Potato Virus X Amplicons in Arabidopsis Mediate Genetic and Epigenetic Gene Silencing

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Amplicon transgenes from potato virus X (PVX) are based on a modified version of the viral genome and are efficient activators of post-transcriptional gene silencing (PTGS). To determine whether *PVX* amplicons activate PTGS in Arabidopsis, we used constructs based on the genome of PVX carrying a green fluorescent protein (*GFP*) reporter gene. Our analysis of the transgene phenotype exploited previous observations indicating that PTGS is associated with short 25-nucleotide RNA species, transgene methylation, and homology-dependent virus resistance. We also used the ability of turnip mosaic virus to suppress gene silencing as a means of dissecting stages of the mechanism. The results showed that a *PVX:GFP* amplicon induces weak PTGS and that this PTGS was enhanced in the presence of a *GFP* reporter gene. Our interpretation of these data is that the PTGS induced by the amplicon was genetically determined and equivalent to the initiation stage of the PTGS mechanism. The PTGS induced by the combined amplicon and reporter gene was equivalent to the maintenance stage and was associated with an epigenetic conversion of the transgene. The distinction between genetic and epigenetic PTGS explains the well-characterized effects of transgene dosage on PTGS that have been previously interpreted in terms of RNA expression thresholds.

INTRODUCTION

Amplicons from potato virus X (PVX) are transgenes based on a modified viral genome in which the key elements are *cis*- and *trans*-acting elements required for virus replication. These elements include the gene for the virus-encoded replication enzyme and the *cis*-acting elements required for replication of the viral RNA. Originally, the amplicon transgenes were introduced into plants as a possible means of achieving high-level expression of genes incorporated into the viral genome. However, from the analysis of transgenic plants expressing a PVX vector with a β-glucuronidase (*GUS*) reporter gene (*PVX:GUS*), we found that there was an unexpectedly low level of *GUS* expression (Angell and Baulcombe, 1997).

Subsequently, it was found that this low level of *GUS* expression was due to activation of a sequence-specific mechanism of RNA degradation by the *PVX:GUS* amplicon. The targets were RNA molecules that shared sequence identity with the amplicon transgene, and the underlying mechanism was the same as that of post-transcriptional gene silencing (PTGS) in plants exhibiting cosuppression and other related phenomena (Angell and Baulcombe, 1997). An important feature of amplicon-mediated PTGS is

the lack of between-line variation that is normally associated with sense or antisense transgenes: in tobacco and tomato, each tested line exhibited a high level of amplicon-mediated PTGS. Therefore, amplicons can be exploited for consistent and efficient transgene silencing. Recently, we have shown that PTGS could be targeted against endogenous RNA by *PVX* amplicons with fragments of endogenous genes and that the silencing phenotype was reproducible in all of the tested tomato and tobacco lines (Angell and Baulcombe, 1999).

There are two features of the *PVX* amplicons that are important for the PTGS phenotypes. First, there is the production of replicating viral RNA that is known, from several systems, to be a potent activator of PTGS (Lindbo et al., 1993; Kumagai et al., 1995; Covey et al., 1997; Ratcliff et al., 1997, 1999; Jones et al., 1998a; Ruiz et al., 1998). Plants probably have an RNA surveillance system that can detect the double strands of these replicating RNAs (Waterhouse et al., 1998) and that is part of an RNA-mediated, anti-viral defense system (Ratcliff et al., 1999). The second feature of PVX influencing the amplicon phenotype is the absence of a virus-encoded suppressor of PTGS. Many viruses encode these suppressors as a strategy to counteract the antiviral defense, but PVX does not (Brigneti et al., 1998; Voinnet et al., 1999). Obviously, if an amplicon transgene encodes a suppressor of silencing, it would compromise the PTGS phenotype.

There are several reports of transgenic plants expressing

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replicating viral RNA or DNA associated with phenotypes that can be explained, at least in part, in terms of PTGS (Kaido et al., 1995; Angell and Baulcombe, 1997; Atkinson et al., 1998). Based on these reports, it seemed likely that amplicon-mediated PTGS could be exploited in host plant–virus combinations other than those with solanaceous species and PVX. To explore this idea, we investigated the phenotype of *PVX* amplicons in Arabidopsis. It was known that PVX is able to replicate in Arabidopsis (T. Dalmay, unpublished data), although it is not able to spread and accumulate systemically. The results presented here confirm that *PVX* amplicon transgenes activate PTGS in Arabidopsis. However, from the analysis of transgene methylation, virus resistance, and the effects of viral suppressors, we have been able to dissect different types of gene silencing, depending on whether the amplicon is present alone or in combination with another homologous transgene. These data indicate the importance of an epigenetic transgene conversion in PTGS and address the roles of RNA thresholds and transgene interactions in PTGS.

RESULTS

PTGS by a *PVX:GFP* Amplicon in Arabidopsis

PVX amplicons are transgene constructs that consistently activate gene silencing in independent lines of tobacco and tomato (Angell and Baulcombe, 1997, 1999). To determine whether *PVX* amplicons also activate PTGS in Arabidopsis, we used the constructs presented schematically in Figure 1. The main elements of these constructs are the cauliflower mosaic virus 35S promoter coupled with the cDNA of the PVX:green fluorescent protein (GFP) vector (35S–*PVX:GFP*). The transcriptional terminator was from the nopaline syn-
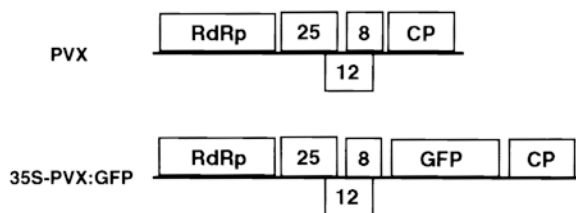
Figure 1. *PVX* and the 35S–*PVX:GFP* Construct.

A schematic but not-to-scale representation of the viral component of the 35S–*PVX:GFP* transgene compared with the genome of unmodified PVX. The *PVX* genes are RdRp, for RNA-dependent RNA polymerase; 25, 12, and 8, the "triple gene block" (encoding proteins of 25, 12, and 8 kD, respectively); and CP, coat protein. In 35S–*PVX:GFP*, the viral cDNA was inserted between the 35S promoter of cauliflower mosaic virus and the terminator sequence of the nopaline synthase gene of the plasmid vector pSLJ755/5.

thase gene, and the construct was incorporated into the T-DNA of an Agrobacterium transformation vector. Nine Arabidopsis transformants carrying this cDNA were generated. DNA gel blot analysis and segregation data (T. Dalmay, unpublished data) revealed that five of these transformants carried a single copy and two carried multiple copies of the transgene. The other two lines carried the *bar* selectable marker gene from the transformation vector, but due to rearrangement of the integrated T-DNA, the 35S–*PVX:GFP* amplicon was not present.

None of the transformed plants carrying the 35S–*PVX:GFP* amplicon exhibited GFP fluorescence, as shown for the Amp243 line in Figures 2A, 2D, 2G, and 2J. In principle, this observation could indicate either that there was PTGS or that the PVX vector replicated at a very low level, if at all, in Arabidopsis. To investigate these possibilities, we crossed the amplicon lines with a line (GFP142) carrying a single copy of a 35S–*GFP* transgene. This line exhibited bright green fluorescence under UV illumination due to *GFP* expression (Figures 2B, 2E, 2H, and 2K). We reasoned that if the phenotype of the plants carrying the 35S–*PVX:GFP* amplicon was due to PTGS, then the 35S–*GFP* transgene would be silenced in the progeny of this cross and there would be little or no GFP fluorescence in these plants. Alternatively, if PVX were not able to replicate in Arabidopsis, the *PVX:GFP* transgene would not cause PTGS, and the progeny would be as fluorescent green as the 35S–*GFP* parent. As a control in these analyses, we also generated plants carrying the 35S–*GFP* transgene combined with a replication defective amplicon (35S–*PVXΔrep:GFP*). The *PVX* cDNA in this construct has a deletion between nucleotides 965 and 2694 in the gene encoding the replication enzyme.

For each of the seven 35S–*PVX:GFP* lines, we made 10 replicate crosses with the 35S–*GFP* plants, and from each cross, 10 progeny were examined for GFP fluorescence. These progeny all exhibited GFP fluorescence that was restricted to the growing points of the shoot (Figures 2C and 2F), flower (Figure 2I), and root (Figure 2L). The other parts of the plant lacked any detectable green fluorescence. We could rule out that the absence of green fluorescence was due to dilution of the 35S–*GFP* transgene, because the progeny of a cross between 35S–*GFP* and nontransgenic plants were fully green fluorescent (data not shown). Moreover, the suppression of the 35S–*GFP* phenotype was dependent on virus replication, because the lines carrying 35S–*GFP* together with the 35S–*PVXΔrep:GFP* transgene were fully green fluorescent (data not shown). Therefore, the *PVX:GFP* amplicon was able to cause silencing of a homologous *GFP* transgene in trans, and as occurred with *PVX* amplicons in tobacco, the silencing was dependent on replication of viral RNA. Also, as occurred with the *PVX* amplicons in tobacco, the silencing phenotype was consistent in independent lines. It is likely that this silencing is dependent on virus replication, because there was no silencing in any of the four lines carrying the 35S–*PVXΔrep:GFP* construct in the GFP background.

Figure 2. GFP Fluorescence in Plants Carrying 35S–*GFP* and 35S–*PVX:GFP* Transgenes.

GFP fluorescence in plants carrying 35S–*GFP* (**[B]**, **[E]**, **[H]**, and **[K]**), 35S–*PVX:GFP* (**[A]**, **[D]**, **[G]**, and **[J]**), or a combination of both transgenes (**[C]**, **[F]**, **[I]**, and **[L]**). The images were produced under UV light in a dissecting microscope, and the red fluorescence is due to chlorophyll. GFP fluorescence appears green, yellow, or blue-green in different tissues and also depends on the photographic exposure time. Nontransformed plants look the same as the Amp243 plants (**[A]**, **[D]**, **[G]**, and **[J]**).

(A) to **(C)** Young seedlings.

(D) to **(F)** Close-up of the growing point of young seedlings.

(G) to **(I)** Immature flowers.

(J) to **(L)** Roots.

There was no detectable fluorescence from the roots of the plants shown in **(A)**, **(D)**, **(G)**, and **(J)**; in the plants shown in **(C)**, **(F)**, **(I)**, and **(L)**, there was GFP fluorescence only in the growing point.

To investigate further the silencing phenotype, we analyzed RNA and transcription in the lines GFP142 (35S–*GFP*), Amp243 (35S–*PVX:GFP*), and the progeny of a cross between these lines. These progeny are referred to hereafter as GFP142 × Amp243. Figure 3 shows the result of RNA gel blot analysis of the respective RNA samples and indicates, as expected, that *GFP* mRNA levels were substantially higher in line GFP142 (Figure 3, lanes 5 to 8) than in GFP142 × Amp243 (Figure 3, lanes 9 to 12). We conclude from these results that the *PVX:GFP* amplicon from Amp243 was able to silence the *GFP* transgene from GFP142.

Figure 3 also shows that in Amp243, the *PVX:GFP* RNA was present as both genomic and subgenomic species (Figure 3, lanes 1 to 4). The subgenomic RNAs would have been produced by the PVX replicase, using negative strand RNA as a template. Thus, the presence of subgenomic RNAs confirms that PVX is able to replicate in Arabidopsis. We obtained further confirmation of this replication by demonstrating that infectious PVX could be isolated from Amp243 and other lines carrying the 35S–*PVX:GFP* construct (data not shown).

From the RNA gel blot, we found, surprisingly, that the *PVX* RNAs were present at a lower level in the GFP142 × Amp243 progeny than in the Amp243 lines (Figure 3, lanes 9 to 12). One interpretation of this result is that the activation of gene silencing required both types of transgene. In this scenario, the absence of GFP fluorescence in Amp243 would be due to poor replication of the *PVX:GFP* RNA rather than gene silencing. A second possibility is that a low level of PTGS in Amp243 was enhanced by the 35S–*GFP* transgene in the GFP142 × Amp243 line. To resolve these alternatives, it was first necessary to determine whether the silencing mechanism in the GFP142 × Amp243 line was transcriptional or post-transcriptional.

To distinguish between transcriptional or PTGS in the GFP142 × Amp243 plants, we used transcription run-off analysis. The data, shown in Figure 4, reveal that the high-level transcription of *GFP* in GFP142 × Amp243 and GFP142 plants does not reflect the marked differential accumulation of *GFP* mRNA in these lines. Therefore, these data indicate that there is silencing of *GFP* at the post-transcriptional level. PTGS was also implicated by the similar levels of PVX run-off transcripts in the Amp243 and GFP142 × Amp243 lines, despite the different levels of *PVX:GFP* RNA (Figure 3).

The transcription analyses included three controls. First, *GUS* DNA was used as negative control. Second, to validate the comparisons of transgene transcription, we showed that ubiquitin transcription was similar in extracts from the different lines. Third, we showed that transcription was almost completely eliminated by the inclusion of actinomycin D as an inhibitor of DNA-dependent RNA polymerase in the transcription run-off analysis (data not shown). In the absence of this third control, it remained possible that incorporation of labeled nucleotide in the run-off assay was due to the RNA-dependent RNA polymerase encoded in the *PVX:GFP* of the amplicon constructs.
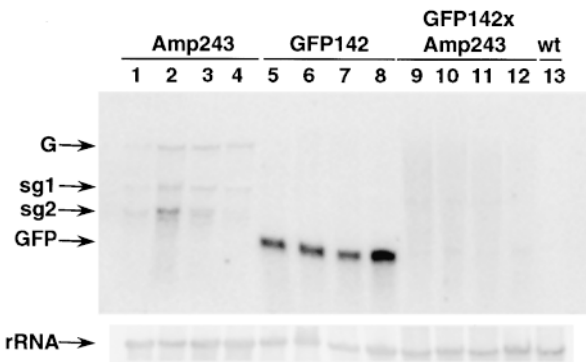
Figure 3. *GFP* and *PVX:GFP* RNA Accumulation.

RNA was isolated from leaves of wild-type plants (wt; lane 13) and lines carrying 35S–*PVX:GFP* (Amp243; lanes 1 to 4), 35S–*GFP* (GFP142; lanes 5 to 8), or a combination of both transgenes (GFP142 × Amp243; lanes 9 to 12). RNA gel blot analysis was conducted using ^{32}P-labeled probes corresponding to the full-length *GFP* sequence (top) and rRNA (bottom). The RNA species detected were the genomic (G) and subgenomic (sg) RNAs of *PVX:GFP*, *GFP* mRNA (GFP), and rRNA.

We noticed that PVX gave a stronger signal than did GFP in these run-off assays with the Amp243 lines (Figure 3). In part, the differential representation may be because the transcripts were hybridized with *PVX* DNA that was longer than the *GFP* DNA. It is likely also that there was premature termination of transcription so that the GFP transcripts from the 3′ end of the transgene would have been underrepresented in the final run-off product.

Virus Infection of Arabidopsis Plants Exhibiting PTGS

In previous analyses, we have used viruses as a tool to dissect the mechanism of PTGS. For example, we have shown that viruses encode suppressors of PTGS and that these suppressors can be used to dissect the underlying mechanism (Brigneti et al., 1998; Voinnet et al., 1999). We have also shown that viruses can be used to report PTGS, provided that there is sequence similarity in the virus and the PTGS transgene (English et al., 1996).

Here, we exploited a viral suppressor of PTGS to determine whether the low level of *GFP* in Amp243 plants was due to PTGS or to inefficient replication of PVX. If there had been inefficient replication, the introduction of a suppressor of PTGS into these plants would not have affected *GFP* expression from the *PVX:GFP* amplicon. However, if there had been PTGS, PVX:GFP accumulation and consequent *GFP* expression would have increased in the presence of the viral suppressor.

The source of the suppressor of PTGS was turnip mosaic virus (TuMV; potyvirus group), which is highly infectious on

Arabidopsis. The TuMV-encoded helper component protease (HC-PRO) is functionally similar to homologous proteins encoded in the genomes of potato virus Y and tobacco etch virus that have been previously characterized as suppressors of PTGS (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998).

Two weeks after TuMV inoculation of Amp243 plants, the systemically infected leaves exhibited uniform green fluorescence, as shown in Figure 5F. The increase in GFP fluorescence was similar but more pronounced on GFP142 × Amp243 plants (Figure 5H), whereas on the mock inoculated or infected wild-type (Figures 5A and 5B) and the mock-inoculated Amp243 or GFP142 × Amp243 plants (Figures 5E and 5G), there was no green fluorescence. TuMV had no effect on the fluorescence of the 35S–*GFP* plants (Figures 5C and 5D).
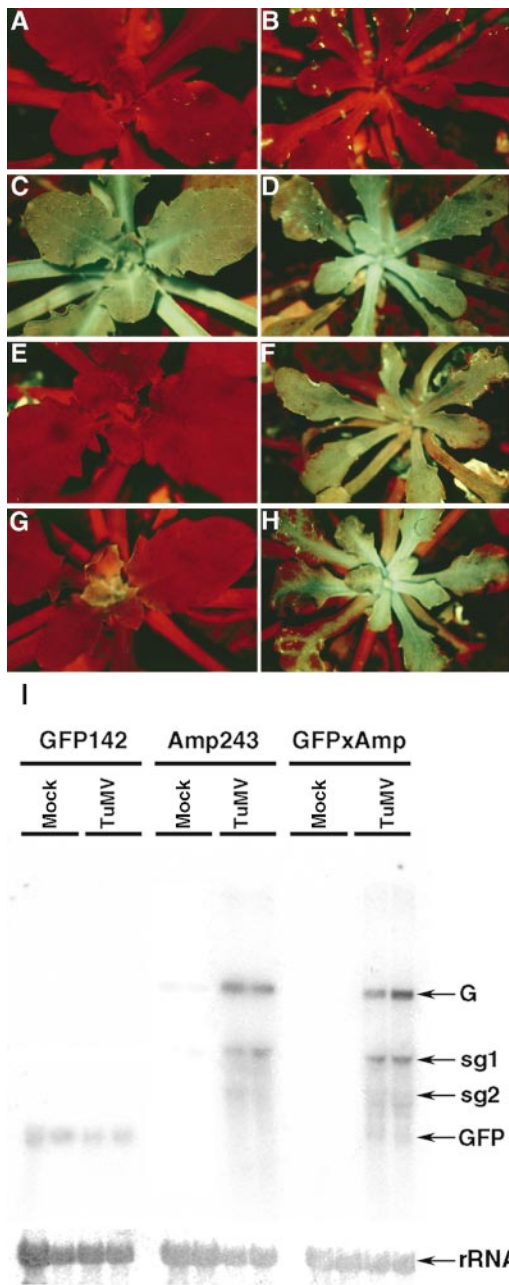
To rule out the possibility that TuMV might increase transcription from the 35S promoter, we analyzed the RNA from infected and noninfected plants. Figure 5I shows the result of RNA gel blot analysis of the respective RNA samples and indicates that TuMV did not increase the level of *GFP* RNA in the nonsilenced GFP142 plants. However, consistent with the fluorescence data, there was a substantial increase in the *PVX:GFP* RNA in the Amp243 plants and of *PVX:GFP* and *GFP* RNA in the GFP142 × Amp243 plants. From these data, we conclude that the TuMV-mediated increase of GFP in GFP142 × Amp243 plants and Amp243 plants is due to suppression of PTGS.

Tobacco and tomato exhibiting PTGS are resistant to viruses that share sequence similarity with the transgene (English et al., 1996). To determine whether PTGS in GFP142 × Amp243 and Amp243 plants can also target viral RNA, we inoculated these plants with tobacco rattle virus (TRV) carrying the *GFP* sequence (TRV:GFP) (Ratcliff et al., 1999). Figure 6 illustrates that the nontransformed plants and GFP142 plants showed green fluorescent spots on the inoculated leaves a few days after inoculation (Figures 6A
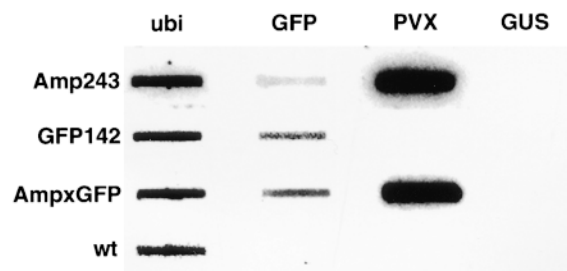
Figure 4. Nuclear Run-Off Analysis of Transgene Transcription.

Ubiquitin (ubi), *GFP*, *PVX*, and *GUS* DNA was fixed onto nitrocellulose membranes that were hybridized with the ^{32}P-labeled in vitro–labeled transcripts of isolated nuclei from lines carrying 35S–*GFP* (GFP142), 35S–*PVX:GFP* (Amp243), or a combination of both transgenes (AmpxGFP). wt, wild type.
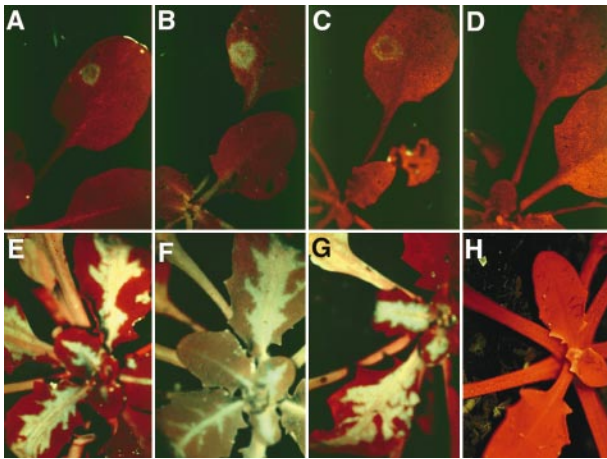
and 6B) and very strong green fluorescence on the systemically infected leaves a week later (Figures 6E and 6F). In contrast, there was no green fluorescence on GFP142 × Amp243 plants (Figures 6D and 6H). These data show that *TRV:GFP* is a potential target of PTGS. However, on the Amp243 plants, despite the PTGS, the appearance of GFP fluorescence indicated that TRV:GFP had accumulated and spread as rapidly as it did on the nontransgenic plants (Figures 6C and 6G). Thus, the data from TuMV infection (Figure 5), *PVX:GFP* RNA analyses (Figure 3), and TRV:GFP susceptibility (Figure 6) collectively indicate that the Amp243 plants exhibit PTGS but that it is weaker than in GFP142 × Amp243 plants.

The 25-Nucleotide RNA

Recently, we reported the presence of a 25-nucleotide-long RNA species in tobacco and tomato plants exhibiting PTGS (Hamilton and Baulcombe, 1999). These short RNAs hybridized with both the plus and minus strands of the PTGS target sequences and were not found in tissues from nonsilenced tomato and tobacco. To further characterize PTGS in the Amp243 and GFP142 × Amp243 plants, we assayed for the short RNAs by RNA gel blotting of samples of low molecular weight RNAs. Figure 7, lane 3, shows that there was no 25-nucleotide *GFP* RNA in the GFP142 plants. However, there was a faint signal in samples from the Amp243 plants (Figure 7, lane 2) and a very strong signal with the sample from GFP142 × Amp243 plants (Figure 7, lane 4). The 25-nucleotide RNAs were detected at a low level in three independent experiments with Amp243. These data confirm that 25-nucleotide RNAs are associated with PTGS in Arabidopsis. Moreover, from the level of these RNAs, there is further evidence that weak PTGS in the Amp243 plants was increased by the addition of the 35S–*GFP* transgene from GFP142.

Although these analyses of the 25-nucleotide RNAs were conducted with *GFP* probes, it also would be expected that there would be short RNA species corresponding to the PVX component of the *PVX:GFP* amplicon. To test this prediction, RNA gel blot analysis of the short RNA was also performed with a *PVX* probe. Surprisingly, and in contrast to the results with the *GFP* probe, the 25-nucleotide RNAs from the Amp243 and GFP142 × Amp243 plants gave equal signals (data not shown). These signals were substantially weaker than those detected with the *GFP* probe. From these data, we conclude that the 25-nucleotide *GFP* RNAs from GFP142 × Amp243 plants were derived mainly from the 35S–*GFP* transgene rather than from the *PVX:GFP* amplicon.

Figure 5. Effect of TuMV Infection on GFP Fluorescence.

(A) to **(H)** Nontransformed (**[A]** and **[B]**), GFP142 (**[C]** and **[D]**), Amp243 (**[E]** and **[F]**), and GFP142 × Amp243 (**[G]** and **[H]**) plants were inoculated with water (**[A]**, **[C]**, **[E]**, and **[G]**) or TuMV (**[B]**, **[D]**, **[F]**, and **[H]**). GFP fluorescence was assessed in the plants under UV light in a dissecting microscope, and the photographs were taken at 14 days after inoculation.
(I) RNA was isolated from the top three or four leaves of mock- or TuMV-inoculated GFP142, Amp243, and GFP142 × Amp243 plants. RNA gel blot analysis was performed using ³²P-labeled probes corresponding to the full-length *GFP* sequence (top) and rRNA (bottom). The RNA species detected were the genomic (G) and subgenomic (sg) RNAs of PVX:*GFP*, *GFP* mRNA (GFP), and rRNA.

Figure 6. GFP Fluorescence Due to TRV:GFP.

(A) and **(E)** Nontransformed plants.
(B) and **(F)** GFP142.
(C) and **(G)** Amp243.
(D) and **(H)** GFP142 × Amp243.
Plants were inoculated with TRV:GFP. GFP fluorescence was assessed in the plants under UV light in a dissecting microscope, and photographs of the inoculated (**[A]** to **[D]**) and systemically infected (**[E]** to **[H]**) leaves were taken at 4 and 11 days after inoculation, respectively.

Transgene Methylation

We previously presented a model of PTGS in which homology-dependent RNA–DNA interactions led to sequence-specific methylation of the participating DNA (Jones et al., 1999). To determine whether these processes could be implicated in the amplicon-mediated PTGS of *GFP* in Arabidopsis, we conducted DNA gel blot analysis with *GFP* transgenes using methylation-sensitive restriction endonucleases and with a *GFP*-specific probe. Figure 8A shows the predicted sizes of the *GFP*-specific HaeIII restriction fragments from 35S–*GFP* reporter and the 35S–*PVX:GFP* amplicon. The data shown in Figure 8B for HaeIII-digested DNA demonstrate that the 35S–*GFP* and 35S–*PVX:GFP* transgenes were not methylated in the GFP142 (Figure 8B, lane 1) and Amp243 plants (Figure 8B, lane 2) but were highly methylated in GFP142 × Amp243 plants (Figure 8B, lane 3). Similar data were obtained with AluI-digested DNA (data not shown). In contrast, when the *GFP* probe was removed and the filter was reprobed for the phytoene desaturase gene (data not shown), the three samples were all digested to the same extent, allowing us to rule out the possibility that there was incomplete digestion of the DNA.

With *GFP* probes, it was not possible to determine whether the methylated DNA in the GFP142 × Amp243 plants was the amplicon or the 35S–*GFP* transgene. However, by using *PVX* probes from the regions flanking *GFP* in

the amplicon, this distinction could be made. It was apparent that the *GFP* sequence in the amplicon was methylated in GFP142 × Amp243 plants (data not shown). In contrast, by using a *PVX* probe from the 5′ end of the viral sequence, it was shown that the *PVX* sequence was not methylated at the 5′ end region in either the Amp243 or GFP142 × Amp243 plants (data not shown). From these results, we conclude that amplicons mediate the homology-dependent transgene methylation that is characteristic of other examples of PTGS. However, the amplicon mediated methylation only in the presence of a 35S–*GFP* transgene, and methylation was restricted to *GFP* sequences. These results indicate that the *PVX:GFP* amplicon could not mediate methylation autonomously.

DISCUSSION

We have shown here that the *PVX* amplicon can mediate gene silencing in Arabidopsis. We have also confirmed that, as in other examples of PTGS, 25-nucleotide RNAs and sequence-specific methylation of DNA are associated with the presence of the amplicon. Our results indicate the potential utility of viral amplicons as a general strategy for gene silencing in transgenic plants, because in Arabidopsis, as in tobacco and tomato, PTGS was observed in all lines tested. However, in Arabidopsis, a nonhost of PVX, amplicon-mediated silencing was weaker than it was in host plants of PVX (Angell and Baulcombe, 1997, 1999). This nonhost effect most likely explains why *PVX* amplicons provoke weak silencing of endogenous genes in Arabidopsis (T. Dalmay, S. Angell, and D.C. Baulcombe, unpublished data). To produce strong gene silencing in Arabidopsis, it will be necessary to generate amplicons based on viruses for which Arabidopsis is a host.
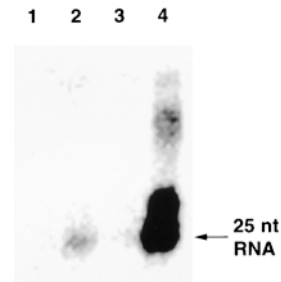
Figure 7. RNA Gel Blot Analysis of Low Molecular Weight RNAs.

Low molecular weight RNA was isolated from leaves of wild-type C24 plants (lane 1), lines carrying 35S–*GFP* (GFP142; lane 3), 35S–*PVX:GFP* (Amp243; lane 2), or a combination of both transgenes (GFP142 × Amp243; lane 4). RNA gel blot analysis was conducted using ^{32}P-labeled probes corresponding to the full-length *GFP* sequence. The ~27-nucleotide (nt) band visible in lane 4 was not detected when the experiment was repeated.

A

B
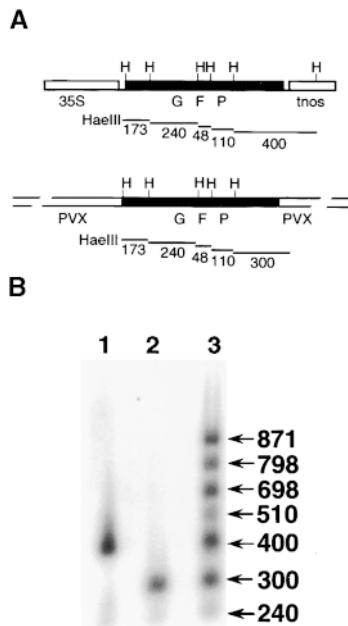
Figure 8. Differences in Transgene Methylation Associated with PTGS.

(A) Structure of the *GFP* transgene in 35S–*GFP* and 35S–*PVX:GFP*. Features shown are the 35S promoter (35S), *GFP* coding sequence (black box; GFP), nopaline synthase terminator (tnos), and the PVX sequence flanking GFP in 35S–*PVX:GFP*. The restriction sites for HaeIII (H) and the lengths (in base pairs) of the expected digestion products are shown.

(B) Gel blot analysis with DNA from leaves of GFP142 (35S–*PVX:GFP*; lane 1), Amp243 (35S–*PVX:GFP*; lane 2), and GFP142 × Amp243 (lane 3) plants using a *GFP*-coding sequence probe. The numbers at right indicate the estimated length (in base pairs) of the hybridizing DNA fragments.

Transgene Interactions Mediated by RNA

Although the PTGS induced by the *PVX:GFP* amplicon was weak in Arabidopsis, it has characteristics that are informative about the mechanism of gene silencing. One of these characteristics is the enhanced PTGS in lines carrying *PVX:GFP* together with a 35S–*GFP* transgene. In principle, this effect could be a consequence of transgene dosage. There are many examples in which PTGS is influenced by the number of homologous transgenes in a genome (Mueller et al., 1995; Goodwin et al., 1996). However, it is unlikely that this explanation applies to the Arabidopsis lines carrying 35S–*GFP* and 35S–*PVX:GFP* transgenes because other lines with two *GFP* transgenes, for example, GFP142 and Amp243 with homozygous transgenes, did not exhibit strong PTGS. A more plausible explanation is based on a homology-dependent interaction between the 35S–*GFP* and 35S–*PVX:GFP* transgenes in GFP142 and Amp243.

It is unlikely that this interaction would operate at the DNA level because there was no gene silencing of the 35S–*GFP*

transgene in plants carrying a 35S–*PVXΔrep:GFP* transgene (data not shown). An alternative and more likely scenario is indicated by the methylation of the *GFP* transgenes in GFP142 × Amp243 plants. In several other experimental systems (Wassenegger et al., 1994; Jones et al., 1998b, 1999; Guo et al., 1999), transgene methylation is an indicator of an RNA–DNA interaction. Thus, the replicating RNA of the 35S–*PVX:GFP* transgenes, either directly or indirectly, could have participated in an interaction with the 35S–*GFP* transgene in GFP142 × Amp243.

Initiation and Maintenance of PTGS

When PVX:GFP was inoculated to *Nicotiana benthamiana* expressing 35S–*GFP*, there was virus-induced silencing of the transgene (Ruiz et al., 1998). Analysis of this manifestation of PTGS revealed that there are separate initiation and maintenance stages. Initiation PTGS is dependent on the presence of the replicating *PVX:GFP*. The maintenance stage follows from RNA–DNA interactions involving the *GFP* sequences and is associated with methylation of the *GFP* DNA. Maintenance PTGS is also associated with elimination of the viral RNA from the inoculated plant. To account for the persistence of PTGS in the absence of the viral RNA, we have suggested that there is an epigenetic conversion of the *GFP* transgene. In the maintenance stage, the epigenetically converted transgene rather than the PVX:GFP would be the mediator of PTGS (Jones et al., 1999).

In these terms, the phenotype of Amp243 represents initiation, as shown schematically in Figures 9A and 9B. The *PVX:GFP* transgene is not methylated in Amp243 (Figure 8B, lane 2), indicating that it has not participated in the RNA–DNA interaction or undergone the epigenetic conversion that is characteristic of the maintenance stage (Jones et al., 1999). In Amp243, PTGS is considered weak because of the low abundance of the 25-nucleotide RNA (Figure 7, lane 2) and the accumulation of *PVX:GFP* RNA (Figure 3, lanes 1 to 4). The absence of resistance against TRV:GFP (Figures 6C and 6G) is also an indicator of weak PTGS. We speculate that PTGS in Amp243 is weak because PVX replicates inefficiently in the nonhost Arabidopsis.

In the GFP142 × Amp243 plants, by comparison with the analysis of virus-induced gene silencing, it seems likely that the PTGS is at the maintenance stage. The transgenes are methylated (Figure 8B, lane 3), and the replicating amplicon RNA has been eliminated (Figure 3). Accordingly, the transgenes in GFP142 × Amp243 plants would have participated in an RNA–DNA interaction (Figure 9C) and undergone the epigenetic conversion (Figure 9D) typical of the maintenance stage of PTGS (Jones et al., 1999). Although initiation of PTGS in GFP142 × Amp243 plants would have involved replication of the amplicon RNA (Figure 9C), in the mature plant the amplicon RNA was eliminated, and PTGS would have been mediated by the 35S–*GFP* transgene (Figure 9E).

The epigenetic conversion in GFP142 × Amp243 plants
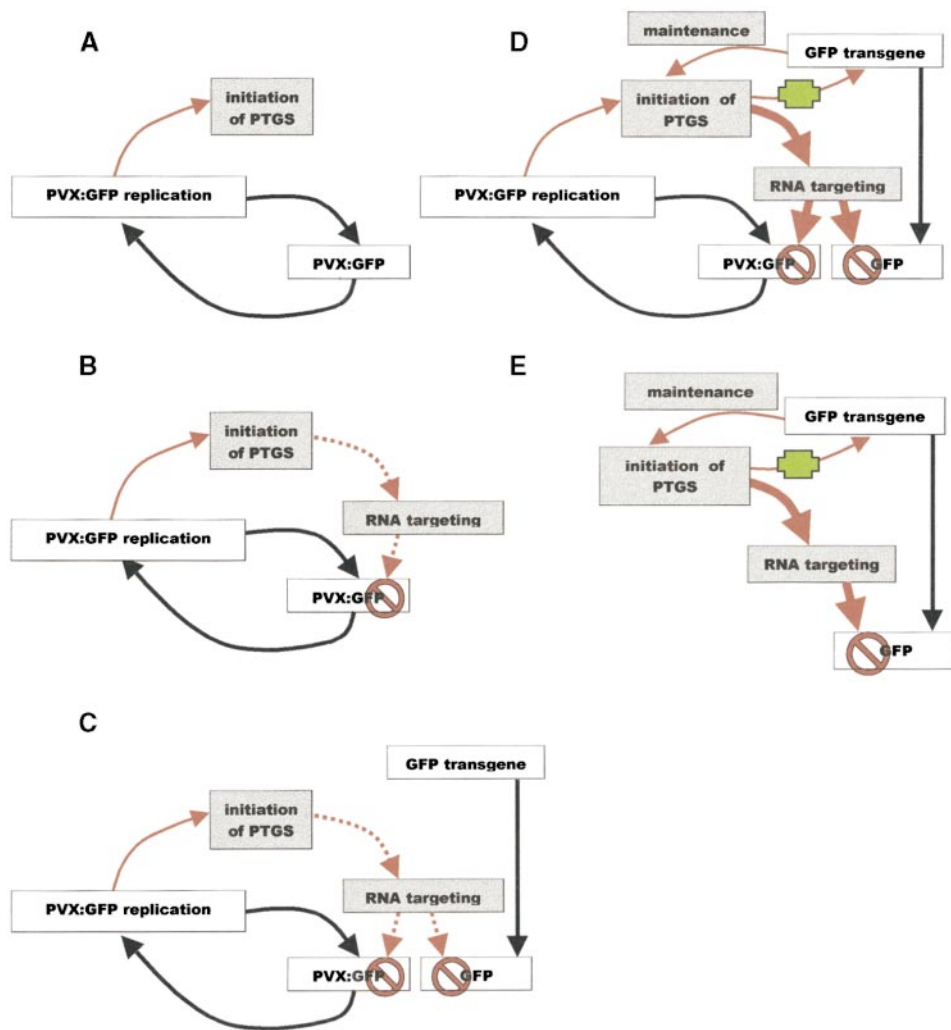
Figure 9. Different Stages of PTGS.

(A) The early initiation stage of PTGS in Arabidopsis cells carrying a *PVX:GFP* amplicon transgene. PVX:GFP replication leads to the accumulation of *PVX:GFP* RNA and initiates PTGS.

(B) The late initiation stage of PTGS in Arabidopsis cells carrying a *PVX:GFP* amplicon transgene. The initiation of PTGS induces an RNA targeting mechanism that constrains accumulation of *PVX:GFP* RNA. The PTGS depends on the continued presence of viral RNA so that there is feedback regulation that prevents the RNA targeting mechanism from eliminating the viral RNA from the cell. This stage represents the PTGS in mature cells of Amp243.

(C) The late initiation stage of PTGS in Arabidopsis cells carrying a *PVX:GFP* amplicon transgene and a *GFP* reporter gene. The situation is as shown in **(B)** except that the RNA targeting mechanism affects the *GFP* mRNA as well as the *PVX:GFP* RNA. There is also an RNA–DNA interaction affecting the *GFP* reporter gene. At this stage, there has not been an epigenetic conversion of the *GFP* reporter gene.

(D) The early maintenance stage of PTGS in Arabidopsis cells carrying a *PVX:GFP* amplicon transgene and a *GFP* reporter gene. The RNA–DNA interaction leads to an epigenetic conversion so that the PTGS is mediated by the *GFP* reporter gene as well as by the *PVX:GFP* RNA of the amplicon. The thick red arrows replacing the dotted arrows reflect the presence of two sources of PTGS resulting in stronger silencing than in **(C)**.

(E) The late maintenance stage of PTGS in Arabidopsis cells carrying a *PVX:GFP* amplicon transgene and a *GFP* reporter gene. The strong PTGS shown in **(C)** results eventually in the complete elimination of *PVX:GFP* RNA from the cell. However, the PTGS persists due to the epigenetic conversion of the *GFP* transgene.

The red lines indicate processes involved in PTGS, the black lines indicate RNA synthesis, and the green cross indicates the RNA–DNA interaction that is proposed to initiate the epigenetic conversion of the transgene. The red "stop" sign indicates RNA degradation.

would explain why the PTGS was stronger than in Amp243 plants: the PTGS would have been determined by the 35S–*GFP* transgene rather than the inefficiently replicating *PVX:GFP* RNA. This stronger gene silencing would then account for the elimination of both *PVX:GFP* and *GFP* RNA (Figure 3, lanes 9 to 12), the resistance against TRV:GFP (Figures 6D and 6H), and the high level of the 25-nucleotide *GFP* RNA (Figure 7, lane 4) in the GFP142 × Amp243 plants. At the stage at which *PVX:GFP* RNA had been eliminated (Figure 9E), the PTGS would be maintained by the epigenetically converted 35S–*GFP* transgene.

We had previously suggested that the HC-PRO suppressors of PTGS in potyviruses act at the maintenance rather than the initiation stage of PTGS (Brigneti et al., 1998). However, the symptoms of TuMV in Amp243 are not consistent with this interpretation because there was suppression of PTGS in Amp243 plants (Figure 5) in which, based on the absence of transgene methylation, we suggest that the maintenance stage is not active (Figures 9B and 9D). To reconcile the interpretations of these data, we now propose that HC-PRO interferes with an RNA targeting mechanism that is part of both initiation and maintenance stages of PTGS (Figure 9). Our previous data, showing that HC-PRO does not prevent DNA methylation associated with PTGS, are consistent with this interpretation (Jones et al., 1999).

Genetic and Epigenetic PTGS

As discussed above, PTGS in GFP142 × Amp243 plants is epigenetic, whereas in Amp243, the ability of the 35S–*PVX:GFP* locus to mediate PTGS is associated with virus replication and is genetically determined. We propose here that this distinction between genetic and epigenetic silencing allows many of the experimental observations about PTGS to be reconciled in variations of the conceptual model presented in Figure 9.

The most obvious examples of epigenetic PTGS are those in which the process is initiated by viruses and in which the virus is eliminated although the silencing persists (Lindbo et al., 1993; Ruiz et al., 1998; Jones et al., 1999). However, there are other examples in which viruses are not involved that may also be epigenetic. For example, there are reports of PTGS that was previously explained in terms of ectopic interactions between homologous transgene loci (Baulcombe and English, 1996). These examples include those in which PTGS follows from increasing the transgene dosage (Mueller et al., 1995; Goodwin et al., 1996). To account for these examples, it was proposed that PTGS requires two transgene functions referred to as silencer and receptor. PTGS would only ensue if silencer and receptor loci were present in the same background or if a single locus could perform both functions. In the present work, the 35S–*PVX:GFP* transgene has the characteristics of the silencer function (Baulcombe and English, 1996), and the 35S–*GFP* transgene, with its propensity to undergo epigenetic conversion, has the predicted attributes of the receptor locus.

It was originally suggested that the ectopic interaction would involve homologous DNAs (Baulcombe and English, 1996). However, in the example described here, the interaction between the *PVX:GFP* and *GFP* transgenes is most likely mediated by RNA because replication-defective amplicons did not cause the epigenetic conversion of 35S–*GFP* or activate PTGS.

Meiotically reversible PTGS (Elmayan and Vaucheret, 1995) and examples in which there is a correlation of transgene methylation with PTGS (Ingelbrecht et al., 1994; English et al., 1996) are likely to have an epigenetic component. It is also possible that delayed or developmentally regulated PTGS involves an epigenetic transgene conversion (Hart et al., 1992; Palauqui and Vaucheret, 1995). In these examples, the epigenetic conversion may be associated with systemic spread of a silencing signal (Palauqui et al., 1997; Voinnet and Baulcombe, 1997; Palauqui and Vaucheret, 1998; Voinnet et al., 1998; Jones et al., 1999).

In contrast, there are likely to be other examples, such as Amp243, in which the PTGS is genetically determined and in which there is no epigenetic component to the mechanism. Candidates for genetic PTGS include the examples with inverted repeat transgenes (Stam et al., 1998). Antisense transgenes may also initiate PTGS without an epigenetic component (Delange et al., 1995). A striking feature of these proposed examples of genetic PTGS is the potential to form double-stranded RNA: with amplicons, such structures are formed as part of the viral replication intermediate; and with inverted repeat transgenes, they could result from intramolecular interactions of complementary RNA. The double-stranded RNA could be formed with antisense transgenes as a result of intermolecular interactions between the sense and corresponding antisense RNAs. Perhaps, then, the initiator of genetic PTGS is double-stranded RNA, as it is in RNA interference in nematodes and other organisms (Sharp, 1999). In epigenetic silencing, the potential for double-stranded RNA formation is less obvious, and it seems likely that initiation may involve forms of aberrant RNA that are not necessarily double stranded.

METHODS

Transgenic Plants

The green fluorescent protein (GFP) insert in pPVX204 (Baulcombe et al., 1995) was replaced with the mGFP5 insert from pBin-35S-mGFP5 (Haseloff et al., 1997). This new potato virus X (PVX)–GFP5 sequence was inserted between the cauliflower mosaic virus 35S promoter and the transcriptional terminator of the nopaline synthase gene and transferred into the binary vector pSLJ755/5. The *PVX-Δrep:GFP* construct was obtained by deleting a BglII fragment (positions 965 to 2694) from the full-length PVX:GFP. pBin-35S-mGFP5 (Haseloff et al., 1997) was used without any change. Transformation of *Arabidopsis thaliana* ecotype C24 with *Agrobacterium tumefaciens* GV3101 carrying 35S–*PVX:GFP* or 35S–*GFP* constructs was

performed as described by Bechtold et al. (1993). The transformants were selected in vitro on medium supplemented with 10 mg/L L-phosphinotricin (Melford, Ipswich, UK) or 50 mg/L kanamycin, respectively.

GFP Imaging

GFP expression was monitored by using an MZ12 dissecting microscope (Leica, Heidelberg, Germany) coupled to an epifluorescence module. Photographs were taken using Kodak Ektachrome Panther (400 ASA) film.

RNA Gel Blot and Nuclear Run-Off Transcription Analyses

RNA gel blot analysis was performed as described previously (Mueller et al., 1995). Polymerase chain reaction–amplified full-length *GFP* DNA fragments were labeled by random-primed incorporation of ^{32}P-dCTP. After hybridization, the signal present in the membranes was analyzed and quantified using Fujix Bio-Imaging Analyzer Bas 1000 (Fuji Photo Film Co., Fuji, Japan) equipment.

Nuclei were isolated as described by Covey et al. (1997), and incorporation of uridine $5'\alpha$-^{32}P-triphosphate (Amersham) was determined by probing 1 μg of the appropriate DNA samples immobilized as slots on Hybond-N+ membranes (Amersham). Incorporation was assessed by using a Fujix Bio-Imaging Analyzer BAS 1000 (Fuji Photo Film Co.), as described by Mueller et al. (1995).

The 25-nucleotide RNAs were extracted and probed as described previously (Hamilton and Baulcombe, 1999). Total RNA was extracted by using an SDS-based extraction solution (Hamilton and Baulcombe, 1999) and precipitated with ethanol. The short RNAs do not bind to silica-based columns such as the RNAeasy columns (Qiagen). The pellet was dissolved in water, heated to 65°C for 5 min, and then placed on ice. Polyethylene glycol (molecular weight 8000; Sigma) was added to a final concentration of 5% and NaCl to a final concentration of 0.5 M. After a 30-min incubation on ice, the RNA was centrifuged at 10,000*g* for 10 min. Three volumes of ethanol were added to the supernatant, and the RNA was precipitated at −20°C for at least 2 hr. The low molecular weight RNAs were pelleted by centrifugation for 10 min at 10,000*g*. The pellet was dissolved in formamide and heated to 65°C for 5 min, and a one-third volume of 4 × loading solution was added (2 × TBE [1 × TBE is 0.09 M Tris-borate, pH 8.0, and 0.002 M EDTA], 40% sucrose, and 0.1% bromophenol blue) before loading on 15% polyacrylamide (19:1) gels containing 7 M urea in 0.5 × TBE. The samples were run until the bromophenol blue reached the bottom of the gel, then blotted to HybondNX (Amersham) membrane, and UV cross-linked. The membrane was prehybridized in 50% formamide, 7% SDS, 50 mM NaHPO$_4$/NaH$_2$PO$_4$, pH 7.0, 0.3 M NaCl, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), and 100 μg/mL sheared, denatured salmon sperm DNA at 40°C for at least 1 hr. Single-stranded ^{32}P-labeled RNA probes were transcribed in vitro from plasmid templates as described by Mueller et al. (1995). The labeled RNA was hydrolyzed by adding 300 μL of 200 mM carbonate buffer (80 mM NaHCO$_3$ and 120 mM Na$_2$CO$_3$) to a 20-μL probe and incubating at 60°C for 2 to 3 hr. Twenty microliters of 3 M NaOAc, pH 5.0, was added to the 320-μL hydrolyzed probe. The probe was then added to fresh hybridization solution. The hybridization was performed at 40°C overnight, and the membrane was subsequently washed at 50°C in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.2% SDS.

DNA Gel Blot Analysis

Genomic DNA was extracted from flowers or leaves, and gel blot analysis was performed as described previously (Jones et al., 1999). ^{32}P-labeled DNA probes corresponded to the entire 812 nucleotides of *GFP5*, a 5′ end sequence of *PVX* (positions 543 to 1528), a downstream viral flanking sequence to the GFP insert in *PVX-GFP* (positions 6469 to 7207), or a 5′ 500-bp cDNA fragment of the Arabidopsis gene encoding phytoene desaturase.

Wild-Type and Recombinant Viruses

A full-length cDNA clone of turnip mosaic virus (TuMV) (Sanchez et al., 1998) was obtained from Dr. F. Ponz (Instituto Nacional de Investigaciones Agrarias, Madrid, Spain). Sap of *Nicotiana clevelandii* plants inoculated with the cDNA was used to infect Arabidopsis plants. The tobacco rattle virus (TRV)-GFP was described previously (Ratcliff et al., 1999).

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