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Local DNA features affect RNA-directed transcriptional gene silencing and DNA methylation

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Summary

Transcription of a nopaline synthase promoter (pNOS) inverted repeat provides an RNA signal that can trigger transcriptional gene silencing and methylation of pNOS promoters *in trans*. The degree of silencing is influenced by the local DNA features close to the target promoter integration sites. Among 26 transgenic *Arabidopsis thaliana* lines harbouring single copies of a T-DNA including a pNOS-*NPTII* reporter gene at different chromosomal loci, *NPTII* RNA levels showed limited variation. When challenged by the silencer transgene providing the pNOS RNA signal, reduction of the *NPTII* RNA levels in the F_1 generation varied by more than 100-fold, ranging from no reduction to reduction to <1% of the non-silenced level. Silencing was generally correlated with proportional DNA methylation in the pNOS region, except for one target transgene showing substantial DNA methylation without adequate silencing. Silencing was progressive through generations. Differences in the degree of silencing among the target transgenes were transmitted at least to the F_3 generation, and seemed to be influenced by transgene-flanking sequences. Apparently, close-by repeats promoted, whereas close-by functional genes diminished, the response to the silencing signal.

Keywords: transgene expression, transcriptional gene silencing, DNA methylation, heterochromatin, position effect, repetitive sequences.

Introduction

In plants, short interfering (si)RNAs can mediate transcriptional gene silencing (TGS), or post-transcriptional gene silencing (PTGS), depending on their homology to the promoter or to the transcribed sequences. In both cases, silencing is usually correlated with RNA-dependent methylation of DNA sequences homologous to siRNA sequences (reviewed by Chan *et al.*, 2005; Matzke and Birchler, 2005).

The nopaline synthase promoter (pNOS), an *Agrobacterium tumefaciens* Ti plasmid-derived constitutive promoter, is used as a model promoter to analyse RNA-directed TGS. In *Nicotiana tabacum* (tobacco; Mette *et al.*, 1999, 2000) and *Arabidopsis thaliana* (Aufsatz *et al.*, 2002a), transcription of double-stranded (ds)RNA from a silencer transgene containing a pNOS inverted repeat (IR) can trigger TGS of homologous target promoters *in trans*. Similar to dsRNA involved in PTGS, this pNOS dsRNA is processed to siRNAs of predominantly 21–24 nucleotides (Mette *et al.*, 2000; Papp *et al.*, 2003). Bisulfite genomic sequencing of silenced target promoters detected substantial DNA methylation at the region homologous to dsRNA (Aufsatz *et al.*, 2002a,b). TGS and DNA methylation at the target pNOS were dependent on the presence of the pNOS dsRNA. After removal of dsRNA, pNOS-driven reporter gene expression was reactivated, and the pNOS DNA methylation in the non-CG sequence context was lost gradually during development, indicating a diminishing level of silencing in the absence of the inducing RNA signal.

In *A. thaliana* (DNA-) METHYLTRANSFERASE 1 (MET1), a *Dnmt1*-class DNA methyltransferase, and DECREASE IN DNA METHYLATION 1, a putative SWI2/SNF2 chromatin remodelling factor, are most likely to be involved in the inheritance of TGS and maintenance of DNA methylation

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(Aufsatz *et al.*, 2002a), whereas *Dnmt3*-related DOMAINS REARRANGED METHYLASE 1 (DRM1), and DRM2, the plant-specific CHROMOMETHYLASE 3 (Cao *et al.*, 2003) and the SNF2-like putative chromatin remodelling protein DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) (Kanno *et al.*, 2004) are needed to establish TGS and *de novo* DNA methylation. Albeit mutations in *MET1* also reduce *de novo* DNA methylation (Aufsatz *et al.*, 2004), and mutations in *DRD1* can also influence the maintenance of DNA methylation patterns (Kanno *et al.*, 2005). The loss of MORPHEUS' MOLECULE 1, another putative chromatin remodelling factor, did not show an effect on RNA-directed TGS (Aufsatz *et al.*, 2002a), whereas the loss of histone deacetylase HDA6 compromised RNA-directed TGS as well as the maintenance of correlated DNA methylation (Aufsatz *et al.*, 2002b).

The possible contribution of the direct chromosomal environment of the target transgene to the RNA-directed TGS process is not yet clear. The target transgene that was used in the initial studies of pNOS silencing in *A. thaliana* was not suitable to investigate this question, as it contained several copies of the original T-DNA construct and was associated with a chromosome rearrangement involving chromosomes 3 and 5 (Aufsatz *et al.*, 2002a). Nevertheless, pNOS copies of the silencer differed in their levels of DNA methylation from those of the target transgene. The IR silencer pNOS copies always showed a higher level of DNA methylation than the target copies (Aufsatz *et al.*, 2002a).

To analyse the influence of transgene integration sites on RNA-directed TGS, we have performed a systematic study using a large collection of well-characterized transgenic *A. thaliana* lines that harboured single T-DNA copies including a pNOS-*NPTII* reporter gene at defined chromosomal loci (Forsbach *et al.*, 2003; Schubert *et al.*, 2004). The results provide clear evidence that RNA-directed TGS and DNA methylation are affected by local DNA features close to the target promoter. Flanking repeats seem to promote inactivation, whereas flanking genes diminish gene silencing to some extent. Silencing and promoter DNA methylation levels were correlated, except for one target transgene showing substantial DNA methylation but only limited inactivation. Apparently, promoter methylation is required but not sufficient for transcriptional inactivation.

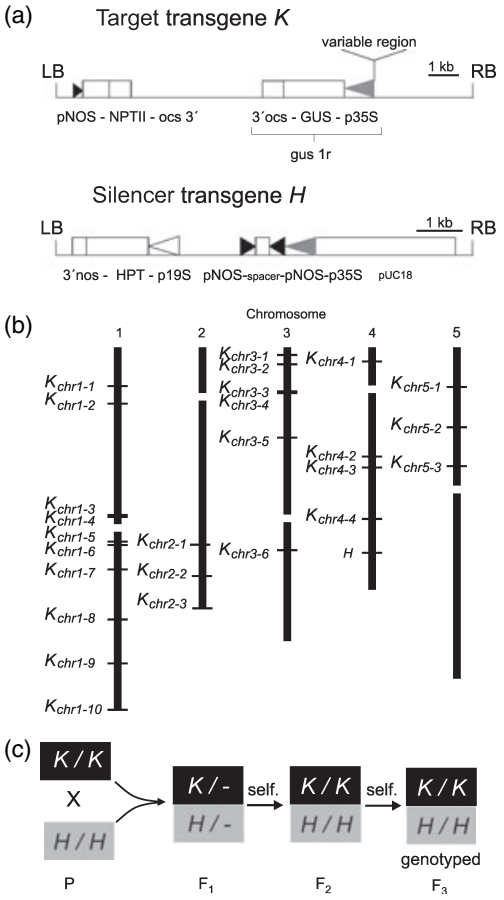

Figure 1. T-DNA constructs and transgene insertion sites.
(a) Target T-DNAs (*K*) contained two reporter genes: a *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPTII*) gene conferring resistance to kanamycin, followed by an octopine synthase polyadenylation signal (ocs 3′) under the control of a nopaline synthase promoter (pNOS, black arrowhead), and a *GUS* gene followed by an ocs 3′ under the control of a cauliflower mosaic virus 35S promoter (p35S, grey arrowhead). The Y-shaped symbol indicates the site of optional sequence blocks (Table S1; Schubert *et al.*, 2004). The silencer transgene *H* contained two copies of the pNOS (black arrowheads) arranged as an inverted repeat separated by a spacer. A flanking p35S (grey arrowhead) induces transcription of the inverted repeat. In addition, a *HYGROMYCIN PHOSPHOTRANSFERASE* (*HPT*) gene conferring resistance to hygromycin, followed by a nopaline synthase polyadenylation signal (nos 3′) under the control of a cauliflower mosaic virus 19S promoter (p19S, open arrowhead), was included (Aufsatz *et al.*, 2002a). LB and RB refer to T-DNA left and right borders; the scale bar represents 1 kb. (b) Positions of target (*K*) and silencer (*H*) transgene insertion sites (horizontal black lines) on the physical map of the five *Arabidopsis thaliana* chromosomes (black bars). Positions of centromeres are indicated by gaps. More detailed information on target integration sites is provided in Table S1. (c) F_1 plants hemizygous for a target transgene and the silencer transgene (*K/–;H/–*) were obtained by crossing parental (P) plants homozygous for a target transgene (*K/K*) with plants homozygous for the silencer transgene (*H/H*). From doubly hemizygous F_1 plants, doubly homozygous F_2 and F_3 plants were obtained by selfing.

Results and discussion

The silencer transgene (Figure 1a, *H*) comprises a transcribed pNOS inverted repeat, providing an RNA signal that can induce RNA-directed TGS and DNA methylation *in trans* at unlinked pNOS copies (Aufsatz *et al.*, 2002a). Schubert *et al.* (2004) described transgenic *A. thaliana* lines harbouring single copy T-DNA insertions and showing stable expression of pNOS-*NPTII* and p35S-*GUS* reporter genes (Figure 1a, *K*). Beside different blocks of sequence elements inserted upstream of the p35S, close to the right border of the cassette, all transgenes contained the same basic T-DNA construct. We selected 26 lines from this collection, with transgene integration sites distributed over all five

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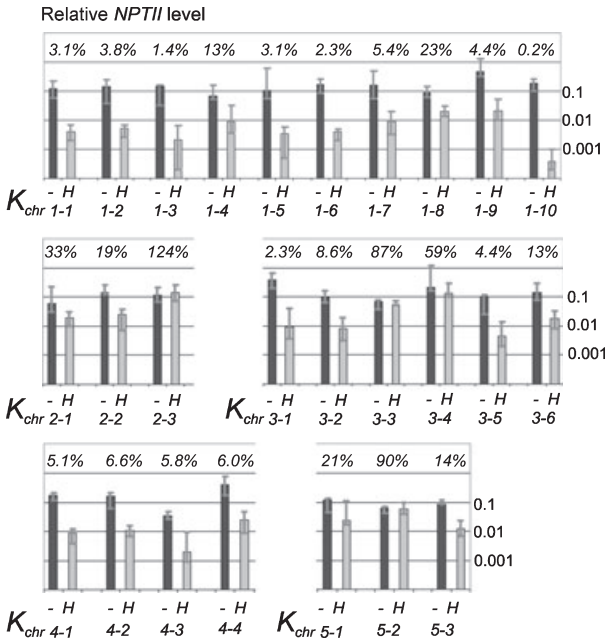

Figure 2. *NPTII* RNA levels for target transgenes at different chromosomal positions in F_1 plants in the non-silenced state, and after introduction of the silencer transgene. *NPTII* RNA levels were calibrated by *ACTIN2*. RNA levels were determined from total leaf RNA by real time RT-PCR. Bar diagrams indicate median *NPTII* RNA levels in F_1 plants in the absence (–; black bars) or presence (*H*; grey bars) of the silencer transgene on a logarithmic scale in arbitrary units. Error bars indicate the highest and lowest values found among measurements from five individual plants. The relative silencing levels were calculated by division of the median *NPTII* RNA level in the non-silenced state by the median value after the introduction of the silencer, and are given as percentages above the panels.

chromosomes of *A. thaliana* (Figure 1b; Table S1), for testing the accessibility of their pNOS-*NPTII* reporter genes to inactivation by the silencer transgene. Target transgenes were combined with the silencer transgene by crosses (Figure 1c).

pNOS-*NPTII* reporter gene activity varies among target transgenes by approximately 10-fold in the non-silenced state, and by more than 100-fold in the silenced state.

NPTII RNA levels were determined for leaf RNA by real time RT-PCR in relation to *ACTIN*2 mRNA. Five individual plants were analysed for each genotype.

The non-silenced activity of the pNOS-*NPTII* reporter genes at the different target loci was determined in hemizygous F_1 plants (Figure 2, black bars). Median *NPTII* RNA levels (third value of five values in ascending order) for different lines varied by a maximum of approximately 14-fold between $K_{chr4\text{-}3}$ (low, 0.035) and $K_{chr1\text{-}9}$ (high, 0.47). For comparison, the variation of *NPTII* RNA levels (calculated as the highest value divided by the lowest value) among individual plants of the same genotype was a maximum of approximately 12-fold ($K_{chr1\text{-}5}$) (Figure 2, error bars for black bars). The mean variation over all 26 target transgenes was 4.3-fold. Thus, in the non-silenced state, the variation of *NPTII* RNA levels among individual plants containing the same target transgene was of the same order of magnitude as the differences of the median *NPTII* RNA levels among different transgenic lines. Therefore locus-specific influences on pNOS-*NPTII* activity in the non-silenced state could not be determined.

To determine the extent of silencing upon induction of TGS, *NPTII* RNA levels were measured in F_1 plants hemizygous for one of the 26 target transgenes and the silencer transgene (Figure 2, grey bars). The greatest difference in median *NPTII* RNA levels was approximately 470-fold between $K_{chr1\text{-}10}H$ (low, 0.0003) and $K_{chr2\text{-}3}H$ (high, 0.14), clearly outranging the maximum approximately 14-fold difference found among non-silenced median *NPTII* RNA levels. Among individual plants of the same genotype, a maximum approximately 33-fold variation of *NPTII* RNA levels was observed (for $K_{chr1\text{-}3}H$), with a mean variation of 7.6-fold calculated over all 26 target transgenes (Figure 2, error bars at grey bars). Thus, after the introduction of the silencer, the variation of *NPTII* RNA levels among individual plants containing the target transgene at the same locus was lower than the differences between plants harbouring target transgenes at different loci. Therefore, comparison of median *NPTII* RNA levels for a target transgene at different loci in the presence vs. absence of the silencer transgene should reflect actual changes in the activity of the pNOS-*NPTII* reporter genes. The higher variation of *NPTII* RNA levels among individual plants of the same genotype, for silenced vs. non-silenced target transgenes, was not caused by increased measurement errors resulting from the lower, on average, *NPTII* RNA levels in the former samples, as no correlation was found between variation and median *NPTII* RNA levels (Figure S1a).

For comparison of silencing efficiencies at different transgene loci, relative *NPTII* levels were calculated by dividing the median *NPTII* mRNA levels in the presence of the silencer by the median *NPTII* mRNA levels in the non-silenced state. Values are given as a percentage (Figure 2, numbers above bars). For the F_1 generation, they range from close to 100% for $K_{chr2\text{-}3}H$ (124%), $K_{chr5\text{-}2}H$ (90%) or $K_{chr3\text{-}3}H$ (87%) to <1% for $K_{chr1\text{-}10}H$ (0.2%). Comparison between *NPTII* RNA levels in the non-silenced state and the relative *NPTII* levels in presence of the silencer did not reveal a clear correlation (Figure S1b).

pNOS-NPTII reporter gene silencing can be progressive

The observed differences in the reaction of target transgenes upon exposure to the silencer transgene could result from differences in the silencing process at several levels (Figure 2). Some target transgenes could generally be resistant to the silencing signal, or their response could increase progressively in later generations. To approach this question, *NPTII* RNA levels of F_3 plants, doubly homozygous

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for target and silencer transgenes, were measured. Target transgenes $K_{chr1\text{-}10}$, $K_{chr2\text{-}3}$ and $K_{chr3\text{-}3}$ were chosen as representative examples (Figure 3a).

Median values of *NPTII* RNA levels in control plants homozygous for target transgenes only were 2.1 for $KK_{chr1\text{-}10}$, 1.3 for $KK_{chr2\text{-}3}$ and 0.72 for $KK_{chr3\text{-}3}$ (Figure 3a, black bars), compared with 0.11 for $K_{chr1\text{-}10}$, 0.11 for $K_{chr2\text{-}3}$ and 0.065 for $K_{chr3\text{-}3}$ in hemizygous F_1 plants. Thus, contrary to what is expected from the double gene dosage of homozygous plants (Matzke *et al.*, 2001), *NPTII* RNA levels were clearly more than twofold higher than those for hemizygous plants. Because the two sets of measurements were separated by approximately one year, this could be the result of inconsistent calibration of real time RT-PCR quantification. Nevertheless, the order of target transgenes with regard to the median *NPTII* RNA levels is comparable between F_1 and F_3 plants. Thus, the relative quantitative differences for the different target transgenes within each of the two datasets should be reliable. The variation of *NPTII* RNA levels among individual plants homozygous for a target transgene was 3.7-fold for $KK_{chr1\text{-}10}$, 3.3-fold for $KK_{chr2\text{-}3}$ and 4.2-fold for $KK_{chr3\text{-}3}$ (Figure 3a, error bars at black bars). This is within the range of the variation among F_1 plants.

For F_3 plants doubly homozygous for target and silencer transgenes, median *NPTII* RNA levels were 0.0098 for $KK_{chr1\text{-}10}HH$, 0.28 for $KK_{chr2\text{-}3}HH$ and 0.065 for $KK_{chr3\text{-}3}HH$ (Figure 3a, grey bars). The variation among individual plants of the same genotype was 140-fold for $KK_{chr1\text{-}10}HH$, 230-fold for $KK_{chr2\text{-}3}HH$ and 10-fold for $KK_{chr3\text{-}3}HH$ (Figure 3a, error bars at grey bars), surpassing the variation for the control plants homozygous for the target transgenes alone, as well as the variation for the F_1 plants hemizygous for target and silencer transgene. Thus, variation among individual plants containing the same target transgene in the silenced state might become more prominent in progressive generations.

The relative *NPTII* levels in the F_3 generation were 0.5% for $KK_{chr1\text{-}10}HH$, 22% for $KK_{chr2\text{-}3}HH$ and 9.1% for $KK_{chr3\text{-}3}HH$ in comparison with 0.2% for $K_{chr1\text{-}10}H$, 124% for $K_{chr2\text{-}3}H$ and 87% for $K_{chr3\text{-}3}H$ in the doubly hemizygous plants of the F_1 generation. For the 'responsive' target transgene $K_{chr1\text{-}10}$ the final inactivation was presumably already reached in the F_1 generation, so that no further inactivation was possible in progressive generations. In contrast, inactivation was much slower for 'recalcitrant' target transgenes and was likely to reach significant levels only in progressed generations. As F_3 plants were doubly homozygous, whereas F_1 plants were doubly hemizygous, for the transgenes involved, it can not be excluded that the gene dosage of target and/or silencer transgenes per haploid genome was also influencing the level of silencing observed, although the relative dosage of silencer transgenes to target transgenes remained constant.

The analysis of *NPTII* mRNA levels was complemented by testing F_3 *A. thaliana* seedlings on medium containing 200 mg l^{-1} kanamycin for resistance conferred by expression of NPTII (Figure 3b). The presence of the silencer compromised kanamycin resistance most strongly for $KK_{chr1\text{-}10}HH$, had a less pronounced effect for $KK_{chr3\text{-}3}HH$ and only a slight effect for $KK_{chr2\text{-}3}HH$, in accordance with the relative *NPTII* levels of 0.5% ($KK_{chr1\text{-}10}HH$), 9.1% ($KK_{chr3\text{-}3}HH$) and 22% ($KK_{chr2\text{-}3}HH$) measured for plants of the same generation. F_3 $KK_{chr1\text{-}10}HH$ seedlings also showed a considerably variable resistance phenotype, which is in agreement with their rather variable *NPTII* RNA levels as mature plants. Thus, in the F_3 generation RT-PCR data and kanamycin resistance tests yielded consistent results, which most likely reflect the actual pNOS-*NPTII* reporter gene activities.

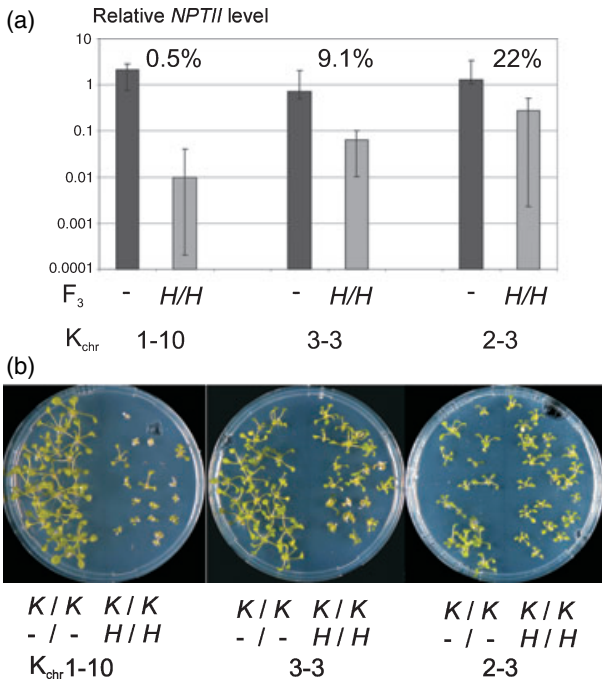

Figure 3. *NPTII* RNA levels and kanamycin resistance for target transgenes $K_{chr1\text{-}10}$, $K_{chr3\text{-}3}$ and $K_{chr2\text{-}3}$ in non-silenced homozygous (*K/K*) plants vs. silenced homozygous (*K/K;H/H)* F_3 plants.
(a) Measurements of *NPTII* RNA levels were performed as described in Figure 2. Bar diagrams indicate the median *NPTII* RNA levels in plants homozygous for a target transgene in the absence (–; black bars) or presence (*H/H*; grey bars) of the silencer transgene on a logarithmic scale in arbitrary units. Five individual plants were tested for each genotype. Error bars indicate the highest and lowest values found among measurements from five individual plants. The corresponding relative *NPTII* levels were calculated by division of the median *NPTII* mRNA level in the non-silenced state by the median value after introduction of the silencer, and are given as percentages.
(b) Growth vigour of the corresponding seedlings on medium containing 200 mg l^{-1} kanamycin as an indicator of *NPTII* expression.

RNA-directed TGS is associated with target promoter methylation

In previous studies RNA-directed TGS was found to be associated with, and to some extent dependent on, DNA methylation of the respective promoter region (Aufsatz *et al.*, 2002a,b, 2004; Kanno *et al.*, 2004; Mette *et al.*, 1999). In

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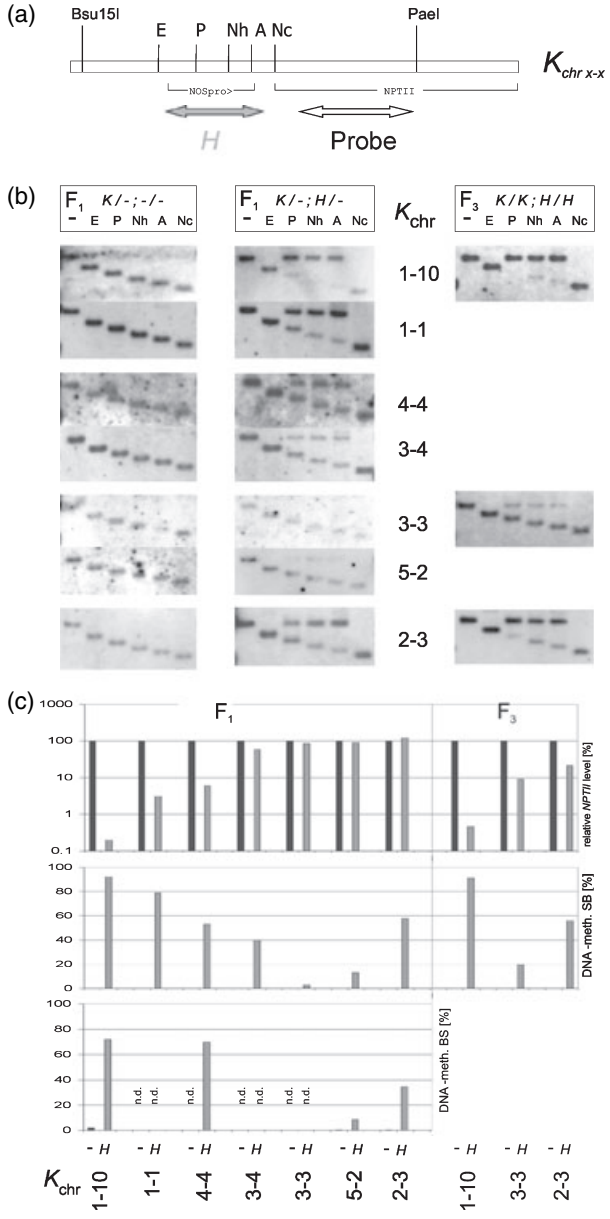
all cases, methylation was almost completely restricted to the region of the target promoter corresponding to the inverted repeat of the silencer transgene, and did not significantly spread into adjacent regions. In order to check this observation for more target transgenes, and to quantify the correlation between TGS and target promoter DNA methylation, pNOS methylation was determined by Southern analysis employing methylation-sensitive restriction enzymes, and by bisulfite genomic sequencing of target transgenes that show different degrees of silencing. Ordered by ascending relative *NPTII* levels in the F_1 generation in presence of the silencer (Figure 2), $K_{chr1\text{-}10}$ (0.2%), $K_{chr1\text{-}1}$ (3.1%), $K_{chr4\text{-}4}$ (6.0%), $K_{chr3\text{-}4}$ (59%), $K_{chr3\text{-}3}$ (87%), $K_{chr5\text{-}2}$ (90%) and $K_{chr2\text{-}3}$ (124%) were selected for methylation analysis.



Results of DNA methylation analysis are summarized in Figure 4 and Figures S2, S3 and S4. Southern hybridization, with a radioactively labelled part of the *NPTII* region upstream of the *Pae*I restriction site as a probe, revealed no DNA methylation at any of the restriction sites in plants hemizygous (Figure. 4b, column *K/–;–/–*) or homozygous (Figure S3) for the target transgene alone. Thus, no evidence of spontaneous DNA methylation at the pNOS of the tested target transgenes was obtained. In F_1 plants doubly hemizygous for target and silencer transgenes (Figure 4b, column *K/–;H/–*), and in F_3 plants doubly homozygous for target and silencer transgenes (Figure 4b, column F_3 *K/K;H/H*; Figure S3), methylation was reproducibly detected at *Psp*1406I, *Nhe*I and *Alw*26I restriction sites. The methylation levels varied according to target transgene line and/or generation in the presence of the silencer, but were consistent among individual plants of the same genotype and generation. Although the applied enzymes were sensitive to methylation in different sequence context (CG for *Psp*1406I, and non-CG for *Nhe*I and *Alw*26I), methylation levels detected by all three enzymes were comparable for each individual plant tested. Comparative analysis of F_1 and F_3 plants indicated progressive DNA methylation for $K_{chr3\text{-}3}$ (F_1 3% vs. F_3 20%), and stable methylation for $K_{chr1\text{-}10}$ (F_1 92% vs. F_3 91%) and $K_{chr2\text{-}3}$ (F_1 58% vs. F_3 56%) (Figure 4b; Figures S2 and S3). No DNA methylation was observed for the *Eco*47I and *Nco*I restriction sites even when the other three sites were densely methylated, i.e. the spreading of DNA methylation out of the region with homology to the pNOS inverted repeat of the silencer had not occurred.

Figure 4. Nopaline synthase promoter (pNOS) DNA methylation analysis at representative target transgene positions in the absence or in the presence of the silencer transgene.

(a) Map of relevant restriction sites on the pNOS-*NPTII* transgene. The region homologous to the pNOS RNA silencing signal is marked by a grey double-headed arrow (*H*), the region in the *NPTII* gene hybridizing to the radioactively labelled probe is marked by a white double-headed arrow. (b) DNA methylation determined by Southern analysis for representative target transgenes in the absence or in the presence of the silencer transgene. In each case, a basic fragment containing the pNOS and part of the *NPTII* gene was released from genomic DNA of leaves by combined *Bsu*15I and *Pae*I cleavage, and either used as a control (–) or incubated with cytosine methylation-sensitive restriction enzymes *Eco*47I (E, GGWCC), *Psp*1406I (P, AACGTT), *Nhe*I (Nh, GCTAGC), *Alw*26I (A, GTCTC, GAGAC) and *Nco*I (Nc, CCATGG) (the methylation-sensitive Cs, according to http://rebase.neb.com/rebase/, are underlined). A shift of a hybridizing band to a smaller size in comparison to the band in the control (–) lane indicates the absence of methylation; a lack of a shift indicates the presence of methylation. From left to right: representative results from control plants lacking the silencer transgene (F_1 *K/–;–/–*) and plants containing the silencer in the first (F_1 *K/–;H/–*) and the third generation (F_3 *K/K;H/H*). Additional results are provided in Figures S2 and S3. (c) Bar diagram for quantitative comparison of relative *NPTII* RNA levels (top panel) and pNOS DNA methylation levels as determined by Southern analysis [middle panel; average value over enzymes P, Nh and A, calculated as a percentage from the intensity of 'cleaved' bands in comparison with the control (–) band], and by bisulfite sequencing (bottom panel; percentage of methylated Cs per total number of Cs in the region analysed), for representative target transgenes in the absence (black bars) and in the presence (grey bars) of the silencer transgene (n.d.: not determined). Detailed results of DNA methylation analysis by bisulfite sequencing are provided in Figure S4.

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To confirm the quantitative interpretation of Southern analysis, and to be sure that the restriction enzymes selected for Southern analysis were representative for the pNOS methylation, bisulfite genomic sequencing of the pNOS top-strand was performed for a responsive ($K_{chr1\text{-}10}$), an intermediate ($K_{chr4\text{-}4}$) and two resistant ($K_{chr5\text{-}2}$, $K_{chr2\text{-}3}$) target transgenes (Figure 4c; Figure S4). DNA preparations from rosette leaves of individual F_1 plants hemizygous for the target transgene alone, or hemizygous for both target and silencer transgene, were analysed. In accordance with the results of Southern analysis, F_1 plants hemizygous for target transgenes $K_{chr1\text{-}10}$, $K_{chr5\text{-}2}$ and $K_{chr2\text{-}3}$ revealed that no more than 1.5% of the cytosines of the pNOS sequence were methylated. The few methylated sites could actually represent false-positive signals resulting from incomplete conversion of non-methylated cytosines by the bisulfite treatment or random base exchanges during PCR steps, which are technical problems not uncommon to the bisulfite genomic sequencing approach. Target DNA methylation detected in F_1 plants with the silencer transgene was almost entirely restricted to the region homologous to the RNA silencing signal. In this region of the pNOS-*NPTII* reporter gene, the cytosine methylation level was 72% for $K_{chr1\text{-}10}$, 70% for $K_{chr4\text{-}4}$, 8.6% for $K_{chr5\text{-}2}$ and 35% for $K_{chr2\text{-}3}$, similar values to those measured by Southern analysis (92% for $K_{chr1\text{-}10}$, 52% for $K_{chr4\text{-}4}$, 12% for $K_{chr5\text{-}2}$ and 58% for $K_{chr2\text{-}3}$). Even for the most densely methylated $K_{chr1\text{-}10}$, only very sparse methylation events were detected outside of the region homologous to the RNA silencing signal, most likely because of false-positive signals. Thus, similar to Southern analysis, bisulfite sequencing detected no spreading of DNA methylation into adjacent sequences.

pNOS promoter inactivation and DNA methylation are correlated, except for target transgene $K_{chr2\text{-}3}$

DNA methylation levels in correlation to relative *NPTII* levels are compiled in Figure 4c. For F_1 plants hemizygous for target and silencer transgenes, increasing gene silencing, as detected by decreasing relative *NPTII* levels, correlated with increasing levels of pNOS DNA methylation for $K_{chr1\text{-}10}$, $K_{chr1\text{-}1}$, $K_{chr4\text{-}4}$, $K_{chr3\text{-}4}$, $K_{chr3\text{-}3}$ and $K_{chr5\text{-}2}$. (Spearman's rank order correlation coefficient −0.739, $P = 0.000$ for CG methylation, and −0.567, $P = 0.014$ for non-CG methylation).

The notable exception was $K_{chr2\text{-}3}$, with no reduction of *NPTII* RNA levels, but with 58% DNA methylation as determined by Southern analysis, and 34% DNA methylation as determined by bisulfite genomic sequencing. A similar pattern appeared in F_3, with $K_{chr2\text{-}3}$ showing some inactivation without further increase in DNA methylation. These observations suggest that promoter methylation is required, but not sufficient, for gene inactivation via RNA-directed TGS.

In line $K_{chr2\text{-}3}$ the T-DNA is inserted into an active gene (At2g47890) encoding a putative CONSTANS-like B-box zinc finger protein. Among five other lines with the target T-DNA inserted into an active gene, $K_{chr1\text{-}8}$ (At1g62260; 23% relative *NPTII* level in F_1), $K_{chr3\text{-}3}$ (At3g11011; 87%), $K_{chr4\text{-}1}$ (At4g02430; 5.1%), $K_{chr4\text{-}4}$ (At4g26580; 6.0%) and $K_{chr5\text{-}1}$ (At5g10030; 21%), three ($K_{chr1\text{-}8}$, $K_{chr3\text{-}3}$ and $K_{chr5\text{-}1}$) showed the same tendency for reduced silencing.

Differences in silencing between target sites are not correlated with varying levels of pNOS-derived RNAs

In order to exclude varying levels of pNOS siRNAs as a reason for the differences in transcriptional inactivation at different target sites, Northern analysis of RNA preparations enriched for small RNAs was performed utilizing a pNOS-specific probe. No differences were detected as to pNOS siRNA abundance between F_1 plants hemizygous for the silencer and target transgenes $K_{chr1\text{-}1}$, $K_{chr1\text{-}10}$ and $K_{chr5\text{-}2}$ as representative examples (Figure S5). Read-through transcripts starting from promoters into sequences flanking target transgenes were suggested by Eike *et al.* (2005) as a possible explanation for differences in expression of transgenes. RT-PCR assays for corresponding pNOS transcripts were negative for $K_{chr1\text{-}10}$, $K_{chr2\text{-}3}$, $K_{chr3\text{-}3}$, $K_{chr3\text{-}4}$, $K_{chr4\text{-}1}$ and $K_{chr5\text{-}2}$ (data not shown).

Location within pericentromeric heterochromatin does not seem to confer particular sensitivity to RNA-directed TGS and DNA methylation

Position–effect variegation in *Drosophila melanogaster* (reviewed by Schotta *et al.*, 2003) provides a paradigm for gene regulation by the surrounding chromatin, i.e. silencing of genes that are placed in the neighbourhood of heterochromatin by chromosomal rearrangements.

Target transgenes $K_{chr1\text{-}3}$ and $K_{chr1\text{-}4}$ had been mapped, by means of their flanking DNA sequences, to the pericentromeric heterochromatin of chromosome 1 (Forsbach *et al.*, 2003). To confirm their localization, and to test for the location of $K_{chr1\text{-}5}$ on the opposite side of the centromere, fluorescence *in situ* hybridization (FISH) with the T-DNA construct and flanking BAC sequences as probes was performed (Figure 5a). FISH signals for $K_{chr1\text{-}3}$, $K_{chr1\text{-}4}$ and $K_{chr1\text{-}5}$ consistently co-localized with one of the intensely 4′,6-diamidino-2-phenylindole (DAPI)-stained chromocenters that represent pericentromeric heterochromatin on meiotic chromosomes (Figure 5b) and in interphase nuclei (Figure 5e,f), whereas the signal for BAC T12C22 (Figure 5b,e), with an insert flanking the pericentromeric region on the bottom arm of chromosome 1, is positioned adjacent to this chromocenter. In contrast, FISH signals for $K_{chr3\text{-}4}$ and $K_{chr4\text{-}4}$, and their flanking BACs, which are not integrated in pericentromeric regions

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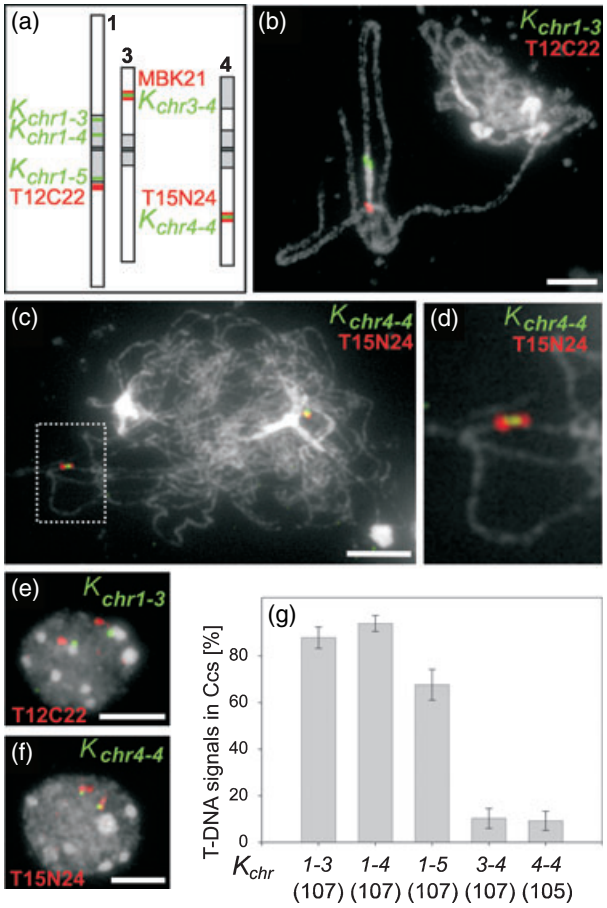

Figure 5. Mapping of target transgenes K_{chr1-3}, K_{chr1-4} and K_{chr1-5} to cytogenetically defined heterochromatin as detected by combined 4′,6-diamidino-2-phenylindole (DAPI) staining and fluorescence *in situ* hybridization (FISH) analysis. Because all analysed plants were homozygous for the target transgenes, (up to) two T-DNA-specific FISH signals per nucleus can be expected.

(a) Physical map of chromosomes 1, 3 and 4, with positions of selected target transgenes (green) and flanking BAC probes (red) in relation to major blocks of repetitive DNA sequences (grey) marked. (b) FISH with a T-DNA-specific probe (green) on pachytene chromosomes of line KK_{chr1-3} showed that the transgene located within the pericentromeric heterochromatin was intensely stained by DAPI. The identity of the intensely DAPI-stained structure was confirmed by the position of the FISH signal with BAC T12C22 (red). (c, d) FISH on leptotene chromosomes of line KK_{chr4-4} revealed the transgene (green) to be localized in a euchromatic region weakly stained by DAPI. The close-up in (d) shows that the T-DNA-specific FISH signal (green) is flanked at both sides by signals of overlapping BAC T15N24 (red). (e, f) Interphase nuclei of lines KK_{chr1-3} and KK_{chr4-4} show T-DNA-specific FISH signals localized either inside (e, green) or outside (f, green) of intensely DAPI-stained chromocenters. Scale bars = 5 μm. (g) Bar diagram indicating fractions of T-DNA-specific FISH signals that co-localized with chromocenters (Ccs). Numbers in parentheses indicate the number of nuclei analysed for each target transgene.

(Figure 1a), did not co-localize with bright DAPI-stained regions (Figure 5c,d,f,g).

Despite their cytogenetically defined heterochromatic localization, K_{chr1-3}, K_{chr1-4} and K_{chr1-5} had been found to actively express their p35S-*GUS* and pNOS-*NPTII* reporter genes in previous studies (Forsbach *et al.*, 2003; Schubert *et al.*, 2004). *NPTII* RNA levels in the silenced state did not stand out from the values for target transgenes at other loci (Figure 6). Similar to active genes in the heterochromatic knob of chromosome 4 of *A. thaliana* (Lippman *et al.*, 2004), target transgenes K_{chr1-3}, K_{chr1-4} and K_{chr1-5} might reside in euchromatic stretches within the heterochromatic pericentromeric region that are too small to be resolved by microscopic methods. Thus, these results do not exclude that location in heterochromatin has the potential to promote silencing of transgenes in plants. As the transgenic lines obtained by Forsbach *et al.* (2003), and in similar studies by De Buck *et al.* (2004) and Nagaya *et al.* (2005), had been selected for kanamycin resistance in the course of transformation, it is conceivable that T-DNA insertions with low *NPTII* expression did not survive. Recently, Francis and Spiker (2005) and Kim *et al.* (2007) used PCR identification of transgenes instead of antibiotic selection to circumvent this limitation. In both studies, an increased frequency of T-DNA integrations into heterochromatic regions was seen in the absence of selection in *A. thaliana*. But as appropriate controls to exclude, for example, the rearrangement of T-DNAs as an alternative reason for low transgene expression were omitted, it is not possible to draw the reverse conclusion that integration into heterochromatin needs to lead to spontaneous T-DNA inactivation.

The local sequence context of the target promoter might determine sensitivity to RNA-directed TGS and DNA methylation

In addition to the global state of the chromatin into which target transgenes are imbedded, influences of the chromosomal context were invoked to explain the puzzling variability of expression of transgenes derived from the same DNA construct at different positions (Day *et al.*, 2000; Jones *et al.*, 1985; Peach and Velten, 1991). This context effect could depend on the presence of particular adjacent genomic sequences. As indicated above (Figure 1a; Table S1), the target transgenes were identical in sequence except for varying repeat sequence elements that were inserted upstream of the 35S-promoter-driven *GUS* gene in the T-DNA construct. Neither the median *NPTII* RNA levels in the non-silenced state nor the relative *NPTII* levels showed correlation with the presence of these particular sequences (Figure S6a,b).

Therefore, we further proceeded to investigate the plant chromosomal sequences within 5 kb of both sides of the target transgenes, with a particular emphasis on sequences directly upstream of the pNOS-*NPTII* reporter gene at the left border side of the T-DNA (Figure S6a). The presence of matrix attachment regions, which are frequently considered as a means to enhance transgene expression in plants by protecting sequences in their proximity from gene silencing (De Bolle *et al.*, 2006 and references therein), does not

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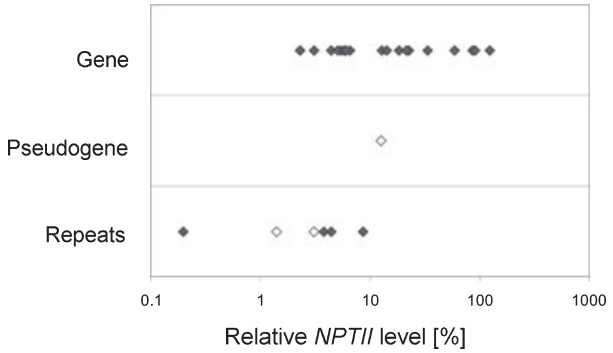

Figure 6. Promotion of RNA-directed transcriptional gene silencing by repetitive sequences flanking target transgenes. The relative *NPTII* levels in F_1 plants (Figure 2) were plotted on a logarithmic scale against a repeat/pseudogene/gene classification of 5-kb genomic sequences immediately flanking the left border of target transgenes. Repeats were defined by REPEATMASKER software (http://repeatmasker.org/); genes and pseudogenes were defined according to annotations in the MatBD database (http://mips.gsf.de/proj/thal/db/). A clear difference with regard to their sensitivity to silencing was identified between target transgenes flanked at the left border side by a gene, a pseudogene or repeats, by Kruskal–Wallis one-way analysis of variance correlation coefficient ($H = 6.541$ with two d.f.; $P = 0.038$). Pairwise multiple comparison by Dunn's method demonstrated a quantitative correlation between the silenced relative *NPTII* levels of the target transgenes and their affiliation to the group flanked by repeats vs. the group flanked by genes (correlation coefficient $Q = 2.473$; $P < 0.05$). Target transgenes $K_{chr1\text{-}3}$, $K_{chr1\text{-}4}$ and $K_{chr1\text{-}5}$ associated with pericentromeric heterochromatin of chromosome 1 (Figure 5) are represented by open diamonds, all other target transgenes are represented by closed diamonds. Student's *t*-test analysis of the two groups (heterochromatic vs. euchromatic) showed that no statistically significant difference was found ($t = 0.901$ with 24 d.f.; $P = 0.376$).

correlate with the level of silencer-mediated pNOS-*NPTII* gene inactivation (Figure S6c). Repetitive sequences in the vicinity of T-DNA insertion sites were identified by the REPEATMASKER program (Smit, AFA, Hubley, R & Green, P. RepeatMasker Open-3.0. 1996-2004 at http://repeatmasker.org), whereas genes were identified at MAtBD (Schoof *et al.*, 2002; http://mips.gsf.de/proj/plant/jsf/athal/index.jsp). A positive correlation was found between efficient silencing in the F_1 generation and the presence of flanking 'low-complexity repetitive sequences', as defined by REAPEATMASKER, at both the left border and the right border sides of target transgenes (Figure S6d). This correlation became even more evident if analysis was solely focused on left border flanking sequences, which are located directly upstream of the pNOS-*NPTII* reporter gene (Figure 6). Repetitive sequences can be a source of endogenous siRNAs, and are preferred targets for DNA methylation. Therefore, flanking regions of target transgenes were scanned for homology with the endogenous siRNAs reported by Lu *et al.*, 2005 (http://mpss.udel.edu/at), and for the presence of DNA methylation in the relevant databases available for *A. thaliana* (Zhang *et al.*, 2006; Zilberman *et al.*, 2007), but no formal correlation could be established (Figure S6e).

How can repetitive sequences promote RNA-directed TGS and DNA methylation? Or, how can vice versa proximity of active genes counteract silencing? Usually, repetitive sequences are by themselves subjected to silencing mechanisms, and might be particularly associated with proteins involved in gene inactivation, such as DNA methyltransferases, histone deacetylases or chromatin remodelling factors. Such an increased local concentration of required factors might allow especially efficient implementation of transcriptional inactivation and DNA methylation in reaction to the sequence-specific RNA signal provided by the silencer transgene. Alternatively, repetitive sequences or the silencing mechanisms acting on them might exclude factors counteracting gene inactivation from their vicinity. The antagonists of silencing might be attracted by active genes. In this view, the reaction of each target promoter to the RNA signal would be determined by the balance of the processes promoting and the processes antagonising its inactivation. The identified reporter transgenes with differing sensitivity to silencing will be employed as versatile experimental material for the further genetic dissection of the underlying processes, by introduction of known and screening for new mutations.

Experimental procedures

Detailed experimental protocols are provided in Appendix S1.

Plant material and growth conditions

Arabidopsis thaliana accession Col-0 was used in all experiments. F_1 plants hemizygous for a target transgene alone were obtained by transfer of pollen from plants homozygous for the target transgene on emasculated wild-type plants, whereas F_1 plants hemizygous for a target transgene and the silencer transgene were obtained by transfer of pollen from plants homozygous for the respective target transgene on emasculated plants homozygous for the silencer transgene. Success of the crosses and identity of the target transgene in the F_1 progeny was confirmed by histochemical GUS staining and genotyping PCR. To obtain doubly homozygous plants, doubly hemizygous F_1 plants were allowed to self-pollinate. The F_2 plants were selfed again and resulting F_3 seeds were collected separately from individual F_2 plants. Seed batches derived from doubly homozygous F_2 were identified by their true breeding for hygromycin resistance and GUS staining. Plants were cultivated on soil at 21°C under a 16-h light/8-h dark (long-day) regime for propagation and seed production, or under an 8-h light/16-h dark (short-day) regime to generate material for RNA and DNA analysis. Kanamycin resistance of the homozygous F_3 plants was tested on germination medium (1 times MS salts; 10 g l^{-1} sucrose) plates with 200 mg l^{-1} kanamycin under long-day conditions.

Transgenic plant lines will be made available on request. Please contact Renate Schmidt for target (*K*) lines and Michael Florian Mette for the silencer (*H*) line.

Quantification of NPTII RNA by real time RT-PCR

NPTII RNA levels were determined by Taq-Man probe-based (Heid *et al.*, 1996) real time RT-PCR measurements of *NPTII* RNA in rela-

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tion to RNA of *ACTIN2* (At3g18780), a nearly constitutively expressed endogenous reference gene (An *et al.*, 1996). Total RNA samples for measurements were extracted from rosette leaves of plants grown for 6–8 weeks under short-day conditions. For each genotype, five individual plants were analysed. Median (third value of five values in ascending order) values are indicated as bar heights, and variations (from lowest value to highest value) of values are indicated as error bars, in Figures 2 and 3a.

DNA methylation analysis

Genomic DNA for methylation analysis was extracted with a DNeasy Plant Maxi Kit (Qiagen, http://www.qiagen.com) from rosette leaves of individual genotyped plants grown for 6–8 weeks under short-day conditions.

Southern analysis

Genomic DNA was incubated with *Bsu*15I and *Pae*I to release a basic pNOS–partial *NPTII* DNA fragment, and additionally with one of the cytosine methylation-sensitive restriction enzymes *Eco*47I, *Psp*1406I, *Nhe*I, *Alw*26I or *Nco*I. DNA blot analysis, using ^{32}P-labelled transcripts of a 535-bp *Nco*I-*Pae*I *NPTII* fragment as a hybridization probe, was performed as described previously (Mette *et al.*, 1999). Southern analysis was performed with at least two individual plants of each genotype.

Bisulfite genomic sequencing

Bisulfite-mediated chemical conversion of DNA was performed using a Qiagen EpiTect Bisulfite Kit (Qiagen), either following the manufacturer's instructions or the method described by Kuhlmann *et al.* (2005). Primers for PCR are indicated in Appendix S1.

Fluorescence in situ *hybridization (FISH)*

Transgene detection by FISH analysis of interphase nuclei and meiotic chromosomes of *A. thaliana* was performed according to the method described by Pecinka *et al.* (2005). Experimental details are given in Appendix S1.

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We thank Christa Fricke, Inge Glaser and Beate Kamm for excellent technical assistance, Armin Meister for support in statistical data analysis, and Barbara Hohn and Ingo Schubert for critically reading the manuscript. This work was supported by German research foundation (DFG) collaborative research centre (SFB) 648 'Molecular Mechanisms of Information Processing in Plants'.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Correlation analysis between *NPTII* RNA levels and measurement variation or relative *NPTII* levels in the presence of the silencer.

Figure S2. DNA methylation analysis by Southern blot on the F_1 generation.

Figure S3. DNA methylation analysis by Southern blot on the F_3 generation.

Figure S4. DNA methylation analysis by bisulfite sequencing on the F_1 generation.

Figure S5. Quantification of pNOS-siRNA by Northern blot.

Figure S6. Correlation analysis between genomic features and relative *NPTII* levels in the presence of the silencer.

Table S1. T-DNA insertion sites of target transgenes.

Appendix S1. Detailed description of materials and methods.

This material is available as part of the online article from http://www.blackwell-synergy.com

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