The EMBO Journal Vol.18 No.1 pp.241–248, 1999

Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters *in trans*

M.F.Mette, J.van der Winden, M.A.Matzke and A.J.M.Matzke[1]

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria

[1]Corresponding author
e-mail: amatzke@imb.oeaw.ac.at

Previous work has suggested that *de novo* methylation of plant nuclear genes can be triggered by an RNA–DNA interaction. To test whether transcription of a promoter would induce *de novo* methylation and silencing of unlinked genes driven by the same promoter, a chimeric 'gene' consisting of a nopaline synthase promoter (NOSpro) positioned downstream of the cauliflower mosaic virus 35S promoter (35Spro) and flanked at the 3′ end by a NOS terminator (NOSter) was constructed and introduced into the genome of a plant that normally expresses an unmethylated NOSpro-neomycinphosphotransferase (*nptII*) gene. Transformants were tested for kanamycin resistance and NOSpro RNA synthesis. Most produced a full-length polyadenylated NOSpro RNA, which did not induce silencing or methylation at the NOSpro-*nptII* target gene. One, however, contained truncated non-polyadenylated NOSpro RNA; in this plant, the NOSpro-*nptII* gene became silenced and methylated in the NOSpro region. Molecular analysis of the NOSpro silencing locus revealed two incomplete copies of the 35Spro-NOSpro gene arranged as an inverted repeat with NOSpro sequences at the center. Reducing NOSpro transcription by crossing a 35Spro-silencing locus partially reactivated *nptII* gene expression and decreased NOSpro methylation at the target locus, thus implicating aberrant NOSpro RNA in this *trans*-silencing phenomenon.

Keywords: aberrant RNA/DNA cytosine methylation/ homology-dependent gene silencing/inverted repeats/ *trans*-silencing

Introduction

Homology-dependent gene silencing in transgenic plants can occur when two homologous transgenes, or a transgene with homology to an endogenous gene, are present in the same genome (Meyer and Saedler, 1996; Depicker and Van Montagu, 1997; Stam *et al*., 1997a; Matzke and Matzke, 1998). Homology-dependent gene silencing is thought to involve two distinct mechanisms that operate at either the transcriptional or post-transcriptional level, respectively. Transcriptional gene silencing occurs when interacting genes share sequence homology in promoter regions, whereas post-transcriptional gene silencing requires homology in transcribed sequences. Both types of homology-dependent gene silencing have been associated with changes in DNA cytosine 5-methylation, which is generally concentrated in promoters in the case of transcriptional gene silencing and at the 3′ end of genes in post-transcriptional gene silencing.

One variant of transcriptional gene silencing is *trans*-silencing, in which a methylated silencing locus induces methylation and inactivation of an unlinked target locus with which it shares DNA sequence identity in promoter regions (Matzke *et al*., 1989; Vaucheret, 1993; Park *et al*., 1996). Silencing loci autonomously acquire stable methylation in promoter sequences. For two *trans*-silencing loci examined so far, spontaneous methylation appears to result from a reiterative and complex structure (Vaucheret, 1993; Matzke *et al*., 1994). *Trans*-silencing and methylation of a target locus in the presence of a stably methylated silencing locus presumably rely on a promoter sequence-specific signal that originates at the silencing locus and directs *de novo* methylation of homologous promoters at the target locus. A sequence-specific *trans*-acting methylation signal could act through DNA–DNA pairing or an RNA–DNA interaction. A precedent for a DNA–DNA association as a means to impose methylation on one sequence from another is provided by a case of meiotic methylation transfer between alleles in the filamentous fungus, *Ascobolus immersus* (Colot *et al*., 1996). There is no direct evidence that DNA–DNA pairing is involved in *trans*-silencing in plants, although it has been invoked to explain many of the observed effects (reviewed in Baulcombe and English, 1996; Meyer and Saedler, 1996; Hollick *et al*., 1997; Bender, 1998; Matzke and Matzke, 1998).

The possibility that an RNA–DNA interaction could induce *de novo* methylation of plant nuclear genes was first suggested by a study in which nuclear cDNA copies of a viroid, a plant pathogen consisting solely of an untranslated, highly base-paired rod-like RNA molecule several hundred nucleotides in length, became methylated *de novo* only during viroid genome replication (Wassenegger *et al*., 1994). This suggested that the replicating viroid RNA, which is restricted to the nucleus, was involved directly in triggering methylation of the viroid cDNA copies integrated into the plant genome. For an RNA signal to be involved in promoter homology-dependent silencing and methylation, promoter sequences at a silencing locus would have to be transcribed. The possibility that this might occur arose from the finding that a target locus that had become methylated following an interaction with a silencing locus failed to acquire silencing activity (Park *et al*., 1996). This indicated that methylation alone is not sufficient to imbue a promoter sequence with *trans*-silencing capability and suggested instead the involvement of a non-transferable property of

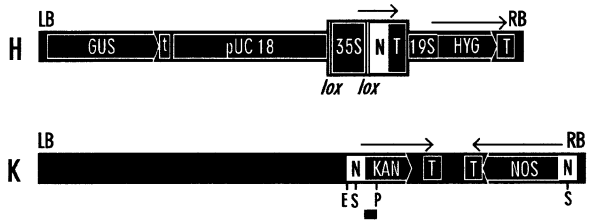

Fig. 1. Transgene constructs. The H construct was designed for transcribing nopaline synthase (*nos*) promoter sequences (black N on white box) by the 35S promoter of CaMV (35S), which was flanked by *lox* sites to allow excision by Cre recombinase, and a 3′ transcription terminator from the *nos* gene (T) (enlarged region). The H construct also contained a hygromycin resistance marker (*hpt* gene) under the control of the 19S promoter of CaMV, pUC18, to facilitate rescue cloning of inserts, and a β-glucuronidase (GUS) gene. The K construct contained two genes under the control of the *nos* promoter, neomycinphosphotransferase encoding resistance to kanamycin (KAN) and an intact *nos* gene (NOS). The *K* locus consists of one copy of the K construct together with several kilobases of non-T-DNA vector sequences that are contiguous with the right T-DNA border (Jakowitsch *et al*., 1999). Arrows show the direction of transcription. E, S and P indicate sites for *Eco*RI, *Sac*II (methylation at this site is consistently correlated with inactivation of the NOSpro) and *Pst*I; these enzymes, together with a Kan probe (black bar), were used for the methylation analysis of the NOSpro-*nptII* gene. t, transcription terminator of the 35S transcript; RB and LB, right and left T-DNA borders, respectively.

the original silencing locus, such as an unusual structural feature or the ability to synthesize transcripts from promoter sequences (Park *et al*., 1996).

To test the hypothesis that transcriptional silencing and methylation of target gene promoters could result from a *trans*-acting promoter RNA, we constructed a chimeric gene consisting of a nopaline synthase promoter (NOSpro) under the control of a cauliflower mosaic virus (CaMV) 35S promoter (35Spro) and used it to transform plants expressing an unmethylated NOSpro-*nptII* target gene. Transformed plants were analyzed for NOSpro RNA synthesis, and for activity and methylation of the NOSpro-*nptII* gene. Although production of a full-length, polyadenylated NOSpro RNA did not lead to inactivation or methylation of the target locus, the synthesis of non-polyadenylated NOSpro transcripts from a 35Spro-NOSpro inverted repeat (IR) was associated with silencing and methylation of the NOSpro-*nptII* target gene. This suggests that aberrant promoter RNAs can mediate the methylation of unlinked homologous promoters *in trans*.

Results

To determine whether an RNA transcribed from promoter sequences could induce *de novo* methylation and *trans*-silencing of unlinked genes under the control of the same promoter, an H construct containing a 35Spro-NOSpro-NOSter chimeric gene and a hygromycin resistance (H) marker was assembled (Figure 1H). The expression of the 35Spro-NOSpro gene in plants could be altered in two ways. First, the 35Spro was flanked by *lox* sites to allow its excision by Cre recombinase (Odell *et al*., 1990). Secondly, the 35Spro could be inactivated by crossing in the *271 trans*-silencing locus, which is a general silencer for 35Spro-driven genes (Vaucheret, 1993). In both cases, NOSpro transcripts should no longer be synthesized, which permitted an assessment of their involvement in silencing of the NOSpro-*nptII* target gene.

The H construct was introduced into a plant that was homozygous for a *K* locus (kanamycin resistance) that contained two NOSpro-driven target genes, neomycin-phosphotransferase (*nptII*) and nopaline synthase (*nos*) (Figure 1K), that are normally expressed and unmethylated. Double transformants (DTs), which contained both a *K* and an *H* locus, were selected on hygromycin-containing medium. Because nopaline can be detected easily in small amounts of transformed callus tissue and can be inactivated coordinately with the NOSpro-*nptII* gene (Matzke *et al*., 1989), the absence of this compound was used in the initial screen for silencing of NOSpro-driven genes following the introduction of the H construct. Subsequent genetic and methylation analyses were carried out with the NOSpro-*nptII* gene. Of nine DTs, eight were NOP-positive, indicating no silencing, and one was NOP-negative, indicating inactivation of the *nos* gene at the *K* locus in this plant. Changes were also observed in the NOSpro-*nptII* gene: a *Sac*II site that was unmethylated in the parental K plant became methylated in the NOP-negative DT but remained unmethylated in the NOP-positive DTs (Figure 2A). In the NOP-negative plant, therefore, both NOSpro-driven genes at the *K* locus were affected after introduction of the H construct. Further analysis of NOSpro-*nptII* expression was performed on selfed progeny of the NOP-negative and NOP-positive plants.

Consistent with methylation of the NOSpro-*nptII* gene in the NOP-negative DT, this gene was silenced in selfed progeny of this plant if they inherited the corresponding *H* locus (referred to hereafter as H_{9NP}). This was indicated by the low percentage of kanamycin resistance (Figure 3B) and the lack of double resistance to kanamycin and hygromycin (Figure 3C). Only kanamycin-resistant seedlings were NOP positive (data not shown). Kanamycin-resistant seedlings indeed lacked the H_{9NP} locus (Figure 2B, Hyg probe, lanes 1–4) and contained an unmethylated NOSpro-*nptII* gene (Figure 2B, Kan probe, lanes 1–4) compared with the parent NOP-negative plant, in which the NOSpro-*nptII* gene was methylated (Figure 2A, NOP^-). Thus, the methylation acquired in the NOSpro of the *nptII* gene at the *K* locus when combined with the H_{9NP} locus in the DT (Figure 2A, NOP^-) was reversed when the two loci segregated in progeny. In contrast, the NOSpro of the *nptII* gene remained methylated in hygromycin-resistant, kanamycin-sensitive seedlings (Figure 2C, Kan probe, lanes 1–4) that had inherited the H_{9NP} locus (Figure 2C, Hyg probe, lanes 1–4). The H_{9NP} locus was thus a strong silencer of unlinked genes driven by the NOSpro, which became methylated in the presence of H_{9NP}.

In accordance with the lack of NOSpro-*nptII* methylation in the NOP-positive DT (Figure 2A, NOP^+ lanes), no silencing of *K* locus genes was observed in offspring of these plants. All selfed progeny were kanamycin resistant (Figure 3E) and ~75% were resistant to both kanamycin and hygromycin (Figure 3F), demonstrating that the *K* locus was active in the presence of these *H* loci. Identical results were obtained with respect to nopaline production (data not shown).

NOSpro RNA was synthesized in both the silenced K/

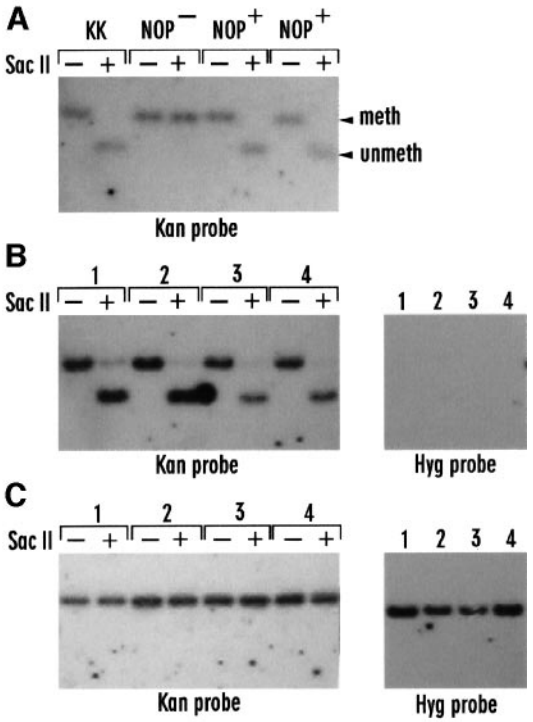

Fig. 2. Methylation analysis of the NOSpro driving *nptII* expression. Double digests with *Eco*RI and *Pst*I ('–' lanes) and triple digests with *Eco*RI, *Pst*I and *Sac*II, which is methylation sensitive ('+' lanes), were performed on DNA samples isolated from the indicated plants. Arrowheads indicate the position of fragments generated in the '+' lanes when the *Sac*II site is either methylated or unmethylated. No change in fragment size upon adding *Sac*II indicates methylation. Blots to the right of (B) and (C) show hybridization to a Hyg probe, confirming the presence or absence of the H_{9NP} silencing locus, of the same plants shown to the left. (**A**) KK: original homozygous parental plant with active, unmethylated NOSpro-*nptII* gene. NOP^- and NOP^+: double transformants (DTs) after introduction of the H construct; the NOSpro is methylated in the NOP^- DT and unmethylated in the two NOP^+ DTs. (**B**) Analysis of four kanamycin-resistant progeny obtained by selfing the NOP-negative DT. These lack the H_{9NP} silencing locus (Hyg probe) and NOSpro copies are mostly unmethylated (Kan probe). The slight residual methylation represents the weak paramutation observed with NOSpro-driven genes (Matzke and Matzke, 1998). (**C**) Analysis of four hygromycin-resistant progeny obtained by selfing the NOP-negative DT. These contain the H_{9NP} silencing locus (Hyg probe), and NOSpro copies are methylated (Kan probe).

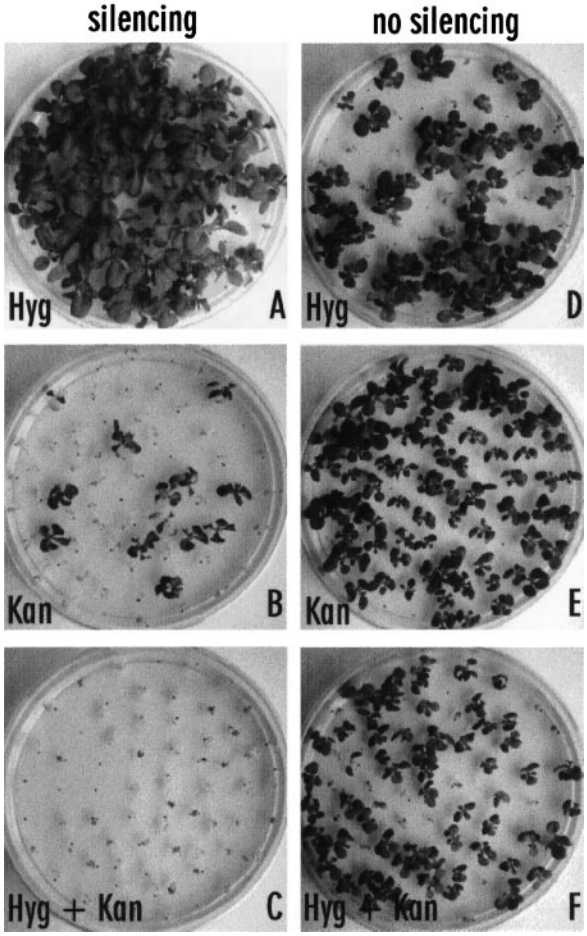

Fig. 3. Segregation of antibiotic resistances (R) in selfed progeny of NOP^- (silenced) and NOP^+ (non-silenced) plants. Seeds obtained from selfing each plant were plated on medium containing hygromycin (Hyg), kanamycin (Kan) or a combination of both (Hyg + Kan). Both plants were hemizygous for a single *H* locus as indicated by 75% Hyg^R (**A** and **D**). In the absence of silencing, selfing should produce 100% Kan^R, 75% Hyg^R and 75% Kan^RHyg^R. If an *H* locus silences the *K* locus, selfing will produce 25% Kan^R, 75% Hyg^R and 0% Kan^RHyg^R (Matzke *et al*., 1989). Silencing of the NOSpro-*nptII* gene at the *K* locus in the presence of H_{9NP} locus was apparent by the low percentage of Kan^R (**B**) and the lack of double resistance (**C**). In contrast, the presence of a non-silencing *H* locus resulted in 100% Kan^R (**E**) and 75% double resistance (**F**). The amount of *hpt* RNA transcribed from the silencing *H* locus was approximately the same in the presence and absence of the *K* locus (data not shown), demonstrating that the silencing of *K* was not due to co-suppression via the transcription terminator homology in the NOSpro-*nptII* and 19Spro-*hpt* genes.

H_{9NP} plant and non-silenced NOP^+ plants. In the former case, however, much of this RNA was smaller than expected (with some RNA preparations, primarily a smear was visible on the Northern blot) (Figure 4A, K/H_{9NP}) and was not polyadenylated (Figure 4B, K/H_{9NP}). In contrast, full-length (~0.65 kb), polyadenylated NOSpro RNA was observed in two NOP^+ non-silenced plants (Figure 4A and B, NOP^+). Silencing and methylation of the NOSpro-*nptII* target gene thus was associated with aberrant [truncated, poly(A)$^-$] NOSpro transcripts originating at the H_{9NP} silencing locus, whereas an mRNA-like NOSpro RNA that was the expected size and polyadenylated did not provoke silencing or methylation of the NOSpro-driven target genes.

To determine whether elimination of NOSpro transcription would alleviate silencing, the K/H_{9NP} plant was crossed with two other plants, one expressing the Cre recombinase, to remove the 35Spro driving transcription of NOSpro sequences, and the second containing the 35Spro silencing locus, *271*, to abolish transcription of the NOSpro. Although it was anticipated that both of these crosses would produce similar results with respect to NOSpro-*nptII* silencing and methylation, this expectation was not completely fulfilled. Significant silencing of the NOSpro-*nptII* gene was still observed in progeny of the $K/H_{9NP}\times$Cre cross, as indicated by the mostly kanamycin-sensitive phenotype (Figure 5A), which was similar to the K/H_{9NP} backcross to normal tobacco (Figure 5B). In contrast, offspring of the $K/H_{9NP}\times271$ cross exhibited moderate kanamycin resistance (Figure 5C) comparable with unsilenced K controls (Figure 5D) and were NOP positive (data not shown), indicating

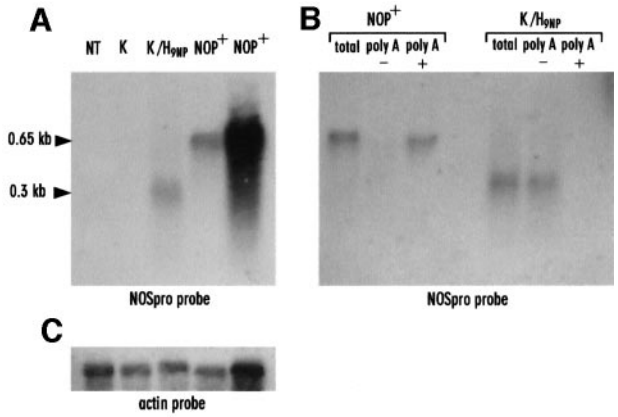

Fig. 4. Northern blot analysis of NOSpro transcripts synthesized from the NOSpro silencing locus H_{9NP} and non-silencing *H* loci present in NOP^+ plants. (A and C) Total RNA; (B) RNA following oligo(dT) chromatography to separate poly(A)$^+$ and poly(A)$^-$ fractions. Varying amounts of NOSpro RNA of the expected size (~0.65 kb) were present in two non-silenced NOP^+ plants (**A**, NOP^+). This NOSpro RNA was polyadenylated (**B**, NOP^+). Smaller NOSpro RNAs, centered around 0.3 kb in this preparation, were present in the silenced plant (A, K/H_{9NP}); this RNA was not polyadenylated (B, K/H_{9NP}). The integrity of the RNA was verified by reprobing the blot in (A) with an actin probe (**C**). NT, normal tobacco; K, original parental plant that does not contain an *H* locus. The expected size of an intact NOSpro RNA was calculated by adding the approximate sizes of the 35S 5′-untranslated region (UTR), *lox* sites and polylinker (90 bp), the NOSpro (300 bp), the 3′-UTR from the transcription termination region (160 bp) and a 100 nucleotide poly(A) tail.

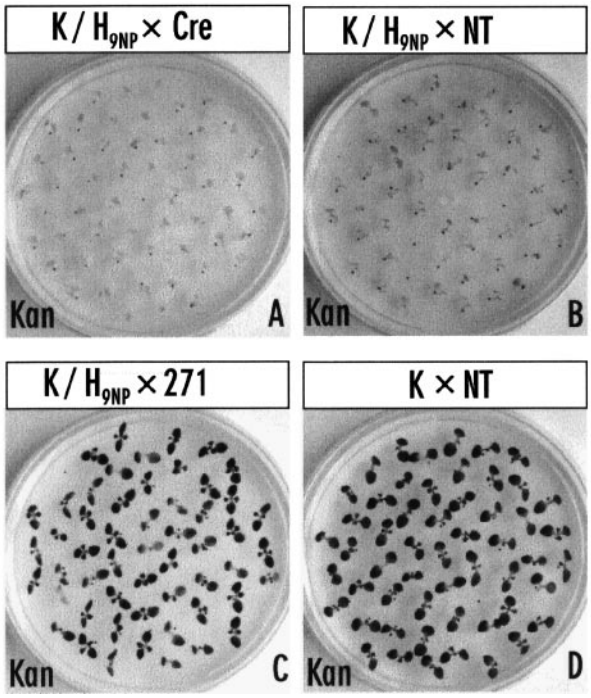

Fig. 5. Effects of removing or repressing the 35S pro on NOSpro-*nptII* silencing in the presence of the H_{9NP} locus. The K/H_{9NP} plant was crossed with a plant expressing Cre recombinase, which should remove copies of the 35Spro flanked by *lox* sites, or containing the *271* locus, which should transcriptionally inactivate the 35Spro. The cross of a plant homozygous for the *Cre* locus to a homozygous K/H9NP plant produced seedlings that remained kanamycin sensitive (**A**), similarly to the backcross to normal tobacco (NT) of K/H_{9NP} (**B**). In contrast, the 271 cross produced seedlings that grew on kanamycin-containing medium (**C**), similarly to seedlings harboring the unsilenced *K* locus (**D**).

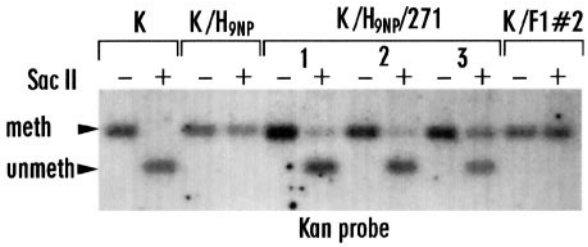

Fig. 6. Decreased methylation of the target NOSpro-*nptII* gene at the *K* locus following introduction of the *271* locus. The strategy for studying methylation at a *Sac*II site in the NOSpro of the *nptII* gene is described in Figures 1 and 2. '–' and '+' lanes show *Eco*RI–*Pst*I digests with and without *Sac*II, respectively. The *Sac*II site is fully methylated in the presence of the H_{9NP} locus (K/H_{9NP}) and the Cre-derived variant F1#2 (K/F1#2), but the smaller fragment indicating incomplete methylation is observed following introduction of the *271* locus (K/H_{9NP}/271, lanes 1–3 indicate individual adult plants). A parental K plant served as an unmethylated control.

appreciable reversal of NOSpro-*nptII*- and *nos*-gene silencing by the H_{9NP} locus following the introduction of the *271* locus.

Consistent with the reactivation of the *nptII* gene after crossing in the *271* locus, the NOSpro-driven expression of this gene was less methylated in K/H_{9NP}/271 plants than in K/H_{9NP} plants (Figure 6). The partial alleviation of NOSpro-*nptII* silencing and reduction of NOSpro methylation was not due to a change in the structure of the H_{9NP} locus in the presence of the *271* locus (see below). Rather, a requirement for NOSpro RNA was suggested by the observation that the amount of NOSpro RNA was reduced in K/H_{9NP}/271 plants (Figure 7).

Although the disparate results from the Cre and 271 crosses with K/H_{9NP} initially appeared contradictory regarding the role of NOSpro transcription in *trans*-silencing, the conflict was resolved when the structure and nucleotide sequence of the H_{9NP} locus were determined. Three overlapping λ clones encompassing this locus and several kilobases of flanking DNA were recovered and sequenced. The H_{9NP} locus contained two copies of the 35Spro-NOSpro gene that lacked NOSter sequences and were arranged as an IR with NOSpro sequences at the center (Figure 8). Only one of the 35Spro copies was complete and flanked by *lox* sites; the second copy was truncated and associated with only one *lox* site. In the progeny of a $K/H_{9NP}\times$Cre cross, therefore, only the intact copy of the 35Spro was removed. Precise excision of this 35Spro copy was demonstrated for one plant, F1#2, by cloning and sequencing a PCR product generated using primers outside the 35Spro region (data not shown). The remaining incomplete copy of the 35Spro in F1#2 is still sufficient to transcribe NOSpro sequences (Figure 7, F1#2) and induce methylation of the NOSpro of the target *nptII* gene (Figure 6, K/F1#2). The silencing that remained following the cross to the Cre-expressing plant can thus be explained by the failure to excise all 35Spro sequences and continued production of aberrant NOSpro RNAs. Both copies of the 35Spro were inhibited by the *271* locus, resulting in decreased or abolished NOSpro RNA synthesis, which was accompanied by reduced silencing and methylation of the NOSpro of the *nptII* target gene. Structural changes in the NOSpro IR did not occur after the introduction of the *271* locus, as

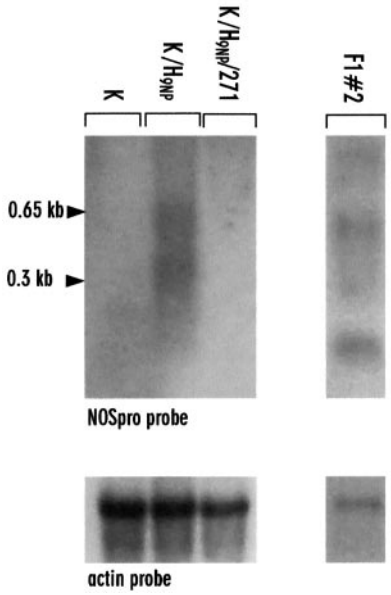

Fig. 7. Northern blot analysis demonstrating the reduction of NOSpro transcripts following introduction of the *271* locus in K/H_{9NP}/271 plants. A NOSpro RNA signal is visible as a smear with stronger bands at 0.3 and 0.65 kb in total RNA isolated from a K/H_{9NP} plant, whereas no NOSpro hybridizing fragments are apparent in RNA isolated from a K/H_{9NP}/271 plant. Within the limit of detection of the Northern blot technique (Materials and methods), this represents at least a 5-fold reduction in the amount of NOSpro RNA. Similar results were obtained with several other plants with these genotypes. A parental K plant that did not contain an H_{9NP} locus was used as negative control. The blot was reprobed with an actin probe (bottom) to confirm the integrity of the RNA. F1#2: NOSpro transcripts originating from the Cre-generated H_{9NP} structural derivative that contained only one 35Spro. Some of these transcripts appear larger than 0.65 kb. Lanes contained 100 μg of total RNA except for F1#2, which contained 20 μg in an independent experiment.

indicated by the consistent appearance of an 849 bp *Eco*RV fragment containing this region (Figure 9). Moreover, the *Sac*II sites in the NOSpro sequences at the H_{9NP} locus were methylated and remained so in the presence of the *271* locus (Figure 9).

Discussion

Signals for *de novo* DNA methylation in plants are not well understood, nor is the mechanism underlying *trans*-silencing, a phenomenon in which a silencing locus directs *de novo* methylation of unlinked homologous target sequences. The homology dependence of the induced methylation of target genes suggests that a sequence-specific methylation signal originates at a silencing locus. Following the demonstration that replication of viroid RNA genomes can trigger methylation of viroid cDNA copies integrated into plant nuclear DNA (Wassenegger *et al*., 1994), an RNA–DNA interaction has been considered a possible signal for *de novo* methylation in plants. Here, we have tested whether a transcriptional *trans*-silencing locus can act by producing transcripts of promoter sequences. Full-size, polyadenylated NOSpro RNA, even when abundant, did not induce *trans*-silencing or methylation of the NOSpro-*nptII* target gene. Silencing and promoter methylation of NOSpro-driven target genes was observed, however, when non-polyadenylated NOSpro RNAs that deviated from the expected size were synthesized from an IR comprising 35Spro-NOSpro sequences. Decreasing NOSpro transcription by repressing the 35Spro partially alleviated silencing and reduced the methylation of the NOSpro-*nptII* target gene, indicating a role for aberrant NOSpro RNA in this *trans*-silencing phenomenon.

Although it is not yet known how aberrant NOSpro transcripts mediate methylation of NOSpro-driven target genes, several possibilities can be considered. The most direct way would involve a diffusible NOSpro RNA that could locate a target NOSpro elsewhere in the nucleus and trigger methylation through an RNA–DNA association. A second possibility is that the NOSpro RNA acts indirectly on the target locus by first inducing local methylation of NOSpro sequences at the silencing locus via an RNA–DNA interaction. The methylated silencing locus could then pair with the NOSpro copies at the target locus and provoke methylation through a DNA–DNA association. This model is not supported, however, by the observation that the *Sac*II sites in the NOSpro IR at the H_{9NP} locus remain methylated when the NOSpro-*nptII* target gene loses methylation and reactivates in the presence of the *271* locus. Therefore, neither the NOSpro IR nor methylation in this region is sufficient to account for the *trans*-silencing ability of the H_{9NP} locus, suggesting the action of a diffusible NOSpro RNA.

Although transcription of NOSpro sequences in the IR appears to be required for the *trans*-silencing effect described here, the exact nature of RNA involved in silencing has not yet been clarified. NOSpro transcripts produced from the H_{9NP} locus are not abundant and are present in varying amounts in different RNA preparations, suggesting that they are unstable. Moreover, a range of sizes of NOSpro RNA can be found in the H_{9NP} and F1#2 plants and it is not straightforward to assign these RNAs to particular regions of the IR. Therefore, it is not clear whether the entire NOSpro IR must be transcribed. The absence of a poly(A) tail in NOSpro transcripts originating from the H_{9NP} locus can be explained by the lack of NOSter sequences in the IR. The absence of polyadenylation and deviations from the expected size contribute to the aberrant character of these NOSpro RNAs. Further work with additional transgene constructs is necessary to ascertain which NOSpro RNAs trigger *trans*-silencing and methylation, and to test whether other promoters can be inactivated similarly. From an applied perspective, producing aberrant promoter transcripts from an IR could provide a novel means to silence individual members of endogenous multigene families that have similar coding regions but distinct promoters.

Trans-silencing ability associated with an IR comprising promoter sequences has a precedent in an endogenous gene system. Several semi-dominant *nivea* alleles in *Antirrhinum* have an inversion of a specific region of the *niv* promoter/leader sequence, which enables them to weaken the expression of the wild-type allele *in trans* (Bollman *et al*., 1991). However, because aberrant or antisense *niv* RNAs were not detected, DNA–DNA pairing was favored as a mechanism to mediate this effect. Depending on the particular alleles or loci involved, *trans*-silencing ability might be based on DNA–DNA associations or RNA–DNA interactions. A detailed structural analysis of another NOSpro silencing locus, H_2, did

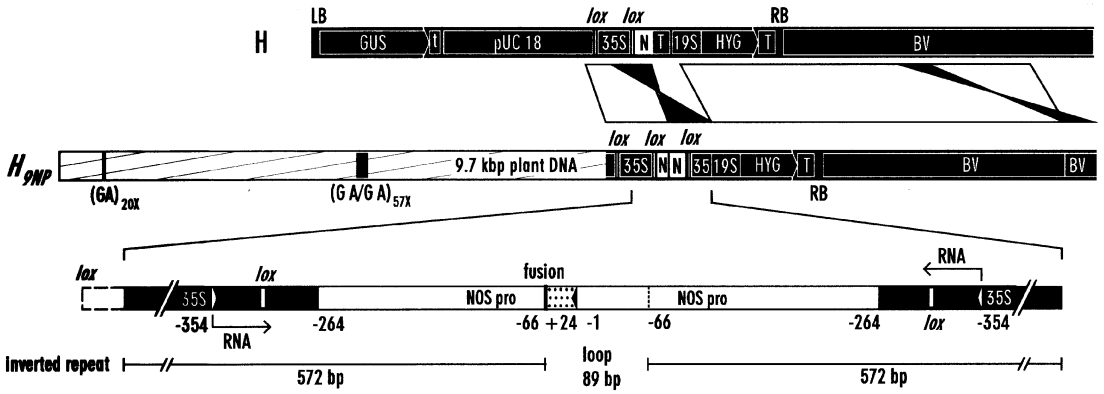

Fig. 8. Structure of the H_{9NP} NOSpro *trans*-silencing locus. The H construct is shown at the top together with non-T-DNA sequences present in the binary vector used for transformation (BV). NOSpro sequences are indicated as a black N on a white box. All other transgene and vector sequences are black; plant DNA is hatched. The structure and nucleotide sequence of the H_{9NP} locus were determined from three independent λ clones that overlapped throughout the region shown. The origins of the transgene sequences from the original construct are indicated by bars between the H and H_{9NP} maps; black regions show sections present in inverse orientation at the H_{9NP} locus relative to their position in the H construct. The IR region containing the 35Spro-NOSpro gene is enlarged at the bottom. The numbers directly below the IR map indicate the distance in base pairs from the transcription start site present in the intact NOSpro (black arrowhead). The transcription start sites of the two 35Spro copies are shown by two white arrowheads; the direction of transcription of NOSpro sequences is given by the arrows above and below the IR map. The 35Spro on the right is incomplete and is flanked by only one *lox* site (white bar). The 35Spro to the left is complete, and is flanked by two *lox* sites. The left-hand section portion of this 35Spro copy that is not duplicated in the IR has a broken outline. The NOSpro to the left is incomplete, stopping at –66 relative to the NOSpro transcription start site. The NOSpro to the right is full length and contains the transcription start site plus 24 bp of 5′-leader sequence (dotted). The fusion of these two NOSpro copies is indicated by the thin black bar. The 35Spro-NOSpro IR could form a cruciform with a stem of 572 bp and a unique loop 89 nucleotides in length. The stem length of a hairpin RNA originating from the 35Spro transcription start sites would be 289 bp. DNA sequences beyond the right T-DNA border (RB) consisted of contiguous BV sequences, which eventually led into BV sequences in inverted orientation. Although no plant DNA to the right was present in the λ clones, 9.7 kb of flanking plant DNA to the left was recovered. The major notable features of this region were a relatively long $[G(A/G)A]_{57X}$ microsatellite and a shorter $(GA)_{20X}$ microsatellite. The complete sequence of the 9.7 kb of plant DNA has been submitted to the DDBJ/EMBL/GenBank databases under accession No. AJ007903. Abbreviations are as in Figure 1.

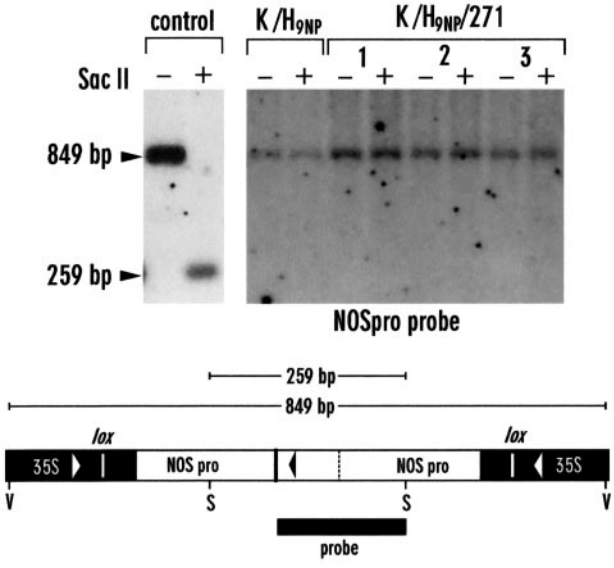

Fig. 9. Stable methylation of NOSpro sequences at the H_{9NP} silencing locus. The structure of the 35Spro-NOSpro IR is shown at the bottom with relevant restriction enzyme sites. The IR can be cut out as an 849 bp *Eco*RV (V) fragment ('–' lanes). If the two *Sac*II (S) sites in the NOSpro sequences are not methylated, a 259 bp fragment should appear in '+' lanes, which are V/S double digests. If the *Sac*II sites are methylated, no shift occurs in the '+' lanes and only the 849 bp fragment is generated. The *Sac*II sites are methylated in H_{9NP} (lane K/H_{9NP}), and these sites remain methylated in the presence of the *271* locus (K/H_{9NP}/271, lanes 1–3; three individual plants). The control consists of an unmethylated plasmid derived from an H_{9NP} genomic λ clone containing the 35Spro-NOSpro IR region shown in the map at the bottom.

not reveal NOSpro IRs similar to the one described at the H_{9NP} locus or any obvious ways to produce NOSpro RNA (Jakowitsch *et al*., 1999). Accordingly, no NOSpro RNA has been detected so far in H_2 plants (M.F.Mette, unpublished data). Although the H_2 locus might act by DNA–DNA pairing, there are not yet conclusive data demonstrating pairing for any *trans*-silencing system in plants.

Transgene sequences arranged as IRs (Hobbs *et al*., 1993; Jorgensen *et al*., 1996; Stam *et al*., 1997b), ill-defined aberrant RNAs (Ingelbrecht *et al*., 1994; Cogoni and Macino, 1997; Jacobs *et al*., 1997; Lee *et al*. 1997; Tanzer *et al*. 1997; Van Houdt *et al*., 1997; Furner *et al*., 1998; Wassenegger and Pélissier, 1998) and non-polyadenylated or double-stranded RNAs (Metzlaff *et al*., 1997) have been implicated in different cases of post-transcriptional gene silencing in plants, although it is not clear how these function to trigger RNA turnover. Transcription through IRs could produce antisense or partially double-stranded RNAs (Montgomery and Fire, 1998). In the case of chalcone synthase co-suppression in petunia, however, promoterless IR transgene loci that strongly elicit co-suppression are apparently not transcribed (Stam *et al*., 1997b). In contrast, our experiments indicated a requirement for transcription of sequences in the 35Spro-NOSpro IR because repressing the 35Spro by crossing in the *271* locus led both to decreased NOSpro transcription and to reduced silencing and methylation of the NOSpro-*nptII* target gene. The incomplete reversal of silencing and NOSpro methylation observed in K/H_{9NP}/271 plants could have been due to incomplete silencing of the 35Spro-NOSpro gene by the *271* locus, resulting in continued production of minute amounts of NOSpro

RNA that were not detectable on Northern blots. Alternatively, NOSpro RNAs could potentiate *trans*-silencing and methylation by acting in conjunction with the 35Spro-NOSpro IR, which could spontaneously form a cruciform structure that independently induces some degree of silencing of the NOSpro-*nptII* target gene by a DNA–DNA interaction.

The possibility that at least some cases of transcriptional and post-transcriptional gene silencing operate through an RNA intermediate has been suggested previously (Park *et al*., 1996; Wassenegger and Pélissier, 1998). Depending on the origins and cellular locations of the aberrant transcripts, different types of silencing would be observed. Aberrant promoter transcripts could act in the nucleus by provoking transcriptional inactivation and promoter methylation that is meiotically heritable to varying degrees. Aberrant transcripts originating from coding regions might trigger methylation in these sequences and fuel a cycle of cytoplasmic RNA turnover that is reset each sexual generation.

It has been argued that gene silencing associated with methylation or cytoplasmic RNA turnover reflects, respectively, distinct plant defense responses to transposable elements (Matzke and Matzke, 1998) or viruses (Covey *et al*., 1997; Ratcliff *et al*., 1997). In this context, it is interesting to consider possible relationships between pathogen defense systems and RNA-mediated DNA methylation. If the NOSpro IR is transcribed in full, the resulting RNA could form a hairpin structure with a stem of 289 bp and a loop of 89 nucleotides (Figure 8). This would approach the size of some NOSpro RNA molecules originating at the H_{9NP} locus (~0.65 kb), and would resemble the size and hairpin structure of viroid oligomeric replication intermediates (Steger *et al*., 1986). This similarity takes on added significance in view of the fact that the original experiments suggesting that an RNA–DNA interaction induced *de novo* methylation of plant nuclear genes involved a viroid system (Wassenegger *et al*., 1994). Double-stranded RNAs have been implicated in co-suppression (Metzlaff *et al*., 1997; Montgomery and Fire, 1998), and RNAs with the potential to form stem–loop structures are encoded by the imprinted *H19* gene and the *Xist* gene in mammals (Pfeifer and Tilghman, 1994). Some mammalian repeats that become methylated produce RNAs that form complex secondary structures (Turker and Bestor, 1997). Aberrant RNAs or RNAs capable of adopting secondary structures are thus emerging as a common theme in a variety of epigenetic effects.

Materials and methods

Plant transformation and marker gene assays

Leaf disk transformation and seed germination assays were performed as described previously (Matzke *et al*., 1989). Nopaline was detected using high-voltage electrophoresis and phenanthrenequinone staining (Matzke *et al*., 1989). With an active *nos* gene, ~50 μg of nopaline are present in 50 μl of 70% ethanol extracts of plant material (made by grinding fresh tissue in 70% ethanol; 100 mg plant tissue/100 μl 70% ethanol). Amounts less than 1 μg are not detectable. Silencing of the *nos* gene thus represents at least a 50-fold reduction in nopaline production. To study kanamycin-resistant phenotypes of seedlings, the germination and growth medium contained 40 mg/l hygromycin and/or 50 mg/l kanamycin. The 271 plants were grown hydroponically using Luwasa Hydroculture (Schmeikal Hydrokulturen, Salzburg, Austria).

Northern blot analysis

Isolation of total RNA from expanding leaves of adult plants and Northern blot analysis were performed as described previously (Park *et al*., 1996). Separation of total RNA into poly(A)$^+$ and poly(A)$^-$ fractions was performed with an mRNA Purification Kit (Pharmacia, Vienna, Austria). Hybridization probes labeled with ^{32}P were synthesized from isolated DNA fragments containing the complete NOSpro sequence or a 0.3 kb *Hin*dIII fragment from exon 3 of tobacco actin Tac9 (Thangavelu *et al*., 1993) using an Amersham Multiprime DNA labeling system (MedPro, Vienna, Austria). The reduction of NOSpro RNA in the presence of the *271* locus was estimated by Northern blot analysis using serial dilutions of NOSpro RNA synthesized *in vitro* with T7 RNA polymerase. The detection limit of this technique was ~100 fg. Approximately 500 fg of NOSpro RNA could be detected on Northern blots containing 100 μg of total RNA isolated from an H_{9NP} plant. Therefore, at least a 5-fold reduction in the amount of NOSpro RNA occurred in the presence of the *271* locus.

DNA blot analysis

Plant genomic DNA for blot analysis was isolated from leaves of adult plants using a Plant DNA Purification Kit (Boehringer Mannheim, Vienna, Austria). DNA blot analysis using ^{32}P-labeled RNA probes and methylation analysis of the *Sac*II site in the NOSpro-*nptII* gene using double (*Eco*RI–*Pst*I) and triple (*Eco*RI–*Pst*I–*Sac*II) digests was performed as described previously (Matzke *et al*., 1989). The cytosine 5-methylation-sensitive enzyme *Sac*II was purchased from Amersham (MedPro, Vienna, Austria). *In vitro* transcripts from the entire 1.0 kb hygromycinphosphotransferase-coding region (Hyg probe), a 0.19 kb 5′ fragment of the *nptII* coding region (Kan probe) or a 0.18 kb 3′ fragment of the *nos* promoter (NOSpro probe) were synthesized in the presence of [^{32}P]UTP (NEN Life Science Products, Vienna, Austria) from pGEM-derived plasmid clones (Promega Biotech, Vienna, Austria) using T7 RNA polymerase (Boehringer Mannheim).

λ cloning and nucleotide sequence analysis

A genomic DNA λ library was obtained from a plant homozygous for the H_{9NP} locus using the Stratagene λ FixII system and screened for the presence of NOSpro-positive clones following the instructions of the manufacturer. The resulting λ clones were used for subcloning and nucleotide sequence analysis with a ThermoSequenase cycle sequencing kit (Amersham) using infrared-labeled oligonucleotides synthesized by MWG Biotech (Ebersberg, Germany) and run on a LI-COR DNA Sequencer Long Read IR 4200 system (LI-COR, Omaha, NB).

Acknowledgements

We thank Dr Christian Kunz, Dr Johannes Jakowitsch and members of the EU Gene Silencing Network for helpful discussions. We are grateful to Dr Hervé Vaucheret for contributing seeds of the 271 plant, to Dr Joan Odell for supplying the Cre-expressing tobacco plant, and to Dr David Lonsdale for the tobacco actin gene. This work has been supported by grants to A.M. and M.M. from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (Grant Nos P10021-MOB and Z21-MED) and the European Union (Contract Nos CHRX-CT94-0540 and BIO4-CT96-0253).

References

Baulcombe,D.C. and English,J.J. (1996) Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants. *Curr. Opin. Biotechnol*., **7**, 173–180.

Bender,J. (1998) Cytosine methylation of repeated sequences in eukaryotes: the role of DNA pairing. *Trends Biochem. Sci*., **23**, 252–256.

Bollman,J., Carpenter,R. and Coen,E.S. (1991) Allelic interactions at the *nivea* locus of *Antirrhinum*. *Plant Cell*, **3**, 1327–1336.

Cogoni,C. and Macino,G. (1997) Conservation of transgene-induced post-transcriptional gene silencing in plants and fungi. *Trends Plant Sci*., **2**, 438–443.

Colot,V., Maloisel,L. and Rossignol,J.-L. (1996) Interchromosomal transfer of epigenetic states in *Ascobolus*: transfer of DNA methylation is mechanistically related to homologous recombination. *Cell*, **86**, 855–864.

Covey,S.N., Al-Kaff,N., Lángara,A. and Turner,D.S. (1997) Plants combat infection by gene silencing. *Nature*, **385**, 781–782.

Depicker,A. and Van Montagu,M. (1997) Post-transcriptional gene silencing in plants. *Curr. Opin. Cell Biol.*, **9**, 373–382.
Furner,I.J., Sheikh,M.A. and Collett,C.E. (1998) Gene silencing and homology-dependent gene silencing in *Arabidopsis*: genetic modifiers and DNA methylation. *Genetics*, **149**, 651–662.
Hobbs,S.L.A., Warkentin,T.D. and DeLong,C.M.O. (1993) Transgene copy number can be positively or negatively associated with transgene expression. *Plant Mol. Biol.*, **21**, 17–26.
Hollick,J., Dorweiler,J. and Chandler,V.L. (1997) Paramutation and related allelic interactions. *Trends Genet.*, **13**, 302–307.
Ingelbrecht,I., Van Houdt,H., Van Montagu,M. and Depicker,A. (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. *Proc. Natl Acad. Sci. USA*, **91**, 10502–10506.
Jacobs,J.J.M.R., Litière,K., van Dijk,V., van Eldik,G.J., Van Montagu,M. and Cornelissen,M. (1997) Post-transcriptional β-1,3-glucanase gene silencing involves increased transcript turnover that is translation independent. *Plant J.*, **12**, 885–893.
Jakowitsch,J., Papp,I., Moscone,E.,van der Winden,J., Matzke,M.A. and Matzke,A.J.M. (1999) Molecular and cytogenetic characterization of a transgene locus that induces silencing and methylation of homologous promoters *in trans*. *Plant J.*, in press.
Jones,A.L., Thomas,C.L. and Maule,A.J. (1998) *De novo* methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. *EMBO J.*, **17**, 6385–6393.
Jorgensen,R.A., Cluster,P.D., English,J., Que,Q. and Napoli,C.A. (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. *Plant Mol. Biol.*, **31**, 957–973.
Lee,K.Y., Baden,C., Howie,W.J., Bedbrook,J. and Dunsmuir,P. (1997) Post-transcriptional gene silencing of ACC synthase in tomato results from cytoplasmic RNA degradation. *Plant J.*, **12**, 1127–1137.
Matzke,A.J.M., Neuhuber,F., Park,Y.-D., Ambros,P.F. and Matzke,M.A. (1994) Homology-dependent gene silencing in transgenic plants: epistatic silencing loci contain multiple copies of methylated transgenes. *Mol. Gen. Genet.*, **244**, 219–229.
Matzke,M.A. and Matzke,A.J.M. (1998) Epigenetic silencing of plant transgenes as a consequence of diverse cellular defense responses. *Cell Mol. Life Sci.*, **54**, 94–103.
Matzke,M.A., Primig,M., Trnovsky,J. and Matzke,A.J.M. (1989) Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. *EMBO J.*, **8**, 643–649.
Metzlaff,M., O'Dell,M., Cluster,P.D. and Flavell,R.B. (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in *Petunia*. *Cell*, **88**, 845–854.
Meyer,P. and Saedler,H. (1996) Homology-dependent gene silencing in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 23–48.
Montgomery,M.K. and Fire,A. (1998) Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. *Trends Genet.*, **14**, 255–258.
Odell,J., Caimi,P., Sauer,B. and Russell,S. (1990) Site-directed recombination in the genome of transgenic tobacco. *Mol. Gen. Genet.*, **223**, 369–378.
Park,Y.-D., Papp,I., Moscone,E.A., Iglesias,V.A., Vaucheret,H., Matzke,A.J.M. and Matzke,M.A. (1996) Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. *Plant J.*, **9**, 183–194.
Pfeifer,K. and Tilghman,S.M. (1994) Allele-specific gene expression in mammals: the curious case of the imprinted RNAs. *Genes Dev.*, **8**, 1867–1874.
Ratcliff,F., Harrison,B.D. and Baulcombe,D.C. (1997) A similarity between viral defense and gene silencing in plants. *Science*, **276**, 1558–1560.
Stam,M., Mol,J.N.M. and Kooter,J.M. (1997a) The silence of genes in transgenic plants. *Ann. Bot.*, **79**, 3–12.
Stam,M., de Bruin,R., Kenter,S., van der Hoorn,R.A.L., van Blokland,R., Mol,J.N.M. and Kooter,J.M. (1997b) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. *Plant J.*, **12**, 63–82.
Steger,G., Tabler,M., Brüggemann,W., Colpan,M., Klotz,G., Sänger,H.L. and Riesner,D. (1986) Structure of viroid replicative intermediates: physico-chemical studies on SP6 transcripts of cloned oligomeric potato spindle tuber viroid. *Nucleic Acids Res.*, **14**, 9613–9628.
Tanzer,M.M., Thompson,W.F., Law,M.D., Wernsman,E.A. and Uknes,S. (1997) Characterization of post-transcriptionally suppressed transgene expression that confers resistance to tobacco etch virus infection in tobacco. *Plant Cell*, **9**, 1411–1423.
Thangavelu,M., Belostotsky,D., Bevan,M.W., Flavell,R.B., Rogers,H.J. and Lonsdale,D.M. (1993) Partial characterization of the *Nicotiana tabacum* actin gene family: evidence for pollen-specific expression of one of the gene family members. *Mol. Gen. Genet.*, **240**, 290–295.
Turker,M.S. and Bestor,T.H. (1997) Formation of methylation patterns in the mammalian genome. *Mutat. Res.*, **386**, 119–130.
Van Houdt,H., Ingelbrecht,I., Van Montagu,M. and Depicker,A. (1997) Post-transcriptional silencing of a neomycinphosphotransferase II transgene correlates with the accumulation of unproductive RNAs and with increased cytosine methylation of 3′ flanking regions. *Plant J.*, **12**, 379–392.
Vaucheret,H. (1993) Identification of a general silencer for 19S and 35S promoters in a transgenic tobacco plant: 90 bp of homology in the promoter sequence are sufficient for trans-inactivation. *C.R. Acad. Sci. Paris*, **317**, 1471–1483.
Wassenegger,M. and Pélissier,T. (1998) A model for RNA-mediated gene silencing in higher plants. *Plant Mol. Biol.*, **37**, 349–362.
Wassenegger,M., Heimes,S., Riedel,L. and Sänger,H.L. (1994) RNA-directed *de novo* methylation of genomic sequences in plants. *Cell*, **76**, 567–576.

Received August 11, 1998; revised September 30, 1998; accepted November 3, 1998

Note added in proof

A recent study has suggested a link between cytoplasmic viral RNA and *de novo* methylation of homologous nuclear sequences (Jones *et al.*, 1998).