating that systemic silencing had been initiated, and the silencing was very prominent at 21 dpi (Fig. 5B). As with other experiments, the appearance of red fluorescence correlated with the disappearance of GFP mRNA (data not shown). As expected, silencing of GFP did not occur in plants coinoculated with a wild-type DI devoid of GFP sequences (Fig. 5B). RNA hybridization analyses of the pTΔp19 plus DI-P combination showed that the early onset of silencing coincided with high levels of DI-P accumulation in inoculated leaves (Fig. 5C).

Suppression capacity of P19. To examine the suppression potency of P19 in the presence of very high levels of silencer (i.e., DI-P), the above results were compared with those ob-

tained when transcripts of pDI-P were coinoculated with RNAs of TBSV producing wild-type P19. Visual inspection under UV light indicated that red fluorescence (i.e., silencing) was also observed on the upper, noninoculated leaves on plants infected with TBSV+DI-P (Fig. 5B). Therefore, the induction of silencing by DI-P was not effectively counteracted by the expression of P19 suppressor from the wild-type virus.

To examine why P19 was unable to suppress silencing induced by DI-P, a molecular analysis for detection of DI-P and P19 was performed. RNA hybridization tests with total RNA from infected plants showed that DI-P RNA readily accumulated in both inoculated and upper leaves of plants infected with TBSV+DI-P (Fig. 5C). When the RNA blot of systemically infected leaves was hybridized with probes specific for GFP (Fig. 5C, left) or TBSV (Fig. 5C, right), two different-sized DIs with (GF)P inserts were detected in the presence of P19 (i.e., TBSV), while only the lower band (with approximately 100 nt deleted) was detected in the absence of P19 (i.e., pTΔp19) (Fig. 5C). The DI RNAs were visible upon ethidium bromide (EtBr) staining of agarose gels (data not shown), con-

firmed that the positive signals obtained with a GFP-specific probe represented DIs containing GFP sequences and not the native GFP mRNA expressed by the transgenic NbGFP plants. RNA hybridizations with TBSV-specific probes showed that RNA from TBSV (+P19) and pTΔp19 (–P19) accumulated to similar levels in *N. benthamiana* (Fig. 5C, right). This is in agreement with Figures 2B and 3C and with separate studies showing that P19 has no effect on replication in this host (Chu et al. 2000; Qiu and Scholthof 2001). As previously reported for wild-type DIs (Scholthof et al., 1995c), the accumulation of TBSV subgenomic (sg) RNAs was preferentially reduced in the presence of DI-P molecules (Fig. 5C). Correspondingly, an immunoblot showed that the level of P19 (suppressor) that is translated from sgRNA2 was present in leaves inoculated with TBSV+DI-P at 6 dpi, but it was undetectable in the upper leaves at 18 dpi (Fig. 5D), although these leaves were systemically infected (Fig. 5C).

Collectively, these results with DI-P illustrate that (i) DIs are very effective inducers of silencing; (ii) the concomitant abundant accumulation of DI-P and reduced levels of P19 interfered

Fig. 2. *Tomato bushy stunt virus* (TBSV)-mediated expression of the green fluorescent protein (GFP) gene. **A**, Schematic diagram of constructs. Rectangles represent TBSV open reading frames (ORFs) and, as indicated, the GFP gene substitutes the coat protein gene. The asterisk (*) denotes the amber stop codon of p33 to permit read-through translation of the p92 replicase gene. The p19 ORF is denoted by the shaded box nested within the p22 cell-to-cell movement gene; the function of the putative pX product is unknown. The transcription initiation sites of two subgenomic RNAs (sgRNA1 and sgRNA2) are indicated by right-angled arrows. In pTG, the p19 gene is inactivated, whereas pTG-p19/wt expresses wild-type P19. **B**, Laser scanning confocal microscopy image showing TBSV-mediated expression of GFP in infection foci on nontransgenic *Nicotiana benthamiana* leaves inoculated with transcripts of pTG or pTG-p19/wt.

Fig. 3. *Tomato bushy stunt virus* (TBSV) vector-mediated gene silencing and its suppression by P19. **A**, Fluorescence upon UV illumination of **1**, green fluorescent protein (GFP)-transgenic *Nicotiana benthamiana* (NbGFP) and **2**, NbGFP 24 days postinoculation (dpi) with pTG RNA. The right panel shows the relative levels of GFP mRNA on an RNA hybridization blot with similar amounts of total RNA from mock-inoculated NbGFP (lane 1) or NbGFP inoculated with RNA from pTG at 24 dpi (lane 2). **B**, **1 and 2**, NbGFP 10 or 24 dpi with pTG RNA; **3 and 4**, NbGFP 10 or 24 dpi with pTG-p19/wt RNA. **4**, The plant shows systemic green fluorescence, but red fluorescence started to appear along the minor veins of newly emerging leaves. **C**, Accumulation of viral RNA in NbGFP 5 days after inoculation with transcripts of either pTG or pTG-p19/wt, or mock-inoculated (M). Hybridization was performed with a randomly primed TBSV cDNA to detect genomic RNA (gRNA). Overexposure also revealed subgenomic RNAs (data not shown).

with suppression, illustrating that suppression is dosage dependent; and (iii) GFP gene silencing does not effectively target DI-P, because systemically invaded tissue contained DIs that had maintained GFP sequences.

DISCUSSION

TBSV and DI-mediated induction of silencing.

In this study, we exploited our TBSV-based vector system (Scholthof 1999) for simultaneous delivery of the silencer (GFP gene) and the suppressor (TBSV P19) on GFP-transgenic *N. benthamiana* (NbGFP). Delivery of GFP with a TBSV CP replacement vector (devoid of P19 expression) onto NbGFP plants initiated systemic silencing about 1 week earlier than observed upon agroinfiltration of NbGFP plants with GFP DNA. The current study confirmed that TBSV vectors rapidly delete foreign inserts (Scholthof 1999; Scholthof et al. 1993), because upper NbGFP leaves became invaded with TBSV RNA devoid of the (intact) GFP gene (data not shown). Nevertheless, the TBSV-mediated VIGS occurred despite the occurrence of deletions. These observations made with the TBSV CP replacement vector illustrate three important features about the silencing process: (i) the initial high levels of GFP RNA transcription from the TBSV vector trigger a systemic silencing reaction; (ii) after silencing has been activated, virus-assisted accumulation of the GFP gene is no longer needed to maintain systemic silencing; and (iii) the TBSV infection is not notably affected by the silencing surveillance in NbGFP plants. Perhaps the TBSV vector escaped the GFP silencing because of its prolific replication, its ability to quickly delete foreign genes from its genome, or both.

Expression of the 3'-proximal GFP sequences from pDI-P transcripts resulted in the rapid onset of silencing, usually visible within 10 dpi. This confirms previous results (Ruiz et al. 1998) that delivery of a portion of the target gene is sufficient to induce silencing of the whole gene. As indicated earlier, DI-P isolated from inoculated leaves (Fig. 5) is directly visible upon EtBr staining of agarose gels, which confirms that it accumulates to very high levels. Thus, this result strengthens the notion that a high dosage of silencer accelerates the silencing process. Another intriguing feature is that GFP sequences were maintained by DI-P, even in the upper systemically infected leaves (Fig. 5). This suggests that these DIs were not very effectively targeted by the GFP-specific silencing. Further ex-

periments are needed to determine if specific inherent characteristics of DIs protect them from the silencing-associated RNA degradation surveillance system.

This study shows that the TBSV CP replacement vector represents an alternative to PVX-based (Burton et al. 2000; Ruiz et al. 1998) and *Tobacco rattle virus*-based (Ratcliff et al. 1999) delivery vehicles for studies on VIGS. Considering its wide host range (Martelli et al. 1988), TBSV or its DIs may serve as useful tools in functional genomic studies in a wide variety of plants. An additional attractive prospect is to use DIs as a vector for silencers and to employ the TBSV vector to supply the suppressor, with the caveat that DI-mediated dosage effects may influence the results. However, the opportunity for simultaneous introduction of silencer and suppressor may permit detailed genetic studies of determinants and interactions that control silencing/suppression-associated processes.

TBSV P19 delays the onset of silencing.

Expression of P19 from a PVX vector on NbGFP plants that were previously silenced through *Agrobacterium*-mediated delivery of the GFP gene resulted in complete suppression of PTGS in stems and newly emerged leaves (Fig. 1). This confirmed and extended a previous report that P19 was capable of reversing gene silencing in vascular-associated tissues (Voinnet et al. 1999). The quantitative differences between that study and our results are reminiscent of variations that were observed in HC-Pro-mediated suppression of the maintenance phase of the host RNA-dependent RNA polymerase (RdRp)-dependent PTGS pathway (Llave et al. 2000). It is known that environmental, physiological, and developmental stages influence susceptibility of plants to virus infection. Perhaps these factors also affect the formation and spread of the silencing signals and the effect of suppressors. Nevertheless, it is clear that TBSV P19 delivered by a PVX vector effectively interferes with PTGS (Fig. 1).

To examine the effect of P19 on VIGS, we used the TBSV vector to simultaneously deliver the silencer (GFP) and suppressor (P19). The results showed that codelivery of GFP and P19 onto NbGFP suppressed the onset of silencing compared with the progress of gene silencing obtained with pTG RNA (Δ P19) (Fig. 3). Interestingly, despite the expression of P19 from the TBSV vector, silencing eventually occurred in newly emerged leaves (Table 1). It is possible that this incomplete suppression by P19 is due to the rapid and abundant appearance of double-stranded TBSV RNAs (Russo et al. 1994;

Table 1. Effects of p19 proteins (P19) on virus induced-gene silencing (grouped in three classes)

P19	Amino acid(s) changed ^a	Biological activities			Suppression of silencing	
		<i>Nicotiana benthamiana</i>		Spinach spread ^d	(days postinoculation)	
		Local ^b	Systemic ^c		12	24
P19/wt	Wild type	+ ^e	+	+	+	+/- ^f
P19/43	R43W	+	-	+	+	+/-
P19/101	R101G	+	-	+	+	+/-
P19/72 ^g	R72G	+	-	-	-	+ & - ^f
P19/71-72	K71A-R72G	+/-	- ^h	-	-	+ & -
P19/85	R85G	+	+	+/-	-	-
Δ P19	No p19	-	-	-	-	-

^a Genotypes and phenotypes were previously published (Chu et al. 2000).

^b Localized necrosis on nontransgenic *N. benthamiana* (Chu et al. 2000) and necrosis along the stems following infection of green fluorescent protein (GFP)-transgenic plants with *Tomato bushy stunt virus* (TBSV) derivatives with the GFP substituting the coat protein gene (Fig. 2).

^c Lethal systemic necrosis on nontransgenic *N. benthamiana* (Chu et al. 2000).

^d Long-distance spread in spinach (Chu et al. 2000).

^e The + and - signs indicate activity that is normal or defective, respectively, compared with wild type. The +/- marking indicates that activity was impaired compared with wild type.

^f The +/- denotes that suppression was not complete and silencing did occur in newly emerged leaves. The + & - sign represents that different branches on the same plant were either silenced or nonsilenced.

^g Indicates the G substitution.

^h Initially, necrosis was induced, but the plants recovered rapidly.

Scholthof et al. 1993), which are thought to be involved in the activation of silencing (Baulcombe 2000; Smith et al. 2000). For example, replicated TBSV genomic RNA can be detected as early as 2.5 h posttransfection of *N. benthamiana* (Qiu and Scholthof 2001). The prolific accumulation of TBSV RNA possibly activates an abundant supply of strong silencing signals that eventually do penetrate into tissues that are not (yet) invaded by the vector and, therefore, devoid of P19. This may be related to the observation that the TBSV vector is somewhat compromised for movement and, unlike wild-type TBSV, does not rapidly and uniformly invade the whole plant.

It is not known if TBSV induces a silencing-related defense reaction in nontransgenic *N. benthamiana* plants and if P19 can suppress such a response. Although P19 is dispensable for initial efficient virus accumulation in *N. benthamiana* (Scholthof et al. 1995b), prolonged systemic infections of this host with TBSV devoid of P19 expression results in a gradual decline of viral titer (H. Scholthof, *unpublished data*). This may be due to silencing, but it cannot be ruled out that unrelated defense responses contribute or that P19 is required for spread through older *N. benthamiana* plants, as it is in some other hosts (Scholthof et al. 1995b). Future experiments incorporating P19 mutants that do not induce a lethal necrosis but suppress silencing may allow for monitoring of viral titers during prolonged infections to examine the contribution of gene silencing during a natural infection.

It appears that P19 is a strong suppressor of PTGS like HC-Pro and moderately functional against VIGS (Carrington et al. 2001; Mallory et al. 2001; Voinnet et al. 2000). It is possible that P19 interferes with the silencing pathway(s) in a manner and at a point that is distinct from what is proposed for HC-Pro (Llave et al. 2000; Mallory et al. 2001) and PVX p25 (Voinnet et al. 2000). P19-mediated suppression might be more similar to that of the *Cucumber mosaic virus* (CMV) 2b host-dependent, long-distance movement protein (Ding et al. 1995; Lucy et al. 2000; Voinnet et al. 1999). Alternatively, the possibility remains that the aggressive pathogenic properties of P19 (Scholthof et al. 1995a) disrupt or prevent (i.e., suppress) the establishment of many normal gene regulatory events (i.e., silencing) in a nonspecific and incomplete manner.

Genetic correlation between P19-mediated suppression and other activities.

Since none of the P19 amino acid substitutions used in this study affected the ability of TBSV to infect *N. benthamiana* (Chu et al. 2000), this provided an opportunity to determine if host-specific p19 genotypes correlated with its ability to suppress silencing in NbGFP plants. In preliminary experiments to test the effect of P19 mutations on PTGS, TBSV p19 mutants with an intact wild-type CP (Chu et al. 2000) were inoculated onto NbGFP plants previously silenced by agroinfiltration. These tests (data not shown) illustrated that the NbGFP plants were overly sensitive to TBSV-induced symptoms compared with nontransgenic *N. benthamiana* plants (Chu et al. 2000). For example, TBSV P19 mutants, as well as coinfections of TBSV with DIs, that induced mild symptoms in nontransgenic *N. benthamiana* plants caused a severe necrosis in NbGFP plants. Perhaps the additive effects of CP (Scholthof et al. 1993), P33 (Burgan et al. 2000), or both on P19-mediated symptom induction are especially prominent in this transgenic NbGFP line for reasons that are not yet understood.

Since RNA of pTG-p19 constructs (with GFP substituting CP, Fig. 2) did not induce a lethal necrosis on NbGFP plants, this permitted a comparison of effects of P19 mutations on VIGS with previous results of genetic studies on P19-mediated biological activities (Table 1). The results showed that the ar-

ginine at position 72 in the center of P19 is involved in all known biological activities of this protein (Chu et al. 2000), and the current data also revealed an interesting role of this amino acid for P19 to function as a suppressor. The P19 proteins produced by pTG-p19/72^s and pTG-p19/71-72 partially suppressed silencing. Both mutants induced an intermediate response whereby some branches of plants were silenced whereas others were suppressed, which correlated with the low or high levels of GFP mRNA, respectively (Fig. 4).

Infections with RNA from the pT19/71-72 mutant (with wild-type CP) on normal *N. benthamiana* plants first caused systemic symptoms that mimicked those observed for wild-type TBSV. However, the plants quickly recovered and displayed symptoms resembling those caused in the absence of P19 (Chu et al. 2000; H. Scholthof, *unpublished data*). The pTG-p19/71-72 mutant (with GFP substituting CP) transcripts caused necrosis along the vascular tissue of NbGFP, but otherwise, symptoms mostly resembled those induced by pTG RNA (devoid of P19 expression) (data not shown). Combined, these results imply that the intermediate suppressor activity by P19/71-72 may be correlated with its intermediate effect on necrosis induction in *N. benthamiana* plants. Although these effects are quite possibly influenced by the reduced levels of P19 accumulation associated with pTG-p19/71-72 (Fig. 4A), the experiments with pTG-p19/72^s (Fig. 4, Table 1) confirm that the arginine at position 72 plays a crucial role. Correlations between suppression and biological activities of mutations other than those affecting position 72 appear more complicated. However, there is a general trend toward a genetic correlation between the ability of P19 to suppress gene silencing and its activities in long-distance movement in spinach and localized necrosis in *N. benthamiana* (Table 1).

Recently, it was shown that HC-Pro determinants necessary for suppression are required for replication maintenance and long-distance movement of *Tobacco etch virus* (Kasschau and Carrington 2001). Those results and our observations imply that the molecular interactions necessary for the suppression of gene silencing may be at the core of virus–host interactions for replication, virus spread, and symptom induction, either alone or in combination.

DIs and P19.

The efficient induction of silencing upon DI-mediated delivery of the 3' end of the GFP gene with DI-P was not suppressed by P19 produced by the wild-type TBSV helper virus (Fig. 5B). The reasons for this effect are likely twofold: an enhanced efficiency of silencing because of a high (GF)P-dosage (Fig. 5C), and a concurrent reduction in P19 accumulation (Fig. 5D) (Scholthof et al. 1995c). In combination with results discussed earlier regarding the presence of silenced and suppressed branches on the same plants, these results imply there is some sort of relay race between silencing and suppression. Changes in the ratio of inducer versus suppressor established by coinfections with DI-P have a profound effect on the outcome of the process. Also, the prolifically replicating DIs may induce gene silencing so rapidly that the signal spreads systemically before sufficient P19 suppressor is produced in trans from the helper virus.

One particularly interesting, but not well-understood, feature associated with the combination of DI-P and helper viruses (Fig. 5) is that, in the absence of P19, a smaller DI-P version accumulated abundantly in systemic tissue. In the presence of P19, this deletion mutant also occurred, but the original DI-P also accumulated. Within this context, it is worthwhile to note that when plants are infected with mutant tombusviruses inactivated for P19 expression, the formation, accumulation, or both of de novo DIs is accelerated compared with infections established by wild-type virus (Rezende et al. 1998; Rochon 1991).

Fig. 4. Effect of P19 amino acid substitutions on suppression of gene silencing. **A**, Immunoblot assay for the detection of P19 in green fluorescent protein (GFP)-transgenic *Nicotiana benthamiana* (NbGFP) leaves 7 days postinoculation (dpi) with transcripts from the constructs denoted above each lane. The upper band presumably reflects a P19 dimer. **B**, Examples of fluorescence patterns on NbGFP plants at 28 dpi with RNAs from indicated constructs. Complete results are summarized in Table 1. **C**, Patterns and progression of gene silencing induced by pTG-p19/71-72 on different branches of the same plant. **1**, Green fluorescence (or complete suppression) on a branch at 26 dpi. **2**, Red fluorescence (i.e., silencing) along the veins on another branch at 18 dpi. **3**, Complete silencing at 26 dpi. **D**, RNA blot analysis of the GFP mRNA levels from leaves showing different patterns of green fluorescence. M = Mock-inoculated NbGFP. Lanes 1, 2, and 3 correspond to **1** to **3** in **C**. Lane 3a represents RNA from a branch showing partial or intermediate red fluorescence compared with samples 2 and 3, at 18 dpi. Lane TG represents RNA from a completely silenced leaf of NbGFP inoculated with pTG, harvested at 26 dpi.

Fig. 5. Defective interfering RNA (DI)-mediated gene silencing. **A**, Schematic representation of the *Tomato bushy stunt virus* (TBSV) genome and the position of the four segments (I to IV) that comprise a prototypic DI. The 3' portion of the green fluorescent protein (GFP) gene (P) was inserted between regions II and III in DI-P. Rectangles represent TBSV open reading frames (ORFs). The asterisk (*) denotes the amber stop codon of p33 to permit read-through translation of the p92 replicase gene. The p19 ORF is nested within the p22 cell-to-cell movement gene; the function of the putative pX product is unknown. **B**, Fluorescence on GFP-transgenic *Nicotiana benthamiana* (NbGFP) plants upon UV illumination at 21 days postinoculation (dpi) with DI or DI-P RNA plus either TBSV RNA or transcripts from pTΔp19, at a 1:1 ratio. **C**, RNA blot assays of total RNA extracted from inoculated (Inoc.) leaves at 5 dpi and systemically infected upper leaves (Syst.) at 10 dpi. The two panels on the left are from membranes that were hybridized with a GFP-specific probe. The blot on the right was hybridized with a TBSV-specific probe. Representative results are shown based on multiple repeats. **D**, Immunoblot assay specific for the detection of P19 showing the abundance of this protein in leaves inoculated (Inoc.) with TBSV+DI-P at 6 dpi and the lack of detectable levels in the systemically infected leaves (Syst.) at 18 dpi. Note: These plants were systemically infected with TBSV and DI-P as shown in **B**, and plants infected with TBSV alone had died by 18 dpi because of high levels of P19 accumulation.

These observations suggest that P19 exerts an inhibitory effect on the generation or accumulation of recombinant DI derivatives. DIs probably originate from replication errors by the viral-encoded RdRp (White and Morris 1999). However, it is not inconceivable that RNA recombination is accelerated in a silencing-associated RNA degradation milieu, and perhaps even low amounts of P19 can interfere with this recombination process.

In conclusion, the results in this study showed that TBSV and its DIs are very effective inducers of VIGS and that P19 is a strong suppressor of PTGS and a moderate suppressor of VIGS. Furthermore, the suppressor activity of P19 is influenced by genetic and dosage features that are also important for P19-associated pathogenesis.

MATERIALS AND METHODS

GFP-transgenic *N. benthamiana* line and *A. tumefaciens* strain.

GFP-transgenic *N. benthamiana* line 16C and *A. tumefaciens* strain C58C1 containing the GFP T-DNA plasmid pCH32 (Voinnet et al. 1998) were generously provided by D. C. Baulcombe (Sainsbury Laboratory, John Innes Center, Norwich, U.K.). The transgenic *N. benthamiana* line 16C is homozygous for the GFP transgene at a single locus. The *A. tumefaciens* carries the same GFP gene as was used to transform *N. benthamiana* (Ruiz et al. 1998; Voinnet et al. 1998). GFP-transgenic plants were grown under conditions of 24°C, 14-h light/10-h dark, and 126 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity. Agroinfiltration of GFP-transgenic plants with *A. tumefaciens* was performed according to a previously described procedure (English et al. 1997).

Chimeric TBSV and DI constructs and transcription.

Recombinant TBSV cDNA plasmids were derived from the original plasmid, pTBSV-100, an infectious full-length cDNA clone of the TBSV cherry strain (Hearne et al. 1990). The GFP gene donor in the cloning experiments was pHS168, which contains the GFP gene of TU#65 (Chalfie et al. 1994) cloned as a *KpnI-EcoRI* fragment into pKAN2 (Scholthof et al. 1995a). This represents the same version of the GFP gene as was used to create transgenic *N. benthamiana* line 16C (Ruiz et al. 1998). To generate pTG (i.e., pHST3-4), the GFP fragment was removed from pHS168 with *EcoRI* and *KpnI*, treated with DNA polymerase I Klenow fragment, and ligated into the *SnaBI* site of the TBSV vector pHST2-14 (Scholthof 1999). The *NheI-PflMI* fragment containing the GFP gene was removed from pTG to substitute the corresponding CP-containing fragment in the plasmids pTBSV-100, pT19/43, pT19/72^g, pT19/71-72, pT19/85, and pT19/101 (Chu et al. 2000). The presence of the GFP gene and P19 expression were confirmed by RNA hybridization and immunoblots.

The chimerical pDI-P was constructed by releasing the *HpaI* to *EcoRI* fragment, covering the 3' portion (nt 489 to 716) of the GFP gene, from pHS168, followed by treatment with DNA polymerase I Klenow fragment and ligation into the *NruI* site of pHS21 (Qiu et al. 2001). Constructs PVX/p19(+) (i.e., pHS142-20) and PVX/p19(-) (i.e., pHS142-22) were previously generated PVX vectors containing the TBSV wild-type p19 gene in plus-sense or minus-sense orientation, respectively (Scholthof et al. 1995a). In vitro transcription reactions to generate capped PVX RNA and uncapped transcripts from TBSV- and DI-derived constructs and inoculation onto plants were previously described (Chapman et al. 1992; Scholthof et al. 1995a, 1995c).

GFP imaging and microscopy.

Visual detection of GFP-emitted green fluorescence was performed by using a 100-W, long-wave UV lamp (Black Ray model B-100 AP; Ultra-Violet Products, Upland, CA, U.S.A.).

Photographs were taken using Kodak Ektachrome 400 ASA film (Kodak, Rochester, NY, U.S.A.) with a Y2 Kenko optical filter (Kenko Co., Tokyo, Japan). Exposure settings were at f/5.6 for 0.5 to 1.5 s, depending on the fluorescence intensity and distance from the plant.

Stem portions were sliced into about 0.5-mm cross sections and placed onto glass slides for fluorescence microscopy examination through a 493-nm blue light filter. The images were captured on Kodak Ektachrome 200 ASA film at 1 to 2 s, depending on the fluorescence intensity.

For confocal microscopy, symptomatic areas of inoculated leaves were harvested with a razor blade and examined as described (Cheng et al. 2000) with a Bio-Rad model 1024ES confocal system (Bio-Rad, Hercules, CA, U.S.A.) attached to a Zeiss Axiokop (Zeiss, Thornwood, NY, U.S.A.) and processed with a Dell PowerEdge 2200 (Dell, Austin, TX, U.S.A.) using Bio-Rad LaserSharp software version 3.1.

Protein and RNA analyses.

Total plant protein and RNA extraction from infected plants inoculated with TBSV RNA transcripts and the immunoblot and northern blot assays were performed as described previously (Scholthof et al. 1993, 1994, 1995a). Total plant RNAs used for GFP mRNA detection were extracted from plant leaf tissues using a hot-phenol procedure (Verwoerd et al. 1989). Approximately 5 μg of total RNAs was denatured at 55°C for 15 min in 20 μl of 0.5 $\times$ morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS, pH 7.0; 5 mM sodium acetate; 0.5 mM EDTA) containing 3.5 μl of formaldehyde (37%) and 10 μl of formamide. The RNAs were fractionated in 1.2% agarose in 1 $\times$ MOPS running buffer. Random primed pHS168 was used as a GFP-specific probe and pTBSV-100 or pBW-1 (Qiu et al. 2001) was used to detect TBSV RNA. A sugar beet cDNA clone was used for the detection of 28S rRNA.

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