RNA-directed DNA methylation in *Arabidopsis*

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In plants, double-stranded RNA that is processed to short RNAs ≈21–24 nt in length can trigger two types of epigenetic gene silencing. Posttranscriptional gene silencing, which is related to RNA interference in animals and quelling in fungi, involves targeted elimination of homologous mRNA in the cytoplasm. RNA-directed DNA methylation involves *de novo* methylation of almost all cytosine residues within a region of RNA–DNA sequence identity. RNA-directed DNA methylation is presumed to be responsible for the methylation observed in protein coding regions of posttranscriptionally silenced genes. Moreover, a type of transcriptional gene silencing and *de novo* methylation of homologous promoters in *trans* can occur if a double-stranded RNA contains promoter sequences. Although RNA-directed DNA methylation has been described so far only in plants, there is increasing evidence that RNA can also target genome modifications in other organisms. To understand how RNA directs methylation to identical DNA sequences and how changes in chromatin configuration contribute to initiating or maintaining DNA methylation induced by RNA, a promoter double-stranded RNA-mediated transcriptional gene silencing system has been established in *Arabidopsis*. A genetic analysis of this system is helping to unravel the relationships among RNA signals, DNA methylation, and chromatin structure.

The term "RNA silencing" refers to epigenetic gene silencing effects that are initiated by double-stranded RNA (dsRNA) (1). Discovered independently in plants, fungi, and animals, RNA silencing phenomena are revealing new ways to repress gene expression and to subdue transposable elements and viruses that produce dsRNA during their replication cycle (2–8). A fundamental step in RNA silencing pathways is cleavage of dsRNA into short RNAs (9), which are believed to act as guides for enzyme complexes that either degrade or modify homologous nucleic acids.

The most familiar type of RNA silencing occurs primarily in the cytoplasm and is termed posttranscriptional gene silencing (PTGS) in plants, quelling in *Neurospora*, and RNA interference (RNAi) in animals. PTGS/RNAi involves a dsRNA that is processed by an RNase III-like enzyme called Dicer into short interfering (si) RNAs 21–22 nt in length. The antisense siRNAs associate with a ribonuclease complex and guide sequence-specific degradation of complementary mRNAs (5–8).

A second form of RNA silencing involves sequence-specific changes at the genome level. RNA-directed DNA methylation (RdDM) (10), which has been described so far only in plants, leads to *de novo* methylation of almost all cytosine residues within the region of sequence identity between the triggering RNA and the target DNA. Similarly to PTGS/RNAi, RdDM requires a dsRNA that is cleaved to short RNAs ≈21–24 nt in length (11). It is not yet certain whether the short RNAs or dsRNA guide methylation of homologous DNA sequences, although the length of short RNAs is consistent with the minimum DNA target size of RdDM (≈30 bp) (12).

RdDM is assumed to be the source of methylation observed in protein coding regions in many cases of PTGS, where it can contribute in an unknown way to the maintenance of silencing (13, 14). In addition, RdDM has been implicated in a type of transcriptional gene silencing (TGS) that is initiated by dsRNAs containing promoter sequences. Promoter dsRNAs that trigger TGS and RdDM of homologous promoters in *trans* can be produced in the nucleus by transcription through inverted repeats (IRs) of promoter sequences (11, 15) or in the cytoplasm by a replicating RNA virus that is engineered to contain sequences identical to the promoter of a nuclear gene (16, 17).

Although the phenomenon of RdDM is well established in plants, a number of questions remain. One concerns the identity of the DNA methyltransferases (MTases) that are required for establishing and maintaining the unusual pattern of methylation characteristic of RdDM. A second issue concerns the relationship between DNA methylation and changes in chromatin structure. Given the close links between DNA methylation, chromatin remodeling (18–20), and histone modifications, such as acetylation (21) and methylation (22, 23), it might be anticipated that alterations in chromatin structure would be required to initiate and/or retain methylation induced by RdDM.

To address these questions, we carried out a genetic analysis of a promoter dsRNA-mediated TGS system that we have established in *Arabidopsis*. In this paper, we describe this system and the impact of several mutations that impair DNA methylation and/or possible chromatin remodeling processes. We discuss whether RdDM might occur in animals and whether RNA might direct chromatin modifications in organisms that do not methylate their DNA.

Experimental Procedures

T-DNA Constructs and Plant Transformation. *Arabidopsis thaliana* plants (ecotype Columbia) were grown at 22°C in a 16 h/8 h day/night cycle. Transformation was done by the floral dip method (24). The nopaline synthase promoter (NOSpro) target construct [NOSpro–*NPTII* (neomycin phosphotransferase) NOSter–NOSpro–*NOS* (nopaline synthase) NOSter] was introduced into *Arabidopsis* by using *Agrobacterium* strain A208 harboring a disarmed Ti-plasmid (25). A line homozygous for the target locus was retransformed by using *Agrobacterium* harboring a binary vector with a NOSpro IR (in which the halves were separated by ≈250 bp of the α' promoter of soybean β-conglycinin; ref. 26) driven by the 35S promoter of cauliflower mosaic virus (35Spro), a pUC18 plasmid vector, and a 19Spro-*HPT* (hygromycin phosphotransferase) gene as selectable marker (11). The 35Spro was flanked by *lox* sites in direct orientation to allow removal by Cre recombinase. Selection of transgenic target and silencer plants was done as described (11).

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Abbreviations: dsRNA, double-stranded RNA; Kan, kanamycin; Hyg, hygromycin; Kan^r, Kan resistant; IR, inverted DNA repeat; MTase, DNA cytosine methyltransferase; NOSpro, nopaline synthase promoter; PTGS, posttranscriptional gene silencing; RdDM, RNA-directed DNA methylation; RNAi, RNA interference; TGS, transcriptional gene silencing; 35Spro, 35S promoter of cauliflower mosaic virus.

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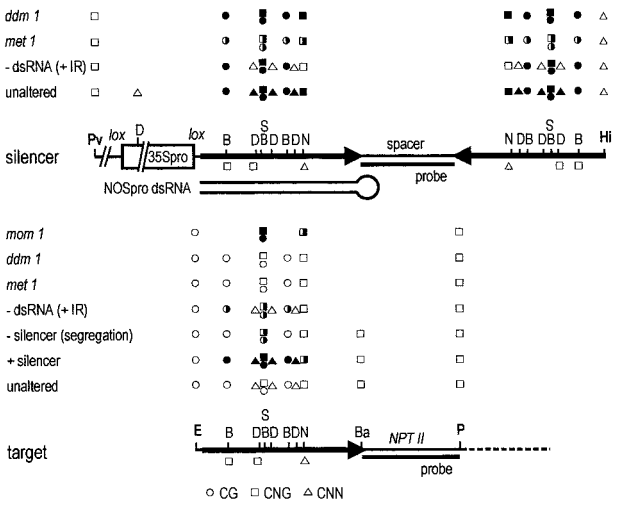

Fig. 1. Structures and methylation analysis of the silencer NOSpro IR (*Upper*) and target NOSpro–*NPTII* gene (*Lower*). NOSpro sequences are depicted as heavy black arrows. Enzymes and probes used for DNA blot analyses are indicated. Abbreviations: Pv, *Pvu*II; D, *Dde*I; S, *Sac*II; N, *Nhe*I; Hi, *Hin*dIII; E, *Eco*RI; B, *Bst*UI; Ba, *Bam*HI; P, *Pst*I. To assess methylation in the target NOSpro, an E and P double digest was performed (the minus lanes in Figs. 4*A* and 6 *A*, *C*, *E*, and *G*) and one of several methylation-sensitive enzymes (B, D, S, N, Ba) was added. Methylation in the NOSpro IR was tested by digesting with Pv and Hi (the minus lanes in Figs. 4*B* and 6 *B*, *D*, *F*, and *H*), together with either B, D, S, or N. Filled, half-filled, and open circles, squares, and triangles (CG, CNG, and CNN, respectively) indicate >90%, ≈50%, and <10% cytosine (C) methylation, respectively. Open squares or triangles below each map for the enzymes D, N, and B indicate that the top and bottom DNA strands contain C residues in different sequence contexts (e.g., CG and CNG, or CNG and CNN). The *Nhe*I site (underlined) is in the sequence context: 5′-CAGCTACG^{m}CAA-3′ (top); and 3′-GT^{m}CGATCGTT-5′ (bottom).

Cre/*lox*-Mediated Deletion of the 35Spro. To delete the 35Spro by Cre recombinase, plants doubly homozygous for target and silencer were supertransformed with a third T-DNA construct expressing Cre recombinase from the 35Spro (27). The Cre construct encodes glufosinate resistance (BAR), which allows the herbicide BASTA to be used for selection directly on soil. Soil-grown triple transformants were selected by spraying with BASTA (Celaflor, Hoechst, Vienna, Austria; 400 mg/liter ammonium glufosinate) twice a week for 2 weeks. T2 seeds from BASTA-resistant T1 plants were plated on MS agar containing 40 mg/liter kanamycin (Kan) (Sigma), and/or 40 mg/liter Kan plus 20 mg/liter hygromycin B (Hyg) (Calbiochem). Resistant T2 seedlings were genotyped by PCR using BAR primers to confirm the absence of the Cre construct because the presence of Cre-recombinase interferes with Southern analysis of DNA-fragments containing *lox* sites (M.F.M., unpublished observations). Genotype-PCR-grade DNA was isolated from *Arabidopsis* leaves as described (28). BAR primers were 5′-CGAGACAAGCACGGTCAACTTC-3′ and 5′-ACCCACGTCATGCCAGTTCC-3′. BAR-negative plants were allowed to set seeds, and DNA was extracted from T3 progeny plants by using a DNAeasy plant maxi kit (Qiagen, Hilden, Germany). The DNA was subjected to restriction digests and Southern hybridization as described in the Fig. 1 and Fig. 6 legends. Data in Fig. 3*C* and Fig. 6 were obtained for plants 2 generations after Cre-mediated removal of the 35Spro.

Mutant Crosses. The following *Arabidopsis* mutants were used in this study: *ddm1-5/som8* (decrease in DNA methylation/somniferous) (29) in ecotype Zürich; *mom1* (Morpheus' molecule) (30) in ecotype Zürich; and *met1/ddm2–1* (DNA methyltransferase 1) (E. Richards, personal communication) in ecotype La-er. Because the strength of NOSpro silencing varied somewhat in different ecotypes (La-er > Col-0 > Zürich), control crosses of double homozygous target/silencer plants with the respective wild-type backgrounds were always performed. Reactivation of the NOSpro–*NPTII* target gene in a given mutant was assessed by survival of seedlings in the F_2 generation and/or advanced generations on Murashige and Skoog (MS) medium containing 40 mg/liter Kan alone or 40 mg/liter Kan plus 20 mg/liter Hyg. The *mom1* and *met1* mutations are not genetically linked to either the target locus or the silencing locus, and could be tested for their effects on NOSpro–*NPTII* silencing immediately in the F2 generation; *ddm1/som8* is linked to the target locus and had to be introgressed into the NOSpro target/silencer line as described below.

ddm1-5/som8 is a fast neutron-generated allele that is distinguishable from the wild-type gene by an 82 bp insertion into the second exon (18). The *ddm1-5/som8* mutation was separated from transgene locus A (which harbors 35S-*HPT* genes) (29) by two outcrosses to ecotype Col-0. Plants homozygous for *ddm1-5/som8* and lacking locus A were screened out from selfed progeny of the second outcross. Genotyping reactions were done by using *som* primers: 5′-AAGCGACGGAGACGACTGTTTG-3′ and 5′-TTTCACAAAGCAACCACACTACG-3′. 35S-HPT primers were 5′-CCCACTATCCTTCGCAAGA-3′and 5′-CGTCTGCTGCTCCATACAAGC-3′. Because the *DDM1* gene is linked to the target transgene locus (the physical distance is ≈270 kb), the *ddm1-5* mutation was introgressed into the genetic background of the NOSpro target/silencer transgenic plant. A *ddm1-5/ddm1-5* plant lacking transgene locus A was crossed with a plant doubly homozygous for the NOSpro target/silencer. An F_2 plant homozygous for the NOSpro silencing locus, hemizygous for the NOSpro target locus and heterozygous for the *ddm1-5* allele was selected. This plant was allowed to self-pollinate, and 193 F_3 seedlings were analyzed for their *DDM1* genotype with the *som* primers described above. Forty-eight F_3 seedlings were homozygous for the *ddm1-5* allele and were further subjected to PCR-genotyping for the presence of target NOSpro–*NPTII* sequences by using NOSpro–*NPTII* primers: 5′-GAGAATTAAGGGAGTCACG-3′and 5′-TCGTCCTGCAGTTCATTC-3′. Two of the 48 *ddm1-5/ddm1-5* plants were hemizygous for the target locus indicating a recombination event between the *ddm1-5* allele and the NOSpro target locus during meiosis of the parental F_2 plant. Progeny seeds from this genotype (i.e., the second generation of homozygosity for *ddm1-5*; the target is still segregating) were analyzed for reactivation of the NOSpro–*NPTII* gene on medium containing Kan (n = 308 seeds) or Kan and Hyg (n = 317). Only weak reactivation was observed in a few progeny. Eventually, in progeny that were third generation homozygous for the *ddm1-5* allele (and homozygous for the target and silencing loci), resistance generally improved and stronger Kan resistant (Kan^R) seedlings appeared stochastically in populations of seedlings that also contained moderately and weakly Kan^R members (Fig. 3*D*).

Plants homozygous for the *mom1* allele without transgene locus A were crossed with a doubly homozygous NOSpro target/silencer plant. F_2 seeds were plated on medium containing Kan, Hyg, or both to test for an immediate effect of the *mom1* mutant allele. No immediate effect was observed. Among the F_3 progeny of this cross, a triple homozygous line for the NOSpro target, NOSpro silencer, and the *mom1* allele was selected. The genotype for the NOSpro silencer was determined by selecting seeds on Hyg-containing medium. The genotype with respect to the *mom1* allele, which is tagged with a T-DNA conferring BASTA resistance, was determined by spraying seedlings with the herbicide as described above. To assess the genotype for the NOSpro target, PCR genotyping with NOSpro–*NPTII* primers (see above) was performed. The progeny F_4 seeds of the triple homozygous plant (i.e., the third generation of homozygosity for

mom1) were plated on medium containing Kan ($n = 440$) or Kan and Hyg ($n = 441$). No resistant seedlings were observed.

met1/ddm2–1 was obtained as progeny from a heterozygous plant and homozygous plants were screened out by using the demethylation assay of centromeric and ribosomal DNA (rDNA) repeats (ref. 31; E. Richards, personal communication). A homozygous *met1* plant was crossed to plants homozygous for both the NOSpro target locus and the NOSpro silencing locus. Resulting F_1 plants were allowed to self-pollinate, and the F_2 seeds were plated on medium containing Kan, Hyg, or both antibiotics. The F_2 seedlings showed weak to moderate resistance on Kan and Kan–Hyg, consistent with a partial release of silencing. Double resistant seedlings were recovered on soil, and *met1* homozygosity was confirmed by using cleaved amplified polymorphic sequence markers (CAPS) markers as described (32). F_4 progeny lines that are triple homozygous for *met1* (i.e., third generation of *met1* homozygosity), the target locus and the silencing locus were selected for further analysis. These seedlings display weak to strong Kan^R (Fig. 3*D*).

Methylation Analysis. Plant genomic DNA was extracted as described (11). For the DNA methylation analyses in *met1*, *ddm1*, and *mom1* mutants, DNA was isolated from plants that had been homozygous for a given mutation for two generations (i.e., F_3 generation for *mom1* and *met1*; F_4 generation for *ddm1*). Methylation in plants containing the Cre-altered, nontranscribed silencing locus were performed after the Cre-expressing locus had been segregated out, eliminating possible background transcription of the Cre locus. Restriction digests were done according to the instructions of the manufacturers. DNA blot analysis using ^{32}P-labeled RNA probes has been described (33). As probes, subcloned 0.19 kb *NPTII* and 0.25 kb α' pro fragments were transcribed *in vitro*. Methylation analysis of centromeric repeats and rDNA repeats was conducted as described by others (32). Bisulfite sequencing was performed as described (12, 34). The following degenerate primers, which allowed for unconverted or converted cytosines, were used to amplify the top strand of the NOS-NPTII target: 5′ primer 5′-YATGAGYGGAGAATTAAGGGAGT-3′ (Y = C or T); 3′ primer 5′-CCRAATARCCTCTCCACCCAA-3′ (R = G or A).

Cloning of Transgene Inserts. Genomic λ clones from target and silencer transgenes were obtained and sequenced as described (35). The silencer transgene complex comprises a single copy of the T-DNA construct with a complete 35Spro–NOSpro IR that was integrated in chromosome 4, BAC clone F10M10 (GenBank accession no. AL035521) with the right border downstream of nucleotide 21681 and the left border upstream of nucleotide 21693. Between plant sequence and the right border, the filler sequence "TTTTT" was inserted. The target locus was originally screened out genetically as a single transgene locus that was readily inactivated when the silencing locus was introduced, and largely reactivated the first generation after segregating away from the silencing locus. More detailed structural analysis by λ cloning revealed that the target locus contains several complete and incomplete copies of the T-DNA construct flanked by *Arabidopsis* DNA from chromosome 5 on the left and chromosome 3 on the right. This finding suggests that a rearrangement of plant DNA, which occasionally occurs during T-DNA integration (36, 37), had occurred. All of the bands visualized in Southern blot analyses using an *NPTII* probe always cosegregated in multiple, independent crosses, which is consistent with a single, multicopy transgene locus. In the absence of the silencing locus, the target locus was stably expressed over multiple homozygous generations. The moderate structural complexity probably enhanced its susceptibility to silencing, as has been observed in a NOSpro silencing system in tobacco (38). Genetic analysis revealing linkage to *ddm1* indicated that the actual T-DNA insertion site was on chromosome 5 (data not shown). Nucleotide 143 of the T-DNA right border region (GenBank accession no. J01826) was fused upstream of nucleotide 66153 of BAC clone F2103 (GenBank accession no. AC009853) on chromosome 3. Nucleotide 98 of the left T-DNA border region (GenBank accession no. J01825) was fused to sequences at the distal end of chromosome 5, BAC clone K9I9 (GenBank accession no. AB013390) upstream of nucleotide 4316. The rearrangement had no visible phenotype effects or impact on target NOSpro expression. Mitotic chromosome counts (39) revealed a normal diploid number of $2n = 10$.

RNA Analyses. Total RNA was extracted, electrophoresed, and blotted as described (40). Control hybridization of tobacco with an actin probe was carried out following published procedures (40). *Arabidopsis* RNA was hybridized with a eukaryotic protein synthesis initiation factor 4A (*eIF-4A*) fragment from *Arabidopsis* (41). Analysis of NOSpro dsRNA and small RNA was carried out as described (11). Transcriptional run-on analysis was performed as described in a former report (42).

Results

A promoter dsRNA-mediated *trans*-silencing system based on the NOSpro was originally established in tobacco (11) and has served as a model for setting up a similar system in *Arabidopsis*. A homozygous line that stably expresses a NOSpro–*NPTII* target gene encoding resistance to Kan (Fig. 1 *Lower*) was produced. The target line was then retransformed with a silencing construct, which contains a NOSpro IR under the control of the 35Spro (Fig. 1 *Upper*) together with a gene encoding resistance to Hyg driven by the 19S promoter of cauliflower mosaic virus (11). The silencing locus produces NOSpro dsRNA (Fig. 2*A*, *Arabidopsis* target + silencer) that is processed into short RNAs ≈21–24 nucleotides in length (Fig. 2*C*, *Arabidopsis* target + silencer), similar to those observed in tobacco transformed with the same construct (Fig. 2 *A* and *C*, tobacco target + silencer) (11).

In the presence of the silencing locus, the target NOSpro–*NPTII* gene is inactivated, as revealed by cultivation of seedlings on media containing different antibiotics. When self fertilized, a plant that is homozygous for the active NOSpro–*NPTII* target gene produces, as expected, 100% Kan^R progeny (Fig. 3*A*, target–Kan). When the silencing locus, which encodes NOSpro dsRNA and resistance to Hyg, is introduced into the target line and is present in the hemizygous condition, selfing produces 75% Hyg-resistant seedlings (Fig. 3*A*, target + silencer-Hyg). Even though the parent is homozygous for the target locus, however, only 25% of the seedlings are Kan^R (Fig. 3*A*, target + silencer-Kan). Any seedling that is Kan^R has not inherited the silencing locus, as indicated by the lack of double resistance (Fig. 3*A*, target + silencer, Kan/Hyg). Conversely, a seedling that has inherited the silencing locus is Kan-sensitive because of silencing of the NOSpro–*NPTII* target gene. Silencing of the target gene occurs at the transcriptional level as demonstrated by a nuclear run-on analysis (Fig. 2*D*).

Transcriptional silencing of the NOSpro–*NPTII* target gene is accompanied by *de novo* methylation of the target NOSpro. When active, the target gene is normally unmethylated in the NOSpro region, as indicated by nearly complete digestion with the methylation-sensitive restriction enzymes *Sac*II ($^mC^mCG^m$-CGG), *Bst*UI ($^mCG^mCG$), and *Nhe*I(GCTAG^{m}C) (Fig. 4*A*, unmeth. control; a superscript "m" indicates a methylated cytosine that can inhibit cleavage). In the presence of the silencing locus, the NOSpro region specifically becomes methylated in both symmetrical (CG and CNG) and nonsymmetrical (CNN) cytosines as demonstrated, respectively, by negligible digestion with *Sac*II and *Bst*UI, and approximately 50% digestion with *Nhe*I (Fig. 4*A*, target + silencer). This pattern of

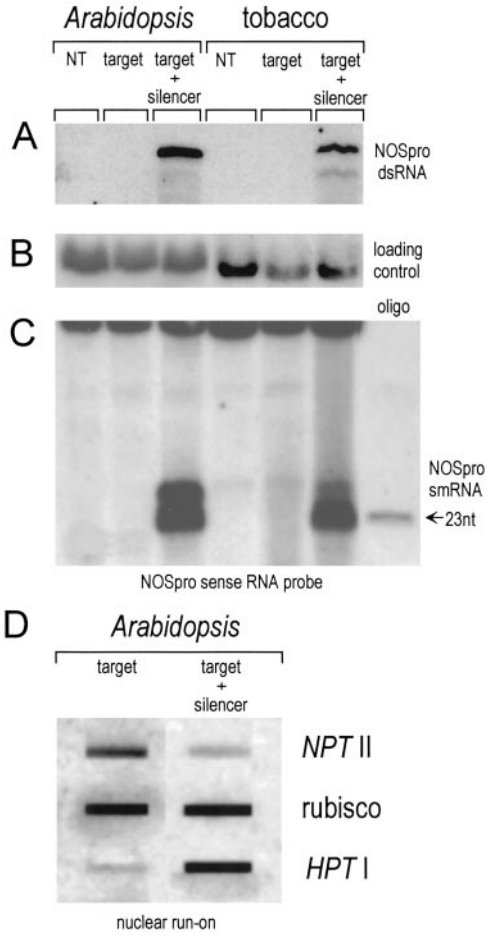

Fig. 2. RNA analysis. (*A*) RNase protection reveals the ≈0.3-kb NOSpro dsRNA transcribed from the silencer NOSpro IR. NT, normal untransformed plants. (*B*) Total RNA used in *A* probed with an actin probe from tobacco and an eIF-4A probe from *Arabidopsis* as loading controls. (*C*) Detection of NOSpro short RNAs (sense probe) produced by means of dsRNA cleavage. Identical results were obtained with an antisense probe. (*D*) Nuclear run-on analysis demonstrating transcriptional down-regulation of the NOSpro–*NPTII* target gene in the presence of the silencing locus, which encodes *HPT* and NOSpro dsRNA. A constitutively expressed ribulose 1,5-bisphosphate carboxylase (rubisco) gene was used as a control. Positive controls in *A* and *C* were prepared from tobacco plants transformed with the 35Spro–NOSproIR construct.

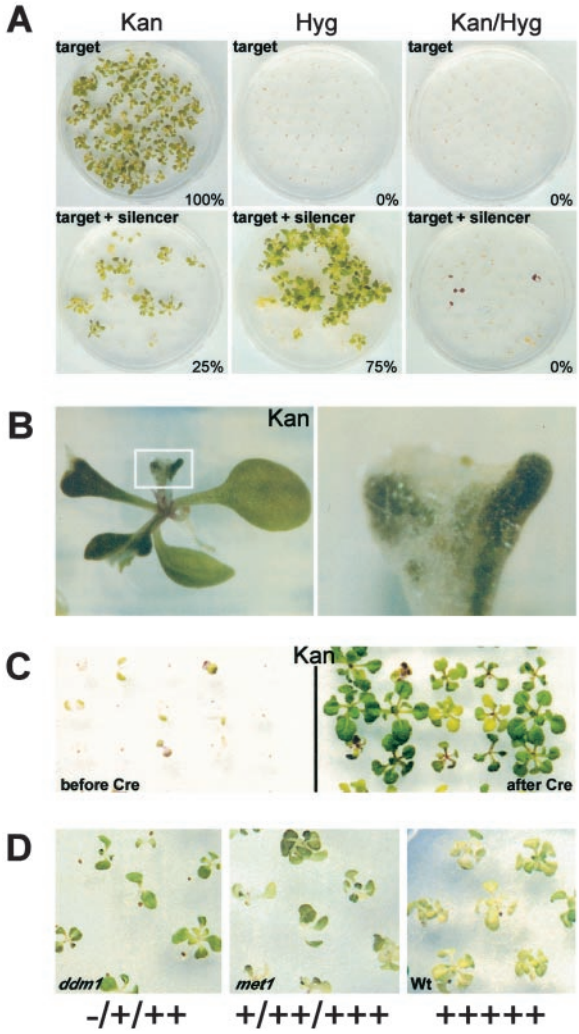

Fig. 3. Phenotypic analysis of silencing. NOSpro–*NPTII* target gene expression is assayed by Kan^R; the silencing locus by Hyg^R. (*A*) Selfing a plant homozygous for an active target gene produces 100% Kan^R progeny. Selfing a plant homozygous for the target locus and hemizygous for the silencing locus, revealed by 75% Hyg^R, produces only 25% Kan^R progeny. Kan^R seedlings lack the silencer, indicated by 0% (Kan/Hyg)^R. (*B*) Mottled Kan^R seedling in the first generation after crossing out the silencing locus (*Right* is an enlargement of the boxed region in *Left*; white and green patches represent Kan^S and Kan^R regions, respectively). (*C Left*) Kan^S seedlings before removing the 35Spro with Cre recombinase. (*Right*) Kan^R seedlings two generations after removing the 35Spro. (*D*) Ranges of phenotypes on Kan-containing medium (plus signs, different degrees of Kan^R; minus sign, Kan^S) in seedlings after three generations of homozygosity for the *ddm1* and *met1* mutations, based on 5 plus signs for wild-type levels of Kan^R in seedlings containing the target locus in the unsilenced state.

methylation, in which C residues in any sequence context are modified specifically in the region of RNA-DNA sequence identity, is characteristic of RdDM and was confirmed when bisulfite sequencing was used to examine methylation in more detail (Fig. 5).

Methylation of the target NOSpro–*NPTII* gene is largely eliminated when the target locus and silencing locus segregate in progeny, as indicated by nearly complete digestion with *Bst*UI and *Nhe*I, and about 50% cleavage with *Sac*II (Fig. 4*A*, target minus silencer). The remaining methylation at the *Sac*II site, which is correlated with the mottled phenotype of many Kan^R seedlings (Fig. 3*B*), is presumably caused by maintenance of some CG and/or CNG methylation through meiosis (42).

A requirement for NOSpro dsRNA in silencing and methylation of the target NOSpro–*NPTII* gene was demonstrated by removing the transcribing 35Spro, which is flanked by *lox* sites (Fig. 1 *Upper*), with Cre recombinase. The removal of the 35Spro and retention of the NOSpro IR at the Cre-modified "silencing" locus was monitored by a shift to a smaller band of the expected size with all enzymes tested in a DNA blot analysis (Fig. 6 *B*, *D*, *F*, and *H*; compare minus lanes in unaltered silencer panels with minus lanes in Cre-altered silencer panels) and confirmed by cloning and sequencing the Cre-modified silencing locus (data not shown).

After deletion of the 35Spro from the silencing locus, NOSpro short RNAs are no longer detectable, even after long exposures of the respective Northern blots (data not shown). Consequently, the target NOSpro–*NPTII* gene is active in the presence of the nontranscribed NOSpro IR, as indicated by the Kan^R phenotype of seedlings that are doubly homozygous for the target locus and Cre-modified silencing locus (Fig. 3*C*, after Cre). Furthermore, methylation of the NOSpro–*NPTII* target gene is reduced ap-

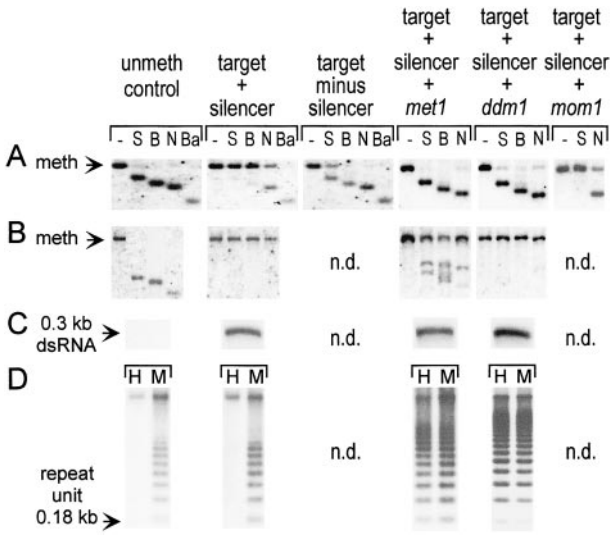

Fig. 4. Methylation analysis. (*A*) Target NOSpro. (*B*) Silencer NOSpro IR. (*C*) NOSpro dsRNA. (*D*) Centromeric repeats. Methylation of the target and silencing loci were analyzed by using the enzymes and probes described in Fig. 1. For *met1*, *ddm1*, and *mom1* mutants, methylation was analyzed by using DNA isolated from plants that had been homozygous for the respective mutation for two generations. Methylation of centromeric repeats was analyzed by using *Hpa*II (H: ^{m}C^{m}CGG) and *Msp*I (M: ^{m}CCGG). The unmethylated control for the silencer NOSpro IR consisted of a λ genomic clone containing the silencing locus. Shifts to the smaller fragments indicate no methylation at a particular site. Arrows in *A* and *B* indicate position of methylated fragment; in *C* and *D*, arrows represent the sizes of the indicated features. n.d., not determined.

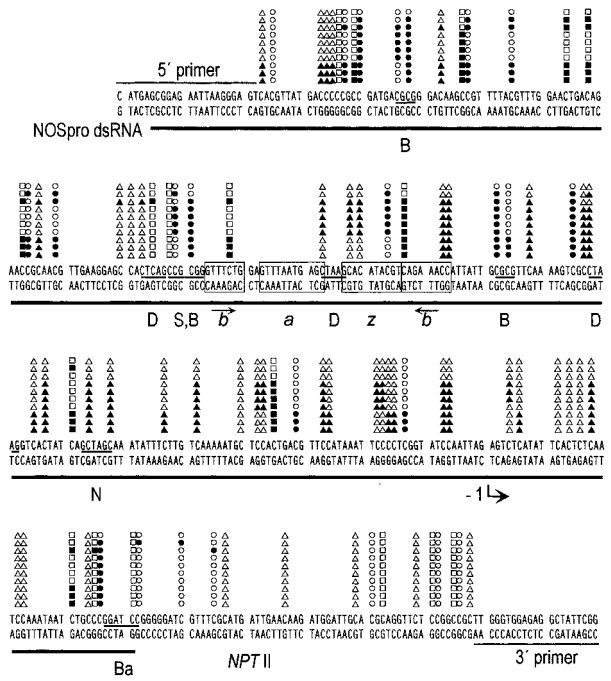

Fig. 5. Bisulfite sequencing. The ≈300-bp NOSpro sequence is shown with the region of identity to NOSpro dsRNA underlined. Methylation (filled symbols) in 10 cloned PCR fragments from the upper DNA strand is indicated. Symbols are described in the Fig. 1 legend. The positions of restriction enzyme sites used in the DNA blot analyses are indicated (abbreviations are given in the Fig. 1 legend). The four boxed regions represent transcriptional regulatory elements (61), which contain short IRs (arrows). The transcription start site is indicated by the bent arrow at −1. The sequence of the primers used is indicated. Methylation does not infiltrate significantly into *NPTII* coding sequences.

proximately 30% at symmetrical Cs in the *Sac*II (Fig. 6*A*, target + Cre-altered silencer) and *Bst*UI (Fig. 6*C*, target + Cre-altered silencer) sites and almost completely at nonsymmetrical C residues in the *Nhe*I site (Fig. 6*E*, target + Cre-altered silencer) and *D*deI sites (^{m}CTNAG) (Fig. 6*G*, target + Cre-altered silencer).

The NOSpro dsRNA not only triggers methylation and silencing of the target NOSpro in *trans*, it also contributes to methylation in *cis* of the NOSpro copies in the IR at the silencing locus. This was demonstrated by examining methylation of the NOSpro IR before and after removing the transcribing 35Spro with Cre recombinase. The transcribed NOSpro IR at the unaltered silencing locus is heavily methylated at both symmetrical and nonsymmetrical Cs within the repeated region as indicated, respectively, by lack of digestion with *Sac*II and *Bst*UI (Fig. 6 *B* and *D*, unaltered silencer panels), and *Nhe*I and *Dde*I (Fig. 6 *F* and *H*, unaltered silencer panels). In contrast, the nontranscribed NOSpro IR at the Cre-altered silencing locus loses methylation at nonsymmetrical C residues, which was revealed by substantial digestion with *Nhe*I and *Dde*I (Fig. 6 *F* and *H*; compare unaltered silencer panel with Cre-altered silencer panels). At the same time, methylation at symmetrical C residues is almost completely retained, as indicated by poor digestion with *Sac*II and *Bst*UI (Fig. 6 *B* and *D*; compare unaltered silencer panel with Cre-altered silencer panels).

The effects of several mutations that release TGS in other systems (14, 17, 29, 30, 32, 43, 44) were tested on the NOSpro dsRNA-mediated TGS system. For these experiments, the double homozygous target/silencer line was crossed with lines homozygous for the following recessive mutations: the *som8* allele (29) of *ddm1*, which encodes a putative component of a SWI/SNF2 chromatin remodelling complex (18); *met1* (*ddm2*), which encodes a DNA MTase (E. Richards, personal communication) that maintains methylation in CG dinucleotides (45); and *mom1*, which encodes a possible chromatin remodeling protein (30). F_1 progeny obtained from these crosses were selfed and the extent of silencing evaluated in the F_2, F_3, and F_4 generations. If a given mutation has no effect (and assuming no linkage between a mutation and the target locus or silencing locus), the percentages of antibiotic resistance in F_2 progeny should be 19% Kan^{R}, 75% Hyg^{R}, and 0% (Kan–Hyg)^{R}. If a mutation releases silencing and is fully penetrant, these percentages would change to 33% Kan^{R}, 75% Hyg^{R}, and 14% (Kan–Hyg)^{R}. In other words, impaired silencing would be indicated by an increase in the percentage of Kan^{R} F_2 progeny and by the appearance of some F_2 progeny that display double resistance.

The *mom1* mutation is the only one of the three tested that did not visibly affect NOSpro silencing, as indicated by no recovery of (Kan–Hyg)^{R} progeny, even in F_3 and F_4 generations (data not shown). Methylation of the target NOSpro is also not reduced in *mom1* mutants, as demonstrated by levels of methylation at the *Sac*II and *Nhe*I sites that approximate those in the silenced state (Fig. 4*A*, compare target + silencer + *mom1* with target + silencer).

The *met1* mutation partially released silencing of the NOSpro–*NPTII* gene in F_2 progeny, as indicated by an increase in the percentage of Kan^{R} seedlings (29%, $n = 428$) and weak resistance of some of these seedlings on medium containing both Kan and Hyg (11% Kan-Hyg^{R}, $n = 912$). Because the *ddm1* mutation is linked to the target locus on chromosome 5, it had to be introgressed into the double homozygous target/silencer line. In the first generation, when the strength of antibiotic resistance could be tested in *ddm1* mutants, sporadic weak reactivation of NOSpro–*NPTII* gene expression was observed (data not shown). In both *met1* and *ddm1* mutants, Kan^{R} resistance could improve

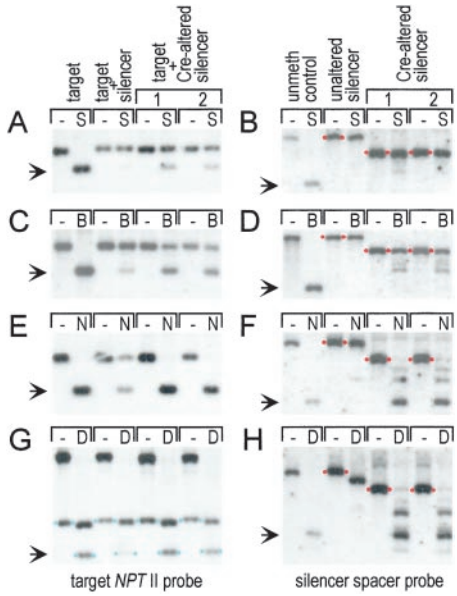

Fig. 6. Methylation analysis in the presence and absence of NOSpro dsRNA. (*A*, *C*, *E*, and *G*) Target NOSpro. (*B*, *D*, *F*, and *H*) Silencer NOSpro IR. The enzymes and probes used are described in Fig. 1. The arrows to the left of each blot show the position of the expected unmethylated fragment. Results from two lines containing the Cre-altered silencer are shown. (*G*) The NOSpro–*NPTII* bands of interest are flanked by blue dots. The large hybridizing fragment in the minus lanes and the fragment in the *D* lanes running just below the NOSpro–*NPTII* band should be disregarded as they are caused by a second *NPTII* gene (not visible in *A*, *C*, and *E*) used for bacterial selection during cloning (25). The size of the fragment in the minus lanes in *B*, *D*, *F*, and *H* is shifted after the Cre cross (indicated by red dots) because of removal of the 35Spro and is independent of methylation. Because of an unmethylated *Dde*I site in the 35Spro (Fig. 1 *Upper*), the size of the fragment of the unaltered silencer shifts after addition of *Dde*I (*H*).

in advanced generations, although the strength of NOSpro–*NPTII* gene expression continued to be nonuniform in genotypically identical seedlings (Fig. 3*D*). The strongest Kan^R plants sustained significant losses of methylation from the target locus, as indicated by substantial digestion with *Sac*II, *Bst*UI, and *Nhe*I (Fig. 4*A*, target + silencer + *met1* and + *ddm1*). These plants are indeed homozygous for the respective mutations, which cause global demethylation, as revealed by the loss of CG methylation at centromeric repeats (Fig. 4*D*, target + silencer + *met1* and + *ddm1* panels) and rDNA repeats (data not shown).

In contrast to the substantial reduction of methylation of the NOSpro target locus in plants showing relatively strong Kan^R in the *met1* and *ddm1* backgrounds, the NOSpro IR at the silencing locus retains considerable methylation in these mutants. This was particularly evident in *ddm1* mutant plants, where—similarly to wild type plants—virtually no digestion by *Sac*II, *Bst*UI, and *Nhe*I was observed (Fig. 4*B*, compare target + silencer + *ddm1* with target + silencer). In *met1* plants, methylation is reduced ≈20–30% at both symmetrical (*Sac*II, *Bst*UI) and nonsymmetrical (*Nhe*I) sites (Fig. 4*B*, target + silencer + *met1* panels). NOSpro dsRNA continues to be synthesized at wild-type levels in the *met1* and *ddm1* mutant plants (Fig. 4*C*).

Discussion

To dissect the mechanism of RdDM and dsRNA-mediated TGS, we have established a two component silencing system based on the NOSpro in *Arabidopsis*. A NOSpro dsRNA transcribed from a NOSpro IR at the silencing locus is processed to short RNAs 21–24 nt in length. Either the dsRNA or the short RNAs can locate and interact with the homologous NOSpro at the unlinked target locus and trigger TGS and promoter methylation. We report here an analysis of methylation of the target NOSpro and the NOSpro IR at the silencing locus in the presence and absence of NOSpro dsRNA, and in several mutants deficient in DNA methylation and/or putative chromatin remodeling proteins. The results are summarized in Fig. 1.

NOSpro dsRNA induces *de novo* methylation of the target NOSpro at Cs in any sequence context within the region of RNA-DNA sequence identity (Fig. 1 target + silencer). Removing the source of the dsRNA, either by segregating away the silencing locus [Fig. 1 target, − silencer (segregation)] or by removing the transcribing 35Spro via Cre/*lox*-mediated recombination [Fig. 1 target, − dsRNA (+IR)], results readily in loss of methylation in nonsymmetrical C residues, indicating that they require continuous *de novo* methylation induced by RdDM. In contrast, methylation in symmetrical CG and CNG nucleotide groups, which can be maintained, respectively, by the DNA MTases MET1 (45) and chromomethylase3 (CMT3) (46, 47), is retained to varying degrees in the target NOSpro after it segregates away from the silencing locus or in the presence of the Cre-altered silencing locus. The persistent target promoter methylation resembles paramutation, which involves meiotically heritable changes in gene expression induced by allelic interactions (48). Indeed, some paramutation-like phenomena are probably caused by maintenance of RNA-induced CG and CNG methylation through meiosis (17), suggesting that MET1 and CMT3 can function during this period. Interestingly, methylation in symmetrical C residues is not lost as readily in the presence of the nontranscribed Cre-altered silencing locus as it is following segregation of the unaltered silencing locus. This difference cannot be ascribed to NOSpro dsRNA, which is not synthesized to detectable levels following Cre-mediated excision of the 35Spro, nor to the somewhat repetitive nature of the target locus, which remains unchanged regardless of the presence of the silencing locus or the Cre-altered derivative. One possibility is that the nontranscribed IR enhances maintenance methylation of the target NOSpro, perhaps through DNA–DNA pairing interactions (49).

The *met1* and *ddm1* mutations, which reduce global methylation and release silencing to varying extents in other TGS systems (14, 17, 29, 32, 43, 44), partially alleviate silencing and reduce methylation of the NOSpro–*NPTII* target gene but these effects can only be considered indirect. With *met1*, partial, nonuniform recovery of Kan resistance was observed in the F_2 generation, followed by progressive improvement in subsequent generations. The *ddm1* mutation appeared somewhat less efficient at releasing silencing than *met1*, though it is difficult to make a strict comparison because the *ddm1* mutation had to be introgressed into our NOSpro target/silencer line. In both mutants, losses of methylation in the target NOSpro can be substantial in F_3 and F_4 progeny that show the strongest Kan^R (Fig. 1 target, *met1*, *ddm1*). Any slight methylation that persists is presumably caused by continued *de novo* methylation induced by NOSpro dsRNA. The lack of effect of *mom1* on NOSpro silencing and methylation (Fig. 1 target, *mom1*) is not unexpected, because this mutation affects a subset of transcriptionally inactivated genes that are silenced by a methylation-independent pathway and, unlike *met1* and *ddm1*, does not cause global demethylation (30, 50).

The two copies of the NOSpro in the IR at the silencing locus are methylated substantially at symmetrical and nonsymmetrical C residues (Fig. 1, unaltered silencer). When dsRNA synthesis terminates following Cre-mediated removal of the transcribing 35Spro, methylation in CG and CNG nucleotide groups is maintained. In contrast, nonsymmetrical CNN methylation is substantially reduced, demonstrating its dependence on transcription of NOSpro dsRNA [Fig. 1 silencer − dsRNA (+IR)]. After withdrawal of the triggering dsRNA, the nontranscribed

IR maintains CG and CNG methylation better than singlet copies of NOSpro at target locus, suggesting that some intrinsic feature of the IR helps to maintain methylation independently of dsRNA. One possibility is that pairing of the IR in *cis* generates an unusual structure (51) that is recognized by the maintenance MTase activities. Consistent with a critical role for maintenance MTases in retaining methylation in the NOSpro IR, reductions in CG and CNG methylation in this region were greater in *met1* than in *ddm1* mutant plants (Fig. 1 silencer, *met1* and *ddm1*). A similar stronger effect of *met1* compared with *ddm1* on methylation of an IR has been noted previously on studies with the *PAI* gene family in *Arabidopsis* (32).

Overall, the results from the mutant analysis indicate that efficient maintenance of methylation triggered by RdDM requires MET1 and the activity of DDM1, perhaps as part of a chromatin remodeling complex. Despite the continued presence of NOSpro dsRNA, significant losses of target NOSpro methylation were observed after several generations in *met1* and *ddm1* mutants. This suggests that in the absence of a maintenance MTase and chromatin restructuring activities, which can help to reinforce silencing, methylation induced by RdDM is lost more rapidly than it can be regenerated *de novo*.

It is not yet known which DNA MTase catalyzes the *de novo* methylation step of RdDM, though MET1 is considered unlikely because of the somewhat delayed influence of the *met1* mutation on our NOSpro silencing system. A CMT was initially a promising candidate for RdDM (1, 52) because of the presence in these enzymes of a chromodomain, which can serve as an RNA-protein interaction module (53). Initial results with the *cmt3* mutation, however, suggest negligible effects on NOSpro silencing in F_2 generation (W.A., X. Cao, S. and Jacobsen, M.M., unpublished results). NOSpro methylation must still be examined in the *cmt3* mutants. Another candidate for RdDM is a member of the domain rearranged (DRM) class, which is the major *de novo* DNA MTase family in plants (54). Mutants defective in DRM2 (X. Cao and S. Jacobsen, personal communication) are currently being tested with the NOSpro system. A final possibility is a member of the Dnmt2 family, which is also present in vertebrates, *Drosophila* and—in a mutated form—in *Schizosaccharomyces pombe* (55). Mutations in this class of putative DNA MTases remain to be assessed in our NOSpro system.

There are so far no reports that RNA directs DNA methylation in animals. This apparent deficiency may reflect differences between plants and animals with respect to specific requirements for RdDM. Factors to consider include whether the unique pattern of methylation triggered by RdDM can be detected at a particular developmental stage in animals, and whether the required DNA MTase is available at that time. In both mammals and *Drosophila*, non-CG methylation, which is conceivably directed by RNA (56), is present in early embryos (57, 58). This methylation might be catalyzed by Dnmt3a, which is the major *de novo* DNA MTase active early in mammalian development (57), or by Dnmt2, which is also primarily active during the initial stages of development in mammals and in *Drosophila* (58). Both of these enzymes have been implicated in the catalysis of non-CG methylation (57, 58), which would be consistent with RdDM. Thus, if RdDM occurs in animals, it might be limited to early stages of development when the appropriate DNA MTase(s) is active. In contrast, the occurrence of RdDM throughout plant development (56) suggests the continuous activity of the necessary DNA MTase, a feature that probably facilitated the detection of RdDM in adult plants.

Even for organisms that do not methylate their DNA, there is growing evidence that chromatin modifications are targeted by components of the RNAi machinery. In *Drosophila*, transgene TGS and PTGS are both dependent on the piwi protein, which is a member of the Argonaute family required for RNAi (59). TGS is associated with complexes of polycomb-group proteins, which are perhaps directed to the transgene promoter by short RNAs containing transcriptional regulatory sequences. In *S. pombe*, homologs of three proteins required for RNAi—dicer, a putative RNA-dependent RNA polymerase, and argonaute—are needed for histone methylation and localization of the heterochromatin protein Swi6 at centromeric repeats (S. Grewal, personal communication). RNAi-based genetic screens to find genes required for RNAi in *Caenorhabditis elegans* identified several ORFs that are predicted to encode chromatin-associated proteins (60).

Genetic screens are required to recover novel mutations affecting NOSpro dsRNA-mediated TGS. We have recently identified one prospective mutant, *rts-1* (RNA-mediated transcriptional silencing), in which silencing is substantially alleviated, whereas target NOSpro methylation is only reduced about 50% (W.A., M.F.M., and A.J.M.M., unpublished results). The *rts-1* mutation does not map to a region of the *Arabidopsis* genome known to encode a DNA MTase, suggesting that it might encode a chromatin factor. Identification of the *RTS-1* gene and further genetic analyses using the NOSpro dsRNA-mediated TGS system should continue to provide insights into the relationship between RdDM and chromatin modifications.

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