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Gene Silencing without DNA: RNA-Mediated Cross-Protection between Viruses

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Previously, it was shown that the upper leaves of plants infected with nepoviruses and caulimoviruses are symptom free and contain reduced levels of virus. These leaves are said to be recovered. Recovery is associated with RNA-mediated cross-protection against secondary virus infection. Here, by analyzing plants infected with viruses that are quite distinct from the nepovirus or caulimovirus groups, we demonstrate that this RNA-mediated defense is a general response to virus infection. Upon infection with a tobravirus, plants exhibited RNA-mediated cross-protection and recovery, as occurs in nepovirus-infected plants. However, upon infection with a potexvirus, plants exhibited RNA-mediated cross-protection without recovery. In both instances, a transient gene expression assay showed that RNA-mediated cross-protection was functionally equivalent to post-transcriptional gene silencing. Combined, these data provide direct evidence that post-transcriptional gene silencing of nuclear genes is a manifestation of a natural defense mechanism that is induced by a wide range of viruses.

INTRODUCTION

Several lines of evidence suggest a link between post-transcriptional gene silencing (PTGS) in transgenic plants and viruses (Baulcombe, 1996a; Pruss et al., 1997). For example, transgene-induced PTGS causes resistance against viruses that have nucleotide sequences similar to that of the transgene (Smith et al., 1994; Mueller et al., 1995; English et al., 1996; Goodwin et al., 1996). This type of transgenic virus resistance is referred to as RNA homology–dependent resistance. Viruses can also induce PTGS of homologous transgenes (Angell and Baulcombe, 1997; Al-Kaff et al., 1998; Atkinson et al., 1998; Kjemtrup et al., 1998; Ruiz et al., 1998). In some transgenic plants, the virus can be both an inducer and a target of gene silencing. The lower leaves of these plants display the normal viral symptoms. However, upper leaves emerging after systemic infection are symptom and virus free. These upper leaves are resistant to secondary infection by the inducing virus and are said to be "recovered" (Lindbo et al., 1993; Guo and Garcia, 1997).

There is also a link between PTGS and viruses in nontransgenic plants. For example, PTGS is induced by recombinant virus vectors carrying inserts that are homologous to endogenous genes. This virus-induced gene silencing may be mediated by tobacco mosaic virus (TMV; a tobamovirus) (Kumagai et al., 1995) and potato virus X (PVX; a potexvirus) (Ruiz et al., 1998) vectors with RNA genomes and by tomato golden mosaic virus (a geminivirus) (Kjemtrup et al., 1998) with a DNA genome. A PTGS-like mechanism is also induced by nepoviruses and caulimoviruses (Covey et al., 1997; Ratcliff et al., 1997) that do not have homology to endogenous genes. In these examples, the infected plants exhibit a response very similar to the virus-induced recovery on transgenic plants in that the upper leaves are symptom free and contain reduced levels of virus. In nepovirus-infected plants, the recovered leaves exhibit homology-dependent resistance to secondary infections (Ratcliff et al., 1997).

Data from plants simultaneously infected with two viruses have also been interpreted in terms of a PTGS-like defense mechanism (Pruss et al., 1997). These mixed infections often result in severe viral symptoms and accumulation of one or both of the viruses at a higher level than in single infections. To explain this synergistic interaction, Pruss et al. suggested that one of the viruses suppresses a host defense mechanism that normally limits the accumulation of the second virus. PTGS has been implicated in this antiviral defense by the finding that the virus-encoded proteins, which are responsible for synergism, also have the ability to suppress PTGS (Anandalakshmi et al., 1998; Beclin et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). Moreover, because suppressors of gene silencing enhance the accumulation of diverse virus types, these findings provide indirect evidence that the PTGS-like resistance is a generalized defense mechanism in plants.

However, from the data described above, it remains

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possible that this PTGS-like mechanism is specific to nepoviruses and caulimoviruses, to transgenic plants, and to artificial situations in which there is sequence similarity between the virus and host genomes. Here, we assess the involvement of a PTGS-like mechanism in plants as a general feature of plant virus infections by using tobacco rattle virus (TRV; a tobravirus) and PVX. TRV is taxonomically distinct from nepoviruses and caulimoviruses, but it induces recovery (Cadman and Harrison, 1959). PVX is also taxonomically distinct and does not induce recovery. Our findings show directly that both TRV and PVX induce a PTGS-like resistance response and that neither recovery nor plant genome homology is essential for induction of this defense mechanism. In addition, we show that this PTGS-like defense response is functionally the same as PTGS of transgenes. These data confirm the role of a PTGS-like mechanism as a generalized antiviral defense response in plants.

RESULTS

The experimental strategy to investigate the possible roles of TRV and PVX as inducers of a PTGS-like defense was an extension of the cross-protection assay used previously to characterize nepovirus recovery (Ratcliff et al., 1997). First *Nicotiana benthamiana* plants were inoculated with the inducing virus, either TRV or PVX. The ability of these viruses to induce a PTGS-like mechanism was then tested by assessing homology-dependent resistance against a second challenge virus. We predicted that the PTGS-like mechanism would only mediate cross-protection against challenge viruses with homology to the inducing virus.

In addition to this cross-protection assay, we also investigated the similarity between PTGS-like virus resistance and PTGS of transgene expression under conditions in which there were no transgenes and in which there was no sequence similarity between the inducing virus and the host plant genomes. First, the PTGS-like resistance response was initiated by a virus that did not have genome similarity with the host. Subsequently, after systemic spread of the virus, transient transgene expression was used to assess PTGS in the virus-infected leaves. We predicted that if the cross-protection mechanism were similar to PTGS, then there would be silencing of the transiently expressed DNA, provided that its sequence was similar to that of the virus.

Recovery Induced by a Tobravirus Is Associated with PTGS

To investigate TRV as an inducer of a PTGS-like resistance mechanism, we used a TRV (strain PPK20) vector, TRV–green fluorescent protein (GFP). RNA 1 of the bipartite TRV genome encodes the essential replication and movement functions. RNA 2 encodes the coat protein and, in the PPK20 strain, two other nonstructural proteins. In RNA 2 of TRV–GFP, the genes encoding these proteins were replaced with *GFP*, as shown in Figure 1A. Neither of these genes is required for infectivity of TRV (Hernandez et al., 1997), and the TRV–GFP vector could accumulate and spread systemically.

The TRV–GFP—infected plants exhibited very mild symptoms. However, from the pattern of GFP fluorescence, it was evident that the virus had spread extensively in the inoculated leaves and in systemically infected regions. Figure 1B shows the systemic GFP fluorescence from TRV–GFP in

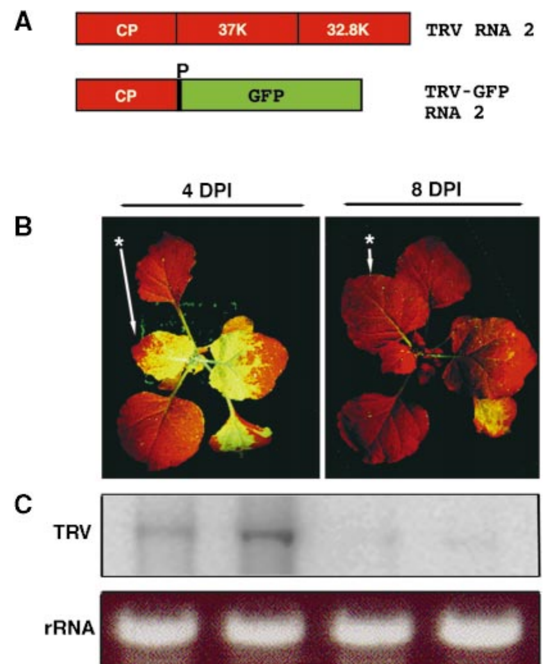

Figure 1. TRV–GFP Recovery on *N. benthamiana*.

(A) Genomic organization of TRV RNA 2 and of TRV–GFP RNA 2. The TRV open reading frames are shown as the coat protein (CP) and 37K and 32.8K proteins. In TRV–GFP RNA 2, GFP is transcribed from the coat protein promoter (P) of pea early-browning virus and replaces the 37K and 32.8K open reading frames.

(B) TRV–GFP recovery on *N. benthamiana*. The same plant was photographed under UV light at 4 and 8 DPI. Leaves equivalent to those marked with an asterisk were used in all experiments on TRV–GFP—recovered tissue.

(C) TRV–GFP RNA accumulation before and after recovery. RNA gel blot analysis of total RNA (10 μg) extracted from two independent TRV–GFP—infected leaves at 4 and 8 DPI, respectively, is shown. The filter was hybridized with a ^{32}P-labeled cDNA probe corresponding to the 3′-terminal 1.4 kb of TRV RNA 1 (see Methods). The TRV RNA 1 genomic band is shown. Ethidium bromide staining of the electrophoresed gel shows the rRNA loading.

N. benthamiana at 4 days postinoculation (DPI). This fluorescence was intense and uniform in infected regions of upper leaves. However, by 8 to 10 DPI, GFP fluorescence disappeared from all fully expanded leaves in all of >100 plants. RNA gel blot analysis (Figure 1C) showed an ~30-fold reduction in TRV RNA levels associated with this loss of GFP fluorescence. In 10 TRV–GFP—infected plants that were observed for an additional 40 days, there was no return of GFP fluorescence.

In a series of cross-protection experiments, illustrated in Figure 2A, the upper leaves of TRV–GFP—infected plants were inoculated with PVX vectors. These vectors, shown in Figure 2B, were PVX–GUS, which carries the β-glucuronidase (*GUS*) reporter gene (Chapman et al., 1992), and PVX–GUSGF. The PVX–GUSGF vector is a derivative of PVX–GUS, with 363 nucleotides of 5′ *GFP* sequence inserted downstream of the *GUS* gene. It was possible to use histochemical staining of GUS to detect infection by these viruses because both vectors carry an intact *GUS* reporter gene. A PTGS-like resistance mechanism should not affect PVX–GUS but may target PVX–GUSGF due to its sequence similarity to TRV–GFP.

In these experiments, *N. benthamiana* plants were initially inoculated with either TRV–GFP or water. At 8 DPI (after disappearance of GFP fluorescence), the upper leaves of six plants were challenge inoculated with in vitro transcripts of PVX–GUS or PVX–GUSGF. Susceptibility to the challenge virus was assessed after another 7 days by analyzing GUS staining of infection foci and by using RNA gel blot analysis. Figure 2C shows that on mock-inoculated and TRV–GFP—infected leaves, there were many PVX–GUS foci and high levels of PVX–GUS RNA. Infection foci and RNA of PVX–GUSGF were abundant on mock-inoculated plants. However, on the TRV–GFP—infected leaves inoculated with PVX–GUSGF, there were no GUS foci, and the PVX–GUSGF RNA was not detectable by RNA gel blot analysis. These data show that TRV–GFP induces effects that are similar to nepovirus-induced recovery in that there was a reduction in virus levels and symptoms in the upper leaves and homology-dependent resistance against a challenge virus. Therefore, RNA-based recovery is not an unusual characteristic of nepovirus-infected plants.

Based on the RNA homology dependency of cross-protection in nepovirus-infected plants, we had previously speculated that the mechanism of recovery was similar to that of PTGS in transgenic plants (Ratcliff et al., 1997). To determine whether this RNA-based mechanism is functionally the same as PTGS in TRV–GFP—infected plants, we used Agrobacterium-mediated transient gene expression, as illustrated in Figure 3A. Transient gene expression has been used as a test of PTGS in several studies (Vaucheret, 1994; English et al., 1997; Voinnet et al., 1998). The assay involved infiltrating leaves with a suspension of Agrobacterium carrying the binary vector pTDB in which the T-DNA includes cauliflower mosaic virus 35S–*GFP* and 35S–*GUS* genes. Agrobacterium transfers the T-DNA of the binary

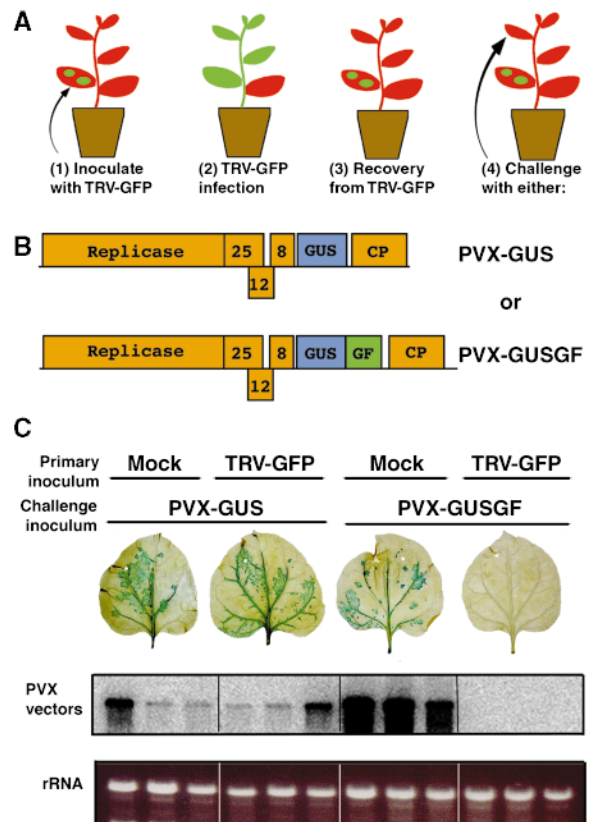

Figure 2. TRV–GFP—Induced Cross-Protection.

(A) Schematic representation of the experimental procedure. Plants are drawn as viewed under UV illumination.

(B) Genomic organization of PVX–GUS and PVX–GUSGF. The PVX open reading frames are shown as replicase (i.e., RNA-dependent RNA polymerase), 25K, 12K, and 8K proteins, and the coat protein (CP). Both PVX–GUS and PVX–GUSGF express the *GUS* reporter gene, in addition to which PVX–GUSGF carries 363 nucleotides of the *GFP* sequence (see Methods).

(C) Susceptibility of TRV–GFP—recovered leaves to PVX–GUS and PVX–GUSGF. Six TRV–GFP—recovered and mock-inoculated plants (8 DPI) were challenge inoculated with in vitro transcripts of PVX–GUS or PVX–GUSGF. After 7 days, accumulation of the challenge virus was assessed on inoculated leaves of three plants by using GUS histochemistry; a typical leaf is shown for each treatment. On the remaining three leaves, accumulation of the challenge virus was assessed by RNA gel blot analysis. RNA (2 μg) from each sample was hybridized with a ^{32}P-labeled DNA fragment corresponding to the *GUS* gene. The main genomic band is shown. Although there was considerable variation in the levels of PVX vector accumulation, qualitatively similar data were obtained in three independent experiments. PVX–GUSGF accumulation was never observed in the upper leaves of TRV–GFP—infected plants. Ethidium bromide staining of the electrophoresed gel shows the rRNA loading.

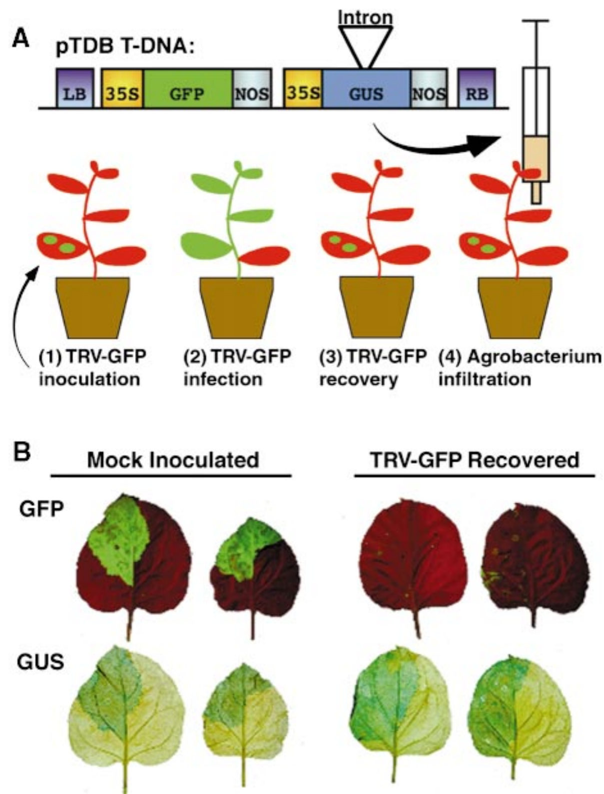

Figure 3. TRV–GFP Recovery Induced PTGS of Transiently Expressed Genes.

(A) Schematic representation of the experimental procedure for transient Agrobacterium-mediated gene expression. The pTDB T-DNA is organized as follows: left border (LB), cauliflower mosaic virus 35S promoter (35S), *GFP*, nopaline synthase terminator (NOS), *GUS*, and right border (RB). The *GUS* gene contains an intron. Plants are drawn as viewed under UV illumination.

(B) Agrobacterium-mediated transient gene expression of *GFP* and *GUS* reporter genes in two mock-inoculated and two TRV–GFP—recovered *N. benthamiana* leaves. Two days after infiltration, leaves were photographed under UV light and then histochemically stained for GUS enzyme activity.

vector to cells within the infiltrated zone. Both reporter genes would normally be expressed. However, if the TRV–GFP had induced PTGS, then expression of the *GFP* reporter would be suppressed.

Figure 3B shows that the infiltrated zones of mock-inoculated plants exhibited GUS staining and GFP fluorescence, demonstrating the activity of both reporter genes, as expected. In contrast, in the TRV–GFP—recovered leaves, the infiltrated zones exhibited GUS staining but no GFP fluorescence. Identical results were found in nine of nine plants tested at 8 DPI and four of four plants tested at 30 DPI. This differential transient gene expression was not a transcriptional effect, because the suppressed *GFP* reporter and the unaffected *GUS* gene have identical promoters. A more likely explanation is PTGS of the *GFP* reporter gene in the TRV–GFP—recovered leaves. This result indicates that TRV-induced recovery and PTGS are likely to involve similar mechanisms.

RNA-Mediated Cross-Protection without Recovery

These experiments with TRV–GFP constructs, together with previous analyses of nepovirus- and caulimovirus-infected plants, established a link among recovery, RNA-mediated cross-protection, and PTGS. However, if a PTGS-like mechanism is a general defense response against viruses, it would be expected that viruses that do not induce recovery should nevertheless induce RNA-mediated cross-protection. To test this prediction, we assessed whether the vectors PVX–GUS and PVX–GUSGF (Figure 2B) could induce RNA-mediated cross-protection, even though they would not cause recovery. The challenge virus in these experiments was a TMV vector (Donson et al., 1991) modified to express the *GFP* reporter gene (TMV–GFP) (Ruiz et al., 1998). The genomic organization of TMV–GFP is shown in Figure 4A.

In these experiments, the inoculation regime differed from that used to assess cross-protection by nepoviruses and TRV–GFP. Instead of applying the challenge virus to systemically infected leaves, in these experiments with PVX and TMV vectors, the two inocula were mixed. The tissue-sampling protocol was also changed. Instead of taking samples from the inoculated leaves, virus accumulation was sampled on leaves that would be systemically infected with both viruses. This modified protocol was used because, unlike TRV–GFP, PVX vectors do not establish uniform infection of the systemically infected leaves (Chapman et al., 1992; Baulcombe et al., 1995). Consequently, any PVX cross-protected leaf would be a mosaic of resistant and susceptible regions.

Ten days after inoculation, virus accumulation was assessed in the upper noninoculated leaves by GFP fluorescence, GUS staining, and RNA gel blot analysis. Figure 4B shows that in plants inoculated with PVX–GUS and TMV–GFP, there were high levels of GUS enzyme activity and GFP fluorescence. RNA gel blot analysis (Figure 4C) showed high accumulation of both TMV–GFP and PVX–GUS RNA. However, on plants inoculated with PVX–GUSGF and TMV–GFP, there was widespread GUS staining, indicating systemic PVX–GUSGF infection, but no GFP fluorescence. Correspondingly, RNA gel blot analysis showed high accumulation of PVX–GUSGF RNA, but TMV–GFP RNA was not detectable. These data show that there was RNA-based sequence-specific cross-protection between PVX–GUSGF and TMV–GFP. Therefore, PVX–GUSGF induced an RNA-mediated defense mechanism, even though it cannot induce recovery.

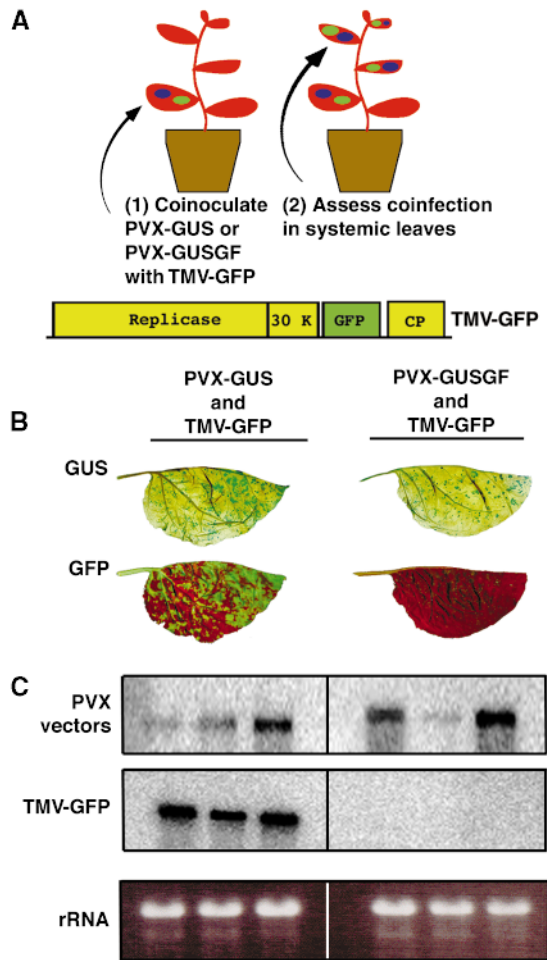

Figure 4. PVX-Induced RNA-Mediated Cross-Protection.

(A) Schematic illustration of experimental procedure. Plants are drawn with UV-illuminated and GUS-stained colors. TMV–GFP genomic organization is shown; TMV open reading frames include those corresponding to replicase (i.e., RNA-dependent RNA polymerase), the 30K protein, and the coat protein (CP). PVX–GUS and PVX–GUSGF are described in Figure 2B.

(B) GUS histochemical staining and GFP fluorescence in systemic leaves of plants inoculated with either PVX–GUS or PVX–GUSGF and TMV–GFP. Leaves of 12 independent plants from each treatment were cut along the midrib at 14 DPI; half of each leaf was photographed under UV light and then GUS stained. Typical results from three plants are shown.

(C) RNA samples (2 μg) from the remaining half of each leaf shown in **(B)** were used for RNA gel blot analysis of virus accumulation. RNA aliquots were hybridized either with ^{32}P-labeled *GUS* DNA to show accumulation of PVX–GUS and PVX–GUSGF or with a ^{32}P-labeled cDNA fragment corresponding to the 5′ 3 kb of TMV to show TMV–GFP accumulation. The major genomic band is shown for each virus. Ethidium bromide staining of an electrophoresed gel shows the rRNA loading.

Following the rationale applied to the analysis of TRV–GFP, we used Agrobacterium infiltration to assess whether the RNA-based mechanism induced by PVX is functionally the same as PTGS. In these experiments, nine plants were either mock inoculated or inoculated with PVX–GF, which carries 465 nucleotides of *GFP* sequence encoding a truncated nonfluorescent protein (Ruiz et al., 1998), as illustrated in Figure 5A. At 8 DPI, upper leaves were infiltrated with Agrobacterium carrying the binary plasmid pTDB (Figure 3A) in the expectation that a PTGS-like defense would specifically suppress GFP expression.

Figure 5B shows UV illumination and GUS histochemical staining of leaves 2 days after infiltration. The infiltrated zone of mock-inoculated leaves showed uniform GFP fluorescence and GUS staining, as previously described (Figure 3B). Leaves infected with PVX–GF also showed uniform GUS staining in the infiltrated zone. However, in these leaves, GFP fluorescence was much less than in the leaves of mock-inoculated plants. Any GFP fluorescence in these leaves was confined to spots, unlike the intense and uniform fluorescence apparent on the leaves of mock-inoculated plants. As in TRV–GFP—recovered leaves, there was a specific reduction in GFP fluorescence. However, unlike the TRV–GFP—induced effect, the suppression of GFP was only partial. We attribute this difference to the nonuniform distribution of PVX vectors in systemically infected leaves. As in the TRV–GFP experiments, we can rule out transcriptional suppression because the 35S promoter was present in both the suppressed *GFP* gene and the unaffected *GUS* gene. Therefore, we conclude that the GFP reporter gene was suppressed by PTGS in PVX–GF—infected areas of the leaf. These data confirm that a PTGS-like mechanism is induced by a virus that does not cause recovery.

DISCUSSION

Based on previous analyses of virus-induced changes and PTGS (Dawson, 1996; Pruss et al., 1997; Ratcliff et al., 1997), it was suggested that antiviral defense in plants exploits the same RNA-mediated mechanism as PTGS. From this suggestion, we predicted that PTGS-like defense would be manifested as an RNA sequence–specific process in cross-protection and gene silencing assays. The results from TRV- and PVX-infected plants (Figures 2 and 4) are consistent with this prediction and extend previous findings that nepoviruses (Ratcliff et al., 1997), caulimoviruses (Covey et al., 1997; Al-Kaff et al., 1998), and viroids (Fernow, 1967; Niblett et al., 1978) induce RNA-mediated defense responses. In addition, from the reporter gene assays (Figures 3 and 5), we have confirmed that the RNA-mediated cross-protection is functionally the same as PTGS. Collectively, therefore, these data provide compelling, direct evidence that viruses from many taxonomic groups can induce a PTGS-like defense response.

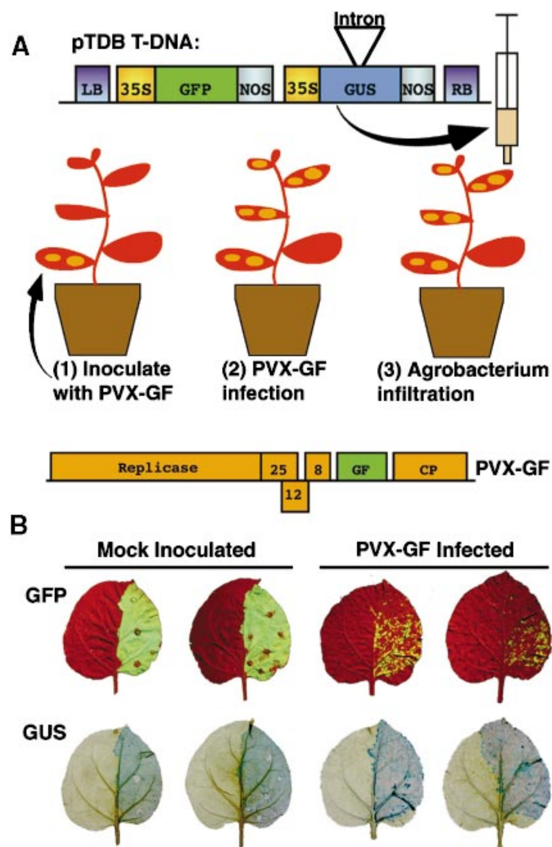

Figure 5. The PVX-Induced RNA-Based Defense Response Resembles PTGS.

(A) Schematic illustration of experimental procedure. The genomic organization of PVX–GF is shown. GF refers to the 5′ part (465 nucleotides) of the *GFP* coding sequence. PVX open reading frames are as given in Figure 2B. The T-DNA organization of pTDB is as given in Figure 3A.

(B) Leaves of mock- and PVX–GF—infected plants 2 days after infiltration with Agrobacterium carrying pTDB. Leaves were photographed under UV illumination (GFP) and then histochemically stained for GUS enzyme activity (GUS).

Mechanisms

Two mechanisms could account for RNA-mediated defense and PTGS. Both of these hypothetical mechanisms involve antisense RNA as the specificity determinant of an RNA degradation mechanism (Baulcombe, 1996b). In the "direct" models, the antisense RNA is produced either by transcription of the silencer transgene (in PTGS) (Grierson et al., 1991) or as the negative strand intermediate of viral replication (in RNA-mediated cross-protection) (Palukaitis and Zaitlin, 1984). In the second category of "indirect" models, the antisense RNA is produced by a host-encoded RNA-dependent RNA polymerase using the transgene or viral RNA as a template (Lindbo et al., 1993; Ruiz et al., 1998).

For PTGS, experimental data from transgenic plants are inconsistent with the "direct" models, and the "indirect" models are generally favored. These data have shown that PTGS requires sense transcription, implying that antisense RNA is insufficient for the silencer activity of a transgene (English et al., 1997; Vaucheret et al., 1997). For RNA-mediated cross-protection, there is no direct evidence to rule out either model. However, many examples of PTGS and RNA-mediated cross-protection can be plausibly brought together by involving double-stranded RNA (dsRNA) in the indirect model. dsRNA is a known intermediate in virus replication (Matthews, 1991) and could be an inducer of PTGS in RNA virus–infected cells. dsRNA has also been implicated in PTGS in petunia and tobacco (Metzlaff et al., 1997; Waterhouse et al., 1998). In contrast, normal cellular RNA is unlikely to contain extensive double-stranded regions because expression of mammalian dsRNA-induced RNase L or of yeast Pac1 dsRNase has no effect on the growth or development of transgenic plants (Mitra et al., 1996; Ogawa et al., 1996; Sano et al., 1997). Perhaps RNA double strandedness is an identifying feature of viral and transgene RNAs in plant cells. This dsRNA could serve as the template for production of antisense RNA, which may in turn be the specificity determinant for the degradation mechanism. It is currently unclear whether dsRNAs could also be involved in the induction of PTGS-like resistance by caulimoviruses and geminiviruses.

Counterdefense Strategies

In addition to the RNA-mediated mechanism, there are several other levels of virus defense in plants, including protein-induced mechanisms and protein-mediated cross-protection (Dawson, 1991). Nevertheless, from the results described here, it is likely that the outcome of many if not all plant–virus interactions is influenced by the RNA-mediated defense response. Viruses that are unable to counter the RNA-mediated mechanism either will be restricted to the site of initial infection or will exhibit only very slow systemic spread. The common observation that most plants are resistant to most viruses may be due, at least partially, to induction of the RNA-mediated mechanism in infected cells.

A straightforward counterdefense strategy, used by potyviruses (Kasschau et al., 1997; Anandalakshmi et al., 1998; Brigneti et al., 1998) and cucumber mosaic virus (Beclin et al., 1998; Brigneti et al., 1998), employs virus-encoded suppressors of PTGS that also suppress the RNA-mediated defense response. In a second type of counterdefense strategy, the virus may evade the RNA-mediated mechanism by replicating rapidly. By the time the defense mechanism becomes active, the virus may have already accumulated to a high level (Pruss et al., 1997). It is possible that PVX, which

does not suppress PTGS of transgenes (Brigneti et al., 1998), exploits this strategy.

Recovery

Clearly, the induction of recovery by nepoviruses and TRV–GFP is associated with the induction of a PTGS-like defense response. However, the demonstration that PVX induces such a response implies that recovery is not an inevitable consequence of the PTGS-like mechanism. For recovery, other factors must be involved. Although we have not identified these factors, we have noted a striking correlation between the ability of a virus to induce recovery and its ability to infect meristems. Normally, plant viruses are excluded from meristematic areas. However, all of the recovery-inducing RNA viruses known to us have the unusual ability to infect meristems. These viruses include nepoviruses (Wingard, 1928; Lister and Murant, 1967), alfalfa mosaic virus (Ross, 1941; Frosheiser, 1974), and TRV (Cadman and Harrison, 1959). In these examples, pollen transmission of the virus is an indicator of meristem entry (Matthews, 1991).

There is also one example of an association between meristem entry and recovery in viroid-infected plants. In this example, with avocado sunblotch viroid, there is high accumulation of viroid RNA but low pollen transmission in symptomatic branches. In contrast, there is a low level of avocado sunblotch viroid RNA and frequent pollen transmission in recovered branches (Wallace and Drake, 1962; Semancik and Desjardins, 1980). Thus, a future challenge is to determine whether recovery requires meristem entry. An alternative hypothesis is that an unidentified factor is independently responsible for both recovery and meristem entry. Clearly, further characterization of recovery will lead to a better understanding of virus resistance and PTGS in plants.

METHODS

Recombinant Virus Material

The tobacco rattle virus–green fluorescent protein (TRV–GFP) is a pseudorecombinant virus consisting of TRV RNA 1 purified from a natural virus infection and TRV RNA 2 from a modified cDNA clone (Mueller et al., 1997). Plasmid pT72K20, containing a full-length cDNA of TRV strain PPK20 RNA 2, was digested with BstEII at a site 170 nucleotides downstream from the start of the 37K gene (nucleotide position 1640) and at an ApaI site introduced immediately downstream of the 32.8K gene (nucleotide position 2910). Introduced into this gap was the coat protein promoter from pea early-browning tobravirus (nucleotide positions 275 to 510) (MacFarlane et al., 1992) in which an NheI site directly follows the AUG initiation codon and the cycle 3 *GFP* gene (Crameri et al., 1996). This construct was linearized with SmaI and transcribed with T7 RNA polymerase. RNA transcripts were combined with TRV RNA 1 (also isolate PPK20) isolated as total plant RNA from an NM-type infection (Harrison and Robinson, 1986). Sap prepared from individual fluorescent foci was used in this study.

Tobacco mosaic virus (TMV)–GFP, potato virus X (PVX)–GF, and PVX–GUS (pGC3) have previously been described (Chapman et al., 1992; Ruiz et al., 1998). PVX–GUSGF was derived from pGC3 by digestion at a ClaI site immediately downstream of the *β-glucuronidase* (*GUS*) gene (nucleotide position 7564) and insertion of a 400-nucleotide TaqI fragment from pTXS–GFP (nucleotide positions 5642 to 6042) (Baulcombe et al., 1995). This 400-nucleotide fragment contained the 5′ 363 nucleotides of the *GFP* sequence (Prasher et al., 1992) and 37 nucleotides from the coat protein promoter of PVX.

TMV–GFP, PVX–GUS, PVX–GUSGF, and PVX–GF were linearized with the appropriate restriction enzyme and transcribed with T7 RNA polymerase, as described previously (Donson et al., 1991; Kavanagh et al., 1992). Transcripts of these constructs were used directly in this study.

All inoculations were conducted on the leaves of 4- to 5-week-old *Nicotiana benthamiana* plants that were lightly dusted with carborundum.

Agrobacterium tumefaciens–Mediated Transient Gene Expression

For Agrobacterium-mediated transient expression of GUS and GFP, cauliflower mosaic virus 35S GFP4 (Haseloff et al., 1997) was introduced to the binary plasmid TDS80, which carries the 35S-driven intron-containing *GUS* gene (Vaucheret, 1994; English et al., 1997), to form pTDB. Transient gene expression was achieved as described previously (English et al., 1997).

GFP Imaging

The GFP was visualized by using a 100-W long-wave UV lamp (Black Ray model B 100AP; UVP, Upland, CA). Photographs were taken on 400 ASA Kodak Ektachrome Panther film through a Wratten 8 filter (Kodak Ltd., Hemel Hempstead, UK), with exposure times of 15 to 90 sec, depending on the distance from the plant.

GUS Histochemistry

Histochemical staining of GUS activity with X-gluc was performed as described previously (Jefferson, 1987). Leaves were incubated for 4 to 12 hr at 37°C before clearing with 70% (v/v) ethanol at 65°C.

RNA Gel Blot Analysis

RNA gel blot analysis was performed as described previously (Mueller et al., 1995). The DNA fragments used as probes were labeled by random-primed incorporation of ^{32}P-dCTP. For analysis of TRV accumulation, we used a BstEII fragment (positions 5345 to 6792) of pTR7116 (Hamilton and Baulcombe, 1989) corresponding to the 3′ 1.44 kb of TRV RNA 1. PVX–GUS and PVX–GUSGF accumulations were analyzed by using a polymerase chain reaction (PCR)–amplified DNA fragment corresponding to the full-length GUS open reading frame as a probe. The PCR primers were 5′-ATGTTACGTCCTGTA-GAAACC-3′ and 5′-ATCAAGCTTATCGATAAGCTT-3′. For analysis of TMV–GFP RNA accumulation, we PCR amplified a cDNA fragment

corresponding to the 5′ 3.35 kb of TMV–GFP and labeled it with ^{32}P. The PCR primers were 5′-ATGGCATACACACAGACAGC-3′ and 5′-TTATTGTGTTCCTGCATCGAC-3′.

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