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Induction of RNA interference in *Caenorhabditis elegans* by RNAs derived from plants exhibiting post-transcriptional gene silencing

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ABSTRACT

The term 'gene silencing' refers to transcriptional and post-transcriptional control of gene expression. Related processes are found across kingdoms in plants and animals. We intended to test whether particular RNA constituents of a silenced plant can induce silencing in an animal. We generated *Nicotiana benthamiana* lines that expressed green fluorescent protein (GFP) from a transgene. Plants in which GFP expression was spontaneously silenced showed siRNAs characteristic of post-transcriptional gene silencing (PTGS). RNA extracts prepared from silenced plants were injected into a GFP-expressing strain of *Caenorhabditis elegans*, where they induced RNA interference (RNAi). Extracts from non-silenced plants were inactive. This directly demonstrates a relationship and a mechanistic link between PTGS and RNAi. Controls confirmed that the silencing agent was an RNA. Size fractionation on denaturing gels revealed that an RNA of ~85 nt was most active in inducing silencing in the worm. Northern blot analysis of the region in question did not detect a prominent GFP-specific RNA of sense or antisense polarity, indicating that the RNA species which induced silencing was present only in low concentration or did not hybridize due to formation of an intramolecular double strand. In view of its high activity, it is possible that this agent is responsible for the systemic spread of silencing in plants and it might represent the aberrant RNA, a previously postulated inducer of silencing.

INTRODUCTION

The term post-transcriptional gene silencing (PTGS) describes an inducible cellular process that directs sequence-specific degradation of homologous RNAs. Various classes of RNAs can be targets, including transcripts derived from endogenous genes and transgenes, as well as virus-derived RNAs. Originally, the process was discovered in plants in the context of antisense inhibition studies, but it is believed that RNA silencing is part of a natural defense mechanism against invading alien RNA. A full coverage of all aspects of RNA silencing in plants can be found in recent reviews (1–9).

RNA silencing is not restricted to plants. Highly related processes are found in a broad variety of different species. The most intensively studied species are *Neurospora crassa* (10–12), where the process is called quelling, *Caenorhabditis elegans* (13–15) and *Drosophila melanogaster* (16,17). For the latter two invertebrates it could be shown that an artificially introduced double-stranded RNA induces a silencing process similar to that in plants, for which the additional term 'RNA interference' and its acronym RNAi have been coined. The number of species in which RNAi/PTGS operates is rapidly expanding from *Escherichia coli* (18) and protozoa (19,20) to vertebrates (21,22), including mammals (23,24).

The occurrence of PTGS across kingdoms indicates that the molecular mechanisms are related. In accordance with this assumption, related genes required for PTGS have been identified in *Neurospora* (10–12), *Drosophila* (25,26), *C.elegans* (27,28) and plants (29–32).

According to current models of the PTGS/RNAi mechanism (3–5,33,34) double-stranded (ds)RNA plays a central role. An RNase III-type enzyme, such as Dicer from *Drosophila* (26) or equivalents in *Arabidopsis thaliana* (35) and *C.elegans* (28), processes precursor dsRNA into short 21–25 nt dsRNA fragments, called 'short interfering (si)RNA'. siRNAs were first described in plants (36) and have recently been identified in *Drosophila* cells treated with dsRNA (37–39) and they have also been detected in *C.elegans* (40,41). They are incorporated into the RNA-induced silencing complex (RISC), a multicomponent ribonuclease conferring sequence specificity (25,26,37–39,42).

Of particular interest in the context of this work is the ability of PTGS to spread systemically throughout a plant. It was shown that local induction of PTGS is sufficient to silence gene expression in the entire plant (43). In accordance with this, silencing is also transmissible by grafting, with 100% efficiency from silenced rootstocks to non-silenced scions (44,45), but far less effective in the reverse direction (46). In view of the sequence specificity, it is expected that the responsible

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signal is a nucleic acid, most likely an RNA. The mobile signal is transported from cell to cell and in the phloem from source to sink tissues. By sophisticated grafting experiments it could be shown that the silencing signal can even pass through plant sections that do not contain the target sequence (47).

siRNAs are obvious candidates to act as a mobile signal. In agreement with this it was recently shown that synthetic siRNAs are effective in guiding RNA degradation in a cell-free system (39) as well as in mammalian cells (48). Synthetic siRNAs can also induce silencing in whole organisms, *C.elegans* (49) and *Drosophila* (50). However, there are two studies in plants which suggest that the siRNAs characteristic of PTGS may not be the responsible signal for spreading PTGS in plants. Mallory *et al*. (51) reported that the occurrence of siRNAs could be suppressed in plants that expressed HC-Pro, a potyvirus-derived suppressor of PTGS. This suppression of siRNAs did not affect the mobile signal that is responsible for induction of PTGS, suggesting that it is a different agent. Further, Voinnet *et al.* (52) showed that p25, a protein encoded by potato virus X (PVX), prevents the spread of silencing, but does not interfere with the accumulation of siRNAs. These findings could indicate that the siRNAs are required for maintenance of silencing at the cellular level, but that the actual signal that transports silencing is most likely a different RNA.

For the current study we intended to test whether a silenced plant produces a signal that can induce silencing in a worm. We reasoned that such an inducing agent could represent the mobile signal responsible for the spread of PTGS in plants. In order to assay RNA fractions for their potential to induce RNA silencing we needed a sensitive test system. Given the common mechanisms of gene silencing across kingdoms we reasoned that microinjection of plant-derived RNAs into *C.elegans* might be useful to identify the mobile signal. As a target we used the mRNA for green fluorescent protein (GFP) of *Aequoria victoria*, because it can be monitored in plants as well as in animals.

MATERIALS AND METHODS

Plasmid

The coding part of the mGFP4 gene (53) was subcloned into the *Bam*HI and *Sac*I sites of pT20 (54), a modified version of pT3T7-lac (Boehringer Mannheim). The resulting plasmid, pT20-GFPm4, was linearized with *Eco*RV or *Sma*I and transcribed with T7 or T3 RNA polymerase for *in vitro* synthesis of sense and antisense RNAs as probes or for the generation of dsRNA.

Plant transformation

Leaf discs for transformation experiments were extracted from young leaves of *Nicotiana benthamiana* (courtesy of the National Tobacco Institute of Greece) grown in a greenhouse. Following co-cultivation with *Agrobacteria* (strain LBA4404) carrying plasmid pBIN35S-mGFP4 (53), discs were placed on MS media (55) containing hormones (1 mg/l 6-benzylaminopurine and 0.1 mg/l α-napthalenacetic acid) and antibiotics (100 mg/l kanamycin and 250 mg/l cefotaxime). Shoots regenerating from the explants were then transferred to MS medium with antibiotics (100 mg/l kanamycin and 250 mg/l cefotaxime), but without hormones, in order to initiate rooting. Plantlets were then transferred to non-aseptic conditions and grown to maturity in a greenhouse at a controlled temperature of 22°C (day) and 18°C (night). Approximately 40 T_1 seeds from each T_0 transgenic plant were surface-sterilized in 10% Na_3PO_4 (15 min), 70% ethanol (1 min) and a 10% solution of a commercial bleach (20 min) and then sown on plates containing MS selection medium (125 mg/l kanamycin). The copy number of the transgene was calculated on the basis of the ratio growing/necrotic seedlings ~15 days post-germination. For the experiments described below, plants homozygous for a single GFP transgene were used (confirmed by a Southern blot; data not shown).

Preparation of plant extracts

Leaves (1 g) from silenced and non-silenced *N.benthamiana* plants transgenic for GFP were ground in a mortar under liquid nitrogen. The frozen powder was collected and 10 ml of a freshly prepared and heated extraction mixture was immediately added. The extraction mixture was composed of 5 ml of extraction buffer (0.1 M LiCl, 100 mM Tris–HCl pH 8.0, 10 mM EDTA, pH 8.0, 1% SDS) and 5 ml of 'acidic phenol' (phenol melted at 65°C, supplemented with 0.1% 8-hyroxyquinoline and equilibrated three times with 50 mM sodium acetate pH 4.0) and was heated prior to usage to 80°C to generate a homogeneous solution without phases. After the extraction mixture, 5 ml of chloroform/isoamyl alcohol (24:1) was added and the suspension was vortexed extensively for ~1 min. After centrifugation (20 min at 4°C, 4000 *g*) the supernatant was collected and re-extracted with 'neutral phenol', followed by extraction with chloroform/isoamyl alcohol (24:1) and precipitation with ethanol after adjusting the solution to 0.2 M sodium. In order to improve the solubility of the extract, 0.5 ml of 4 M LiCl was added and suspended for ~3 h with rotation. This was followed by ethanol precipitation. In this way, some side products were eliminated from the extract. The dried pellet extract was redissolved in TE buffer and adjusted to a concentration of ~1 mg/ml. Extracts prepared by this protocol did not contain large quantities of DNA. Preparative extracts were scaled up to 5 g of leaf material, which was taken, however, from a second silenced and a second non-silenced plant, respectively. The LiCl fractionation with 8 M LiCl was done as described previously (56).

Control treatments

Treatment of 10 µg extract with DNase-free RNase A (10 µg), RNase-free DNase I (10 U) and calf intestinal phosphatase (10 U) as well as other standard procedures were done as described (57).

Size fractionation and northern analysis

Standard slab polyacrylamide gels containing 8 M urea were used to separate 10 µg extract per lane. Five lanes were loaded together with appropriate DNA markers. The gel was stained with ethidium bromide and zones as indicated in the text were excised. The RNA was recovered by the isotachophoresis procedure as described (58). The samples were treated with phenol, ethanol precipitated and dissolved in 20 µl of TE buffer. Note: if there was 100% recovery this procedure would represent an enrichment, because 50 µl of extract produced a gel-purified sample of 20 µl; however, the recovery of RNA in the total procedure is less than half, so that the original concentration for each RNA species is roughly maintained.

Northern analysis for the detection of siRNAs was done as described (56), but at 53°C. The analysis in Figure 1C was done with the addition of 50% formamide and at 65°C.

Nematode strains

We reared and maintained *C.elegans* strains at 20°C as described (59). We constructed N2 Is[$p_{myo\text{-}3}$GFP*rol-6*(*su1006*)] transgenic lines expressing GFP in body wall muscle cells by injecting plasmid pPD93.97 (60,61) at 100 ng/µl using standard protocols (62,63). This plasmid carries a GFP reporter gene driven by *myo-3* regulatory sequences and is included in the Fire lab plasmid collection kit (60). In all experiments we additionally injected plasmid pRF4, which harbors the dominant *rol-6*(*su1006*) allele that causes a readily distinguished roller phenotype in transgenic animals, as a co-transformation marker (64). Extrachromosomal arrays resulting from genetic transformation by microinjection were stably integrated into the genome by means of UV irradiation (62,63).

RNA interference assays

For standard RNAi, dsRNA preparations were injected into N2 Is[$p_{myo\text{-}3}$GFP*rol-6*(*su1006*)] adults as described (13). The RNAi assay was performed as described previously (15). Briefly, for each injection group 10–15 animals were injected in pools of five animals each. The first batch of eggs laid within 1 day post-injection were discarded. The reason for this is that these eggs, fertilized prior to injection, are not affected by RNAi. The second batch of eggs collected 2 days post-injection was used for the analysis. **All** progeny animals from **all** injection groups were observed for interference effects under a UV microscope (500–700 animals per injection sample). At this stage the homogeneity of interference effects among individuals was assessed by simple visual inspection, and 50–70 animals from each injection group showing the strongest interference were picked and transferred to new plates. Pictures of 10 animals from each of these populations were taken under the same magnification and UV illumination conditions. These pictures were subsequently used to quantify the interference. For each photographed animal, three muscle cells on the same focal plane were chosen (anterior, middle and posterior parts of the body) and the intensity of the GFP fluorescent signal from each cell was measured in Adobe Photoshop (Adobe Systems, CA) and NIH Image (NIH, Bethesda, MD). A total of 30 cells were examined for each injection sample. We used Microsoft Excel 2000 software (Microsoft, CA) to carry out statistical analysis of the data. The 'silencing potential' is given as the percentage reduction in GFP expression. For example, 80% GFP expression compared with the control corresponds to a silencing rate of 20%.

RESULTS

Characterization of silenced and non-silenced GFP plants

We generated 14 transgenic lines of *N.benthamiana* that expressed GFP systemically from the 35S promoter of cauliflower mosaic virus. Visualization under UV light confirmed expression of GFP in young plants of the T_1 generation. The transgenic lines showed different degrees of GFP expression (Fig. 1A). Some plants continued to express GFP for their entire lifespan (non-silenced). Some other plants showed spontaneous silencing as early as the six-leaf stage. These plants appear red under UV light due to the fluorescence of chlorophyll. The red color is almost as intense as in GFP-deficient wild-type plants. Micrographs of silenced and non-silenced plant tissues are shown in Figure 1B. Silenced and non-silenced plants were analyzed for GFP mRNA and its concentration correlated with the intensity of GFP expression (Fig. 1C). Almost no mRNA could be detected in silenced plants. Next, we tested for the occurrence of the ~21–23 nt short RNAs that are a hallmark of RNA silencing in plants (36). Silenced plants showed GFP-specific siRNAs of at least two size classes of sense and antisense polarity, whereas no short RNAs were detectable in plants that showed GFP expression (see Figs 1D and 4). This analysis confirmed that we could discriminate between GFP silenced and non-silenced plants, a prerequisite for the following experiments.

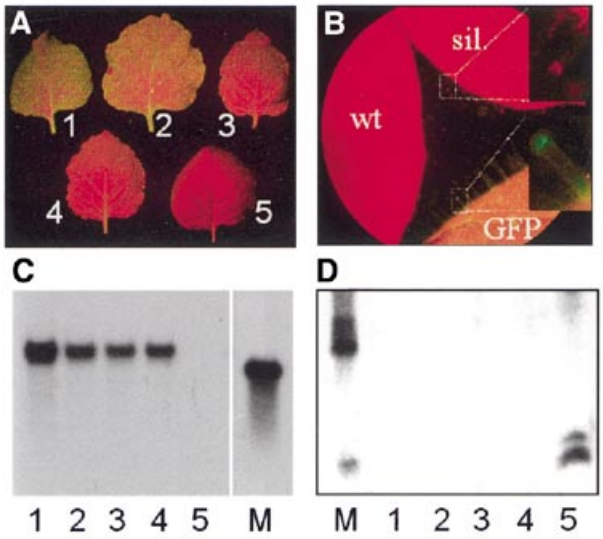

Figure 1. Analysis of GFP expression in different transgenic lines. (**A**) *Nicotiana benthamiana* leaves from five different plants under UV light. Leaf 1 is expressing GFP strongly, leaves 2–4 express GFP with degreasing intensity; leaf 5 is from a silenced plant. (**B**) Micrographs under UV light of a leaf of a non-transgenic (wt), a silenced (sil.) and a GFP-expressing plant (GFP) are shown; the green fluorescence of GFP expression is best seen in the close-up of a trichome. (**C**) Northern analysis of the GFP mRNA on a denaturing 1.2% formaldehyde agarose gel. The level of GFP expression correlates with the visual impression of (A). Leaf 5 does not contain detectable GFP mRNA; M, an *in vitro* synthesized GFP sense marker transcript of ~750 bases. (**D**) Northern analysis of the same RNAs on a 12% polyacrylamide gel. Leaf 5 contains siRNAs. M, marker; the lower signal is a radiolabeled synthetic 22mer RNA.

Testing crude extracts for silencing in *C.elegans*

Analytical RNA extracts were prepared from silenced and non-silenced leaves. To test their potential to induce silencing, samples were adjusted to a concentration of ~1 µg/µl and injected into *C.elegans* that expressed GFP in body wall muscle cells. Analysis of the progeny of injected animals showed that the extract derived from non-silenced plants reduced GFP expression only marginally (Fig. 2A and B). This indicates that the total plant RNAs, including GFP mRNA and its potential degradation products, did not interfere with GFP expression in the receiving animal. The slight reduction in GFP activity was not significant, since it was also seen in

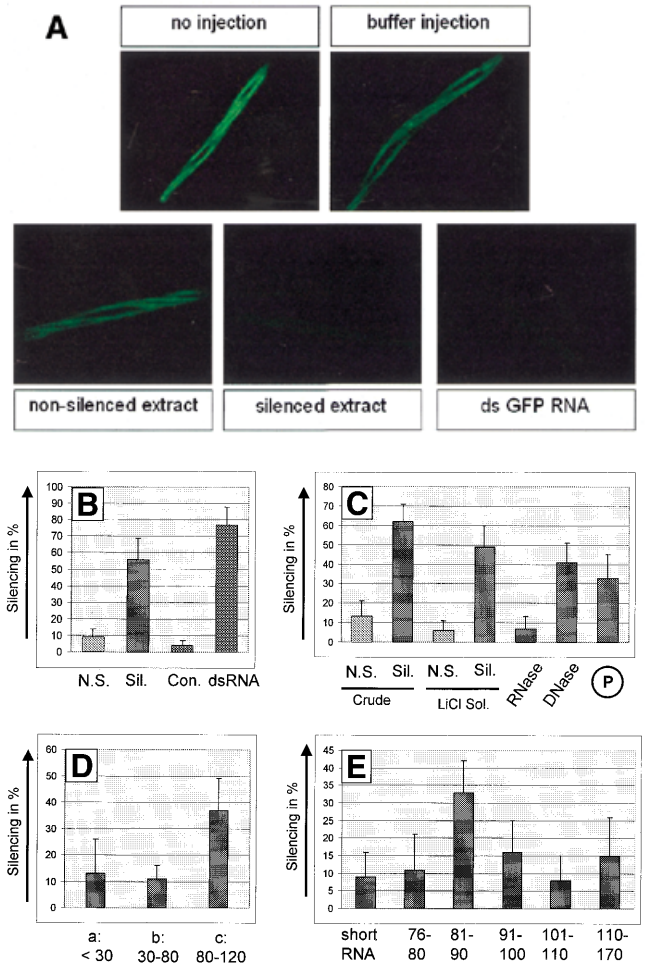

Figure 2. Silencing potential of plant RNAs on GFP expression in *C.elegans*. (**A**) Representative examples of micrographs of nematodes taken under UV illumination for quantification of GFP expression. The treatments are indicated. It should be noted that the differences in GFP expression seen between non-injected and the buffer-injected animal (top) are within the variation limits amongst individuals. To remove this variability, 10 pictures were analyzed as described in Materials and Methods to determine the silencing data in each of the columns of (B)–(E), which indicate percent silencing compared with the non-injected control; standard deviation is indicated by bars. (**B**) Silencing data from analytical extracts from a non-silenced (N.S.) and silenced (Sil.) plant. Injections of buffer (Con) and of GFP dsRNA are indicated. The samples correspond to the pictures displayed in (A). (**C**) Silencing data for preparative extracts of silenced and non-silenced plants, the crude fractions and the LiCl-soluble fractions. The right three columns show the LiCl-soluble fraction after treatment with RNase, DNase and phosphatase. (**D**) Silencing data for the excised fractions in Figure 3A; a–c refer to the zones given there; nucleotide numbers are given. (**E**) Silencing data for the excised fractions in Figure 3B. The assumed size range of the extracted RNAs is indicated in nucleotide numbers.

mock-injected animals (Fig. 2A and B). In contrast, GFP activity was reduced substantially after injection of total extracts derived from a silenced plant (Fig. 2A and B). A control injection of *in vitro* synthesized GFP dsRNA (also at 1 µg/µl) did not give much higher inhibition (Fig. 2A and B). Considering that the main constituents of the crude extract were tRNA and rRNA, the agent that induced RNAi must have been highly active given its low concentration.

In view of these initial results we made a preparative extract from leaves of a silenced and a non-silenced plant (different individuals of the same line). In this case also only the extract originating from the silenced plant had substantial inhibitory potential (Fig. 2C). As a first step towards characterization of the silencing agent, both extracts were subjected to LiCl fractionation in order to enrich them for small RNAs. The LiCl-soluble fraction, which contained low molecular weight RNAs (compare Fig. 3A), was tested. Again, only the fraction originating from the silenced plant was active (Fig. 2C). This suggested that a shorter RNA (<200 nt) was responsible for the silencing, although activity was slightly reduced compared with the crude extract. The silencing-competent LiCl-soluble fraction was subjected to further controls.

Control treatments of the extracts

Treatment with RNase A essentially abolished silencing activity, whereas pre-treatment with DNase I had little effect (Fig. 2C), confirming that the silencing agent was an RNA. Pre-treatment with phosphatase slightly diminished the silencing activity (Fig. 2C), without inactivating it completely. It is possible that the silencing RNA had a 5′- and/or 3′-phosphate, which supported its activity, or provided some protection against exonucleases. It should be noted that the occurrence of a 5′-phosphate in siRNAs is consistent with the RNase III mechanism (65). In accordance with this we had previously observed that phosphorylation of synthetic siRNAs resulted in higher silencing activity (50).

Size fractionation of the extracts

In order to get an idea as to whether a particular RNA size was responsible for the silencing activity, we fractionated the LiCl-soluble RNA on a denaturing polyacrylamide gel. Three zones of the gel, corresponding to different size classes, were excised (Fig. 3A, a–c). The first zone contained short RNAs up to ~30 nt. Based on molecular hybridization we knew that the extract contained GFP-specific siRNAs, however, the concentration of this size category of RNAs was extremely low. In accordance with this there was only marginal silencing activity in this RNA fraction (Fig. 2D). This was not surprising. First, the siRNA would need to re-anneal and, second, siRNAs are required in about the same amounts as dsRNAs to induce silencing in *Drosophila* (50), definitely much more than was available in the extracts. Further, siRNAs seem to have an even lower potential to induce RNAi in *C.elegans* (49). The second gel-purified fraction derived from the LiCl-soluble fraction of the extract covered the size range ~30–80 nt, including tRNAs (Fig. 3A), and was inactive (Fig. 2D). A third zone was excised that contained RNAs larger than tRNA, ranging up to ~120 nt (Fig. 3A). This fraction had clear silencing activity (Fig. 2D).

In view of the finding that relatively long RNAs are responsible for the silencing activity, we could not exclude that RNAs >120 nt would likewise be active. Further, we could not be sure whether the LiCl fractionation had eliminated some of the active fraction. Therefore, we performed a further gel fractionation, but this time loading the crude RNA extract.

The short RNAs and five further zones of RNA were excised and analyzed (Fig. 3B). Again, the short RNAs that were recovered from the gel were not active (Fig. 2E). The residual five zones represented a more detailed analysis of the size range from 76 (above tRNAs) to ~170 nt (compare Fig. 3B). The first fraction contained the zone from just above tRNAs to just under the next visible band, which corresponds to ~80 nt.

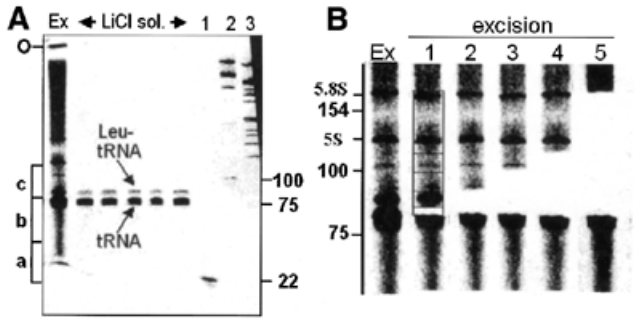

Figure 3. Electrophoretic separation of RNA extracts on a denaturing 5% polyacrylamide gel. Visualization was with ethidium bromide, however, the negative is displayed to increase contrast. (**A**) Separation of the crude extract (Ex) and the LiCl-soluble fraction, which was loaded in five lanes. The positions of the bulk of the tRNA and the larger $tRNA^{Leu}$ are indicated. Lane 1, synthetic 22mer RNA; lane 2, 100 bp DNA ladder; lane 3, pBR322 × *Hin*fI (which is incompletely denatured). The left side indicates the origin of the gel (O) and the three zones (a–c) excised from the five lanes separating the LiCl-soluble material. (**B**) Separation of the crude extract (lane Ex). This lane was part of a preparative gel, in which several lanes were run in parallel, similar to the LiCl-soluble fraction in (A). Gel slices corresponding to different size classes were excised. After each of the five excisions the gel was photographed. The panel shows one particular lane after each round of excision, as indicated at the top of the lane. The boxes in excision lane 1 illustrate the excised zones. The numbers on the left refer to the sizes of the DNA fragments, as in (A). The positions of 5S and 5.8S rRNA are indicated (corresponding to 120 and 170 bases). The signal slightly larger than 100 bases represents 4.5S chloroplast rRNA.

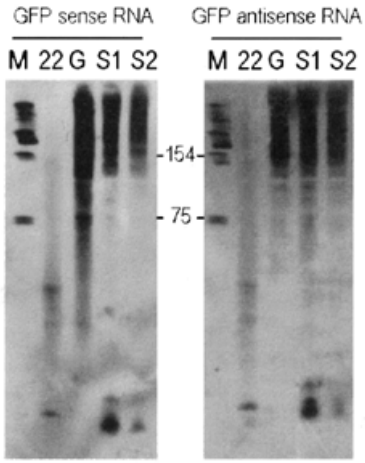

Figure 4. Northern blot analysis of extracts after separation on a denaturing 12% polyacrylamide gel. The left panel shows hybridization with a GFP antisense probe, the right with a GFP sense probe. Lane M, pBR322 × *Hin*fI; 22, an end-labeled 22mer RNA; G, extract from a GFP-expressing (non-silenced) plant; S1 and S2, extracts from two plants with silenced GFP. Extract of plant S2 had been shown to be active in inducing silencing in *C.elegans* and had been used for the fractionation in Figure 2B. As expected, the silenced plants showed siRNAs with both probes. The silenced plants contained less GFP sense RNA than the non-silenced control. The higher molecular weight signals detected with the GFP sense probe may be non-specific, due to the low stringency conditions applied for detection of siRNAs. However, in neither of the silenced plants was a specific sense and antisense signal detected in the size range 81–90 nt, indicating that the active RNA was present only in low concentration.

This fraction had no substantial silencing activity (Fig. 2D). The next gel slice contained the stainable RNA. Based on the size we assume that this product represents a group of plant $tRNA^{Leu}$, some of which are known to be longer than ordinary tRNAs (for example 86 nt, compare accession no. TOBCPLB3). This fraction containing RNAs between ~81 and 90 nt was clearly able to induce gene silencing in *C.elegans* (Fig. 2E), whereas the residual three larger fractions had only marginal activities slightly above background levels (Fig. 2E). This detailed analysis confirmed the initial result that an RNA larger than tRNA was responsible for silencing.

The assignment of silencing activity to a particular size class prompted us to carefully analyze the GFP-specific RNAs of this length in the extract. Extracts of two silenced plants, including the one from which the silencing-active fraction was derived, and an extract derived from a non-silenced plant were separated on a denaturing 12% polyacrylamide gel and transferred to two membranes for northern analysis with GFP sense and antisense RNA. As expected, both probes detected the short RNAs in the extracts from silenced plants (Fig. 4). It was also expected that the extract from the non-silenced plant contained more GFP sense RNAs. The signals in the higher molecular weight zone observed with the sense probe were most likely non-specific and due to the low stringency applied in order to visualize the siRNAs. However, in the region from which the silencing-competent RNA was excised, no particular signal could be detected with either of the two probes. This indicates that the silencing-inducing RNA was present in only low concentration. It is also conceivable that the RNA escapes detection by northern blotting because it is able to form an intramolecular double strand, such that the probe cannot hybridize. Control experiments (not shown) with an *in vitro* synthesized RNA molecule that consisted of sense and antisense sequences of ~40 nt and was closed by a short loop revealed that such an RNA species is hardly detectable by northern blotting.

DISCUSSION

In this study we have provided evidence that silenced plants contain an RNA agent that is able to induce RNAi in *C.elegans*. This directly verifies that PTGS and RNAi are related mechanisms across kingdoms. Thus, the nematode can be used as a sensitive test system to study the signal that is responsible for spreading silencing in plants. When used at a concentration of 1 μg/μl, the crude extract was almost as active in inducing silencing in the nematode as was pure *in vitro* synthesized dsRNA. Considering that GFP-related RNAs represented only a small fraction of the extract, the plant-derived RNA that induced silencing in *C.elegans* must have had a higher 'specific activity' than ordinary *in vitro* synthesized dsRNA. The observation that the silencing activity could be separated and purified from a denaturing gel also argues against a conventional dsRNA, although re-annealing would be possible in principle.

Additionally, our experiments suggest that silencing was not induced primarily by siRNAs, at least not with high efficiency. Although it has been shown that synthetic siRNAs are able to induce silencing in tissue culture (39,48), in *Drosophila* embryos (50) and also in *C.elegans* (49), it is unlikely that the siRNAs were responsible for inducing the silencing process. Two reasons argue against the siRNAs: first, the low degree of activity obtained here with siRNAs derived from silenced plants and, second, the quantities required to induce RNAi by

siRNAs. In *Drosophila* we observed that synthetic siRNAs are active at about the same concentration as dsRNA (50), but in *C.elegans* synthetic siRNAs are less active than dsRNAs (49). However, even if siRNAs were similarly active in *C.elegans* as dsRNA, the plant extracts injected in this study would contain too minute quantities of siRNAs to be responsible for silencing.

Unlike the short RNAs, we identified considerable silencing activity in a RNA fraction slightly larger than the main fraction of tRNAs. We estimate the length of the RNA to be ~85 ± 5 nt. No particular RNA species could be identified in this size range on the two northern blots (Fig. 4). This could indicate that the concentration is extremely low or that the RNA species is not detectable because it forms an intramolecular duplex RNA so that it is inaccessible for the molecular sense or antisense probe or simply does not bind to the membrane. Attempts to detect a circular RNA in the active fraction by analysis on a 2-dimensional gel followed by molecular hybridization have so far failed (data not shown). It is possible, however, that the silencing-competent RNA has a distinctive structure, base modifications or defined end groups. In view of the involvement of RNA-dependent RNA polymerase (RdRp) in RNAi, it is conceivable that the RNA in question is a template (or a product) of RdRp, an assumption supported by the recent finding that RdRp accepts strands from siRNA as primers (66,67). Further, it is tempting to assume that this RNA represents the postulated 'aberrant' RNA (68), which is considered the trigger of the silencing reaction. This would be in agreement with the recent data that connect RNA silencing with DNA methylation, reviewed in Bender (8) and Matzke *et al*. (9). As summarized recently by Bender (8), aberrant RNA may be produced from a methylated target gene and could be responsible for the change in methylation status, amplification of the trigger RNA and the maintenance of silencing. Methylation has so far not been observed in *C.elegans*. However, Ketting *et al.* (69) discussed the possibility that activation of an RNA-degrading complex through dsRNA may direct chromatin changes to chromosomal locations with homology to the dsRNA, to prevent the further production of repetitive mRNA molecules.

In view of the high specific activity in inducing RNAi in *C.elegans* it is possible that the 85mer RNA is also the signal responsible for the systemic spread of silencing in plants. This would be consistent with previous experiments which suggested that the mobile signal is distinct from the siRNAs (51,52). We are currently preparing larger quantities of the silencing-competent RNA that will allow biochemical characterization.

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