# High molecular weight RNAs and small interfering RNAs induce systemic posttranscriptional gene silencing in plants

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**Posttranscriptional gene silencing (PTGS) in transgenic plants is an epigenetic form of RNA degradation related to PTGS and RNA interference (RNAi) in fungi and animals. Evidence suggests that transgene loci and RNA viruses can generate double-stranded RNAs similar in sequence to the transcribed region of target genes, which then undergo endonucleolytic cleavage to generate small interfering RNAs (siRNA) that promote degradation of cognate RNAs. The silent state in transgenic plants and in *Caenorhabditis elegans* can spread systemically, implying that mobile silencing signals exist. Neither the chemical nature of these signals nor their exact source in the PTGS pathway is known. Here, we use a positive marker system and real-time monitoring of green fluorescent protein expression to show that large sense, antisense, and double-stranded RNAs as well as double-stranded siRNAs delivered biolistically into plant cells trigger silencing capable of spreading locally and systemically. Systemically silenced leaves show greatly reduced levels of target RNA and accumulate siRNAs, confirming that RNA can induce systemic PTGS. The induced siRNAs represent parts of the target RNA that are outside of the region of homology with the triggering siRNA. Our results imply that siRNAs themselves or intermediates induced by siRNAs could comprise silencing signals and that these signals induce self-amplifying production of siRNAs.**

Posttranscriptional gene silencing (PTGS) is an epigenetic form of mRNA degradation important in the defense of plants against virus infection and widely used as a tool for inactivating gene expression (1–3). Discovered in plants, PTGS or the closely related phenomenon RNA interference (RNAi) occurs in many organisms, including *Neurospora crassa*, *Trypanosoma brucei*, *Caenorhabditis elegans*, *Drosophila*, and mammals. The underlying mechanisms are thought to be highly conserved in evolution (2, 4). RNAi in animals is initiated by double-stranded RNAs (dsRNAs) similar in sequence to the transcribed region of target genes. These dsRNAs undergo endonucleolytic cleavage to generate 21- to 23-nt-long small interfering RNAs (siRNAs), which then promote RNA degradation (5–7)

Remarkably, the silent state in transgenic plants and in *C. elegans* can spread from cell to cell and even systemically throughout the organism, implying the existence of mobile silencing signals (2, 8). Little is known about the chemical nature of these signals, but it seems likely that the sequence-specific component is an RNA (8–11). The finding that siRNA and dsRNA accumulate in silent tissues, together with studies of informative stable transformants and PTGS induced by RNA viruses, supports the view that dsRNAs and siRNAs have key roles in plant PTGS (12–14). Nevertheless, direct evidence that these or other RNAs can induce systemic PTGS or comprise silencing signals in plants is lacking.

In the present study, we used a positive marker system and real-time monitoring of green fluorescent protein (GFP) expression to show that double-stranded siRNAs, large sense, antisense, and double-stranded RNAs delivered biolistically into plant cells trigger PTGS capable of spreading locally and systemically. The introduced siRNAs trigger the production of siRNAs derived from sequences both 3′ and 5′ of the inducing siRNAs. Our findings support the hypothesis that siRNAs themselves or intermediates induced by siRNAs could comprise silencing signals and are generated in a self-amplifying fashion.

## Materials and Methods

**Transgenic Plants.** The *Nicotiana benthamiana* line designated Nb GFP is the line 16c carrying an mGFP-ER reporter gene with a cauliflower mosaic virus 35S RNA promoter and Nos terminator described by Ruiz *et al.* (15). The *Nicotiana tabacum* line designated Nt TET$^R$GUS was obtained by *Agrobacterium*-mediated leaf-disk transformation (16) of homozygous tobacco line R7 containing a 35S-TET$^R$ transgene and hygromycin-resistance marker (17) with the plasmid pTX-GUS carrying an *Escherichia coli* β-glucuronidase (GUS) reporter gene regulated by a tetracycline repressor (TET$^R$)-repressible promoter and a kanamycin-resistance marker (18). Plants homozygous for a single TX-GUS T-DNA locus were obtained by selfing primary regenerates and selecting for kanamycin-resistant progeny. Plants were raised from seed in 10-cm-diameter Petri dishes containing agar-solidified Linsmaier and Skoog medium (19) at 28°C in constant light (3,000 lux), and then grown on soil in a phytotron at 25°C (16 h 12,000-lux light/8 hr dark). Cells with GUS activity were detected by histological staining (20). When indicated, 3-week-old, hydroponically grown, Nt TET$^R$GUS plants were fed through the roots with 15 μg/ml anhydrotetracycline (17) for 2 days and then stained for GUS.

**Biolistic Delivery.** Plasmids were prepared by standard methods (21). Plasmid p35S-TET$^R$ is the *Eco*RI–*Hin*dIII fragment of pTET1 (22) containing TET$^R$ with a 35S RNA promoter and ocs terminator cloned into pBSKSII (Stratagene) cut with *Eco*RI and *Hin*dIII. The truncations p35S-TET$^R_{0–414}$, p35S-TET$^R_{414–716}$, and p35S-TET$^R_{610–716}$ contain the *Acc*65I–*Nsi*I, *Bam*HI–*Nsi*I and *Bam*HI–*Nde*I fragments, respectively, of p35S-TET$^R$ in sense and antisense orientation. Plasmid p35S-GFP is pUC18 containing mGFP-ER with a 35S promoter and Nos terminator (23). The truncations p35S-GFP$_{0–313}$ and p35S-GFP$_{313–818}$ contain the

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Abbreviations: dsRNA, double-stranded RNA; GFP, green fluorescent protein; GUS, *Escherichia coli* β-glucuronidase; PTGS, posttranscriptional gene silencing; RdRP, RNA-dependent RNA polymerase; RNAi, RNA interference; siRNA, small interfering RNA; TET$^R$, tetracycline repressor.

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*Bam*HI–*Nde*I and *Nde*I–*Sac*I fragments, respectively, of the mGFP-ER transcribed region used for *in vitro* transcription.

RNA transcripts were produced with the relevant fragments of the transcribed regions of TET$^R$ and mGFP-ER cloned into pBS-SK− as templates and treated with DNase by using a Megascript transcription kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Typical yields were 50 $\mu$g of RNA when 1 $\mu$g of DNA template was used. Integrity of the transcripts was verified by agarose-gel electrophoresis under denaturing conditions. RNA transcripts were annealed by heating at 95°C for 2 min and slowly cooling to 37°C over a period of 5 min. To test for RNase sensitivity, single-stranded or annealed RNA was precipitated with ethanol and then incubated in 40 $\mu$g/ml RNase A/200 mM NaCl/100 mM LiCl/1 mM EDTA/10 mM Tris buffer, pH 7.5, for 30 min at 25°C.

siRNAs representing regions of TET$^R$ (22), mGFP-ER (23), and sGFP (24) transcripts were purchased from Mycrosynth (Balgach, Switzerland). Positions of 5′ and 3′ ends relative to the 5′ end of the transcripts are indicated in parentheses. Sense TET$^R$ siRNA, 5′(517)-UGAUAGUAUGCCGCCAUUAUU-3′(537); antisense TET$^R$ siRNA, 5′(535)-UAAUGGCG-GCAUACUAUCAGUA-3′(514); sense mGFP-ER siRNA, 5′(556)-AGAACGGCAUCAAAGCCAACU-3′(576); antisense mGFP-ER siRNA, 5′(574)-UUGGCUUUGAUGC-CGUUCUUUU-3′(553); sense sGFP siRNA, 5′(193)-UUCAC-CUACGGCGUGCAGUGC-3′(213); antisense sGFP siRNA, 5′(211)-ACUGCACGCCGUAGGUGAAGGU-3′(190).

Double-stranded siRNAs with 2- and 3-nt 3′ overhangs were obtained by spontaneous annealing of mixtures of the antisense and sense oligoribonucleotides at room temperature.

Axenically grown plants 12 days after germination and with one true leaf were bombarded by using a biolistic PDS-1000/He particle gun (Bio-Rad). DNA and RNA were loaded on gold particles and were delivered at 1,100 psi, following the manufacturer's recommendations. Silencing of TET$^R$ and GFP transgenes was detected, respectively, by histological staining of GUS and by visual inspection of plants illuminated with a 100-W "blue light" lamp model B100-AP (Ultraviolet Products, Upland, CA). Images were collected with a Powershot Pro 70 digital camera (Canon, Japan).

**RNA Analyses.** Total RNA and fractions enriched in small RNAs were prepared from plant tissues and analyzed by RNA-blot hybridization essentially as described (25). DNA probes were prepared by using a Megaprime kit (Amersham Pharmacia) and [$^{32}$P]dATP and [$^{32}$P]dCTP. The templates used were the *Bam*HI–*Ecl*136II fragment for the full-length cDNA probe, the *Bam*HI–*Hin*cII fragment for the 5′ probe and the *Mfe*I–*Ecl*136II fragment for the 3′ probe of mGFP-ER, respectively.

## Results

**A Positive Marker System for Detecting Silencing in Individual Cells.** We used biolistic delivery to test the ability of RNAs to trigger silencing. Initiation of gene silencing in individual cells is difficult to detect against a background of highly expressing cells. To overcome these problems, we developed a positive marker system based on the silencing of a transcriptional repressor. Tobacco plants were transformed sequentially with a chimeric gene (*35S-TET$^R$*) encoding a bacterial tetracycline repressor (TET$^R$) regulated by the cauliflower mosaic virus 35S RNA promoter (17), and then with a chimeric GUS reporter gene regulated by the TET$^R$-repressible TX promoter (18). Fig. 1 *A–C* illustrates the principle of the assay. If TET$^R$ is highly expressed in these Nt TET$^R$GUS transformants, then transcription of the TET$^R$-repressible target gene will be blocked and no GUS should be detected by histological staining. In contrast, if expression of the *TET$^R$* gene is silenced, then the TET$^R$-repressible reporter gene will be transcribed, GUS will accumulate, and the silenced cells will exhibit a blue coloration.

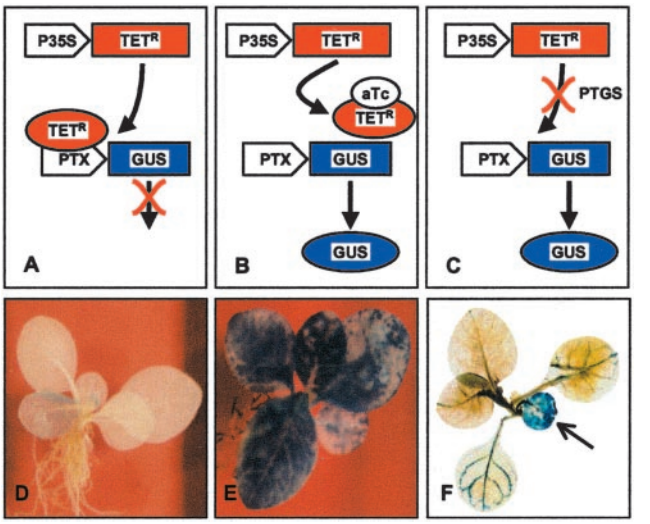


**Fig. 1.** A positive marker system for detecting PTGS. (*A–C*) Cartoons illustrating how expression of a TET$^R$-repressible *TX-GUS* reporter gene (*A*) can be restored by treatment with anhydrotetracycline (aTc) (*B*), and by silencing of a *35S-TET$^R$* transgene (*C*). Elliptical symbols represent proteins. (*D* and *E*) Representative examples of untreated (*D*) and 15 $\mu$g/ml aTc-treated (*E*) Nt TET$^R$GUS plants stained for GUS 2 days later. (*F*) An Nt TET$^R$GUS plant stained 1 month after bombardment with *35S-TET$^R$* plasmid DNA, showing intense staining of the bombarded leaf (arrow) and staining along the veins of nonbombarded leaves indicative of systemic silencing.

We selected a low-background line that showed pale diffuse areas of blue coloration (Fig. 1*D*) in meristematic and root tissues only. Substantial blue coloration indicative of high GUS activity was observed only after plants were treated with anhydrotetracycline to inactivate the TET$^R$ (Fig. 1*E*). To validate the system we confirmed that biolistic delivery of additional transgene copies triggers local and systemic silencing (Fig. 1*F*), as was reported for negative marker systems (26–28). High-level GUS expression indicative of silencing occurred both in leaves bombarded with *35S-TET$^R$* DNA and along the veins of some nonbombarded leaves (Fig. 1*F*).

The efficiency of silencing was determined by bombarding 12-day-old plants and staining for GUS 12 days later. Plants showing blue regions on bombarded leaves that were comparable or more intense than those obtained after anhydrotetracycline induction were judged to express local silencing. Fig. 2*A* shows that *ca.* 79% of plants bombarded with plasmid DNA carrying the full-length *35S-TET$^R$* gene exhibited local silencing. A lower incidence of silencing was detected with constructs containing *ca.* 300 bp of 3′-transcribed region in sense as well as in antisense orientation. No silencing was detected with constructs containing *ca.* 100 bp of 3′-transcribed region in either orientation or with gold particles that were not loaded with DNA. These results confirm earlier reports (26–28) that silencing can be triggered by biolistic bombardment with additional copies of a resident transgene; that the efficiency of induction decreases with size of the transcribed region; and that sequences transcribed in both orientations are effective. Thus, it appears that the TET$^R$GUS system provides a reliable positive marker for silencing.

**Biolistically Delivered RNA and siRNA Can Trigger Silencing.** RNA preparations obtained by *in vitro* transcription and then treated with DNase were tested for silencing activity. dsRNA representing the entire transcribed region of the *TET$^R$* gene gave a high,

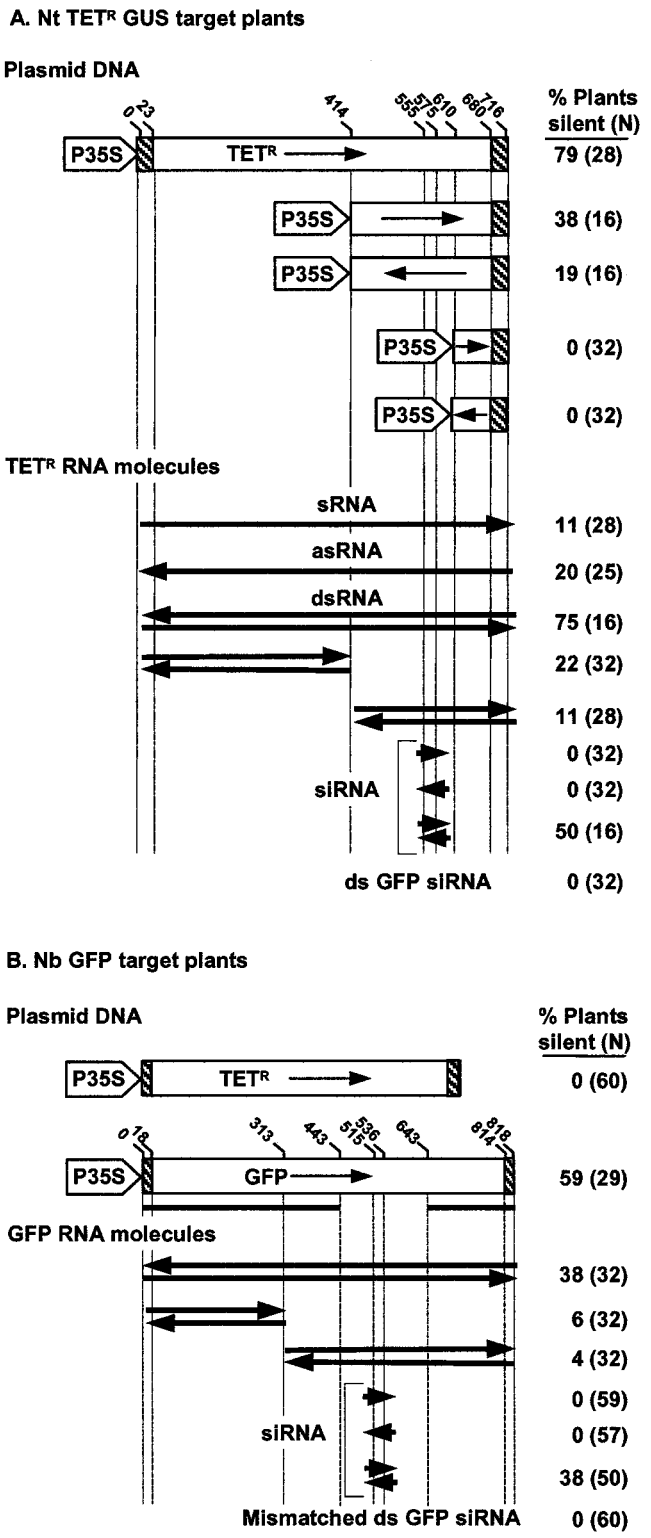


**Fig. 2.** The incidence of silent plants after biolistic delivery of plasmids and RNA into tobacco and *N. benthamiana* transformants. (*A*) The incidence of silencing of *35S-TET*$^R$ in a Nt TET$^R$GUS transformant bombarded with *35S-TET*$^R$ plasmids and RNA molecules representing the *TET*$^R$ transcribed region. Plantlets (12 days old with one true leaf) were bombarded with 5–10 $\mu$g of nucleic acid and stained for GUS *ca.* 12 days later. The incidence of silencing is expressed as percentage of plants showing blue GUS staining obtained in at least three independent experiments for the number of plants indicated in parenthesis. The sense and antisense siRNAs represent positions 517–537 and 535–514 of the transcribed region, respectively. Double-stranded siRNA was obtained by spontaneous annealing of the single-stranded siRNAs at room temperature. The promoter (open bar), 3′- and 5′-untranslated region (hatched bars), coding region (open bar with arrows showing orientation) are indicated for the DNA constructs. The length and orientation (solid arrows) of RNA molecules are indicated. Positions are relative to 5′-end of the RNA. (*B*) The incidence of silencing of *35S-GFP* in an Nb GFP transformant bombarded with *35S-GFP* plasmids and RNA molecules representing the *GFP* transcribed region. The conditions are the same as in *A*. The sense and antisense siRNAs represent positions 556–576 and 574–553 of the transcribed region, respectively. The double-stranded siRNA with mismatches at 6 of 19 positions represents a transcribed region of a related *sGFP* gene (24). The horizontal lines represent positions 0–443 and 643–818 used to prepare DNA probes for RNA-blot hybridization.

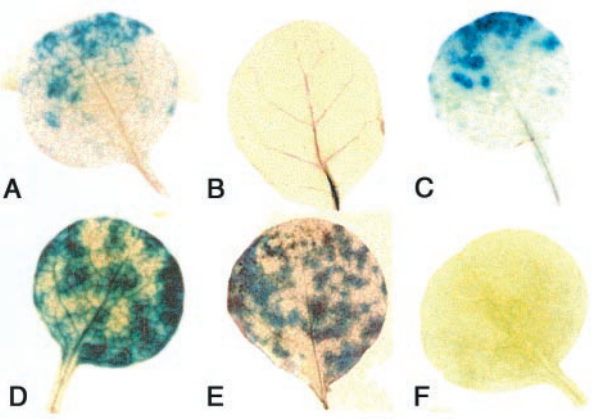


**Fig. 3.** The effect of RNase A treatment and double-stranded siRNA sequence mismatch on the induction of silencing: Representative examples of bombarded leaves of Nt TET$^R$GUS plants stained for GUS 12 days after bombardment. Leaves were bombarded with *TET*$^R$ sense RNA (*A*), RNase A-treated *TET*$^R$ sense RNA (*B*), *TET*$^R$ dsRNA (*C*), RNase A-treated *TET*$^R$ dsRNA (*D*), double-stranded *TET*$^R$ siRNA (*E*), or unrelated double-stranded *GFP* siRNA (*F*).

*ca.* 75%, incidence of silencing (Fig. 2*A*). Substantially lower efficiencies were obtained with shorter, 414- and 303-nt-long, dsRNAs representing the 5′- and 3′-ends of the transcribed region. The high, 50-fold, yield of RNA product relative to DNA template obtained by *in vitro* transcription, the fact that RNA preparations were treated with DNase, and the finding that comparable amounts of dsRNA and plasmid DNA exhibit similar silencing efficiencies (data not shown) make it unlikely that the silencing activity of the RNA preparations is due to traces of DNA. Full-length sense and antisense RNAs also exhibited silencing activity, but at considerably lower efficiencies than those obtained with full-length dsRNA (Fig. 2*A*). Fig. 3 *A–D* shows that the activity of single-stranded RNA, but not that of dsRNA, was abolished by incubating the preparations with RNase A, supporting the conclusion that single-stranded RNA can induce silencing and that silencing obtained with dsRNA preparations was not due to contamination with the single-stranded RNAs.

Double-stranded siRNAs can trigger degradation of target RNAs in *Drosophila* extracts (6, 7) and in cultured mammalian cells (29). We tested the ability of chemically synthesized 21-nt sense, 22-nt antisense, and double-stranded siRNA with 2- and 3-nt 3′-overhangs to silence genes in intact plants. Whereas double-stranded *TET*$^R$ siRNA exhibited substantial activity (Figs. 2*A* and 3*E*), no silencing activity was detected with single-stranded *TET*$^R$ siRNAs in either orientation. Fig. 3*F* shows that a double-stranded siRNA of the same length but unrelated in sequence did not result in silencing, demonstrating that the induction is sequence specific.

We confirmed and extended our most important RNA results

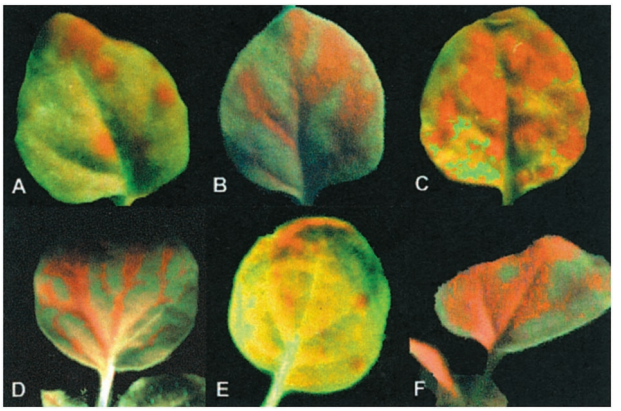


**Fig. 4.** Representative patterns of silencing in Nb GFP plants bombarded with *35S-GFP* DNA (*A* and *B*); *GFP* dsRNA (*C* and *D*); and, double-stranded *GFP* siRNA (*E* and *F*). (*A*, *C*, and *E*) Local, spreading silencing in bombarded leaves photographed 5 days after bombardment. (*B*, *D*, and *F*) Systemic silencing in nonbombarded leaves photographed 1 month after bombardment of plants. Red fluorescence of leaves irradiated with blue light is indicative of *GFP*-gene silencing.

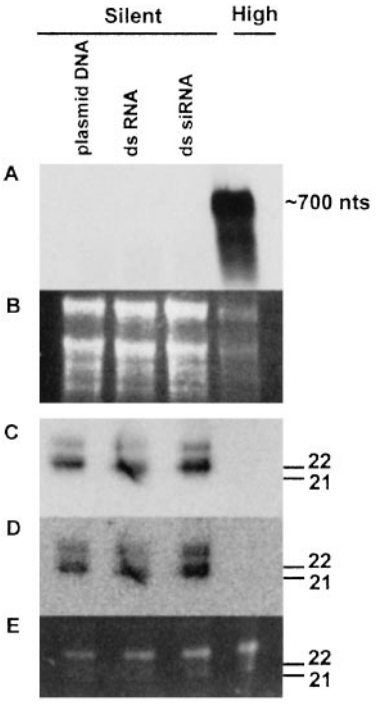


**Fig. 5.** RNA-blot hybridization. Leaves were harvested for RNA isolation 1 month after bombardment. Leaves were chosen that were not present at the time of the bombardment, but were completely silent as judged from the absence of GFP fluorescence (Silent) or from control plants showing high GFP fluorescence (High). (*A*) Total RNA hybridized with a probe for the entire *GFP* transcript. The small RNA fraction hybridized with probes for the 5′ (*C*) and 3′ (*D*) regions of *GFP* mRNA indicated in Fig. 2*B*. Note that the probes do not include the region identical in sequence to the double-stranded siRNA used to trigger silencing. The positions of *GFP* mRNA in *A* and the 21- and 22-nt standards in *C* and *D* are shown on the right. Ethidium bromide staining (*B*) and (*E*) is shown as loading controls for the total RNA blot (*A*) and siRNA blot (*C* and *D*), respectively.

for the *N. benthamiana* transformed line 16c (Nb GFP), which carries an unrelated, chimeric *35S-GFP* gene (15). Fig. 2*B* shows that Nb GFP bombarded with additional copies of *35S-GFP* plasmid DNA, with high molecular weight *GFP* dsRNA, and with double-stranded *GFP* siRNAs triggered silencing. The efficiency of silencing was considerably lower for shorter dsRNAs representing the 5′ and 3′ regions of the transcript. No silencing was observed when a double-stranded *GFP* siRNA with mismatches at 6 of 19 positions was used, indicating that silencing triggered by siRNAs is highly sequence specific. Oligodeoxyribonucleotides equivalent in sequence to the inducing siRNAs were also ineffective (data not shown).

Previous studies have shown that introduction of transcribed as well as nontranscribed transgenes can trigger silencing in tobacco and *N. benthamiana*, which spreads systemically to nonbombarded leaves (26–28). GUS staining of entire plants one month after bombardment revealed that silencing triggered by *35S-TET*$^R$ DNA can spread systemically to the veins of nonbombarded leaves (Fig. 1*F*). Although similar results were obtained with single- and double-stranded *TET*$^R$ RNA and double-stranded *TET*$^R$ siRNA (data not shown), it was not technically feasible to assay systemic silencing in numerous large plants by GUS staining. Therefore, we used real-time monitoring of Nb GFP plants to follow systemic spread on a routine basis. Fig. 4 shows similar silencing patterns on leaves 5 days after bombardment with *35S-GFP* plasmid DNA, *GFP* dsRNA, and *GFP* siRNAs. Systemic spread into leaves not present at the time of bombardment was also comparable, independent of the nucleic acid used. Silencing, occasionally detected as early as 1 day after bombardment, first appeared as patches on bombarded leaves, which after 3–4 days increased in size. Systemic spread, starting with the veins of nonbombarded leaves, was first detected in plants 2 weeks after bombardment (data not shown) and after 1 month was clearly evident in nonvascular tissues (Fig. 4 *B*, *D*, and *F*).

**Double-Stranded siRNA Induces PTGS and the Accumulation of Newly Formed siRNA in Nonbombarded Leaves.** RNA-blot hybridization was used to compare the accumulation of *GFP* mRNAs in highly GFP-expressing leaves of Nb GFP plants and in completely silenced nonbombarded leaves of Nb GFP plants bombarded with *35S-GFP* plasmid DNA, high molecular weight *GFP* dsRNA, and double-stranded *GFP* siRNA. Fig. 5*A* shows that silencing of systemic leaves was correlated with a dramatic decrease in *GFP* mRNA accumulation. We also assayed the leaves for siRNAs, which are a hallmark of PTGS (10, 13). Fractions enriched for small RNAs were hybridized with DNA probes representing the 3′ and 5′ regions of *GFP* mRNA indicated in Fig. 2*B*. Fig. 5 *C* and *D* shows that siRNAs approximately 21 and 23 nt in length representing both regions of *GFP* mRNA accumulated in systemically silent leaves obtained by bombardment with plasmid DNA, dsRNA, and double-stranded siRNA, but not in highly expressing leaves. Together, these results confirm that the RNAs tested induce systemic silencing at the posttranscriptional level. The 3′- and 5′-probes used for RNA-blot hybridization do not include the region of *GFP* mRNA identical in sequence to the siRNA used to induce PTGS. This fact indicates that biolistically delivered siRNA induces the *de novo* formation of siRNAs that accumulate in systemically silenced tissues.

## Discussion

Earlier studies have shown that the local introduction of additional gene copies and high molecular weight viral RNAs can induce systemic silencing (26–28). We used a biolistic approach to test directly the capacity of RNA molecules to trigger systemic PTGS. This approach offered several advantages. For example, potential effects of viral RNA replication, expression of viral RNA-dependent RNA polymerases (RdRPs), transcription of delivered DNA, or the delivered DNA itself are excluded. Our approach also excludes interactions between PTGS and signaling

pathways in pathogen-induced defense responses (30). As judged from the induction of pathogenesis-related (PR) proteins, these responses are strongly induced by pathogen-based delivery systems such as *Agrobacterium tumefaciens* (31) but are not induced by bombardment (data not shown).

Here we demonstrate that several likely RNA components of the PTGS pathway can induce systemic PTGS. Although it had been established that high molecular weight dsRNAs can trigger silencing confined to bombarded cereal cells (32) and systemic silencing in *C. elegans* (33), it had not previously been shown that *ca.* 21-nt siRNAs as well as high molecular weight single-stranded RNAs of both polarities can trigger silencing able to spread from cell to cell and systemically. Quantitative differences in silencing obtained with different RNAs are difficult to interpret because of variation in the efficiency of delivery and the likelihood that size, concentration, sequence, and secondary structure influence stability of exogenous RNAs. Nevertheless, effects of RNA polarity, length, and strandedness were roughly in agreement with those deduced from the delivery of DNA in transient assays systems (26–28).

The present study bears on the mechanisms underlying systemic PTGS. Indirect evidence supports a branched model in which sense, antisense, and ill-defined aberrant RNAs feed at the dsRNA step into a common pathway similar to RNAi in animals (14, 34, 35). It is commonly assumed that the sequence-specific component of mobile silencing signals is RNA. The source of this RNA in the silencing pathway is not known. Studies with potato virus X have shown that the lower size limit of complete sequence identity between viral and target RNAs required for PTGS corresponds to the size of siRNAs (36). We have shown directly that siRNAs can trigger systemic silencing, suggesting that production of siRNA is sufficient for systemic silencing. This finding leads us to propose that mobile signals comprise siRNAs or RNAs derived from siRNAs as suggested by Hamilton and Baulcombe (10). Although we have not ruled out the possibility that single-stranded RNAs and dsRNA are components of the mobile signals, we believe these RNAs are effective because they give rise to siRNAs. On the other hand, Mallory *et al.* (37) have reported that expression of the tobacco etch virus protein P1/HC-Pro blocks PTGS and siRNA accumulation in tobacco, but not the capacity to generate systemic signals. They concluded that siRNA is not necessary for systemic silencing, and, hence, that the mobile signal arises upstream of siRNA. At present, we cannot readily explain the apparent discrepancy between our findings and those of Mallory *et al.* Direct evidence that any silencing-related RNA species can move from cell to cell and trigger silencing is still lacking.

Genes encoding proteins related in sequence to RdRP are essential for transgene-induced PTGS (38–40). Our finding that single-stranded RNAs of either polarity can trigger silencing suggests that at least one site of RdRP action is upstream of both the dsRNA step and production of systemic signals. Several current models hold that the silent state is maintained by a self-sustaining cycle involving RNA intermediates such as dsRNA and siRNA (34, 41). We found that double-stranded siRNAs can trigger formation of nonhomologous siRNAs representing 3′ and 5′ regions of the target RNA, implying that siRNAs trigger *de novo* production of siRNAs in silent tissues. Similar conclusions have been drawn from recent studies of silencing induced by virus vectors carrying partial transgenes (42). Studies with *Drosophila* embryo extracts (43) and *C. elegans* (44) suggest that siRNAs can serve as primers for generating dsRNAs from the target RNA mediated by RdRP, and that new siRNAs are generated in a cycle of dsRNA synthesis and degradation. We speculate that a similar mechanism capable of amplifying siRNA signals operates in systemically silenced plants.

This hypothesis is, however, difficult to reconcile with our finding that siRNAs complementary to the region 3′ of the inducing 21/22-nt RNAs are present in systemically silenced leaves. Possible explanations include the following: (*i*) the circularization of part or all of the mRNA to allow the production of dsRNAs with homology to the entire transcript; (*ii*) the presence of small amounts of antisense RNA in the Nb GFP plants, which serve as substrates for dsRNA formation; and (*iii*) the production and amplification of siRNA by using single-stranded RNA as the substrate without a siRNA primer.

PTGS is important for protecting plants against infection with viruses and has been proposed as a surveillance system for recognizing potentially deleterious foreign nucleic acid sequences (2, 3). Recent studies of PTGS mutants and viral suppressors of PTGS suggest that PTGS-like mechanisms may also play a role in down-regulating plant genes during development (3). Our results with the $TET^R$ system illustrate how silencing of repressors might also serve as a mechanism for stable, systemic activation of gene expression.

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