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Suppression of Virus Accumulation in Transgenic Plants Exhibiting Silencing of Nuclear Genes

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Homology-dependent gene silencing contributes to variation between transgenic plants with respect to transgene and/or endogenous gene expression levels. Recent studies have linked post-transcriptional gene silencing and virus resistance in plants expressing virus-derived transgenes. Using a potato virus X vector, we present three examples in which silencing of nonviral transgenes prevented virus accumulation. This effect was dependent on sequence homology between the virus and the silenced transgene. Analysis of potato virus X derivatives carrying bacterial β-glucuronidase (*GUS*) sequences showed that the 3′ region of the *GUS* coding sequence was a target of the silencing mechanism in two independent tobacco lines. Methylation of the silenced *GUS* transgenes in these lines was also concentrated in the 3′ region of the *GUS* coding sequence. Based on this concurrence, we propose a link between the DNA-based transgene methylation and the RNA-based gene silencing process.

INTRODUCTION

Gene silencing in transgenic plants is manifested as decreased accumulation of specific mRNAs and occurs most often when there are multiple copies of a particular sequence present in the genome (Jorgensen, 1990; Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990; Hobbs et al., 1993; Dehio and Schell, 1994; Ingelbrecht et al., 1994; Matzke et al., 1994). The repeated sequences may consist of coding sequences, promoter regions, or both. Endogenous genes and/or introduced transgenes can be affected. Although some examples of gene silencing are due to decreased transcription of the affected gene(s) (Brusslan et al., 1993; Meyer et al., 1993), other examples are due to post-transcriptional mechanisms (Dehio and Schell, 1994; Smith et al., 1994; Van Blokland et al., 1994; Mueller et al., 1995).

Recent studies have linked post-transcriptional gene silencing and virus resistance in transgenic plants expressing virus-derived sequences. The association of gene silencing and virus resistance is based on observations that resistance has been achieved with untranslatable as well as translatable transgenes and in plants that accumulate low levels of the transgene mRNA and protein product (Lindbo et al., 1993; Smith et al., 1994; Mueller et al., 1995). Using transgenes based on the RNA-dependent RNA polymerase (RdRp) gene of potato virus X (PVX), we showed that the resistance-conferring locus can also silence in *trans* the expression of a homologous transgene that does not confer resistance (Mueller et al., 1995).

These observations led us (Mueller et al., 1995) and others (Smith et al., 1994) to propose that the same basic mechanism could account for certain types of gene silencing and virus resistance. The proposed mechanism involves increased turnover (degradation) of RNA species homologous to the silenced transgene, including the transgene mRNA itself. Implicit in this model is the idea that the effect is post-transcriptional.

There are likely to be different mechanisms of gene silencing in plants (Jorgensen, 1992; Flavell, 1994), and it is possible that gene silencing associated with virus resistance operates through a mechanism that is specific to viral sequences. In this article, we describe experiments that test whether transgenic virus resistance and silencing of nonviral genes operate through similar mechanisms.

These experiments utilized PVX, a positive-strand RNA virus that infects solanaceous plants, modified with inserted marker genes. A PVX derivative carrying the bacterial β-glucuronidase gene (PVX–*GUS*) (Chapman et al., 1992) was inoculated onto previously described transgenic tobacco lines carrying the *GUS* gene under the control of the cauliflower mosaic virus 35S promoter (Hobbs et al., 1990). Line T19 accumulates high levels of *GUS* mRNA and *GUS* enzyme activity. In comparison, line T4 accumulates approximately eightfold lower levels of *GUS* mRNA and GUS enzyme activity (Hobbs et al., 1990; J.J. English and D.C. Baulcombe, unpublished data). When the two transgene loci are combined in F_1 plants, the T4 phenotype is epistatic to the T19 phenotype. That is, the T4 transgene can silence the T19 transgene, resulting in low levels of *GUS* activity and *GUS* mRNA. In this article, we show that T4 plants also inhibit accumulation of PVX–*GUS* and use this property to examine gene silencing in more detail. In addition, PVX derivatives expressing the tomato polygalacturonase gene (PVX–*PG*) or the bacterial neomycin phosphotransferase gene (PVX–*NPT*) were inhibited on tomato and tobacco lines, respectively, in which homologous

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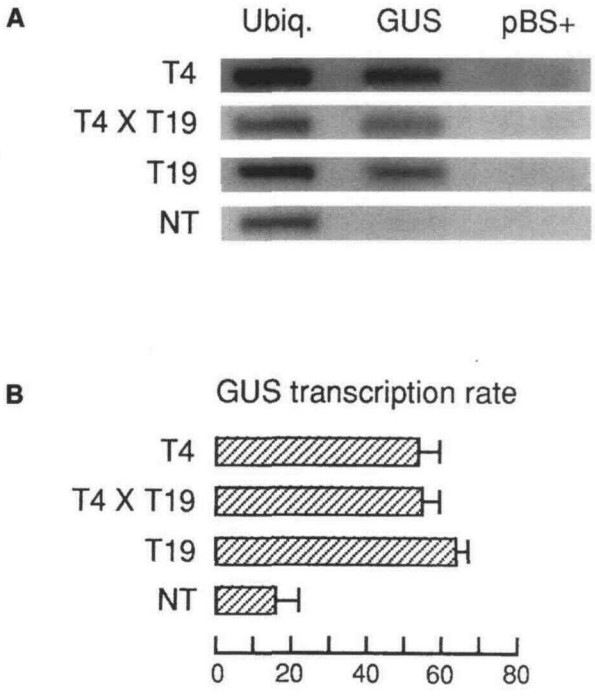

Figure 1. Transcription Rate of *GUS* Transgenes Estimated by Nuclear Run-Off Assays.

(A) ^{32}P-labeled run-off transcripts were prepared using nuclei from T4, T19, T4 × T19 F_1, and nontransformed (NT) plants. The transcripts were hybridized to slot blots containing 1 μg each of a ubiquitin cDNA–containing plasmid (Ubiq.), a *GUS*-containing plasmid (GUS), and pBluescript (pBS+). After washing, filters were exposed to x-ray film or analyzed on a Fujix Bio-Imaging Analyzer Bas 1000.

(B) *GUS* transcription rate was determined by quantification of the hybridization signals, using a Fujix Bio-Imaging Analyzer Bas 1000 and normalizing the *GUS* signal to the ubiquitin signal in each experiment. Three experiments were performed by using independently isolated sets of nuclei. The average *GUS* transcription rates (percentage of ubiquitin) from these experiments are shown. Error bars correspond to one standard deviation.

transgenes were silenced. These results support the view that transgenic virus resistance and gene silencing operate through similar mechanisms and that experiments with virus constructs may reveal new details of these mechanisms.

RESULTS

Post-Transcriptional Gene Silencing in Tobacco Line T4

To determine whether the gene silencing mechanism in line T4 operates at the post-transcriptional level, we estimated *GUS* transcription rates by nuclear run-off assays. Nuclei were isolated from T4, T19, T4 × T19 F_1, and nontransformed plants. Radioactive run-off transcripts were detected by hybridization to plasmid DNA containing the *GUS* coding sequence. A ubiquitin-containing plasmid (Mueller et al., 1995) was used as an internal standard, and pBluescript (Stratagene) served as a negative control.

Figure 1A shows the results of a typical nuclear run-off experiment. Figure 1B shows the average of data from three separate experiments in which the rate of *GUS* transcription was standardized to ubiquitin. By using this assay, we determined that plants with low (T4 and T4 × T19 F_1) and high (T19) *GUS* mRNA levels transcribed the *GUS* transgene at similar rates. Any slight difference was not large enough to account for the approximately eightfold difference in *GUS* mRNA accumulation between lines T4 and T19 (Hobbs et al., 1990; J.J. English and D.C. Baulcombe, unpublished data). We concluded that the different levels of *GUS* mRNA in lines T4 and T19 were regulated at the post-transcriptional level and that this would be appropriate material for examining the relationship between transgenic virus resistance and gene silencing.

Inhibition of PVX-*GUS* on Tobacco Line T4

Figure 2 shows the structures of PVX and several derivatives that have been modified to express foreign marker genes. To determine whether silencing of the *GUS* transgene could affect virus accumulation, we inoculated nontransformed, T4, T19, and T4 × T19 F_1 plants with PVX–*GUS* (Figure 2; Chapman et al., 1992). Figure 3A shows histochemical staining of noninoculated leaves from each genotype. Line T19 had a high level of *GUS* activity, whereas *GUS* staining was barely detectable in T4 and T4 × T19 F_1 plants. On leaves inoculated with PVX–*GUS*, numerous large *GUS*-positive lesions were seen on the nontransformed and T19 plants (Figures 3A and 3B). Each lesion corresponded to a site in which the hybrid virus had established infection and spread from the inoculated cell. On line T19, the *GUS*-positive viral lesions were superimposed over a background of *GUS* activity from the nuclear transgene. When PVX–*GUS* was inoculated onto T4 plants and T4 × T19 F_1 plants, there were few *GUS*-positive lesions (Figures 3A and 3B).

RNA gel blot analysis of inoculated leaves showed that PVX–*GUS* genomic RNA accumulated to high levels on T19 and nontransformed plants, whereas little or no PVX–*GUS* genomic RNA was detected on the low-expressing lines (Figure 3C). Thus, the transgene in line T4 not only suppressed *GUS* expression from the transgene in line T19, but it also prevented accumulation of PVX–*GUS*. Two other 35S–*GUS* tobacco lines were used in this study: T5, a high expresser, supported PVX–*GUS* infection; and T7, a low expresser (Hobbs et al., 1990, 1993), did not (data not shown). Thus, suppression of PVX–*GUS* was not unique to line T4.

In these experiments, the inoculum was either infectious PVX

RNA generated in vitro or virus particles extracted from *Nicotiana clevelandii* plants that had been infected previously with the infectious RNA. Similar results were obtained with both types of inoculum.

Inhibition of virus accumulation was dependent on sequence homology of the inoculated virus and the silencing transgene. A PVX derivative expressing the jellyfish green fluorescent protein (PVX–*GFP*) (Figure 2; Chalfie et al., 1994; Baulcombe et al., 1995), which has no homology with *GUS*, accumulated equally well on T4, T19, T4 × T19 F_1, and nontransformed tobacco plants, as shown by the morphology and number of *GFP*-positive lesions (Figures 3D and 3E) and RNA gel blot analysis (data not shown).

Other Examples of Post-Transcriptional Gene Silencing Affecting Virus Accumulation

To establish whether the effect on virus accumulation is a general feature of post-transcriptional gene silencing, we tested two other PVX constructs on plants with gene-silencing activity. A PVX derivative carrying part of the tomato polygalacturonase cDNA (PVX–*PG*; Figure 2) was inoculated onto tomato line E109-C1.10, which exhibits post-transcriptional silencing of a 35S–*PG* transgene, and the nontransformed line E109-C1.5 (C. Bird, unpublished data). The resulting RNA gel blots are presented in Figure 4. PVX–*PG* genomic RNA accumulated to high levels on the leaves of the nontransformed line, but there was no detectable accumulation on the leaves of the *PG*-silenced line (Figure 4). To determine whether the effect on PVX-*PG* was sequence specific, PVX–*GFP* and PVX–*GUS* were also used as controls. RNA gel blot analysis indicated that PVX–*GFP* accumulated to similar levels on the *PG*-silenced line and on the nontransformed line (Figure 4). Histochemical staining for GUS activity showed that PVX–*GUS* also infected both genotypes equally well, producing an average of 21.8 lesions per leaf on the *PG*-silenced line (SD = 6.9) and 23.8 lesions per leaf on the nontransformed line (SD = 9.4).

PVX–*NPT* was unable to infect tobacco plants exhibiting post-transcriptional silencing of a 35S–*NPT* transgene (lines GVCHS(320)-1 and GVCHS(300)-6; Ingelbrecht et al., 1994; data not shown). PVX–*GFP* and PVX–*GUS* accumulated to high levels on these lines, confirming that the effect on PVX–*NPT* is sequence specific (data not shown). Thus, three independent examples have shown that post-transcriptional silencing of nonviral transgenes can inhibit accumulation of PVX derivatives homologous to those transgenes.

Target Specificity within the *GUS* Coding Sequence

Because the *GUS* gene is dispensable for PVX–*GUS*, it was possible to test the effect of deletions within the *GUS* coding sequence without affecting pathogenicity. This deletion analysis addressed whether particular sequence motifs or regions or a minimal length of homology are required for the virus to

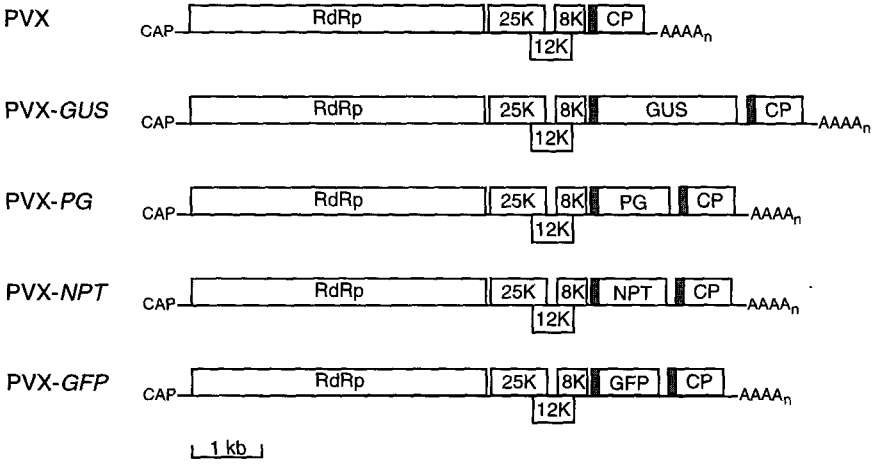

Figure 2. Structure of PVX and Derivatives.

PVX is a single-strand RNA virus with a cap structure at its 5′ end and polyadenylated at its 3′ end. Open reading frames (ORFs) corresponding to RdRp, 25K, 12K, 8K, and coat protein (CP) are shown as boxes. PVX–*GUS* and PVX–*GFP* have the marker genes β-glucuronidase and jellyfish green fluorescent protein, respectively, inserted between the 8K ORF and the CP ORF (Chapman et al., 1992; Baulcombe et al., 1995). PVX–*PG* and PVX–*NPT* have the marker genes polygalacturonase and neomycin phosphotransferase, respectively, inserted between the 8K ORF and the CP ORF. Expression of inserted marker genes is controlled by a duplicated CP promoter (shaded boxes).

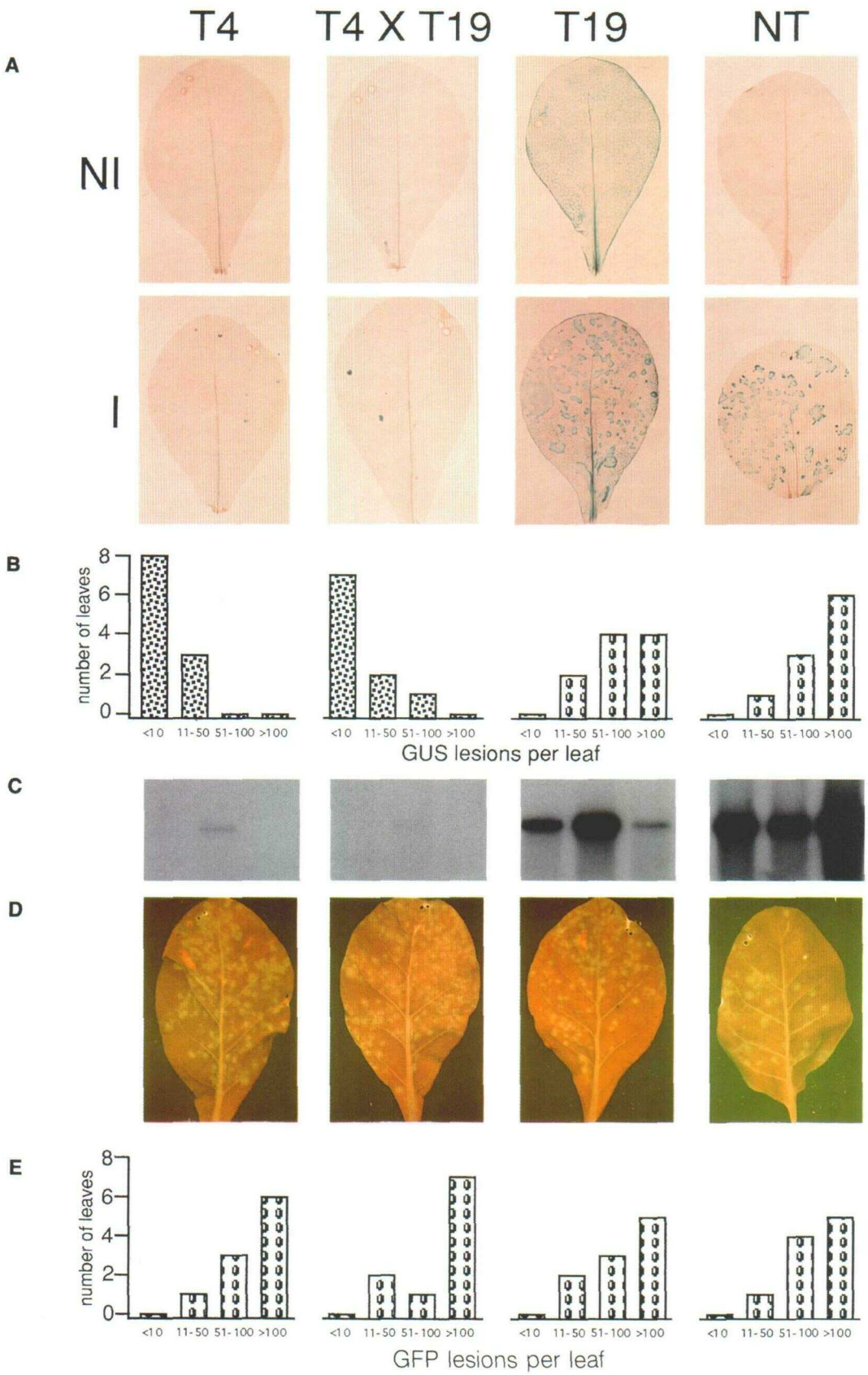

Figure 3. Infection of Wild-Type and Transgenic Tobacco Lines with PVX–*GUS* and PVX–*GFP*.

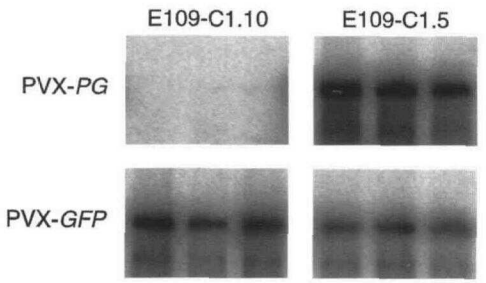

Figure 4. *Infection of Nontransformed and PG-Silenced Tomato Lines with PVX–PG and PVX–GFP.*

Accumulation of PVX–*PG* or PVX–*GFP* genomic RNA was assayed by RNA gel blot analysis 11 days postinoculation. Line E109-C1.5 is the untransformed control, and line E109-C1.10 has a silenced *PG* transgene, resulting in a 99% reduction of *PG* expression (C. Bird, personal communication). The three lanes shown for each tomato line represent samples from three different plants.

be a target of the gene-silencing mechanism. Figure 5 shows the 3′ region of PVX–*GUS* and three derivatives, each of which has ~700 bases deleted from the *GUS* coding sequence.

Infectious RNA from each construct was inoculated onto T4, T19, and nontransformed tobacco. RNA was extracted from the inoculated leaves 7 days postinoculation, and virus genomic RNA accumulation was assessed by RNA gel blot analysis. PVX–*GU* (3′ terminal deletion) replicated equally well on T4, T19, and nontransformed tobacco (Figure 5B). In contrast, PVX–*GS* (internal deletion) and PVX–*US* (5′ terminal deletion) accumulated to high levels on T19 and nontransformed tobacco but not on T4 tobacco (Figures 5C and 5D). The PVX–*GUS* deletion derivatives behaved similarly on T5 (high expresser) and T7 (low expresser) to T19 and T4, respectively (data not shown). The interpretation of these data is that the 3′ region of the *GUS* coding sequence is required for the virus to be a target of gene silencing in lines T4 and T7.

Methylation of the *GUS* Transgene

Hobbs et al. (1990) showed that complete methylation of an AvaI restriction site at the 3′ terminus of the *GUS* coding sequence was correlated with silencing of the *GUS* transgenes in lines T4 and T7. In contrast, there was little or no methylation of AvaI and PstI sites at the 5′ terminus of the *GUS* coding sequence and within the 35S promoter (Hobbs et al., 1990, 1993). These observations, combined with our finding that the 3′ region of the *GUS* coding sequence is important for PVX–*GUS* inhibition, prompted us to examine methylation of the *GUS* transgene in lines T4 and T7 in more detail.

As shown in Figure 6A, a number of cytosine methylation–sensitive restriction enzyme recognition sequences were identified throughout the *GUS* coding sequence. DNA gel blot analyses were performed using SnaBI, BstBI, and MluI in combination with EcoRI, which is not sensitive to cytosine methylation, and a probe corresponding to the *GUS* coding sequence. An EcoRI-SnaBI digest would give rise to a 1.7-kb fragment if the SnaBI site was not methylated. The 1.7-kb fragment was observed for T4, T7, T5, and T19, indicating that the SnaBI site was not methylated in any of these genotypes (Figure 6B and data not shown). An EcoRI-BstBI digest would give rise to 1.0- and 3.0-kb fragments if the BstBI site was not methylated. The 1.0- and 3.0-kb fragments were observed for T5, T7, and T19 (Figure 6B and data not shown). In line T4, a 4-kb fragment was observed, indicating that the BstBI site was methylated. An EcoRI-MluI digest would give rise to 0.7- and 0.85-kb fragments if each of the three MluI sites was not methylated. The 0.7- and 0.85-kb fragments were observed for T5 and T19 (Figure 6B and data not shown). A 1.55-kb fragment was observed for T4 and T7, corresponding to a fragment extending from the EcoRI site to the MluI site near the 5′ end of the *GUS* coding sequence (Figure 6B and data not shown). This result indicated that the two MluI sites near the 3′ end of *GUS* were methylated in lines T4 and T7.

These data and previously reported AvaI and PstI site methylation data (Hobbs et al., 1990, 1993) are summarized in Figure 6A, where highly methylated restriction sites are marked by asterisks. Additional DNA gel blot analyses were performed using EcoRI plus the cytosine methylation–sensitive restriction enzymes BstUI, HhaI, and HpaII, which have 18, 12, and 9 sites, respectively, distributed throughout the *GUS* coding sequence and nopaline synthase 3′ end. This analysis confirmed that the 3′ region of the *GUS* coding sequence was extensively methylated in lines T4 and T7 (data not shown).

DISCUSSION

Gene Silencing and Virus Resistance

Previous models of post-transcriptional gene silencing were based on the phenotypes of transgenic plants expressing viral

Figure 3. (continued).

(A) Noninoculated (NI) and inoculated (I) leaves stained histochemically for GUS activity.
(B) Histograms showing the number of leaves of each line with the indicated numbers of *GUS*-positive lesions 7 days postinoculation with PVX–*GUS*.
(C) Accumulation of PVX–*GUS* genomic RNA 7 days postinoculation assayed by RNA gel blot analysis. The three lanes shown for each tobacco line represent samples from three different plants.
(D) Tobacco leaves 7 days postinoculation with PVX–*GFP*, illuminated with UV light (396 nm).
(E) Histograms showing the number of leaves of each line with the indicated numbers of *GFP*-positive lesions 7 days postinoculation with PVX–*GFP*. Different shadings in the histograms indicate the morphology of viral lesions: small spots indicate small lesions; larger spots indicate larger lesions. NT, nontransformed plants.

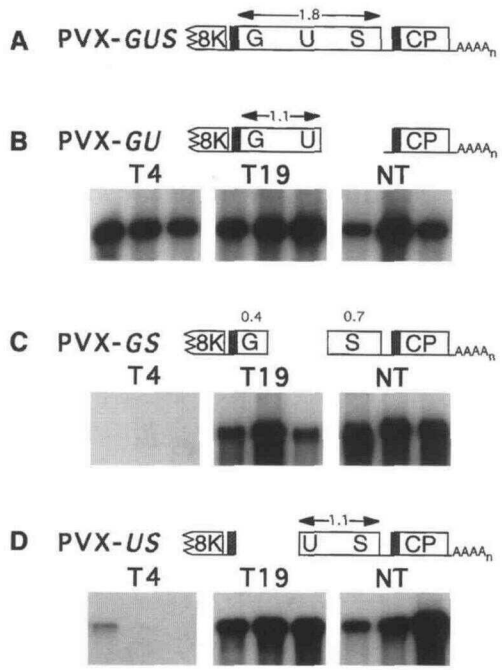

Figure 5. Infection of T4, T19, and Nontransformed Tobacco with PVX–*GUS* Deletion Derivatives.

(A) The 3′ region of PVX–*GUS*.
(B) The 3′ region of PVX–*GU* and RNA gel blot analysis of T4, T19, and nontransformed (NT) tobacco leaves 7 days postinoculation.
(C) The 3′ region of PVX–*GS* and RNA gel blot analysis of tobacco leaves 7 days postinoculation.
(D) The 3′ region of PVX–*US* and RNA gel blot analysis of tobacco leaves 7 days postinoculation.
Approximate lengths of segments of the *GUS* coding sequence (in kilobases) are shown above. Shaded and open boxes, CP, and *GUS* are as given in Figure 2. The three lanes shown for each tobacco line represent samples from three different plants.

sequences (Smith et al., 1994; Mueller et al., 1995). Gene silencing, according to these models, requires an actively transcribed transgene and is mediated by RNA degradation. The affected RNAs could include mRNA from the silenced transgene, mRNA from a homologous (trans)gene, and viral RNA: any RNA homologous to the silenced transgene would be a potential target. These models explain the association of gene silencing and virus resistance conferred by transgenes based on viral sequences. In addition, because the resistance suppresses viruses that accumulate in the cytoplasm, the models introduce the novel idea that gene silencing is a cytoplasmic process.

A prediction of these models is the potential for virus resistance in any plant with a post-transcriptionally silenced transgene. Resistance would depend on sequence homology between the silenced transgene and the virus. This prediction was fulfilled with PVX–*GUS*, PVX–*PG*, and PVX–*NPT* in plants exhibiting silencing of homologous transgenes (Figures 3 and 4, and data not shown). These new data provide additional support for the concept that post-transcriptional gene silencing and virus resistance result from an RNA-based mechanism that is active in the cytoplasm (Smith et al., 1994; Mueller et al., 1995). The finding that nuclear β-1,3-glucanase RNA levels are unaffected by cosuppression in tobacco (de Carvalho et al., 1995) is also consistent with the idea that post-transcriptional gene silencing occurs in the cytoplasm.

In the deletion analysis of PVX–*GUS*, we exploited the effect of gene silencing on virus accumulation to identify the target of the proposed RNA degradation mechanism. This analysis revealed that the 3′ region of the *GUS* coding sequence was the target of gene silencing in tobacco lines T4 and T7 (Figure 5 and data not shown). This sequence specificity could indicate that there are preferred sequence motifs or secondary structures of the RNA degradation mechanism within the 3′ region of the *GUS* coding sequence. A similar suggestion was based on analyses of RNA from tomato in which PG was cosuppressed (Smith et al., 1990) and from tobacco displaying homology-dependent resistance to tobacco etch virus (Goodwin et al., 1996). In both examples, there were small RNAs of discrete sizes from the silenced genes that could be products of the degradation mechanism. An alternative explanation of localized RNA degradation is that the target region is defined by its position in the RNA molecule. There is precedent for position-dependent RNA degradation from the finding that natural RNA degradation in plants can be initiated at the 3′ end of the molecule (Green, 1993).

DNA Methylation and Post-Transcriptional Gene Silencing

The data reported here (Figures 1 and 6) add to a growing number of examples in which DNA methylation is associated with post-transcriptional gene silencing (Ingelbrecht et al., 1994; Smith et al., 1994; E. Mueller and D.C. Baulcombe, unpublished results). However, the significance of DNA methylation associated with post-transcriptional gene silencing is not known (Matzke and Matzke, 1995). The current report presents two findings that may advance our understanding of this relationship: (1) DNA methylation within the silenced *GUS* transgenes was concentrated in the 3′ region of the coding sequence (Figure 6); and (2) the RNA target of the silencing mechanism was also in this region (Figure 5). These new results are incorporated into the model for gene silencing in tobacco lines T4 and T7 shown in Figure 7. Aspects of this model may also apply to other examples of post-transcriptional gene silencing and to examples of homology-dependent virus resistance.

Briefly, gene silencing would be initiated by an aberrant form of the transgene RNA (aRNA). The aRNA would serve as a template for a plant-encoded RdRp that would produce antisense RNA (asRNA) molecules. The asRNA would be confined to the 3′ region of the *GUS* coding sequence, and

RNAs homologous to this region would be targeted for destruction. Transgene methylation could be a cause and/or an effect of the silencing mechanism. The major components of this model–aRNA, asRNA, and DNA methylation—are discussed in more detail.

aRNA and asRNA

The degree of gene silencing varies among transformed lines. Some lines may be strongly silenced, whereas other lines with the same trangene show little or no gene silencing (Hobbs et al., 1990). This variability is heritable, meaning that there must be a feature of the DNA at or close to the silencing locus that influences, either directly or indirectly, the RNA degradation mechanism responsible for gene silencing. The current model for gene silencing in lines T4 and T7 (Figure 7) proposes that this DNA feature affects production of an aRNA. The aRNA would transmit the signal for gene silencing from the DNA to the RNA level, at which the silencing mechanism acts.

A previous model (Smith et al., 1994) suggested that gene silencing would be a correction for gene overexpression and that accumulation of transgene-homologous RNAs above a threshold level would be a quantitative "aberration" that triggers the silencing mechanism. This assertion was based on nuclear run-off assays that suggested that silencing potato virus Y–derived transgenes were transcribed at four to five times the rate of nonsilenced transgenes. However, the data reported here (Figure 1) and previously (Mueller et al., 1995) show that silenced and nonsilenced transgenes can be transcribed at similar rates. Therefore, we suggest that a qualitative feature of the aRNA can initiate gene silencing. This idea of a qualitatively aberrant RNA fits well with the earlier suggestion that gene silencing is mediated via asRNA produced by a plant-encoded RdRp (Smith et al., 1994). The structural aberration in the aRNA could make it a preferred template for the RdRp, whereas normal pre-mRNA and mRNA would not serve as efficient templates for the RdRp. The idea of a qualitatively aberrant RNA does not exclude the possibility that a quantitative element may affect gene silencing. Obviously, if more aRNA were produced, the RNA degradation system would be more efficient.

The idea that asRNA is involved in post-transcriptional gene silencing is based on the potential for many different RNA

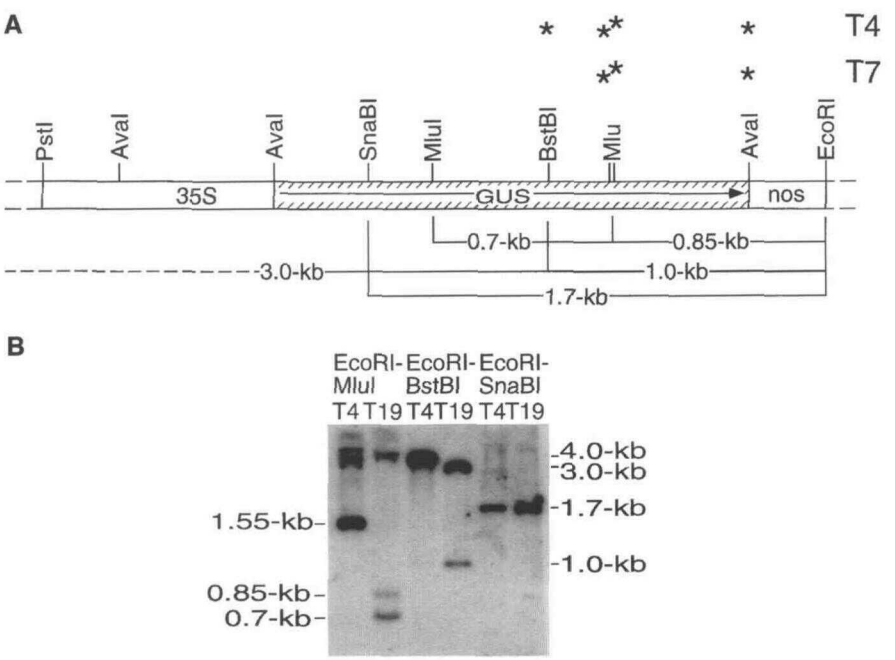

Figure 6. Methylation of the *GUS* Transgene in Lines T4 and T7.

(A) Restriction map of the *GUS* transgene present in lines T4 and T7. Lengths of predicted restriction fragments are shown below. Restriction sites that are highly methylated in lines T4 and T7 are marked by asterisks. SnaBI, BstBI, and MluI site methylation data are presented in **(B)** (data not shown). PstI and AvaI site methylation data are from Hobbs et al. (1990, 1993). AvaI methylation data have been confirmed by us (J.J. English and D.C. Baulcombe, unpublished data). nos, nopaline synthase.

(B) DNA gel blot analysis of tobacco lines T4 and T19, using EcoRI plus methylation-sensitive restriction enzymes. Lengths of the observed bands are given at left and right. Reprobing of this blot with a chloroplast probe showed that T4 and T19 DNA samples had been digested to the same extent (data not shown).

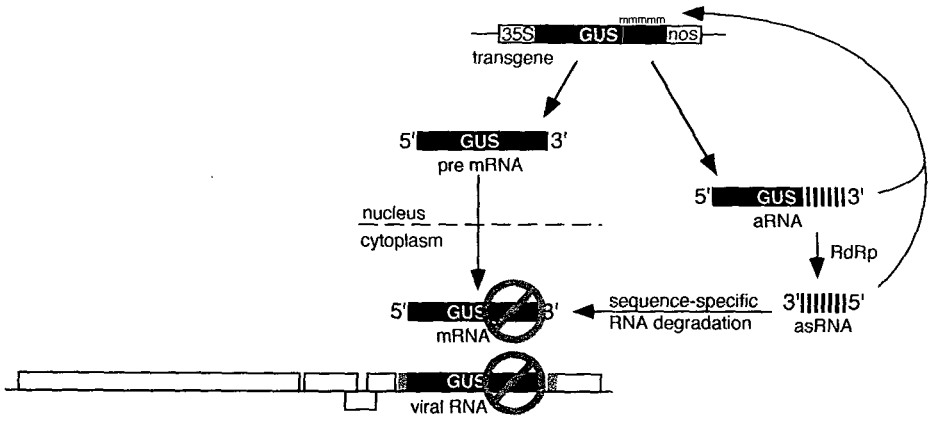

Figure 7. Model for Post-Transcriptional Gene Silencing in Tobacco Lines T4 and T7.

A 35S–*GUS* transgene is shown in the nucleus. DNA methylation is represented by mmmmm. Normal pre-mRNA and aRNA are produced. The pre-mRNA is processed and transported to the cytoplasm as mature mRNA. The aRNA serves as a substrate for a plant-encoded RdRp, and asRNA corresponding to the methylated region of the transgene is produced. The asRNA molecules target complementary sequences, including the mRNA and homologous viral RNA, for sequence-specific degradation. Arrows from the aRNA and the asRNA to the *GUS* transgene represent the idea that RNA-mediated DNA methylation might occur. nos, nopaline synthase.

species to be targeted in a homology-dependent manner and invokes asRNA as the specificity determinant of the RNA degradation mechanism. From deletion experiments with PVX–*GUS* (Figure 5), we infer that asRNA molecules would be concentrated at the 3′ end of the *GUS* coding sequence in tobacco lines T4 and T7 (Figure 7).

Transgene Methylation

The concurrence of DNA methylation in the silenced *GUS* transgenes in lines T4 and T7 with the RNA target of the silencing mechanism is striking. We envisage two ways in which DNA methylation could be involved with post-transcriptional gene silencing, either or both of which may apply. Transgene methylation could lead directly to the formation of aRNA (Figure 7), possibly due to transcript termination within the methylated region. Transcript termination could give rise to aRNAs with different 3′ ends distributed throughout the methylated region. If asRNAs were short and localized near the 3′ ends of their aRNA templates, there would be extensive overlap between the methylated region and the RNA target of the silencing mechanism. There are precedents from the analysis of methylated fungal DNA and viral sequences in mammalian cells for termination of transcription in highly methylated regions of DNA (Keshet et al., 1985; Barry et al., 1993).

Alternatively, DNA methylation could result from the silencing mechanism via interactions of the aRNA and/or the asRNA with the chromatin (Figure 7). Support for this idea comes from the finding that RNA-directed methylation of nuclear DNA occurred in plants carrying transgenes derived from the potato spindle tuber viroid (Wassenegger et al., 1994).

These alternatives are not mutually exclusive. If DNA methylation is both a cause and a consequence of post-transcriptional gene silencing, the processes depicted in Figure 7 would form a cycle, and the degree of gene silencing and transgene methylation would increase with the age of the plant. This cycle could be initiated by any process leading to even a low level of aRNA or asRNA formation, such as DNA methylation due to ectopic pairing (Flavell, 1994; Matzke and Matzke, 1995). The cycle could be broken by processes that prevent transgene methylation or asRNA formation, such as resetting of DNA methylation at meiosis. These ideas are consistent with examples in which transgene silencing increases throughout development and is reset at meiosis (Dehio and Schell, 1994; de Carvalho et al., 1995; H. Vaucheret, personal communication).

The aRNA and asRNA remain elusive components of the model depicted in Figure 7. They may be difficult to detect if they are heterodisperse and/or if they are present in very small amounts. By showing that a particular region of the transgene RNA may be targeted by gene silencing, we have opened up a new perspective that should facilitate the search for aRNA and asRNA. We predict that the asRNA will be complementary to the target region of the transgene RNA only and that aRNA may be altered within this region. A search for these RNA species will be a next step in our continuing attempt to understand post-transcriptional gene silencing and the related phenomenon of homology-dependent resistance to viruses.

METHODS

Nuclear Run-Off Assays

Isolation of nuclei and ^{32}P-labeling of nascent transcripts were performed as described by Mueller et al. (1995). Labeled transcripts were extracted twice with phenol–chloroform and ethanol precipitated. Slot

blots were prepared using Hybond-N nylon membranes (Amersham). Sample DNAs were the β-glucuronidase gene (*GUS*) coding sequence cloned in pBluescript KS+ (Stratagene), a ubiquitin cDNA clone from *Antirrhinum majus* (Mueller et al., 1995), and pBluescript. One microgram of linear DNA was used per slot and fixed to the membrane by UV cross-linking. Filters were prehybridized as given in Mueller et al. (1995), labeled transcripts were added at a concentration of 10^6 cpm/mL, and hybridization was allowed to proceed overnight. Filters were washed at a final stringency of 0.15 M sodium chloride, 0.015 M sodium citrate, 0.5% SDS at 65°C, blotted to remove excess liquid, and exposed to x-ray film or analyzed on a Fujix Bio-Imaging Analyzer Bas 1000 (Fuji Photo Film Co., Ltd., Fuji, Japan).

Virus Constructs

Wild-type potato virus X (pTXS) (Kavanagh et al., 1992), PVX–*GUS* (Chapman et al., 1992), and PVX–*GFP* (Baulcombe et al., 1995) have been described previously. PVX–*GU* was derived from PVX–*GUS* by deleting the DNA between a unique BstBI site (position 1093 within the GUS coding sequence) and a ClaI site at the 3′ terminus of *GUS*. PVX–GS was derived from PVX–*GUS* by deleting the DNA between a unique SnaBI site (position 383 within the *GUS* coding sequence) and BstBI. PVX–*US* was made by cloning a polymerase chain reaction product carrying *GUS* sequences from position 787 to the 3′ terminus into the PVX vector P2C2S (Baulcombe et al., 1995). PVX–*PG* was made by cloning a polymerase chain reaction product carrying tomato polygalacturonase cDNA sequences from position 59 to 1060 (Grierson et al., 1986) into PVX vector P2C2S (Baulcombe et al., 1995).

In Vitro Transcription and PVX Infection

In vitro transcription reactions to produce infectious PVX RNA were performed as described previously (Chapman et al., 1992). For RNA analysis, infectious RNA was rubbed onto the leaves of 5- to 7-week-old tobacco plants or 10- to 14-day-old tomato plants in the presence of a small amount of carborundum. For GUS histochemistry, virus that had been bulked up by infection of *Nicotiana clevelandii* was used.

GUS Histochemistry

Histochemical staining of leaf material was performed using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as described by Jefferson (1987). Leaves were vacuum infiltrated with a solution containing 0.5 mg/mL X-gluc, 50 mM sodium phosphate, pH 7, 1 mM EDTA, and 0.5% Triton X-100 and incubated for 16 to 18 hr at 37°C before clearing the tissue by using chloral hydrate solution.

RNA Gel Blot Analysis

RNA was extracted from inoculated leaves 7 to 11 days postinoculation, as described previously (Mueller et al., 1995). RNA samples (1 μg) were electrophoresed on 0.8% (w/v) agarose–formaldehyde gels, according to Sambrook et al. (1989), transferred to Hybond-N membranes, and hybridized with a ^{32}P-labeled RNA probe corresponding to the 3′ terminal 1562 bases of PVX.

GFP Visualization

GFP activity was visualized as described previously (Baulcombe et al., 1995). Inoculated leaves were illuminated with UV light (396 nm) for photography and for counting of *GFP*-positive lesions.

DNA Gel Blot Analysis

DNA was extracted from 5- to 7-week-old plants (i.e., similar in age to plants used for PVX infection), and DNA gel blot analysis was performed as described previously (Mueller et al., 1995).

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REFERENCES

Barry, C., Faugeron, G., and Rossignol, J.-L. (1993). Methylation induced premeiotically in *Ascobulus*: Coextension with DNA repeat lengths and effect on transcript elongation. Proc. Natl. Acad. Sci. USA **90,** 4557–4561.

Baulcombe, D.C., Chapman, S., and Santa Cruz, S. (1995). Jellyfish green fluorescent protein as a reporter for virus infections. Plant J. **7,** 1045–1053.

Brusslan, J.A., Karlin-Neumann, G.A., Huang, L., and Tobin, E.M. (1993). An Arabidopsis mutant with a reduced level of *cab*140 RNA is a result of cosuppression. Plant Cell **5,** 667–677.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green fluorescent protein as a marker for gene expression. Science **263,** 802–805.

Chapman, S., Kavanagh, T.A., and Baulcombe, D.C. (1992). Potato virus X as a vector for gene expression in plants. Plant J. **2,** 549–557.

de Carvalho Niebel, F., Frendo, P., Van Montagu, M., and Cornelissen, M. (1995). Post-transcriptional cosuppression of β-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. Plant Cell **7,** 347–358.

Dehio, C., and Schell, J. (1994). Identification of plant genetic loci involved in posttranscriptional mechanism for meiotically reversible transgene silencing. Proc. Natl. Acad. Sci. USA **91,** 5538–5542.

Flavell, R.B. (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA **91,** 3490–3496.

Goodwin, J., Chapman, K., Swaney, S., Parks, T.D., Wernsman, E.A., and Dougherty, W.G. (1996). Genetic and biochemical dis-

section of transgenic RNA-mediated virus resistance. Plant Cell **8,** 95–105.

Green, P.J. (1993). Control of mRNA stability in higher plants. Plant Physiol. **102,** 1065–1070.

Grierson, D., Tucker, G.A., Keen, J., Ray, J., Bird, C.R., and Schuch, W. (1986). Sequencing and identification of a cDNA clone for tomato polygalacturonase. Nucleic Acids Res. **14,** 8595–8603.

Hobbs, S.L.A., Kpodar, P., and DeLong, C.M.O. (1990). The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol. Biol. **15,** 851–864.

Hobbs, S.L.A., Warkentin, T.D., and DeLong, C.M.O. (1993). Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. **21,** 17–26.

Ingelbrecht, I., Van Houdt, H., Van Montagu, M., and Depicker, A. (1994). Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc. Natl. Acad. Sci. USA **91,** 10502–10506.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The *GUS gene fusion system.* Plant Mol. Biol. Rep. **5,** 387–405.

Jorgensen, R.A. (1990). Altered gene expression in plant due to *trans* interactions between homologous genes. Trends Biotech. **8,** 340–344.

Jorgensen, R.A. (1992). Silencing of plant genes by homologous transgenes. AgBiotech News Info. **4,** 265–273.

Kavanagh, T.A., Goulden, M., Santa Cruz, S., Chapman, S., Barker, I., and Baulcombe, D.C. (1992). Molecular analysis of a resistance-breaking strain of potato virus X. Virology **189,** 609–617.

Keshet, I., Yisraeli, J., and Cedar, H. (1985). Effect of regional DNA methylation on gene expression. Proc. Natl. Acad. Sci. USA **82,** 2560–2564.

Lindoo, J.A., Silva-Rosales, L., Proebsting, W.M., and Dougherty, W.G. (1993). Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. Plant Cell **5,** 1749–1759.

Matzke, A.J.M., Neuhuber, F., Park, Y.-D., Ambros, P.F., and Matzke, M.A. (1994). Homology-dependent gene silencing in transgenic plants: Epistatic silencing loci contain multiple copies of methylated transgenes. Mol. Gen. Genet. **244,** 219–229.

Matzke, M.A., and Matzke, A.J.M. (1995). How and why do plants inactivate homologous (trans)genes? Plant Physiol. **107,** 679–685.

Meyer, P., Heidmann, I., and Niedenhof, I. (1993). Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia. Plant J. **4,** 89–100.

Mueller, E., Gilbert, J.E., Davenport, G., Brigneti, G., and Baulcombe, D.C. (1995). Homology-dependent resistance: Transgenic virus resistance in plants related to homology-dependent gene silencing. Plant J. **7,** 1001–1013.

Napoli, C., Lemieux, C., and Jorgensen, R.A. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans.* Plant Cell **2,** 279–289.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Smith, C.J.S., Watson, C.F., Bird, C.R., Ray, J., Schuch, W., and Grierson, D. (1990). Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Mol. Gen. Genet. **224,** 447–481.

Smith, H.A., Swaney, S.L., Parks, T.D., Wernsman, E.A., and Dougherty, W.G. (1994). Transgenic plant virus resistance mediated by untranslatable sense RNAs: Expression, regulation, and fate of nonessential RNAs. Plant Cell **6,** 1441–1453.

Van Blokland, R., Van der Geest, N., Mol, J.N.M., and Kooter, J.M. (1994). Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. Plant J. **6,** 861–877.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M., and Stuitji, A.R. (1990). Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2,** 291–299.

Wassenegger, M., Heimes, S., Riedel, L., and Sänger, H.L. (1994). RNA-directed de novo methylation of genomic sequences in plants. Cell **76,** 567–576.