

RESEARCH ARTICLE

# RNA–DNA Interactions and DNA Methylation in Post-Transcriptional Gene Silencing

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Post-transcriptional gene silencing (PTGS) is a homology-dependent process that reduces cytoplasmic RNA levels. In several experimental systems, there is also an association of PTGS with methylation of DNA. To investigate this association, we used plants carrying a transgene encoding the green fluorescent protein (GFP). Gene silencing was induced using potato virus X RNA vectors carrying parts of the coding sequence or the promoter of the *GFP* transgene. In each instance, homology-based, RNA-directed methylation was associated with silencing. When the *GFP*-transcribed region was targeted, PTGS affected both transgene and viral RNA levels. When methylation was targeted to a promoter region, transgene RNA levels were reduced; however, viral RNA levels were unaffected. For comparison, we induced PTGS of the gene encoding the endogenous ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) small subunit (*rbcS*) by inoculation with potato virus X-*rbcS*. In this example, no methylation of the *rbcS* DNA was associated with the reduction in *rbcS* transcript levels, and viral RNA levels were unaffected. Finally, we investigated DNA methylation by using *GFP*-transformed plants in which PTGS was induced by localized introduction of a T-DNA carrying *GFP* sequences. In these plants, there was methylation of a *GFP* transgene associated with systemic spread of a gene-silencing signal from the infiltrated part of the plant. This transgene methylation was not affected when systemic PTGS was blocked by suppressors of silencing encoded by potato virus Y and cucumber mosaic virus. Combined, these data support an epigenetic model of PTGS in which transgene methylation is associated with an RNA–DNA interaction that ensures that PTGS is maintained.

## INTRODUCTION

Post-transcriptional gene silencing (PTGS) is a genetic control mechanism that operates at the level of sequence-specific RNA degradation and acts against transgenes, endogenous genes, and viruses (reviewed in Depicker and Van Montagu, 1997; Stam et al., 1997a; van den Boogaart et al., 1998). Although PTGS originally was identified in plants, recent evidence suggests that a similar mechanism exists in animals (Fire et al., 1998; Kennerdell and Carthew, 1998).

In transgenic plants, particularly in lines that carry multiple transgene copies, PTGS may be initiated spontaneously (Hobbs et al., 1993; Goodwin et al., 1996; Stam et al., 1997b). PTGS also can be triggered by viruses carry-

ing homology to nuclear sequences (Lindbo et al., 1993; Kumagai et al., 1995; Jones et al., 1998a; Kjemtrup et al., 1998; Ruiz et al., 1998), by using biolistic (Voinnet et al., 1998; Palauqui and Balzergue, 1999) or Agrobacterium-mediated delivery of DNA (Voinnet and Baulcombe, 1997). These observations have led to proposals that ectopic interactions between homologous nucleic acids lead to the onset of PTGS (Baulcombe and English, 1996; Selker, 1999).

Several studies have indicated an association between PTGS and coding region methylation in plants (Ingelbrecht et al., 1994; English et al., 1996; Sijen et al., 1996; van Houdt et al., 1997). Indeed, methylation may play a role in controlling gene expression in many organisms (reviewed in Martienssen and Richards, 1995; Selker, 1997; Finnegan et al., 1998) and may affect transcript accumulation through the action of methylcytosine binding proteins (Kass et al., 1997) and/or by altering chromatin structure

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(Razin, 1998). Based on these observations, a model for PTGS in plants was proposed whereby DNA methylation in transcribed regions causes production of aberrant transcripts. According to this model, the aberrant transcripts would be implicated in sequence-specific RNA degradation, which is integral to the mechanism of PTGS (English et al., 1996).

Transgene methylation also has been implicated in PTGS induced by viruses. In a previous study, virus-induced gene silencing (VIGS) initiated by an RNA virus, pea seed-borne mosaic virus (PSbMV), was associated with de novo methylation of homologous nuclear DNA sequences (Jones et al., 1998b). Similarly, viroid RNA also has the ability to direct methylation (Wasseneger et al., 1994; Pélissier et al., 1999). Together, these studies suggest that methylation associated with PTGS may have resulted from a direct interaction between homologous RNA and DNA sequences, although it was not clear whether DNA methylation was a cause or a consequence of PTGS.

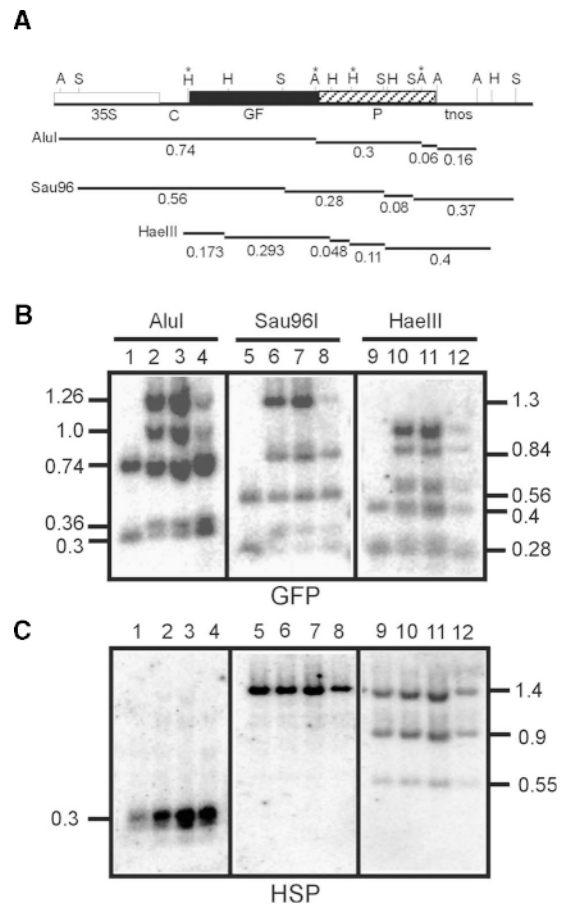
To gain further insight into the role of RNA–DNA interactions and de novo methylation, we have investigated PTGS of a transgene and an endogenous gene in *Nicotiana benthamiana*. We also have used viral suppressors of silencing to dissect the pathway of events leading to PTGS. Our findings demonstrate that DNA methylation is an indicator of an RNA–DNA interaction and that this interaction may be involved in an epigenetic transition associated with many examples of PTGS.

## RESULTS

### De Novo Methylation Associated with VIGS of a Transgene Encoding the Green Fluorescent Protein

It had been shown previously that PSbMV-induced PTGS in peas was associated with methylation of the corresponding PSbMV transgene (Jones et al., 1998b). To investigate whether this RNA-directed methylation of DNA applied more generally, we investigated VIGS in *N. benthamiana* lines 16c and 8a, which carry single copies of the cauliflower mosaic virus 35S:green fluorescent protein (*GFP*) transgene (Ruiz et al., 1998). For this study, recombinant potato virus X (PVX) carrying the full-length (PVX-GFP), the 5' 453 nucleotides (PVX-GF), or the 3' 359 nucleotides (PVX-P) of *GFP* was used to promote VIGS. As in the previous study (Ruiz et al., 1998), PVX-GFP- and PVX-GF-induced silencing were extensive at 20 days postinoculation (DPI). PVX-P also induced VIGS of the *GFP* transgene after the same time period, whereas plants infected by nonrecombinant PVX showed no silencing of *GFP* (data not shown).

To analyze the association between VIGS and methylation of the *GFP* transgene, we prepared DNA samples from tis-



**Figure 1.** Methylation Associated with VIGS of *GFP*.

**(A)** Structure of the *GFP* transgene, including the 35S promoter (35S; open box); the chitinase endoplasmic reticulum targeting signal sequence (C); 5' 453 bp of *GFP* (GF; solid box); 3' 359 bp of *GFP* (P; hatched box); the nopaline synthase terminator (tnos); sites for the restriction endonucleases AluI (A), HaeIII (H), and Sau96I (S); and sizes of expected digestion products in kilobases. Restriction sites marked by asterisks contain cytosines in a symmetrical (CpG or Cp-NpG) configuration.

**(B)** and **(C)** DNA gel blot analysis of samples from nonrecombinant PVX-infected, nonsilenced leaves (lanes 1, 5, and 9) and PVX-GFP-silenced (lanes 2, 6, and 10), PVX-GF-silenced (lanes 3, 7, and 11), and PVX-P-silenced (lanes 4, 8, and 12) leaves of 16c plants. Results are representative of at least three separate experiments. DNA samples were digested with AluI, Sau96I, or HaeIII as indicated. The blot was probed with a full-length *GFP*-specific probe **(B)** followed by reprobing with a *N. benthamiana hsp70*-specific probe **(C)**. Results for lines 16c (data shown) and 8a (data not shown) were identical. Sizes (in kilobases) of relevant DNA fragments are indicated.

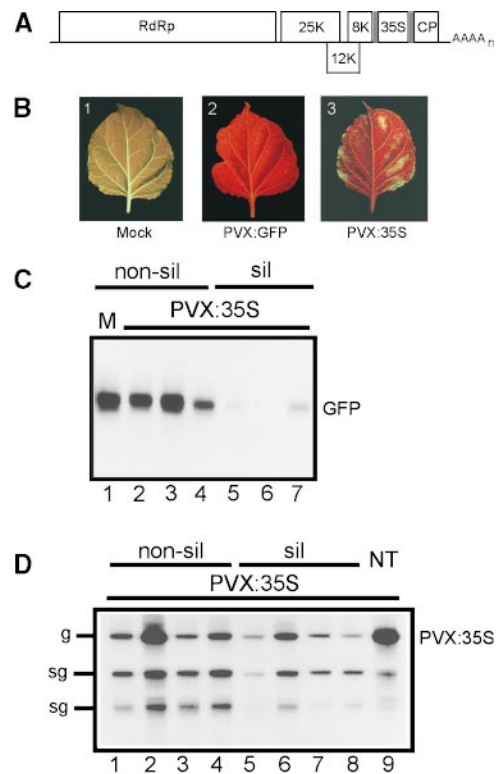
sues in which the *GFP* transgene was silenced. This DNA was digested with the methylation-sensitive restriction enzymes AluI, Sau96I, and HaeIII and analyzed after gel blot hybridization with a *GFP* probe. Figure 1A shows the organi-

zation of the 35S:*GFP* transgene, the location of restriction sites, and the sizes of digestion products of a nonmethylated *GFP* transgene.

The *GFP* DNA of nonsilenced PVX-infected tissue was completely digested with these enzymes, indicating that the transgene was not methylated (Figure 1B, lanes 1, 5, and 9). In contrast, in samples prepared from tissue silenced by PVX-*GFP*, PVX-*GF*, or PVX-*P*, there were hybridizing fragments of higher molecular weight, indicating that the *GFP* DNA was methylated. In nonsilenced samples, for example, *AluI* fragments of 0.74 and 0.3 kb were detected (Figure 1B, lane 1), whereas in the three samples from *GFP*-silenced leaves, there were additional fragments of 1.26, 1.0, and 0.36 kb (Figure 1B, lanes 2 to 4). *Sau96I* digestion gave fragments of 0.56 and 0.28 kb for nonsilenced samples (Figure 1B, lane 5) and additional fragments of 1.3, 0.84, and 0.36 kb for silenced samples (Figure 1B, lanes 6 to 8). Similarly, *HaeIII* digestion of the nonsilenced sample produced hybridization products of 0.4 and 0.29 kb and shorter fragments that could not be resolved on this gel (Figure 1B, lane 9), whereas samples from the *GFP*-silenced tissue showed additional fragments of 1.0, 0.85, and 0.63 kb (Figure 1B, lanes 10 to 12).

To rule out the possibility that the differences in hybridization pattern between silenced and nonsilenced samples were due to incomplete digestion, we removed the *GFP* probe and reprob the filter with the *N. benthamiana* heat shock protein *hsp70* gene (M. Aranda and A.J. Maule, unpublished data). Figure 1C shows that for each restriction enzyme, nonsilenced (lanes 1, 5, and 9) and silenced (lanes 2 to 4, 6 to 8, and 10 to 12) samples had identical hybridization patterns using the *hsp70* probe. This result confirms that all samples were digested to the same extent. Thus, the additional *GFP*-hybridizing fragments in the samples from *GFP*-silenced leaves could be explained by the presence of cytosine methylation within recognition sites for *AluI*, *HaeIII*, and *Sau96I*.

There were four important features of the *GFP* DNA methylation data (Figure 1). First, from the presence of smaller *GFP* DNA fragments, we conclude that the methylation of the *GFP* DNA was never complete in these examples of PTGS. Second, we noted that the methylation profile was similar, irrespective of whether silencing was triggered by PVX-*GFP*, PVX-*GF*, or PVX-*P*. Thus, sites within the P portion of the transgene were affected when silencing was triggered by PVX-*GF*, and likewise, sites within the GF portion were affected when the initiator of silencing was PVX-*P*. This result indicates that methylation extends beyond the region of identity between the viral and transgene sequences. A third feature is that the methylation may be restricted to the transcribed region of the gene: sites in the 35S promoter and 3' end of the nopaline synthase terminator were not affected (Figure 1A). However, because few restriction sites were available for analysis in the promoter and terminator regions, the extent of methylation was not determined precisely. Finally, the fourth feature of these



**Figure 2.** VIGS of 35S:*GFP* by PVX-35S.

(A) Genomic organization of the PVX vector carrying the 35S promoter sequence. The PVX open reading frames are shown as RdRp (RNA-dependent RNA polymerase), 25K (25-kD protein), 12K (12-kD protein), 8K (8-kD protein), and CP (coat protein). The 35S insert was a 347-bp fragment carrying the 35S promoter sequence inserted downstream of a duplicated coat protein promoter (dark boxes).

(B) Upper leaves of 16c plants that were either mock-inoculated (leaf 1) or inoculated with PVX-*GFP* (leaf 2) or PVX-35S (leaf 3). It is clear that silencing exhibited by PVX-35S plants is not as complete as that exhibited by PVX-*GFP* plants. The leaves were photographed at 30 DPI.

(C) *GFP* mRNA levels in mock-inoculated (M; lane 1) or PVX-35S-inoculated (lanes 2 to 7) 16c plants. Lanes 2 to 4 contain samples prepared from tissue of infected plants that was not exhibiting GFP silencing (non-sil). Lanes 5 to 7 are samples from tissue that was exhibiting GFP silencing (sil). Five micrograms of total RNA was run per lane, and a probe specific for *GFP* was used for detection.

(D) PVX-35S RNA levels in systemically infected leaves. Samples were prepared from tissue of infected 16c plants that was not (non-sil; lanes 1 to 4) or was (sil; lanes 5 to 8) exhibiting silencing of the *GFP* transgene, and from infected nontransformed *N. benthamiana* (NT; lane 9). One microgram of total RNA was loaded per lane, and a probe specific for the 35S promoter was used for detection. The genomic (g) and major subgenomic (sg) RNA species are labeled.

data is that methylcytosine residues were detected at both symmetrical (CpG and CpNpG) and nonsymmetrical sites, because both types of site were analyzed and shown to be affected (Figure 1A).

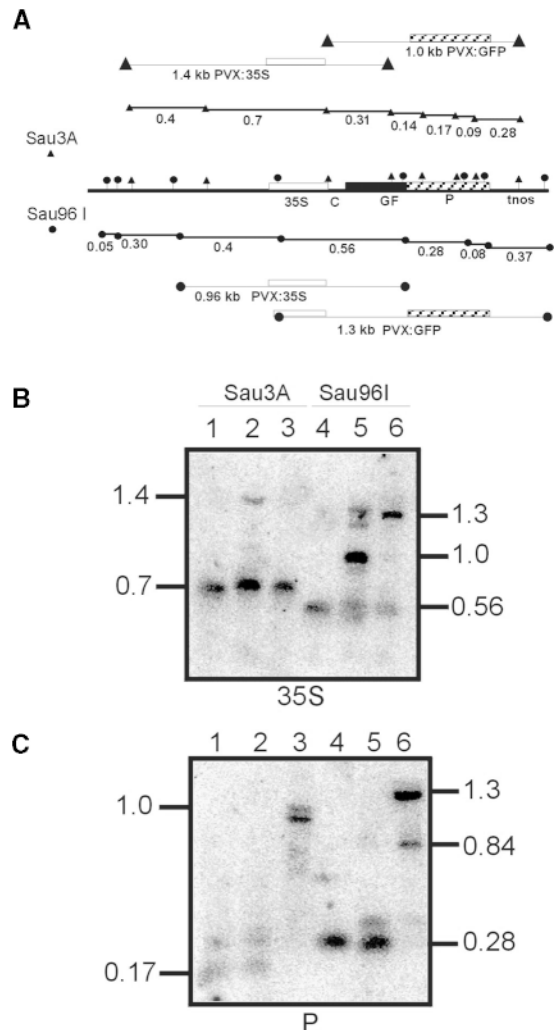
### VIGS of *GFP* by a PVX Vector Carrying the 35S Promoter Sequence

In the experiments described above, we have shown a strong correlation between VIGS of the *GFP*-transcribed region and methylation. To further investigate the mechanism and effects of de novo methylation, we decided to use PVX to target a nontranscribed region of the transgene. Figure 2A shows the construction of a PVX vector that carried a 347-nucleotide fragment of the 35S promoter sequence (PVX-35S). This construct was used to inoculate line 16c or nontransformed *N. benthamiana* plants. At 20 DPI, loss of green fluorescence was observed in systemically infected 16c plants. However, unlike leaves infected with PVX-GFP that were completely silenced, green fluorescent sectors still were visible (Figure 2B).

RNA and DNA samples were prepared from PVX-35S-infected tissue at 20 DPI to assess *GFP* and PVX RNA levels and to determine whether the transgene was methylated in the silenced tissue. Figure 2C shows that *GFP* mRNA levels were reduced in silenced tissue of infected 16c plants (lanes 5 to 7) compared with levels in noninfected 16c tissue (lane 1) and in nonsilenced tissue of infected plants (lanes 2 to 4). This result confirms the visual observations that PVX-35S induces silencing of the 35S:*GFP* transgene.

Using a probe specific for the 35S promoter region, we compared viral RNA levels in infected nontransgenic tissue (Figure 2D, lane 9) to levels in nonsilenced (Figure 2D, lanes 1 to 4) and silenced (Figure 2D, lanes 5 to 8) 16c tissue that was infected. This RNA gel blot analysis detected high levels of PVX-35S in all samples tested. Due to the between-sample variation, it was not possible to determine whether the levels were the same in the silenced and the nonsilenced samples. However, if there was a reduction in viral RNA, it was only slight and much less than the 100-fold reduction of PVX-GFP RNA detected in leaves exhibiting VIGS of *GFP* (Ruiz et al., 1998).

For methylation analysis, DNA samples were prepared from 16c plants in which the *GFP* transgene had been silenced by either PVX-35S or PVX-GFP. The DNA was digested with *Sau*3A or *Sau*96I, and the products were analyzed by DNA gel blotting using probes specific for either the 35S promoter or the 3' 359 nucleotides of *GFP* (P). Figure 3A shows the location of restriction sites relevant for this analysis. After digestion with *Sau*3A and probing with the 35S promoter, a 0.7-kb fragment was detected in all samples (Figure 3B, lanes 1 to 3). However, there was an additional fragment of 1.4 kb in the PVX-35S-silenced sample (Figure 3B, lane 2). Reprobing of this filter with the 3' 359 nucleotide P probe detected fragments of 0.28 and 0.17 kb for nonsilenced and PVX-35S-silenced samples (Figure 3C, lanes 1 and 2) and additional fragments of 1.0 and 0.9 kb in the PVX-GFP-silenced sample (Figure 3C, lane 3). These results indicate that *Sau*3A sites within the *GFP* sequence are methylated when silencing is induced by PVX-GFP and not when induced by PVX-35S. Conversely, *Sau*3A sites within and upstream of the 35S promoter are methylated when si-



**Figure 3.** Differences in Methylation Associated with VIGS of 35S:*GFP* Induced by PVX-35S and PVX-GFP.

(A) Structure of the *GFP* transgene. Features shown are the 35S promoter (35S; open box), chitinase signal sequence (C), 5' 453 bp of *GFP* (GF; solid box), 3' 359 bp of *GFP* (P; hatched box), nopaline synthase terminator (tnos), restriction sites for *Sau*3A (filled triangles) and *Sau*96I (filled circles), and expected digestion products. Also illustrated are the largest methylated products for each enzyme detected by either the 35S (open boxes) or P (hatched boxes) probes, when silencing is induced by PVX-35S or PVX-GFP.

(B) and (C) DNA gel blot analysis of samples from nonsilenced (lanes 1 and 4), PVX-35S-silenced (lanes 2 and 5), and PVX-GFP-silenced (lanes 3 and 6) 16c tissue. DNA samples were digested with *Sau*3A or *Sau*96I as indicated and probed with the 35S promoter sequence (B) or the P 3' 359 bp of *GFP* (C). Sizes (in kilobases) of relevant DNA fragments are indicated.

lencing is induced by PVX-35S but not when it is induced by PVX-GFP (Figure 3A).

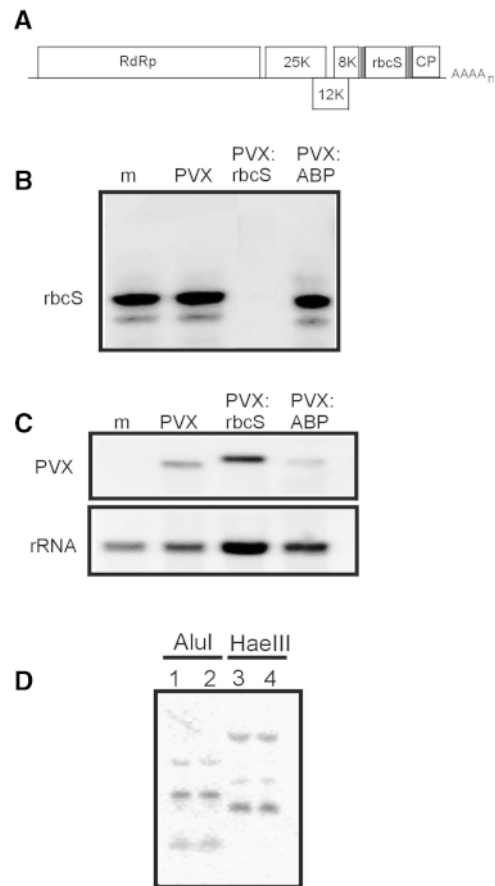
A similar conclusion was obtained by analyzing *Sau96I* digestion products. Probing with the 35S promoter sequence detected a hybridization product of 0.56 kb for the nonsilenced sample (Figure 3B, lane 4). In the PVX-35S-silenced sample, there were additional fragments of 1.31, 1.26, and 0.96 kb (Figure 3B, lane 5). The 0.96-kb fragment was most predominant, indicating that the *Sau96I* site within the 35S promoter was more often methylated than the upstream sites. The 1.3-kb fragment in the PVX-GFP-silenced sample (Figure 3B, lane 6) probably is a consequence of methylation of the *Sau96I* sites in the transcribed region of the transgene. Reprobing with the P probe detected a fragment of 0.28 kb for nonsilenced and PVX-35S-silenced samples (Figure 3C, lanes 4 and 5) and additional fragments of 1.3 and 0.84 kb for PVX-GFP (Figure 3C, lane 6). From these data, combined with the data in Figure 1, we conclude that viral RNA can mediate methylation of both the transcribed and promoter regions of the GFP transgene. Methylation can spread within the transcribed region when silencing is initiated by PVX-GFP. When silencing is initiated by PVX-35S, methylation is directed to the promoter but does not extend downstream into the transcribed region.

#### De Novo Methylation Is Not Associated with VIGS of an Endogenous Gene

To determine whether VIGS always is associated with methylation of the corresponding target gene sequences, we analyzed RNA and DNA levels in plants infected with PVX carrying an insert corresponding to 500 nucleotides of the *N. benthamiana* ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) small subunit (*rbcS*) gene. Figure 4A shows the construction of PVX-*rbcS*. At 20 DPI, the plants infected with PVX-*rbcS* exhibited a stunted chlorotic phenotype that is very similar to the phenotype of plants carrying an antisense Rubisco transgene (Rodermel et al., 1988) and is likely due to the VIGS of *rbcS* in the infected plants. The controls in these experiments were wild-type PVX and PVX carrying an insert from the gene encoding the auxin binding protein (PVX-ABP). Both of these constructs induced mild symptoms on infected plants.

Figure 4B shows the results of an RNase protection experiment using a probe to detect endogenous *rbcS* transcripts. Levels of *rbcS* mRNA in leaves exhibiting the chlorotic phenotype were at least 10-fold lower than in mock-inoculated leaves or in control leaves infected with PVX or PVX-ABP. RNA gel blot analysis with a PVX-specific probe showed that viral RNA levels were high in tissue infected with PVX-*rbcS*, wild-type PVX, or PVX-ABP (Figure 4C). These results suggest that the endogenous *rbcS* transcript, but not PVX-*rbcS*, is a target of VIGS.

The possible methylation of *rbcS* DNA was investigated in DNA samples prepared at 25 DPI from *N. benthamiana*



**Figure 4.** VIGS Induced by PVX-*rbcS*.

(A) Genomic organization of the PVX vector carrying 500 nucleotides of the gene encoding the Rubisco small subunit (*rbcS*). The genome is labeled as given in the legend to Figure 2A.

(B) *rbcS* mRNA levels in mock-inoculated (m) or wild-type PVX and PVX-*rbcS*- (PVX:*rbcS*) and PVX-ABP- (PVX:ABP) infected plants. RNA was prepared from systemically infected leaves at 25 DPI, and detection was by RNase protection with a probe specific for endogenous *rbcS* transcripts.

(C) RNA gel blot analysis of mock-inoculated (m) and PVX, PVX-*rbcS* (PVX:*rbcS*), and PVX-ABP (PVX:ABP) levels in systemically infected leaves at 25 DPI. Probes specific for viral RNA (top) and ribosomal RNA (bottom) were used. Migration of PVX-*rbcS* RNA is slower than that of wild-type PVX, indicating that the accumulated RNA had not developed the ability to overcome VIGS due to loss of the *rbcS*-derived insert.

(D) DNA gel blot analysis of samples from *N. benthamiana* tissue systemically infected by PVX-*rbcS* (lanes 1 and 3) or PVX-GUS (lanes 2 and 4) at 25 DPI. DNA was digested with AluI or HaeIII as indicated. Filters were probed with the same fragment of *rbcS* as that carried in PVX-*rbcS*.

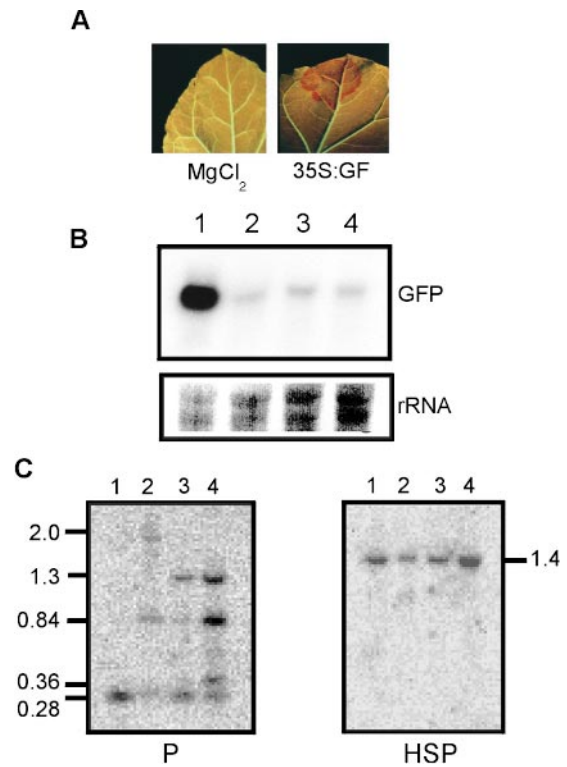
infected with either PVX- $\beta$ -glucuronidase (PVX-GUS) or PVX-*rbcS*. The DNA was digested with *AluI*, *HaeIII* (data shown), *Sau3A*, or *Sau96I* (data not shown) and probed with an *rbcS*-specific probe. Figure 4D shows that for each enzyme, there was an identical hybridization profile in the two samples, indicating that *de novo* methylation is not associated with VIGS of *rbcS*. Once again, equal digestion of samples was confirmed by reprobing of the filter with *hsp70* (data not shown).

### De Novo Methylation Is Associated with Systemic Silencing

To determine whether *GFP* PTGS is associated with transgene methylation in the absence of virus infection, we exploited the experimental system described previously based on infiltration with *Agrobacterium* (Voinnet and Baulcombe, 1997). This system involves initiation of silencing in the infiltrated tissue followed by systemic spread of a silencing signal and maintenance of silencing in tissues that have received the signal.

The oldest mature leaf on *N. benthamiana* line 16c plants that had three fully expanded leaves was infiltrated with *Agrobacterium* carrying the 35S:GF T-DNA, in which the 3' part of the *GFP* coding sequence was deleted. Using 35S:GF allowed us to distinguish between the *GFP* transgene and any introduced GFP sequences by using the P probe, which comprises the 3' 359 nucleotides of GFP, in DNA gel blot analysis. Figure 5A shows that 6 days postinfiltration there was silencing of GFP in the infiltrated patch. Control infiltrations by using  $MgCl_2$  (Figure 5A) or *Agrobacterium* that did not carry the *GFP* sequence did not cause silencing. After 20 DPI, silencing of GFP was visible in all newly emerging tissue, and eventually (~35 DPI) even mature, noninfiltrated leaves became silenced.

RNA and DNA samples were prepared from tissue infiltrated 7 days earlier with either  $MgCl_2$  (sample 1) or *Agrobacterium* 35S:GF (sample 2) and from systemically silenced tissue at 35 DPI that either was (sample 3) or was not (sample 4) mature at the time of infiltration. Figure 5B shows the results of gel blot analysis of these RNA samples. As expected, *GFP* RNA levels were lower in tissues in which GFP fluorescence was visibly reduced (Figure 5B, lanes 2 to 4) compared with unsilenced tissue (Figure 5B, lane 1). The corresponding DNA samples were digested with the methylation-sensitive enzyme *Sau96I*, and the products were analyzed by DNA gel blotting with the P probe (Figure 5C). Similar to the results described for VIGS of GFP, the predicted (unmethylated) hybridization pattern was observed only for unsilenced tissue, and all silenced tissue showed additional hybridizing fragments. Thus, a fragment of 0.28 kb was detected in the sample from unsilenced tissue (Figure 5C, lane 1). Mature tissue that had been infiltrated with 35S:GF showed additional fragments of 2.0 and 0.84 kb (Figure 5C, lane 2), and the tissues that were systemically si-



**Figure 5.** Methylation Associated with Systemic Silencing of GFP after *Agrobacterium* Infiltration of GFP Sequences.

**(A)** Mature leaves of 16c plants that were infiltrated with either  $MgCl_2$  (left) or *Agrobacterium* carrying 35S:GF (right). Silencing of GFP is visible as a patch of red fluorescence in leaves infiltrated with 35S:GF. Leaves were photographed at 6 DPI.

**(B)** RNA gel blot analysis of RNA samples prepared from tissue infiltrated with  $MgCl_2$  (lane 1) or 35S:GF (lane 2) and systemically silenced tissue, which was mature (lane 3) or undeveloped (lane 4) at the time of infiltration. Samples 1 and 2 were taken at 7 DPI, and samples 3 and 4 were taken at 35 DPI. Five micrograms of total RNA was loaded per lane, and a probe specific for the entire *GFP* sequence was used. Ethidium bromide staining of the electrophoresed gel shows the rRNA loading.

**(C)** and **(D)** DNA gel blot analysis of samples prepared from tissues described in **(B)**. DNA was digested with *Sau96I* and probed with the 3' 359 bp of GFP **(C)** followed by reprobing with the *N. benthamiana hsp70* gene **(D)**. Sizes (in kilobases) of relevant DNA fragments are indicated.

lenced showed additional fragments of 1.3, 0.84, and 0.36 kb (Figure 5C, lanes 3 and 4). The sizes of these fragments indicate that the integrated transgene was methylated both in the 5' region corresponding to the infiltrated GF sequence and in the nonoverlapping 3' regions (cf. Figure 1). To confirm equal digestion of samples, we stripped and reprobbed the blot with the *N. benthamiana hsp70* gene. Figure 5D shows that all four samples had identical hybridization patterns, indicating that all samples were digested to the same

extent and that the differences in hybridization pattern with the 3' 359-bp P probe are likely due to methylation.

These results demonstrate that *GFP* DNA methylation occurs in the developed tissue in which silencing is triggered initially and also is associated with systemic silencing in both developed and developing tissue. Methylation of *GFP* DNA never was observed in unsilenced leaves (data not shown), indicating that de novo methylation is not simply a consequence of normal development.

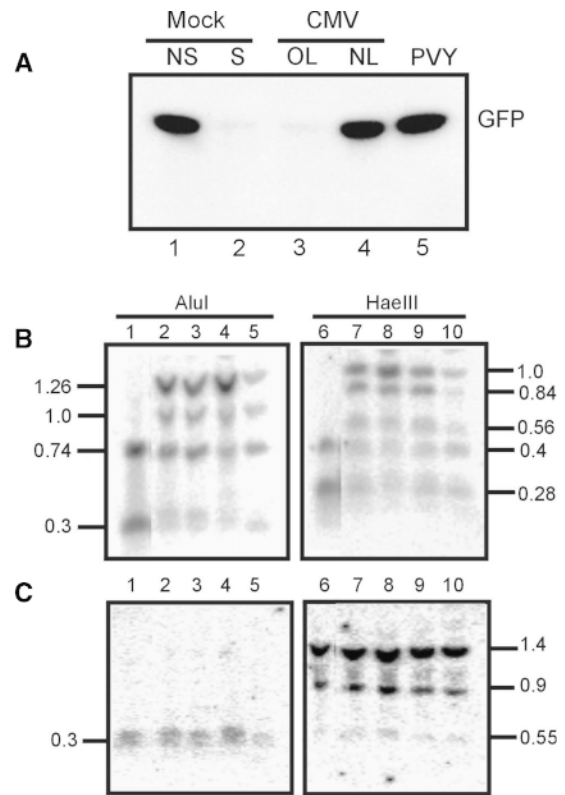
### Suppression of Gene Silencing Does Not Reverse DNA Methylation

Certain viruses encode proteins that act as viral suppressors of gene silencing (Anandalakshmi et al., 1998; Béclin et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). For example, the HC-Pro protein of potyviruses can reverse silencing in tissues in which silencing already has been set, and the 2b protein of cucumber mosaic virus (CMV) prevents initiation of gene silencing at the growing points of the plant. Thus, although both proteins suppress PTGS, it is likely that they do so via different mechanisms and thus may provide useful tools to dissect the pathway of events leading to establishment of the silenced state.

PTGS of the *GFP* transgene in line 16c seedlings was initiated by infiltration by using *Agrobacterium* 35S:*GFP*. Once silencing was established at 20 DPI, plants were inoculated with either the potyvirus potato virus Y (PVY) or CMV. As described in Brigneti et al. (1998), PVY infection suppressed silencing in all infected tissue. However, for CMV infection, only leaves that emerged after systemic spread of the virus exhibited green fluorescence, whereas older infected leaves continued to fluoresce bright red. DNA and RNA samples were prepared from nonsilenced and silenced 16c plants and from silenced plants infected by PVY or CMV. For CMV-infected plants, samples were taken from the older infected silenced leaves that had emerged before systemic infection and from new nonsilenced leaves that emerged after the infection was established.

Figure 6A shows the results of RNA gel blot analysis confirming earlier findings that GFP fluorescence correlates with levels of *GFP* mRNA. Thus, *GFP* mRNA levels in nonsilenced plants (lane 1) were similar to levels in the new leaves infected by CMV (lane 4) and in PVY-infected tissue (lane 5). Levels of *GFP* mRNA were low in silenced plants (lane 2) and in the old leaves of a CMV infection (lane 3).

To investigate whether suppression of silencing was associated with a change in DNA methylation status, we digested the DNA samples with *AluI* and *HaeIII*, and the products were analyzed using a *GFP*-specific probe. In the sample from nonsilenced tissue, in which silencing had not been triggered, there were fragments of 0.74 and 0.3 kb for *AluI* (Figure 6B, lane 1) and of 0.4 and 0.28 kb for *HaeIII* digestion (Figure 6B, lane 6). These are the sizes that would be expected if there were no methylation of the *GFP* DNA



**Figure 6.** Suppression of GFP Silencing by PVY and CMV.

**(A)** RNA gel blot analysis of samples prepared from nonsilenced (NS) and silenced (S) 16c plants and silenced plants that were infected with either CMV (OL and NL) or PVY. For CMV infection, RNA was taken from old leaves (OL) that had emerged before systemic infection with CMV and from new leaves (NL) that had emerged after systemic infection. Five micrograms of total RNA was loaded per lane, and hybridization was to a probe specific for *GFP*.

**(B)** and **(C)** DNA gel blot analysis of samples prepared from tissue as described in **(A)**: nonsilenced (lanes 1 and 6), silenced (lanes 2 and 7), CMV-infected old (lanes 3 and 8) and new (lanes 4 and 9), and PVY-infected (lanes 5 and 10) leaves. DNA was digested with *AluI* or *HaeIII* as indicated. The blot was probed with a full-length *GFP*-specific probe **(B)** followed by reprobing with the *N. benthamiana hsp70* probe **(C)**. Sizes (in kilobases) of relevant DNA fragments are indicated.

(cf. Figure 1A). The samples from CMV- and PVY-infected tissue showed identical additional hybridizing fragments of 1.26, 1.0, and 0.36 kb for *AluI* (Figure 6B, lanes 3 to 5) and of 1.0, 0.85, and 0.63 for *HaeIII* digestion (lanes 8 to 10). This pattern of additional hybridizing fragments is the same as that observed for systemic silenced samples (Figure 6B, lanes 2 and 7). As for the previous experiments, the filter was reprobed with the *N. benthamiana hsp70* gene to



confirm equal digestion of samples (Figure 6C). Thus, the methylation associated with PTGS is maintained after infection by CMV or PVY, even though silencing was suppressed.

## DISCUSSION

From the data reported here, it is shown that viral RNA has the potential to interact with homologous transgenes and that sequence-specific methylation of DNA is associated with this interaction. This type of interaction had been implicated by previous reports. However, it remained possible that there could be either DNA–DNA interactions between homologous transgenes (Mette et al., 1999) or RNA–RNA interactions between viral or viroid RNA and the nascent RNA transcript of a homologous transgene (Wasseneger et al., 1994; Jones et al., 1998b; Guo et al., 1999). In the example described here, with PVX-35S, the only interaction leading to methylation of the 35S transgene promoter would have been between RNA and the promoter DNA. Thus, we have established that a direct RNA–DNA interaction can mediate methylation.

Using VIGS, we found that both transcribed and promoter regions participate in the RNA–DNA interaction and were targeted for methylation. However, the consequences of the interaction varied, depending on the region involved. Viral RNA interactions with the transcribed region led to PTGS of the transgene and elimination of the virus from infected cells, whereas interactions with the promoter led to transcriptional silencing, and virus accumulation remained high (Figure 2).

Previous analyses showed that VIGS of *GFP* induced by PVX-GFP involves separate initiation and virus-independent maintenance stages. Initiation is characterized by simultaneous accumulation of the virus and suppression of the *GFP* transgene RNA, whereas, in the maintenance stage, PTGS of the transgene persists even though the viral RNA has been eliminated. The previous analyses also showed that PVX induces PTGS in the absence of homologous DNA. This DNA-independent PTGS (Ratcliff et al., 1997, 1999) is targeted against the replicating virus and represents an RNA-mediated system of antiviral defense that limits virus accumulation in infected cells. However, because activation of the mechanism is dependent on virus replication, this defense system does not cause complete suppression of virus accumulation.

Combining the findings from these previous analyses with the results presented here, we now propose that the initiation stage of VIGS is due to RNA-mediated defense and does not require any sequence similarity between the virus and the genome of the host plant. We further propose that the maintenance stage requires the presence of a transgene that is similar to the viral RNA and is dependent on an RNA–DNA interaction leading to methylation of the transcribed part of the transgene. Thus, with PVX-GFP and derivatives,

progression into the maintenance stage would follow an interaction between *GFP* RNA derived from the virus and the homologous transgene. In this scenario, the RNA–DNA interaction forms the basis for an epigenetic change in gene expression that ensures maintenance of PTGS in the absence of the continued presence of the virus.

It remains to be determined how an RNA interaction could lead to DNA methylation and how the interaction would influence PTGS. In principle, transcription could be affected by the formation of an RNA–DNA duplex or triplex or by DNA methylation. From the pattern of *GFP* DNA methylation (Figure 1), it is likely that transcription of the *GFP* transgene is involved. As demonstrated using PVX-GF and PVX-P to initiate VIGS, the methylated DNA extends beyond the region of identity between viral RNA and the *GFP* transgene but appears to be restricted to the transcribed region. In terms of RNA-directed methylation, this pattern could be explained if the initial methylation corresponds to this region of identity. Aberrant transcripts of this DNA then would mediate more extensive secondary methylation that corresponds to the transcribed region of the *GFP* transgene. This cycle of RNA–DNA interactions and production of aberrant RNA may account for maintenance of methylation at nonsymmetrical cytosines that is otherwise difficult to explain in terms of a conventional maintenance methylase.

Such a cyclical process also can explain the failure of VIGS initiated by PVX-35S or PVX-*rbcS* to progress into the virus-free maintenance stage (Figures 2 and 4). For VIGS of *GFP* by using PVX-GFP, silencing is maintained in the absence of virus accumulation, whereas for PVX-35S and PVX-*rbcS*, silencing of the corresponding nuclear sequence always correlated with the presence of high levels of virus. With PVX-35S, the maintenance stage of VIGS would be absent because the targeted DNA was not transcribed (Figures 2 and 3). With PVX-*rbcS*, there was no detectable methylation of the *rbcS* DNA (Figure 4), indicating that this endogenous gene did not participate in the RNA–DNA interaction; therefore, the ability to perform PTGS was not imprinted.

## Systemic Silencing

We previously described the systemic spread of gene silencing and invoked an RNA signal molecule to account for the nucleotide specificity of this phenomenon (Voinnet and Baulcombe, 1997; Voinnet et al., 1998). In making this suggestion, the possibility that the signal molecule shares some properties with viroids was introduced. Like the silencing signal molecule, these pathogenic RNA species are able to spread systemically. Here, through the characterization of RNA-mediated methylation and the association of systemic silencing with transgene methylation, we provide further support for an RNA component of the signal molecule.

In the infiltrated leaf, in which the systemic silencing was initiated, there was methylation of the integrated *GFP* trans-



gene due to an interaction either with the infiltrated T-DNA or with transcripts of this DNA (Figure 5). We interpret this methylation as an indicator that the *GFP* transgene would participate in the cycle involving transcription and RNA–DNA interactions, as proposed above for the maintenance stage of VIGS. From the methylation of the *GFP* transgene in the noninfiltrated leaves (Figure 5), we infer that this cycle subsequently was repeated in cells receiving the signal of systemic silencing.

Further support for the involvement of the RNA–DNA interaction cycle in systemic silencing is based on the distribution of methylated cytosine residues in the integrated *GFP* transgene (Figure 5): when systemic silencing was initiated by the 5' part of the *GFP* sequence, there were methylated residues in the nonoverlapping 3' part of the integrated *GFP* transgene. Correspondingly, the target of systemic silencing extended beyond the sequence used to initiate systemic silencing. Thus, the 3' part of the *GFP* sequence was a target when systemic silencing was initiated by the nonoverlapping 5' region and vice versa.

### Viral Suppressors

We suggested previously that virus-encoded suppressors of silencing could be used to dissect the mechanism of PTGS. For example, from the ability of the potyvirus-encoded HC-Pro to reverse PTGS, we had suggested that this protein blocks the maintenance stage of the gene-silencing mechanism. In contrast, the 2b protein of CMV was unable to reverse PTGS but was able to prevent the establishment of PTGS in leaves emerging from the meristem. Therefore, we suggested that the 2b protein was able to prevent the initiation stage of silencing or to block systemic movement of a silencing signal.

According to these suggestions, if HC-Pro was able to block maintenance of silencing, it should have interfered with the cycle of RNA–DNA interactions, and the level of *GFP* DNA methylation would have been reduced in the leaves of PVY-infected plants. Similarly, if the 2b protein was able to block movement of the silencing signal or to prevent initiation of silencing in the differentiating leaves, the *GFP* transgene in the newly emerging leaves of CMV infected plants would have been unmethylated.

However, both of these predictions were wrong. The *GFP* transgene was as highly methylated when systemic silencing of *GFP* was suppressed by PVY or CMV as it was in noninfected plants, thereby showing that PTGS and methylation can be uncoupled. Because methylation is maintained in the absence of PTGS, we conclude that methylation is not a consequence of PTGS. However, we cannot distinguish between the possibilities that methylation is a cause of silencing or that methylation and PTGS represent separate pathways that share a common trigger. To account for our observations, we now consider that both HC-Pro and 2b act at a stage in the

PTGS mechanism that is downstream of the cycle of RNA–DNA interactions involved in maintenance and signal production. To account for the ability of HC-Pro to suppress silencing in all parts of the plant, we conclude that this protein targets a labile component of the PTGS pathway. In contrast, the 2b protein must act against a stable component of the PTGS pathway. Presumably, because the 2b protein cannot reverse systemic silencing in the old leaves of the plant, it prevents formation of this component, at the level of either synthesis or assembly.

### Epigenetic and Genetic PTGS

In this article, we describe PTGS that is inducible by a virus and by *Agrobacterium* infiltration. The examples with *GFP* can be considered epigenetic because, once established, maintenance of PTGS did not require continued presence of the inducing agent. For this epigenetic PTGS, we provide strong evidence that RNA–DNA interactions and transgene methylation are associated with the mechanism. In transgenic plants, there are examples of spontaneous PTGS that also may have an epigenetic basis. We consider that PTGS with a delayed onset or involving a systemic signal is likely to be epigenetic. However, in other examples of transgenic PTGS that do not require an activating stimulus the mechanism may be determined by genetic rather than epigenetic factors. For example, inverted transgene repeats often are associated with PTGS, and it is proposed that the palindromic sequence organization promotes production of aberrant RNAs that are targeted for degradation (Stam et al., 1997b; Selker, 1999). In these cases, PTGS will be maintained due to the structural determinants of the transgene.

The difference between genetic and epigenetic PTGS is likely to involve the various ways that the gene-silencing mechanism is activated and maintained, although the components that target and degrade RNA may be identical. Similarly, in fungi and animals, there may be certain components, including double-stranded RNA and a host-encoded RNA-dependent RNA polymerase (Cogoni and Macino, 1999), that are common, but the initiation mechanism may vary in each system. For example, in *Neurospora crassa*, PTGS can be independent of DNA methylation, suggesting that silencing is genetically determined (Cogoni et al., 1996). In *Caenorhabditis elegans* and *Drosophila*, the reported examples of PTGS are induced and therefore are more likely to be determined epigenetically rather than genetically, despite the lack of DNA methylation in these organisms (Fire et al., 1998; Kennerdell and Carthew, 1998). In these examples, the inducing agent is double-stranded RNA, and it is possible that the PTGS mechanism is DNA independent and maintained by an RNA-dependent RNA polymerase (Baulcombe, 1999). Alternatively, the inducing RNA may be able to imprint the corresponding nuclear gene in a methylation-independent manner.

## METHODS

### Plant Material

Transgenic *Nicotiana benthamiana* lines 16c and 8a, carrying single 35S:green fluorescent protein (*GFP*) transgenes, were described previously (Ruiz et al., 1998).

### Wild-Type and Recombinant Viruses

Cloned copies of wild-type potato virus X (PVX), as well as PVX-GFP, PVX-GF, and PVX-P, have been described previously (Baulcombe et al., 1995; Ruiz et al., 1998; Voinnet et al., 1998). PVX-35S was generated by cloning a 347-bp Asp718-HindIII fragment from pJIT121 (Guerineau et al., 1992) into the SmaI site of the PVX vector construct pGR107. This vector is based on the cDNA of PVX strain UK3 under the control of the cauliflower mosaic virus 35S promoter and is similar to pPVX201, except that it is in an *Agrobacterium* binary vector (L. Rui and D.C. Baulcombe, unpublished data) and was transformed into *Agrobacterium*. Inoculation of the *Agrobacteria* with a toothpick led to infection of the plant (L. Rui and D.C. Baulcombe, unpublished data).

PVX-*rbcS* carries a 500-bp fragment of an *N. benthamiana* ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) small subunit (*rbcS*) cDNA (nucleotides 2 to 502) that was polymerase chain reaction-amplified using primers based on the *N. tabacum rbcS* sequence (GenBank accession number X02353).

### Agrobacterium Induction of Post-Transcriptional Gene Silencing

GFP-expressing 16c seedlings were infiltrated, as described previously, with a hypervirulent strain of *Agrobacterium* carrying a binary Ti plasmid into which a 35S:GF cassette had been inserted. The 35S:GF construct was derived from 35S:*GFP* by deletion of a PmlI-SacI restriction fragment carrying the 3' 359 nucleotides of *GFP* (Voinnet and Baulcombe, 1997).

### GFP Imaging

Visual detection of GFP fluorescence in whole plants and photography was performed as described previously (Voinnet et al., 1998).

### DNA Extraction and Gel Blot Analysis

Genomic DNA was extracted from leaves by using the DNeasy plant DNA extraction kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. DNA gel blot analysis was performed as described previously (Jones et al., 1998a). <sup>32</sup>P-labeled DNA probes corresponded to the entire 812 bp or 3' 359 bp of GFP, the 347-bp Asp718-HindIII fragment carrying the 35S promoter, the 500-bp cDNA fragment of *rbcS* described above, or a 450-bp cDNA fragment of the *N. benthamiana* heat shock *hsp70* gene.

### RNA Extraction, Gel Blot Analysis, and RNase Protection Assay

Extraction of total RNA was achieved using RNA isolator (Genosys Biotechnologies Inc., The Woodlands, TX) according to the manufac-

turer's instructions. RNA gel electrophoresis and gel blot analysis were performed as described previously (Jones et al., 1998b). Probes used are described above. RNase protection was as described by Hamilton et al. (1998).

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## RNA–DNA Interactions and DNA Methylation in Post-Transcriptional Gene Silencing

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