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A Viral Movement Protein Prevents Spread of the Gene Silencing Signal in *Nicotiana benthamiana*

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Summary

In plants, viruses induce an RNA-mediated defense that is similar to posttranscriptional gene silencing (PTGS) of transgenes. Here we demonstrate with potato virus X (PVX) that PTGS operates as a systemic, sequence-specific defense system. However, in grafting experiments or with movement defective forms of PVX, we could not detect systemic silencing unless the 25 kDa viral movement protein (p25) was made nonfunctional. Investigation of p25 revealed two branches to the PTGS pathway that converge in the production of 25 nucleotide RNAs corresponding to the target RNA. One of these branches is unique to virus-induced PTGS and is not affected by p25. The second branch is common to both virus- and transgene-induced PTGS, is blocked by p25, and is likely to generate the systemic silencing signal.

Introduction

Posttranscriptional gene silencing (PTGS) in transgenic plants is related to an anti-viral defense system that operates at the level of RNA (Baulcombe, 1999). It is thought that PTGS is activated when a surveillance system recognizes viral or transgene RNA. Subsequent to the recognition event, these RNAs are degraded in a sequence-specific manner. The specificity of the degradation mechanism may be mediated by 25 nucleotide RNA species corresponding to the target RNA that accumulate in tissues exhibiting PTGS (Hamilton and Baulcombe, 1999). In infected cells, PTGS is targeted against the viral RNA and causes its accumulation to slow down or stop at late stages in the infection process (Ratcliff et al., 1999). In transformed plants, PTGS is targeted against transcripts of the transgene and any similar endogenous genes so that the corresponding gene products accumulate at a low level (Vaucheret et al., 1998).

The role of PTGS in virus protection is illustrated by the phenotype of sgs2 mutant Arabidopsis plants infected with cucumber mosaic virus (CMV) (Mourrain et al., 2000). These plants are defective in PTGS and hypersusceptible to the virus. In addition, with caulimo-, nepo-, potex-, and tobraviruses there is evidence that PTGS accounts, at least in part, for cross protection against infection with a second virus (Covey et al., 1997; Ratcliff et al., 1997; Ratcliff et al., 1999). The first virus induces PTGS so that the infected cells are primed to resist the second virus in a nucleotide sequence–specific manner. Silencing of endogenous genes in plants infected with tobamo-, potex-, and gemini-virus vectors carrying elements of host sequences is also an indication that PTGS is an antiviral defense mechanism (Baulcombe, 1999). Following the onset of virus replication, PTGS is targeted against sequences in the viral genome and expression of the corresponding endogenous genes is suppressed.

Consistent with the widespread occurrence of PTGS in virus-infected cells, it has been shown that some viruses are able to overcome or prevent PTGS. The first demonstration of this property came from analysis of potyviral synergistic interactions with other viruses (Pruss et al., 1997), leading to the identification of the Hc-protease (HcPro) as a suppressor of PTGS (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). A second factor, the 2b protein of CMV, was also identified as a suppressor of PTGS (Brigneti et al., 1998). Importantly, these combined studies revealed that HcPro and the 2b proteins have distinct targets in the silencing mechanism. It was subsequently established that suppression of PTGS is a widespread property of RNA and DNA viruses of plants (Voinnet et al., 1999). Presumably, PTGS is a basic response to virus infection and the production of a suppressor represents a necessary adaptation by the virus if it is to replicate and spread in a plant.

One of the most intriguing features of PTGS in transgenic plants is that it is not cell autonomous. A signal of gene silencing can move between cells through plasmodesmata and over long distances through the vascular system, directing sequence-specific degradation of target RNAs (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). The nature of the signal is not known but, based on the specificity of its action, it is thought to incorporate a nucleic acid. The discovery that PTGS is transported systemically in transgenic plants has prompted speculation that it also operates in a non-cell autonomous manner during natural virus infections (Voinnet et al., 1998; Jorgensen et al., 1998; Carrington, 1999; Lucas and Wolf, 1999). A virus-induced silencing signal could migrate cell-to-cell in advance of the infection front and be transported over long distances through the phloem. The effect of this intercellular signaling would be to potentiate RNA sequence-specific virus resistance in noninfected tissues and, consequently, to delay spread of the virus through the plant.

Here we provide evidence to support this idea using PVX-based-experimental systems in which movement of a virus-induced signal could be uncoupled from movement of the virus. We show that the antiviral signal molecule can be transported over several centimetres from the infected cells and accumulates in and around the veins of recipient leaves. In the course of developing this study, we discovered that the 25 kDa movement protein of PVX (p25) is a suppressor of the systemic PTGS response. Further experiments with p25 allowed

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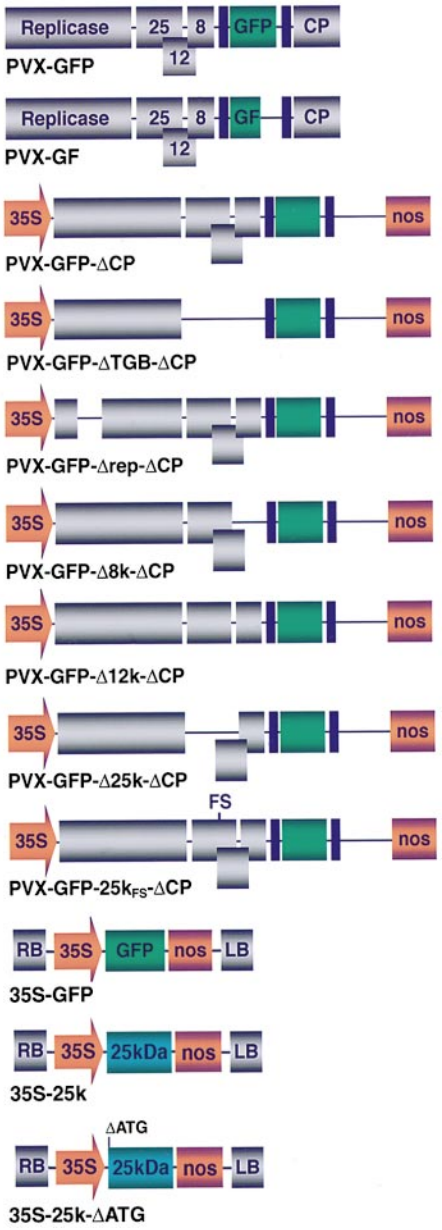


Figure 1. Viral and Transgene Constructs Used in This Study

PVX-GFP and PVX-GF were described previously (Ruiz et al., 1998). Expression of the inserts in the PVX vector is controlled by a duplicated coat protein (CP) promoter indicated in blue. The Replicase ORF is essential for replication of viral RNAs; the 25, 12 and 8 kDa proteins are all strictly required for cell-to-cell movement of viral RNAs; the CP is essential for encapsidation as well as cell-to-cell and systemic movement of viral RNAs. All other viral constructs were based on the PVX-GFP construct coupled to the 35S promoter and nos terminator and inserted into the T-DNA of the pBin19 binary vector plasmid. PVX-GFP-ΔCP carries a deletion spanning the entire CP ORF; PVX-GFP-ΔTGB-ΔCP and PVX-GFP-Δrep-ΔCP are based on PVX-GFP-ΔCP and carry a deletion spanning all the TGB ORFs and an in-frame deletion in the replicase ORF, respectively. The PVX-GFP-Δ12k-ΔCP and PVX-GFP-Δ25k-ΔCP constructs carry deletions into the 12 kDa and 25 kDa ORF, respectively. PVX-GFP-Δ8k-ΔCP carries a frameshift mutation that prevents translation of the 8 kDa ORF. PVX-GFP-25k$_{FS}$-ΔCP carries a frameshift mutation in the 25 kDa ORF, indicated by "FS" (see Experimental procedures for details). The 35S-GFP construct was described previously (Voinnet and Baulcombe, 1997). The 35S-25k and 35S-25k-ΔATG constructs are based on the PVX 25 kDa ORF coupled to the 35S promoter and the 35S terminator and inserted into the T-DNA of pBin19. The start codon of the 25 kDa ORF has been removed in 35S-25k-ΔATG, as indicated by "ΔATG". LB and RB indicate left and right border of the pBin19 T-DNA, respectively.

us to differentiate two branches of the PTGS pathway. One branch is activated by replicating viral RNA and is not affected by p25. The second branch can be activated by non replicating RNA of viral or transgene origin and is suppressed by p25. Our analysis indicates that the systemic signal of silencing is produced in this second, p25-sensitive, branch of the pathway and is likely a precursor of the 25 nucleotide RNAs.

Results

To test the hypothesis that signaling of PTGS is a systemic anti-viral defense, we designed grafting experiments in which virus movement would be uncoupled from transport of a silencing signal. The experiments used line 16c of *Nicotiana benthamiana* carrying a highly expressed green fluorescent protein (GFP) transgene. These plants are bright green under ultra-violet (UV) illumination, whereas non-transformed (NT) plants are red due to chlorophyll fluorescence. Transgene-induced, systemic silencing of the GFP transgene was initiated by localized infiltration of a strain of *Agrobacterium tumefaciens* carrying a 35S-GFP T-DNA construct (35S-GFP, Figure 1). Virus-induced PTGS of the GFP transgene was initiated by infection with a PVX vector carrying 450 nucleotides from the 5′ end of the GFP reporter gene (PVX-GF, Figure 1).

The rootstocks in these experiments were GFP transgenic plants that had been inoculated with PVX-GF five days previously. These plants exhibited the early signs of PTGS of GFP (Figure 2A). The scions carried a GFP transgene together with the *Rx* gene which confers extreme resistance against PVX (Bendahmane et al., 1999). The presence of *Rx* would prevent replication of PVX-GF in the scions but should have no effect on systemic transport of a silencing signal (Figure 2A).

Within 20 days after grafting, PTGS of GFP was extensive in the rootstocks, as indicated by the loss of green fluorescence under UV illumination (Figure 2B, panel 1). As expected, there was no spread of PVX-GF into the *Rx*/GFP scions, indicated by the absence of PVX symptoms and by the failure to detect PVX-GF RNAs by Northern analysis (data not shown). Also as expected, there was spread of PVX-GF and of gene silencing into the GFP scions without *Rx* (Figure 2B, panel 4, data not shown). However, there was no systemic silencing of GFP in the *Rx*/GFP scions in any of ten grafts tested. The scions remained green fluorescent (Figure 2B, panel 1) and the levels of GFP mRNA were high, as in non-infected GFP plants (data not shown).

The absence of systemic spread of GFP silencing into the GFP/*Rx* scions could result if *Rx* was able to interfere with systemic silencing. However, when silencing had been induced in the stocks by *Agrobacterium* infiltration, there was spread into GFP/*Rx* scions in 8 out of

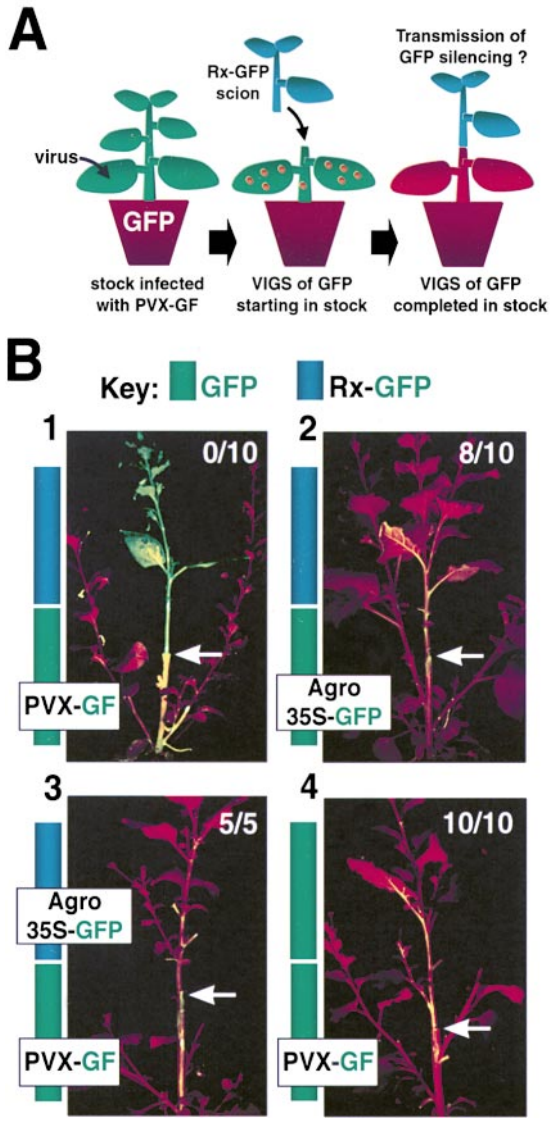


Figure 2. Test for Graft-Transmission of PVX-GFP-Induced Signal in GFP Transgenic *N. benthamiana*

(A) Schematic description of the grafting experiments.

(B) Young GFP transgenic rootstocks were either inoculated with PVX-GF (panels 1,3,4) or infiltrated with the 35S-GFP strain of *Agrobacterium* (panel 2). Five days later, transgenic scions carrying either the GFP transgene (panel 4) or both the GFP and the *Rx* transgenes (*Rx*-GFP, panels 1, 2, 3) were wedge-grafted onto the rootstocks. Graft-transmission of GFP silencing was then scored under UV illumination throughout time (scores are depicted in the right-hand top corner of each panel) . The photographs in panels 1–4 were taken 4 weeks after grafting. The arrow indicates the graft union. Note that GFP can appear yellow because of the long exposure times required for imaging.

10 graftings tested; these scions had lost green fluorescence, and GFP mRNA could not be detected (Figure 2B, panel 2, data not shown). In a further control to assess the effect of *Rx* on silencing, we infiltrated *Agrobacterium* cells carrying the 35S-GFP T-DNA directly into GFP/*Rx* scions that had been grafted onto PVX-GF-infected plants. In all five of these tests, GFP silencing was induced and spread through the GFP/*Rx* scions (Figure 2B, panel 3), indicating that *Rx* had no effect either on initiation or systemic spread of PTGS. It seemed likely, therefore, that the failure of systemic silencing to spread out of the PVX-GF-infected stocks was due to a factor, presumably a protein, encoded in the PVX genome.

The PVX-Encoded 25 kDa Protein Prevents Systemic Silencing

To determine whether PVX-encoded proteins are able to prevent or interfere with systemic silencing, we carried out experiments with deletion mutants of PVX-GFP (Figure 1). These mutant viruses would have been confined to the initially infected cell because they were all defective for the coat protein (CP) that is required for cell-to-cell and long distance movement of PVX. If, as predicted, a PVX-encoded protein prevented systemic silencing, PTGS initiated by the corresponding PVX-GFP mutant would be manifested away from the inoculated cells. In contrast, silencing initiated by PVX-GFP constructs carrying mutations in any other open reading frame (ORF) would be restricted to the inoculated area.

We first tested the ability of PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP (Figure 1) to induce systemic silencing of the GFP transgene. These constructs are similar to the PVX-GFP vector (Figure 1), except that there is a deletion in the CP ORFs. In addition to the CP mutation, PVX-GFP-ΔTGB-ΔCP carries a deletion spanning all three ORFs of the triple gene block (TGB). The TGB encodes three proteins that are strictly required, in addition to the CP, for cell-to-cell movement of PVX (Verchot et al., 1998).

In order to generate high titer inocula of these mutant viruses, we used the pBin19 Ti-plasmid vector (Bevan, 1984), in which the PVX-GFP constructs were coupled to a 35S promoter. *Agrobacterium* cultures carrying these constructs were infiltrated into leaves of GFP transgenic plants. Transfer of the T-DNA would allow a high proportion of cells inside the infiltrated area to become infected with the movement-defective mutants of PVX-GFP.

At 3 days post inoculation (dpi) with PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP, there was strong expression of GFP manifested as bright green fluorescence in the infiltrated regions (data not shown). However, starting at 5–6dpi, the infiltrated regions became red-fluorescent, suggesting that local PTGS of GFP had been initiated by both of these constructs (Figure 3A, panels 2–3). The development of this local silencing was as rapid as in leaves infiltrated with the 35S-GFP construct (Figure 3A, panel 1).

With PVX-GFP-ΔTGB-ΔCP, systemic silencing was initiated in 100% of the GFP plants and developed as quickly and extensively as with the 35S-GFP construct (Figure 3B, graph and panel 1). In contrast, systemic silencing initiated with the PVX-GFP-ΔCP construct was delayed (Figure 3B, graph), appeared in only 30% of the inoculated plants, and, in those plants, was restricted to the veins in a few leaves (Figure 3B, panel 2). Because the difference between PVX-GFP-ΔTGB-ΔCP and PVX-GFP-ΔCP involved the TGB ORFs, these results suggested that one or more of the TGB proteins prevented systemic silencing from the PVX-GFP-ΔCP-infected cells.

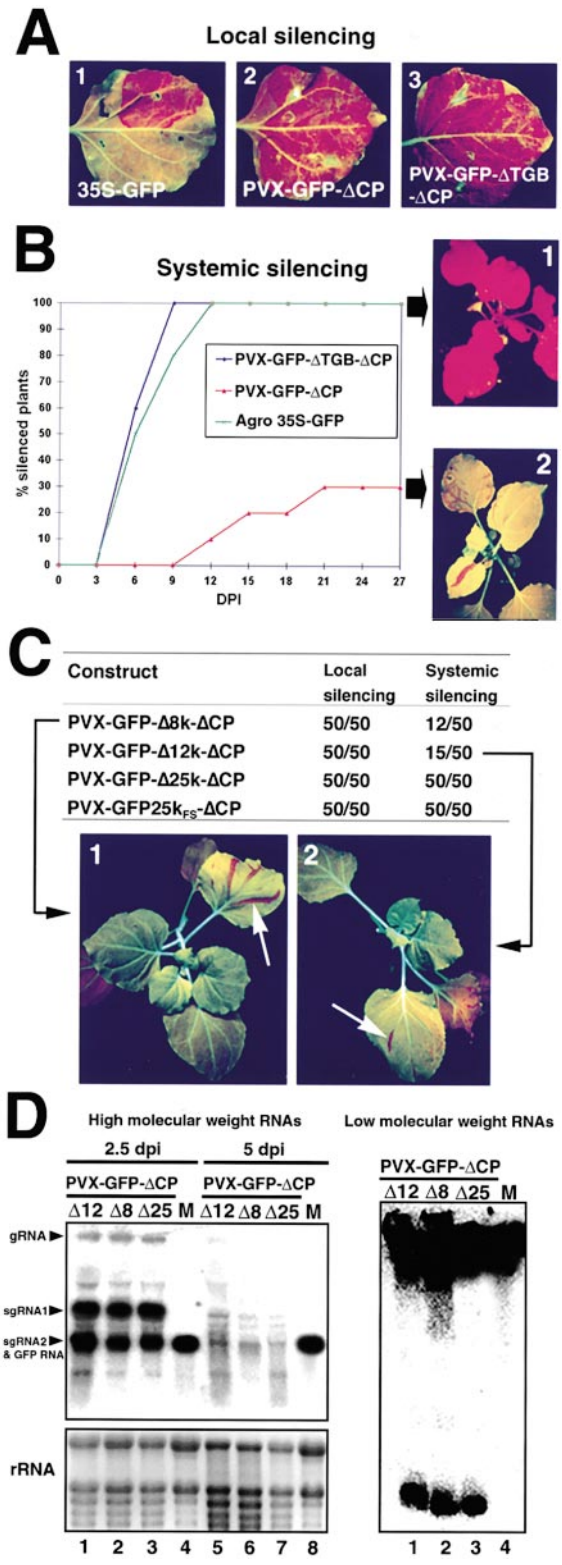


Figure 3. Effect of the TGB Proteins on Systemic Movement of the GFP Silencing Signal

(A) Following strong transient expression of GFP, the area infiltrated with either 35S-GFP, PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP become progressively red fluorescent under UV illumination. The photos in panels 1–3 were taken at 12 days postinfiltration and suggest that GFP silencing was initiated to the same extent by either constructs.

(B) Kinetics of GFP systemic silencing. Each point in the graph represents the percentage of plants exhibiting GFP systemic silencing as assessed under UV illumination. The average values are from 30 individual plants tested in 3 independent experiments, for each treatment. Plants were scored as silenced even if the systemic silencing was confined to small areas near the veins of a few leaves (i.e., panel B2, at 21 dpi). All of the PVX-GFP-ΔTGB-ΔCP-inoculated plants showed extensive systemic silencing at 21dpi (i.e., panel B1). Note that GFP can appear yellow because of the long exposure times required for imaging.

(C) GFP systemic silencing induced by individual TGB mutants of PVX-GFP-ΔCP. The number of plants exhibiting local and systemic silencing of GFP is indicated alongside the total number of plants tested in 3 independent experiments. The arrows show restriction of systemic silencing in the veins of single leaves of PVX-GFP-Δ8k-ΔCP and PVX-GFP-Δ12k-ΔCP inoculated plants, at 21 dpi. Plants exhibiting such phenotype were scored as silenced in the assay. Systemic silencing induced by PVX-GFP-25k$_{FS}$-ΔCP and PVX-GFP-Δ25k-ΔCP was always extensive (i.e., panel B1).

(D) Northern analysis of high and low molecular weight RNAs. Total RNA was extracted at 2.5 and 5dpi from leaves of GFP plants that had been infiltrated with either PVX-GFP-Δ8k-ΔCP, PVX-GFP-Δ12k-ΔCP, PVX-GFP-Δ25k-ΔCP, or water (Mock: M). Northern analysis was carried out on 10μg of the high molecular weight RNA fraction, to detect accumulation of the PVX-GFP and transgene GFP RNA, using a probe corresponding to the central region of the GFP cDNA (Left panel). Ethidium bromide staining of the electrophoresed gel shows rRNA loading. Northern analysis of the low molecular weight fraction (right panel) was carried out to detect accumulation of 22–25nt antisense GFP RNAs. Loading in lanes 1–4 was standardized with ethidium bromide staining and quantification of tRNAs in each sample. The probe used corresponded to the full-length GFP cDNA.

Similar experiments were carried out with PVX-GFP-ΔCP derivatives in which the TGB ORFs were mutated individually (PVX-GFP-Δ25k-ΔCP, PVX-GFP-25k$_{FS}$-ΔCP, PVX-GFP-Δ12K-ΔCP and PVX-GFP-Δ8K-ΔCP; Figure 1). With all of these mutants, the infiltrated region became red-fluorescent, suggesting that there was initiation of local PTGS of GFP (Figure 3C). However, the only TGB mutants that produced extensive systemic silencing were those carrying either a deletion (PVX-GFP-Δ25k-ΔCP, Figure 1) or a frameshift mutation (PVX-GFP-25k$_{FS}$-ΔCP, Figure 1) in the ORF of the 25 kDa protein (p25) (Figure 3C).

The viruses carrying mutations in the ORFs of the 12 kDa and 8 kDa proteins (PVX-GFP-Δ12K-ΔCP and PVX-GFP-Δ8K-ΔCP, respectively, Figure 1) encode a functional 25 kDa protein and, like PVX-GFP-ΔCP, were poor inducers of systemic silencing. Most of the GFP plants inoculated with these constructs did not exhibit any systemic silencing of GFP (Figure 3C). However, as with PVX-GFP-ΔCP, about 25% of the inoculated plants exhibited partial silencing of GFP. At 21 dpi, this partial silencing was restricted to the regions in and around the veins of some upper leaves (Figure 3C, arrows in panels 1 and 2) and did not develop further.

In principle, the contrasting silencing phenotypes triggered by the PVX-GFP TGB mutants could be a direct effect of p25. Alternatively, there could be an indirect effect if the mutations affected replication or the ability of these mutants to induce PTGS of GFP in the inoculated leaves. To resolve these alternatives, we carried out northern analysis of RNA from the infiltrated leaf tissues at 2.5 and 5dpi, using a GFP-specific probe.

At 2.5 dpi, with PVX-GFP-Δ12K-ΔCP, PVX-GFP-Δ8K-ΔCP and PVX-GFP-Δ25k-ΔCP, the extracts contained four major RNA species (Figure 3D, left panel, tracks 1–3) detected with the GFP probe. The genomic viral RNA (gRNA) was the least and the viral subgenomic (sg) RNA1 was the most abundant. The sgRNA2 co-migrated with and could not be differentiated from the GFP transgene mRNA (Figure 3D, left panel, tracks 1–4). At 2.5 dpi, these RNAs were all abundant in the PVX-GFP-Δ12K-ΔCP, PVX-GFP-Δ8K-ΔCP and PVX-GFP-Δ25k-ΔCP-infected tissues. At 5dpi, however, with all three TGB mutants, the levels of these RNA species were markedly reduced. This reduction was dependent on the virus because, in mock inoculated tissue, the GFP mRNA was at the same level at 2.5 and 5dpi (Figure 3D, left panel, tracks 4 and 8). Thus, this change in RNA abundance was likely due to PTGS that was targeted against both viral and transgene GFP RNA species (Figure 3D, left panel, tracks 5–8, sgRNA3 & GFP RNA).

As an additional test of PTGS induced by the TGB mutants, we assayed for 22–25nt antisense GFP RNAs at 5dpi. In other systems, the relative amount of those small antisense RNAs correlates with the level of PTGS (Hamilton and Baulcombe, 1999; Dalmay et al., 2000)). As expected, these 22–25nt GFP RNAs were absent in the extract of mock-infiltrated leaves (Figure 3D, right panel, track 4). However, in PVX-GFP infected tissues, these RNAs were present and their levels were unaffected by mutations in the TGB ORFs (Figure 3D, right panel, tracks 1–3). This data indicate that all three TGB mutants were efficient inducers of PTGS of GFP.

Combined, these results show that all of the TGB mutants replicated and activated intracellular PTGS to a similar extent. However, systemic spread of silencing only occurred when the PVX-GFP constructs carried mutations in the p25 ORF. It is unlikely that this block was an RNA-mediated effect because systemic silencing was initiated by a PVX-GFP mutant with a frameshift mutation in the p25 ORF. Therefore, we conclude that the p25 protein was able to prevent systemic PTGS of the GFP transgene.

Systemic Silencing in Nontransgenic Plants

The experiments described above were not directly informative about the extent of systemic silencing in virus-infected plants because they involved GFP transgenes integrated in the plant genome and in the T-DNA of the infiltrated *Agrobacterium*. Any virus-induced effects would have been amplified and relayed by these transgenes, as shown previously (Voinnet et al., 1998), so that systemic silencing would have been more extensive than in non transgenic plants. Therefore, to obtain a more accurate picture of the systemic signaling caused by virus infection, we carried out a series of experiments in non transgenic plants. The PTGS in these experiments was targeted against the endogenous gene encoding the ribulose bisphosphate carboxylase small subunit (rbcs). As shown previously, this gene is a potential target of PVX-induced PTGS, but, unlike transgenes, it does not participate in the initiation, amplification, or maintenance of the mechanism (Jones et al., 1999). Therefore, it was likely that systemic silencing of rbcs would indicate the extent of signal spread from the virus-infected cells (Figure 4A).

The constructs in these experiments were derivatives of PVX-GFP-ΔCP (Figure 1), in which a 500nt fragment of the rbcs cDNA was inserted into the GFP ORF (Figure 4A). These derivatives are collectively referred to as PVX-rbcs-X in which "X" indicates the various mutations carried by each individual construct (Figure 4C). The *Agrobacterium* infiltration procedure was used to inoculate these PVX constructs to non transgenic plants. Inoculation of the PVX-rbcs-ΔTGB-ΔCP derivative led to systemic silencing of rbcs that was manifested as yellow-green chlorosis in and around the veins of systemic leaves (Figure 4B). In contrast to the extensive GFP silencing (Figure 3B, panel 1), the rbcs silencing remained restricted to the vicinity of the veins and was only evident in leaves that emerged within 10–16 dpi. This phenotype was consistent with the lack of relay-amplification associated with PTGS of the rbcs gene and was likely a direct indicator of the virus-induced signal.

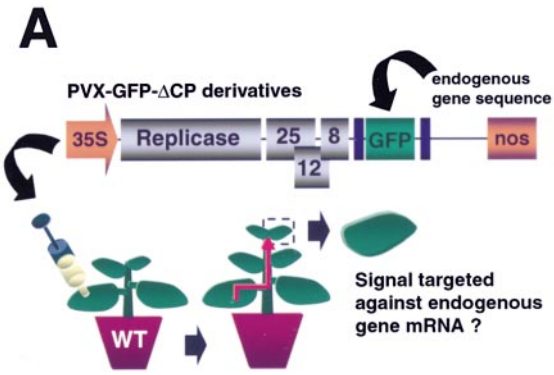
As with GFP silencing, the rbcs systemic effect required mutation of the 25 kDa ORF (in PVX-rbcs-ΔTGB-ΔCP and PVX-rbcs-Δ25k-ΔCP, Figure 4C). A construct in which the 25 kDa ORF was intact (PVX-rbcs-Δ12K-ΔCP) did not induce systemic silencing (Figure 4C). From these results we conclude that, in the absence of a transgene, a virus-induced silencing signal can move several centimeters from infected cells and is primarily localized in the vicinity of the veins. Importantly, the replication-defective PVX-rbcs-Δrep-ΔCP failed to induce systemic silencing of rbcs (Figure 4C). This result suggests that in non transgenic plants, production of the signal is dependent on the replication competence of the viral genome responsible for its induction.

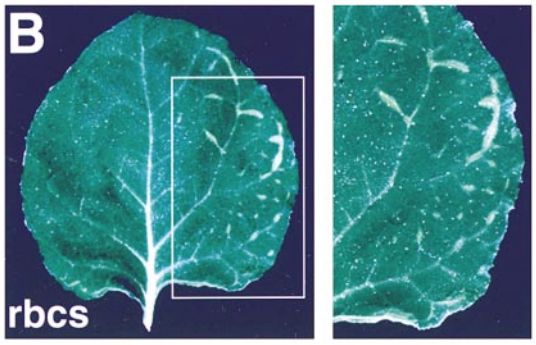
Similar results were obtained with PVX-GFP-ΔCP derivatives targeted against the phytoene desaturase (PDS) gene. As for rbcs, the systemic silencing of PDS, manifested as photobleaching, was only transient and localized around the veins of some new emerging leaves (Figure 4D). It was also dependent on PVX replication (Figure 4E). The PDS mRNA is several orders of magnitude less abundant than the rbcs mRNA (data no shown). We can therefore rule out that the level of target gene expression influenced the vein pattern and persistence of systemic silencing in non transgenic plants.

Ectopic Expression of p25 and Systemic Silencing

The analyses with mutant PVX (Figures 3 and 4) did not rule out that other virus-encoded proteins, in addition to p25, are involved in preventing systemic silencing. To address this possibility, we induced systemic silencing in the presence of p25 expressed independently of other virus encoded proteins (Figure 5A). Induction of systemic silencing was by *Agrobacterium* infiltration of leaves of GFP transgenic plants. The *Agrobacterium* strains carried the 35S-GFP construct or, as a reference, the PVX-GFP-Δ25k-ΔCP construct (Figure 1). These strains were mixed with a second strain containing either the 35S-25k construct or the 35S-25k-ΔATG construct in which the start codon of the p25 ORF is removed (Figure 1).

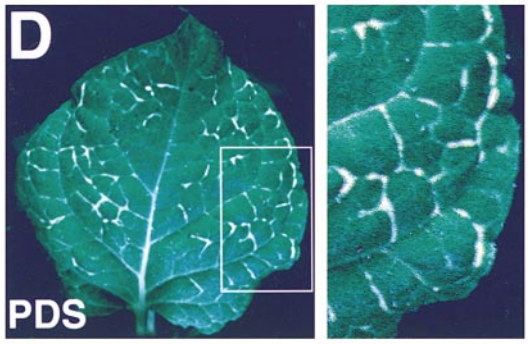
The construct combinations with 35S-25k-ΔATG in-





C

Construct	rbcs systemic silencing
PVX-rbcs-ΔTGB-ΔCP	35/40
PVX-rbcs-Δrep-ΔTGB-ΔCP	0/40
PVX-rbcs-Δ12k-ΔCP	0/40
PVX-rbcs-Δ25k-ΔCP	36/40



E

Construct	PDS systemic silencing
PVX-PDS-ΔTGB-ΔCP	35/40
PVX-PDS-Δrep-ΔTGB-ΔCP	0/40

Figure 4. Systemic Silencing in Nontransgenic Plants

(A) The diagram summarizes the order of events described in (B) and (D).

(B) Systemic silencing of rbcs. First, one or two expanded leaves of a non transformed seedling were infiltrated with a strain of *Agrobacterium* containing either PVX-GFP-ΔTGB-ΔCP or PVX-GFP-Δrep-ΔCP (Figure 1), in which a 500-nucleotide fragment from the ribulose bisphosphate carboxylase small subunit (rbcs) cDNA was inserted in the GFP ORF. Fourteen days later, systemic, new emerging leaves were inspected for silencing of rbcs. The picture represents a typical systemic leaf from a plant inoculated with the PVX-rbcs-ΔTGB-ΔCP derivative showing yellow-green chlorosis in and near the class II and III veins.

(C) Influence of p25 and PVX replication on systemic silencing of rbcs. The table indicates the number of plants exhibiting systemic silencing of rbcs and the total number of plants tested for each construct.

(D) Systemic silencing of PDS. The principle of the experiment is similar to that in (A-B) except that a 415-nucleotide fragment from the central region of the phytoene desaturase (PDS) cDNA was inserted into the GFP ORF of the corresponding PVX-GFP derivative (Figure 1). The picture represents a typical systemic leaf from a plant inoculated with the PVX-PDS-ΔTGB-ΔCP derivative and shows photobleaching associated with PDS silencing near the class II and III veins.

(E) Influence of PVX replication on systemic silencing of PDS. The table indicates the number of plants exhibiting systemic silencing of PDS and the total number of plants tested for each construct.

duced systemic silencing as rapidly and as extensively as with the 35S-GFP construct alone (Figure 5B and data not shown). In contrast, systemic silencing of GFP occurred in only a few plants that had been infiltrated with the 35S-25k combinations (Figure 5B). Moreover, in those plants, systemic silencing was incomplete and was restricted to the veins of a few leaves, as in the experiments involving PVX-GFP derivatives with an intact p25 ORF (Figure 5B, Figure 3B, and 3C). From these results we conclude that, of the PVX-encoded proteins, p25 was sufficient to interfere with systemic silencing of the GFP transgene.

Ectopic Expression of p25 and Local Silencing

The effect of p25 on systemic silencing could result from a block of signal production in the infiltrated cells. Alternatively, this protein could prevent movement of the signal out of the cells in which it was produced. To investigate these alternatives, we monitored the local effects of p25 on RNA levels and GFP fluorescence in the leaves where PTGS had been initiated. If signal movement was targeted, the local silencing in inoculated cells would be unaffected. However, an effect of p25 on signal production would likely affect initiation of local silencing.

By 5dpi, in leaves infiltrated with the (35S-25k-ΔATG+35S-GFP) combination or with the 35S-GFP construct alone, there was loss of GFP fluorescence, as expected, indicating the onset of local PTGS (data not shown). Correspondingly, the levels of GFP RNAs in those tissues were lower than in mock-infiltrated tissues (Figure 5C, tracks 2 and 3 compared with track 4) and the GFP 25nt antisense RNAs were abundant (Figure 5D, tracks 2–3).

In contrast, infiltration with the (35S-25k+35S-GFP) combination caused the green fluorescence to increase in the infiltrated leaf (data not shown). The GFP RNA was also much more abundant in those tissues than in the mock-infiltrated tissues, presumably because the integrated and the ectopic 35S-GFP transgenes were both expressed (Figure 5C, track 1 compared to track 4). Correspondingly, the GFP 25nt antisense RNAs were more than five times less abundant than in tissues infiltrated with 35S-GFP or with (35S-GFP+35S-25k-ΔATG)

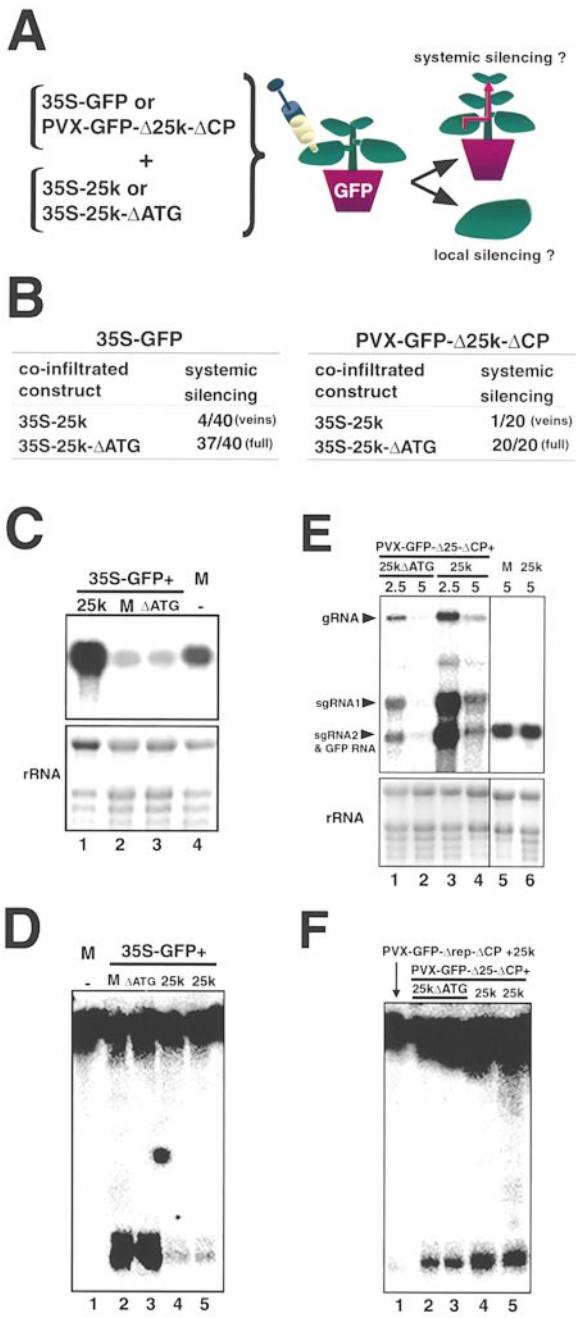


Figure 5. Ectopic Expression of p25

(A) Principle of the experiments described below. A culture of an *Agrobacterium* strain containing the 35S-25k or the 35S-25k-ΔATG construct (Figure 1) was mixed (equal volume) with a culture of an *Agrobacterium* strain containing either the 35S-GFP or the PVX-GFP-Δ25k-ΔCP construct (Figure1). The corresponding suspension was then infiltrated into one or two leaves of young GFP transgenic seedlings, and the onset of local and systemic silencing of the GFP transgene was monitored throughout time.

(B) Systemic silencing of GFP induced with the 35S-GFP transgene (left table) or with PVX-GFP-Δ25k-ΔCP (right table) in combination with 35S-25k or 35S-25k-ΔATG. The values are from independent experiments involving 10 plants each. "Veins" indicates that systemic silencing was only manifested in the veins of a few leaves at 21dpi. "Full" indicates extensive systemic silencing of GFP at 21 dpi.

(C) Northern analysis of high molecular weight RNAs. Total RNA was extracted at 2.5 and 5dpi from leaves of GFP plants that had been infiltrated with the 35S-GFP construct in combination with either the 35S-25k construct (25k), the 35S-25k-ΔATG construct (ΔATG), or water (Mock: M). Northern analysis was carried out on 10μg of the high molecular weight RNA fraction, to detect accumulation of the GFP RNA, using a probe corresponding to the full length GFP cDNA. Ethidium bromide staining of the electrophoresed gel shows rRNA loading.

(D) Northern analysis of low molecular weight RNAs was carried out to detect accumulation of 22–25nt antisens GFP RNAs in the 5dpi samples analyzed in (C). Loading in lanes 1–5 was standardized with ethidium bromide staining and quantification of tRNAs in each sample. The probe used corresponded to the full-length GFP cDNA.

(E) Northern analysis of high molecular weight RNAs. Total RNA was extracted at 2.5 and 5dpi from leaves of GFP plants that had been infiltrated with the PVX-GFP-Δ25k-ΔCP construct in combination with either the 35S-25k construct (25k), the 35S-25k-ΔATG construct (ΔATG), or water (Mock: M). Northern analysis was as described in (C).

(F) Northern analysis of low molecular weight RNAs. This analysis was performed at 5 dpi, as described in (D). Track 1 shows low levels of 25nt RNAs accumulating in tissues from leaves inoculated with the non replicating PVX-GFP-Δrep-ΔCP in combination with 35–25k.

(Figure 5D tracks 4 and 5). Collectively, these results indicate that ectopic, constitutive expression of p25 prevented transgene-induced silencing of the GFP transgene in the infiltrated region.

When the inducer of silencing was the replicating PVX-GFP-Δ25k-ΔCP construct, the effects of p25 were more complex. In the (PVX-GFP-Δ25k-ΔCP+35S-25k) samples, at 2.5dpi, the levels of all high molecular weight RNAs were substantially higher than in the control (Figure 5E, track 3 compared to track 1). These data suggest that p25 caused suppression of PTGS at this early time point. However, by 5dpi, even in the presence of p25, the target RNAs had all declined to lower levels than at 2.5dpi (Figure 5E, tracks 2 and 4). The GFP mRNA from the transgene was masked by one of the viral subgenomic RNAs, but it was clearly less abundant than in the mock-infiltrated tissue (Figure 5E, track 4 compared to track 5, sgRNA2 and GFP RNA). This decline in the levels of target RNAs was observed in at least three independent experiments and indicates that, between 2.5 and 5dpi, local PTGS triggered by PVX-GFP-Δ25k-ΔCP had overcome the initial effect of p25.

The failure of p25 to prevent PTGS in PVX-GFP-Δ25k-ΔCP-infiltrated tissues was confirmed by the analysis of 25nt GFP RNAs. At 5dpi, these RNAs were 2.5 times more abundant in the presence of 35S-25k than in the presence of 35S-25k-ΔATG (Figure 5F, tracks 2–3 compared to tracks 4–5), corresponding to the similar difference in PVX-GFP-Δ25k-ΔCP RNAs levels (Figure 5E, track 2 compared to track 4). Thus, these 25nt GFP RNAs were likely generated primarily from replicating viral RNAs. In agreement with this idea, there was only a low level of 25nt RNAs in tissues that had been infiltrated with the non replicating PVX-GFP-Δrep-ΔCP construct (Figure 1) together with 35S-25k (Figure 5F, track 1).

Collectively, these results indicate that the ectopically expressed p25 prevented systemic silencing irrespective of whether the inducer was a non replicating transgene construct (35S-GFP or PVX-GFP-Δrep-ΔCP) or replicating RNA (PVX-GFP-Δ25k-ΔCP, Figure 5B). In contrast, local silencing was only suppressed by p25 if

the inducer was a non replicating transgene construct (Figure 5C, 5D and 5F, track 1). In this situation, the block on PTGS was associated with reduced accumulation of the 25nt GFP RNAs and, presumably, was targeted against either synthesis or processing of the precursor of these 25nt RNAs.

Discussion

Systemic Signaling of PTGS and Virus Movement

The conventional models of virus movement involve opening of channels between cells; some viruses open plasmodesmata so that particles or viral ribonucleoproteins can move through; others produce tubules that extend from the surface of the infected cell and introduce virions into adjacent cells (Carrington et al., 1996). According to these models, the p25 protein of PVX has been characterized as a movement protein and was considered as a facilitator of channel gating (Angell et al., 1996). However, from the demonstration here, that replication of PVX induces systemic PTGS (Figure 4), it may be necessary to develop more refined models of virus movement (Carrington, 1999). These models will need to accommodate the anti-viral effect of systemic PTGS and the ability of viruses to suppress this process, as shown here with p25.

We anticipate that many viruses, like PVX, will have the potential to induce signaling of PTGS. It is possible that some of these viruses will resemble PVX in that they will prevent propagation of the silencing host response out of the infected cells. However, others may not have this capacity and would induce signaling of PTGS, as observed with PVX-rbcs-Δ25k-ΔCP and PVX-PDS-Δ25k-ΔCP (Figure 4B, 4D). In these situations, the PTGS signal could influence virus movement into and around cells that are several centimeters from the zone of infected cells.

A likely manifestation of virus movement influenced by systemic PTGS is in plants infected with nepo-, tobra-, caulimo-, and other groups of virus (Covey et al., 1997; Ratcliff et al., 1997; Ratcliff et al., 1999) that exhibit a pattern of symptom development referred to as recovery. Initially, these plants exhibit severe symptoms and the viruses are abundant in the infected tissues. Later, when the plants recover, the symptoms are mild and the viruses accumulate at lower levels. In these plants, we consider that the initially infected leaves could act as a source of signal that would potentiate resistance in the recovered leaves. Supporting this idea, we have shown that there is RNA sequence-specific immunity against secondary infection in recovered leaves (Ratcliff et al., 1997; Ratcliff et al., 1999).

Signaling of PTGS may also play a role when viruses exhibit restricted movement on infected plants. For example, in Arabidopsis plants carrying the RTM1 and RTM2 loci (Whitham et al., 1999), tobacco etch virus (TEV) can replicate and move from cell to cell in the inoculated leaf but cannot spread systemically. It is striking that this effect is specific for TEV. Conceivably, the products of RTM1 and RTM2 could affect the ability of TEV to prevent systemic spread of the sequence-specific silencing signal (Chisholm et al., 2000). Similarly, in the many examples where viruses replicate efficiently but are restricted to the initially infected cells (Matthews, 1991), it is possible that the silencing signal plays an important role. These subliminal infections could result if the signal moves ahead of the virus and prevents virus accumulation in cells that are adjacent to the site of initial infection.

Experiments involving double virus infection are also consistent with the widespread involvement of a PTGS signal (reviewed in Atabekov and Taliansky, 1990). These experiments demonstrate that movement-competent viruses could assist cell-to-cell trafficking of movement-defective viruses even when they were from different viral genera. Previously, these findings were interpreted in terms of common mechanisms of virus trafficking. However, as the complementing pairs of viruses included combinations of tubule forming and plasmodesmatal gating viruses, a more plausible interpretation could involve interference with the PTGS signal.

Two Branches of the PTGS Pathway

Transgene-mediated PTGS in Arabidopsis involves production of 25nt RNA and requires an RdRP homolog encoded by Sde1; in contrast, PTGS induced by some viruses appears to be independent of Sde1, although it also involves 25nt RNA (Dalmay et al., 2000). To explain these findings, we proposed that PTGS in plants is a branched variation of the pathway leading to RNA interference in Drosophila. This pathway involves processing of double stranded (ds) RNA into short 21–25nt RNAs that serve as the guide RNA for a sequence-specific nuclease (Zamore et al., 2000).

Our previous suggestion was that, in plants, there are SDE1-dependent and SDE1-independent branches of the PTGS pathway (Dalmay et al., 2000). Both branches are dependent on synthesis of dsRNAs and converge at, or before, production of 25nt RNA. The dsRNA in the SDE1-independent branch would be produced through replication of the virus and would thus be dependent on the viral-encoded RdRp (Figure 6A). In this model, the Sde1-dependent branch of the pathway is unaffected by viral RNA (Figure 6A).

To interpret the effects of p25 in terms of this model, we differentiate local and systemic PTGS. The local PTGS was suppressed by p25 if it was induced by a 35S-GFP transgene (Figure 5C, 5D) but not if the inducer was the replicating PVX-GFP-Δ25k-ΔCP (Figure 3, Figure 5E, 5F). Therefore, according to the model (Figure 6A), p25 would be a suppressor of the Sde1-dependent branch of the pathway. In contrast, systemic PTGS was suppressed by p25 irrespective of whether the inducer was the 35S-GFP transgene (Figure 5B) or the replicating PVX-GFP-Δ25k-ΔCP (Figures 3 and 5B). Thus, this effect of p25 on virus-induced systemic silencing is difficult to reconcile with the model presented in Figure 6A, in which the Sde1-dependent branch of the pathway is a transgene-specific process and is not affected by viral RNA.

In principle, these data could indicate that there are separate pathways, with multiple p25 targets, leading to local and systemic silencing. We cannot rule out that possibility, although it seems unlikely, because it requires that a virus-encoded protein would suppress the local PTGS induced by a transgene but not by a replicat-

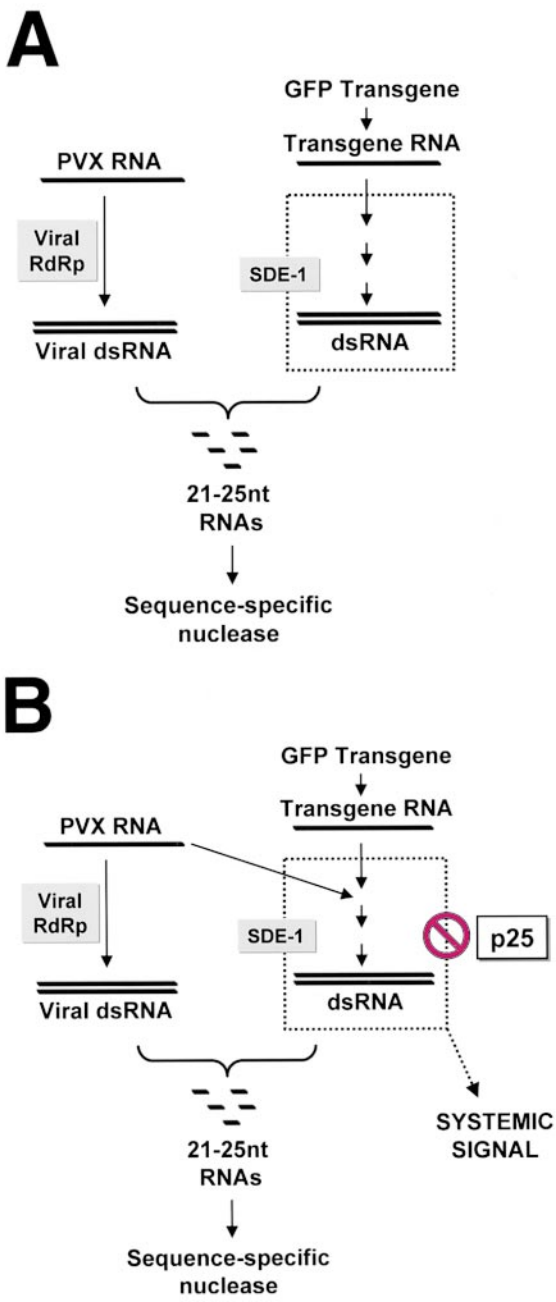


Figure 6. A Model for the Mode of Action of p25

(A) The two SDE1-dependent and SDE1-independent branches of the PTGS pathway, as proposed previously (Dalmay et al., 2000). In the SDE1-independent branch, the viral RNA is copied into double stranded RNA (viral dsRNA) by the virus-encoded RNA-dependent RNA polymerase (viral RdRp). Transgene transcripts are processed into dsRNA through a series of steps that involve SDE1. Both viral and transgene dsRNAs are then processed to 21–25nt RNAs giving specificity to a sequence-specific nuclease that mediates PTGS (Zamore et al., 2000). Note that, in this model, the SDE1-dependent branch is unaffected by viral RNA.

(B) A refined model of PTGS based on the effects of p25 on local and systemic silencing. This model recognizes participation of viral RNA in the SDE1-dependent branch. This branch is involved in production of the systemic PTGS signal and is suppressed by the PVX-encoded p25.

ing virus. Instead, we favor an alternative explanation in which p25 has a single target required for both local and systemic silencing. According to this explanation, the systemic signal would be produced in the SDE1-dependent branch of the pathway and, therefore, would be a precursor of the 25nt RNAs (Figure 6B).

This 'single target' explanation involves a refinement of the previous PTGS model (Figure 6A) in which the SDE1-dependent branch is not influenced by viruses. In the refined model (Figure 6B), the virus-induced local PTGS would involve the SDE1-independent, p25-insensitive branch of the pathway, as previously. However, the SDE1-dependent, p25-sensitive branch is now recognized as being virus-induced (Figure 6B). As a result of this change, the model accommodates the finding that systemic signal production is influenced by PVX replication (Figure 4) and is suppressed by p25.

A further attraction of this refined model is that it resolves an apparent discrepancy between our results with Sde1 and those of Mourrain et al., (2000) with Sgs2, which is identical to Sde1. In our analysis, we found that mutation of Sde1/Sgs2 does not affect susceptibility to tobacco mosaic virus, tobacco rattle virus and turnip crinkle virus (Dalmay et al., 2000) whereas Mourrain and colleagues found that mutations at this locus resulted in hypersusceptibility to CMV (Mourrain et al., 2000). Presumably, the two sets of data differ because, of the viruses tested, CMV is the only one for which RNA accumulation is strongly limited by systemic PTGS. The other viruses are most likely limited by local PTGS which, as discussed above, is not dependent on Sde1.

Dissection of PTGS Using Viral Suppressors

Our earlier characterization of viral suppressors involved infection of plants exhibiting transgene-induced PTGS of GFP. With PVY and other viruses there was an increase of GFP in some or all of the infected tissues, indicating that the corresponding virus encoded a suppressor of PTGS (Brigneti et al., 1998; Voinnet et al., 1999). In contrast, in PVX-infected plants, there was no reversal of PTGS, and we originally concluded that this virus does not encode a suppressor.

However, in the light of data presented here, in particular from the ectopic expression of p25 (Figure 5), it is clear that PVX does encode a suppressor of PTGS. It is likely that this property of PVX was not evident in the earlier experiments because the p25 protein of PVX and the other suppressors of PTGS, including HcPro of PVY, act on different stages in the gene silencing mechanism.

The clearest indication that HcPro and p25 target different stages in gene silencing is from their differential ability to suppress virus-induced PTGS. The HcPro suppresses virus-induced PTGS of GFP (Anandalakshmi et al., 1998) whereas it is clear from the present and previous studies that p25 does not (Ruiz et al., 1998; Figure 3D and 5E). Thus, according to the scheme of Figure 6B, HcPro should act on PTGS at some point after the convergence of the SDE1-dependent and SDE1-independent branches. Since we have proposed that signal production takes place before the convergence of the two branches, we predict that HcPro would not suppress systemic PTGS. Recent data from grafting experiments confirm this prediction (Vicki Vance, personal

communication) and thereby illustrate how analysis of the different viral suppressors of PTGS can be informative about the underlying mechanisms.

In future, the use of the transient assay described in this paper should make it possible to position the various suppressors in the PTGS pathway and to determine if these proteins can act synergistically. Meanwhile, analysis of cellular factors interacting with the suppressors should allow identification of components of PTGS that would not be accessible by conventional mutagenesis.

Experimental Procedures

Plant Material, *Agrobacterium* Infiltration and Grafting Procedure

Transgenic *N. benthamiana* homozygous for the GFP transgene (line 16c) and the *Agrobacterium* infiltration method were described previously (Voinnet et al., 1998). For co-infiltrations, equal volume of both Agrobacterium cultures ($OD_{600}=1$) were mixed before infiltration. For single infiltration, cultures containing the 35S-25k construct were also diluted up to $OD_{600}=1$ to avoid toxicity to the plant cells. The *Rx*-GFP plants resulted from a cross between transgenic *N. benthamiana* homozygous for the Rx locus (Bendahmane et al., 1999) and line 16c. Graftings were performed according to Palauqui et al., 1997.

Construction of PVX-GFP Derivatives

All the constructs used in this study were based on pPVX204, which is a PUC19-based vector in which the full length PVX vector is inserted between the 35S promoter and the Nos terminator. The construct referred here to as PVX-GFP is a derivative of pPVX204 carrying the mGFP5 insert from pBin-35-mGFP5 (Ruiz et al., 1998). PVX-GF was derived from PVX-GFP. The entire coat protein ORF was removed from PVX-GFP by digestion with SalI and XhoI and subsequent religation, leading to PVX-GFP-ΔCP. PVX-GFP-ΔTGB-ΔCP was generated by digestion of PVX-GFP-ΔCP with AvrII and EagI, which removed the 3′ end of the replicase ORF, the entire TGB, and the 3′ end of the GFP5 ORF. To restore the replicase and GFP functions, a 3-way ligation was performed with two DNA fragments that had been PCR amplified from PVX-GFP-ΔCP and digested as described below.

Amplification with 5′-GCACAGATTTTCCTAGGCACGTTATC and 3′-GAAAGAAATTGGgccggctcttgaac (EagI site underlined) led to a DNA fragment corresponding to the 3′ end of the replicase ORF that was subsequently digested by AvrII and EagI; amplification with 5′-cagaaaccggccgctagcGGGCCATTGCCG (EagI site underline) and 3′-TGTACTGCTTGAGATTTACAGCT led to a DNA fragment corresponding to the 5′ end of GFP5 ORF that was subsequently digested by EagI. PVX-GFP-Δrep-ΔCP and PVX-Δrep-GFP-ΔTGB-ΔCP were generated by digesting PVX-GFP-ΔCP and PVX-GFP-ΔTGB-ΔCP, respectively, with BglII and religation, generating a 1729-nt deletion in the replicase ORF. Individual TGB mutants were generated by introducing previously characterized mutations into the PVX-GFP-ΔCP background. PVX-GFP-Δ12k-ΔCP was made by inserting an ApaI-BstBI restriction fragment of PVX-GFP-12D (Verchot et al., 1998) into ApaI-BstBI digested PVX-GFP-ΔCP. PVX-GFP-Δ8k-ΔCP was generated by inserting an ApaI-BstBI restriction fragment of pTXS-Δ8K-GFP (kindly provided by Simon Santa Cruz, SCRI, Dundee) into ApaI-BstBI digested PVX-GFP-ΔCP. pTXS-Δ8K-GFP has a mutation in the start codon (M→T) of the 8 kDa protein that also introduces an in-frame STOP codon without altering the coding capacity of the overlapping 12 kDa protein ORF. PVX-GFP-Δ25k-ΔCP was generated from pTXS-GFP-ΔApa/Apa that has a 354-nt deletion in the 25 kDa ORF, between an ApaI site inserted by mutation of nucleotides 4588–4591 in the PVX genome and an ApaI site existing naturally at position 4945. The deletion was then introduced as an AvrII-BstBI fragment into AvrII-BstBI digested PVX-GFP. Finally, the PVX-GFP-25kFS-ΔCP construct was generated by inserting an AvrII-BstBI restriction fragment from pTXS-GFP3A (kindly provided by Simon Santa Cruz, SCRI, Dundee) into AvrII-BstBI digested PVX-GFP-ΔCP. pTXS-GFP3A carries a 4bp deletion, resulting from removal of the 3′ overhang (T4 DNA polymerase) of an ApaI digestion at nucleotide 4945 in the PVX genome. This mutation causes a frameshift in the 25 kDa ORF starting at amino acid 154 and introduces an in-frame STOP codon at amino acid 159, leading to a truncated protein (C-terminal deletion of 73 amino acids). Constructs carrying fragments of endogenous genes (PDS and Rbcs) were all derivatives of the above vectors. The unique PmlI blunt site in GFP5 was used to clone the corresponding inserts (see figure legends). All the constructs described here were confirmed by sequencing and inserted as SacI fragments into the T-DNA of the pBin19 vector plasmid (Bevan, 1984). The 35S-25k and 35S-25k-ΔATG constructs are based on pBin19 containing the 35S expression cassette of pJIT61 (JIC). The 25 kDa inserts were PCR fragments amplified from pPVX204, using the PFU polymerase (Promega). For 35S-25k-ΔATG, the start codon was omitted in the forward primer. Both constructs were confirmed by sequencing.

General Procedures

RNA isolation and Northern analysis of high and low molecular weight RNAs were as described (Dalmay et al., 2000). Viral inocula of PVX-GFP and PVX-GF were described previously (Ruiz et al., 1998).

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