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RESEARCH ARTICLE

Genetic analysis of RNA-mediated gene silencing in *Arabidopsis thaliana*

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Abstract In plants, a particular class of small non-coding RNAs, short interfering RNAs, can serve as a signal to induce cytosine methylation at homologous genomic DNA regions in the nucleus. If the targeted DNA regions have promoter function, this RNA-directed DNA methylation (RdDM) can result in transcriptional gene silencing (TGS). RNA-directed transcriptional gene silencing of transgenes provides a versatile system for the study of epigenetic gene regulation in plants. In our experimental setup in *Arabidopsis thaliana*, transcription of a promoter-inverted repeat provides a RNA signal that triggers *de novo* cytosine methylation and TGS of a homologous nopaline synthase promoter (*proNOS*) *in trans*. Utilising this two component transgene system in a forward-genetic screen for "suppressor of silencing" mutations, we were able to identify new candidates for factors involved in RdDM of transgenic as well as endogenous target regions.

Keywords RNA-dependent DNA methylation · Transcriptional gene silencing · *Arabidopsis thaliana* · Mutagenesis · NOS promoter

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1 Introduction

DNA methylation in general and the mechanism of RNA-directed DNA methylation (RdDM) as well as related transcriptional gene silencing (TGS) in particular have been extensively studied in the model plant *Arabidopsis thaliana* (Matzke et al. 2009; Law and Jacobsen 2010). As in other plants, DNA methylation in *A. thaliana* can occur in symmetric CG and CNG as well as in asymmetric CHH (where H is A, C or T) context. In total, about 7 % of cytosines are methylated but methylation is not distributed randomly. 24 % of cytosines in CG, but only 6.7 % of cytosines in CNG and 1.7 % of cytosines in CHH context are methylated (Cokus et al. 2008).

In each round of the cell cycle, cytosine methylation patterns need to be re-established in genomic DNA, as only unmethylated cytosines are incorporated in DNA replication and thus no methylation is present on the *de novo* synthesized DNA strand. Cytosine methylation in CG context is maintained by METHYLTRANSFERASE 1 (MET1), a plant member of the Dnmt1 class of methyltransferases, while methylation in CNG context is maintained by the CHROMOMETHYLASE3 (CMT3). Cytosine methylation in CHH context persists by constitutive *de novo* DNA methylation involving the DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), a plant member of the Dnmt3 class of methyltransferases.

The target specificity of *de novo* DNA methylation by DRM2 is achieved by a class of small non-coding RNAs, the short interfering (si)RNAs. The generation of these 24 nucleotides (nt) long RNAs is initiated by a plant-specific DNA-dependent RNA-polymerase, Pol IV, that transcribes regions undergoing RdDM. Two

Pol IV subunits, NUCLEAR RNA POLYMERASE D1 and D2 (NRPD1 and NRPD2) were identified as essential for RdDM of endogenous target sequences (Huang et al. 2009; He et al. 2009; Lahmy et al. 2009; Bies-Etheve et al. 2009). The single stranded "aberrant" transcripts made by Pol IV are thought to be recognized by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) and used as substrate to generate double stranded (ds)RNA. This dsRNA is cleaved by DICER-LIKE 3 (DCL3) into 24 nt fragments. One strand of the DCL3 cleavage products is then bound by ARGONAUT 4 (AGO4) and transferred to another plant-specific DNA-dependent RNA-polymerase, Pol V (Henderson and Jacobsen 2007; Matzke et al. 2009). Here, AGO4 interacts with the subunit NUCLEAR RNA POLYMERASE E1 (NRPE1). By interacting either with a Pol V-derived transcript or with the DNA itself, the siRNA bound by AGO4 then provides the sequence specificity to the *de novo* methylation of DNA by DRM2.

Beside NRPE1, the Pol V complex includes NUCLEAR RNA POLYMERASE E2 (NRPE2), the elongation factor SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L), the chromatin remodelling factor DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) and the factor DEFECTIVE IN MERISTEM SILENCING 3 (DMS3). These factors are supposed to support the recruitment of the Pol V complex to the site of RdDM. The formation or recruitment of the complex is further aided by SET domain proteins SUPPRESSOR OF VARIEGATION 3–9 HOMOLOGUE SUVH2 and SUVH9.

To find new relevant factors and increase the insight in the mechanism of RdDM and related TGS, we made use of a *A. thaliana* line containing two transgenes, a target transgene (*K*) and an unlinked silencer transgene (*H*) (Fig. 1, Aufsatz et al. 2002). The employed target transgene $K_{chr1-10}$ contains an intact single copy T-DNA insertion including 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 (*proNOS*) and a *GUS* gene followed by an ocs 3′ under the control of a cauliflower mosaic virus 35S promoter (*pro35S*) (Fischer et al. 2008). The silencer transgene (*H*) consists of two copies of the *proNOS* oriented as inverted repeat (IR) under the control of a constitutive *pro35S* promoter and a hygromycin resistance gene controlled by *pro19S*. Transcription of this silencer *proNOS* IR leads to formation of *proNOS* dsRNA that is processed to siRNAs of predominantly 21–24 nucleotides in length (Papp et al. 2003). Just as endogenous 24 nt siRNAs, these transgene-derived siRNAs guide *de novo* DNA methylation to the *proNOS* copies and lead to transcriptional silencing.

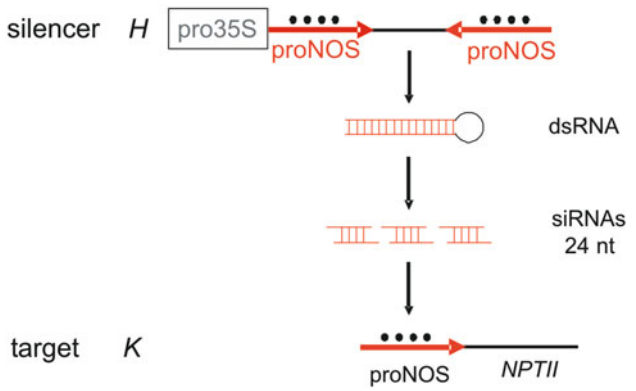


Fig. 1 Transgene system for the analysis of RNA dependent DNA methylation and related transcriptional gene silencing in *Arabidopsis thaliana*. *H* silencer transgene contains two copies of the nopaline synthase promotor (*proNOS*) in inverted repeat (IR) orientation under the control of a cauliflower mosaic virus 35S promoter (*pro35S*). Transcription of the IR generates self-complementary RNA that folds back to form double stranded RNA (dsRNA). The dsRNA is cleaved to 21–24 nt long small interfering (si)RNAs, of which the 24 nt species serves as signal for the methylation (*black dots*) of *proNOS in trans*. *K* target transgene, contains a *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPTII*) gene under the control of a *proNOS*. *NPTII* expression confers kanamycin resistance; methylation of the *proNOS* (*black dots*) will result in transcriptional gene silencing and sensitivity to kanamycin

2 Material and methods

2.1 Plant material and growth conditions

Target and silencer transgenes were introduced separately into *A. thaliana* accession Columbia (Col-0) by *Agrobacterium tumefaciens* mediated transformation and were combined by crossing plants homozygous for target or silencer, respectively (Fischer et al. 2008).

About 10^5 seeds from plants homozygous for target and silencer transgene were submitted for custom ethyl methanesulfonate (EMS) mutagenesis to Lehle Seeds (Round Rock, TX, USA). The seeds were divided into 32 batches ($\sim 3 \times 10^3$ seeds per batch), treated with EMS, and germinated on soil. For each batch, the resulting M_1 plants ($\sim 1.5 \times 10^3$ assuming a survival rate of 50 %) were grown and allowed to selfpollinate to generate M_2 seeds, which were harvested in bulk. About 2×10^4 M_2 seeds per batch (thus, ~ 13 M_2 seeds per M_1 plant) were germinated on ½ Murashige and Skoog medium (Murashige and Skoog 1962) containing 200 mg/l kanamycin under

long day conditions (16 h light, 8 h dark, 21 °C) to screen for reactivation of kanamycin resistance. After 3 weeks, the most vigorous plants (10 per batch) were transferred to soil and allowed to selfpollinate. Release of silencing of the *NPTII* gene was confirmed by testing the M_3 generation for viability on kanamycin containing medium. Lines with a survival rate of at least 95 % among M_3 seeds were considered as true candidates for "suppressor of silencing" mutants.

Plants for expression and DNA methylation analysis were grown on soil for ~6 weeks under short day conditions (8 h light, 16 h dark, 21 °C).

2.2 DNA isolation

Genomic DNA was extracted from leaves using DNeasy® Plant Mini and Maxi Kit (Qiagen) according to the manufacturer's protocol.

2.3 Confirmation of the presence of target and silencer transgene by PCR

PCR indicative for the presence of transgenes was performed with the primers indicated in Table 1. Primer pair pNOS-for and pNOS-rev was used to demonstrate the presence of the target transgene, primer pairs p19s-for and Hyg-rev to demonstrate the presence of the silencer transgene. The heterozygous (signal present) versus homozygous (signal absent) state of the silencer transgene was tested by PCR with flanking primers HinS-for and HinS-rev.

2.4 NPTII ELISA

NPTII protein levels in leaves were determined using a PathoScreen® kit for neomycin phosphotransferase II from Agdia according to the manufacturer's protocol. The data read out was performed on an infinite200 plate reader (Tecan) with supplied software (Tecan i-control).

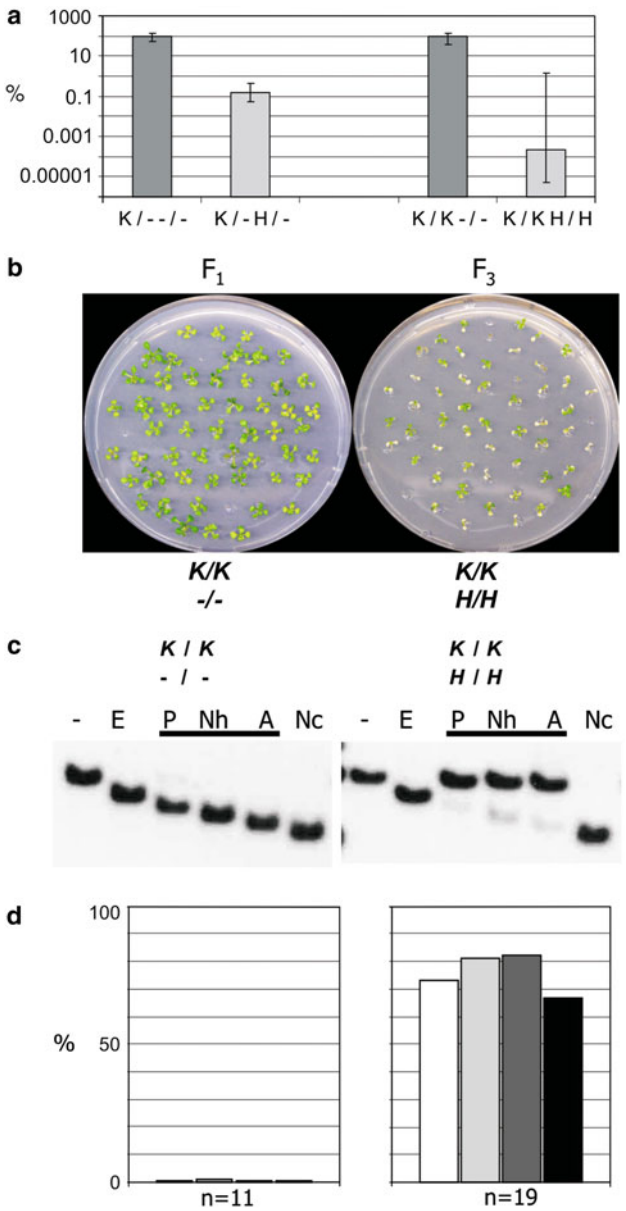
2.5 DNA methylation analysis

Quantitative (q) PCR was performed after cleavage of genomic DNA with methylation-sensitive restriction enzymes. About 0.1 μg of genomic DNA was diluted in 500 μl H_2O. Aliquots of 90 μl of this stock were diluted to a total volume of 100 μl of the appropriate reaction buffer and incubated with 30 units of restriction enzyme (Fermentas) at 37 °C over night. After incubation, restriction enzymes were inactivated by incubation at 65 °C for 5 min and the reaction mixture diluted 1:10 with H_2O. 10 μl of the dilution were added to 12.5 μl SyBr green Supermix® (Bio-Rad cat. no. 170-8882) and 1.25 μl of each primer solution (of 10 pmol/μl) according manufacturer's protocol to perform qPCR in a final volume of 25 μl on an iQ5 cycler (Bio-Rad). The primers used for amplification of the proNOS-fragment (460 bp) were pNOSfor (5′-GATAGTTGGCGAAATTTTCAAAGT-3′) and pNOSrev (5′-CAATCCATCTTGTTCAACCATGG-3′). The PCR program included an initial step of 5 min 95 °C followed by 40 cycles of 15 s 95 °C, 30 s 62 °C and 30 s 72 °C (detection during elongation). Calibration using a serial dilution of genomic DNA resulted in a correlation coefficient of 0.999 and a PCR efficiency of 99.7 % for the quantification.

Bisulfite mediated chemical conversion of DNA was done using an EpiTect Bisulfite Kit (Qiagen) following the manufacturer's instructions. Primers used for the amplification of a particular *AtSN1* copy from bisulfite converted genomic *A. thaliana* DNA were ATS150 (5′-ACCAACGTGCTGTTGGCCCAGTGGTAAATC-3′) and AtSN1-F4 (5′-AAAATAAGTGGTGGTTGTACAA GC-3′). The PCR program included an initial step of 5 min 95 °C followed by 40 cycles of 15 s 95 °C, 30 s 60 °C and 30 s 72 °C. PCR amplification products were cloned with a Strataclone™ PCR Cloning kit (Stratagene) and the resulting plasmid clones were sequenced using a Thermo Sequenase Cycle Sequencing kit (USB) and a LI-COR 4300 DNA analyser.

Table 1 Primer pairs for the amplification of PCR products specific to target and silencer transgene

Forward	Sequence (5′ → 3′)	Reverse	Sequence (5′ → 3′)	Product (bp)
Target				
pNOS-for	ACAAGCCGTTTTACGTTTGG	pNOS-rev	GGAACGTCAGTGGAGCATTT	230
Silencer				
p19S-for	AGGAACCGACAACCACTTTG	Hyg-rev	GACATATCCACGCCCTCCT	332
p35S-for	CGCACAATCCCACTATCCTT	Spac-rev	TTCACCAACTCAACCCATCA	677
Spac-for	TGATGGGTTGAGTTGGTGAA	Term-rev	CAAAGTGGTTGTCGGTTCCT	692
HinS-for	GAGATAGTGGAGCAATCTCTGAGATG	HinS-rev	TTCATACGAGACCCTCTGTTTTGG	500



◀ **Fig. 2** Transcriptional gene silencing and RNA dependent DNA methylation of the target *proNOS-NPTII* reporter gene. **a** Remaining *NPTII* mRNA in presence of the silencer (*light grey*) relative to *NPTII* mRNA in absence of the silencer (*dark grey*) was detected by real time RT-PCR and is given in percent on a logarithmic scale. RNA was prepared from leaf tissue of individual hemizygous F_1 ($N = 5$) and homozygous F_3 ($N = 5$) plants (Fischer et al. 2008). *Error bars* show the total deviation. **b** Homozygous seedlings carrying the *proNOS-NPTII* target (*K*) in the absence (*left*) and the presence (*right*) of the silencer (*H*) on medium containing kanamycin. **c** Methylation analysis of the target *proNOS* in homozygous F_3 plants in the absence (*left*) and the presence (*right*) of the silencer by Southern with methylation sensitive restriction enzymes. A basic fragment containing the *proNOS* was released by *Bsu*15I and *Pae*I cleavage (−). Methylation was tested by additional incubation with methylation-sensitive restriction enzymes *EcoR*47I (E, GGWCC), *Psp*1406I (P, AACGTT), *Nhe*I (Nh, GCTAGC), *Alw*26I (A, GTCTC, GAGAC) and *Nco*I (Nc, CCATGG). The *underlined* enzymes are recognition sites in the region covered by *proNOS* siRNAs derived from the silencer. **d** Methylation analysis of the target *proNOS* in homozygous F_3 plants in the absence (*left*) and the presence (*right*) of the silencer by bisulfite sequencing analysis in percent of cytosines in total (*white*), CG context (*light grey*), CNG context (*dark grey*) and CHH context (*black*) (*K*/*K*: $N = 11$, *K*/*K*; *H*/*H*: $N = 19$)

Southern analysis in combination with methylation-sensitive restriction enzymes to determine DNA methylation in the proNOS was performed as described by Fischer et al. (2008).

3 Results

3.1 Identification of "suppressor of silencing" mutants releasing RNA-directed transcriptional gene silencing

The presence of the silencer transgene *H* reduces the level of *NPTII* transcripts from target transgene $K_{chr1-10}$ ~100-fold (Fig. 2a, Fischer et al. 2008). Consistent with this, plants are resistant to kanamycin if they contain the target transgene $K_{chr1-10}$, but become sensitive if they contain, additionally, the silencer transgene *H* (Fig. 2b). Analysis of the DNA methylation by Southern blot analysis with methylation-sensitive restriction enzymes (Fig. 2c) and bisulfite sequencing (Fig. 2d) revealed that ~80 % of the cytosines in the *proNOS* of the target transgene were methylated. The methylation was evenly distributed among cytosines independent of their sequence context, which is a hallmark of RdDM.

Ethyl methanesulfonate mutagenesis of seeds homozygous for target and silencer transgenes ($K_{chr1-10}/K_{chr1-10}$; *H*/*H*) in the Col-0 accession was performed by Lehle Seeds (Round Rock, TX, USA). From the obtained 32 batches of M_2 seeds, (each batch representing the progeny from 1,500 M_1 plants), 20,000 seeds per batch were germinated on medium containing kanamycin (200 mg/l) (Fig. 3). Resistant M_2 plants were transferred to soil and allowed to set seeds by selfing. The suppression of TGS was confirmed by germinating resulting M_3 seeds on kanamycin containing selective medium. True candidate lines for "suppressor of silencing" mutants showed root growth and development of primary leaves in all progeny. The lines passing these criteria were then tested by PCR for the presence of intact target and silencer transgenes (data not shown). The first round of screening resulted in six independent M_3 lines from different seed batches which contained

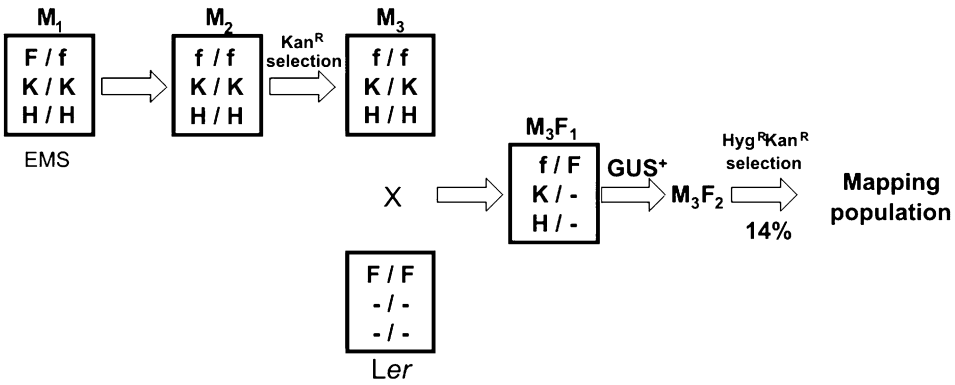


Fig. 3 Genetic screen for factors involved in RNA-directed transcriptional gene silencing. *K*: target transgene; *H*: silencer transgene; *f*/*f*: functional/non-functional allel of factor involved in RdTGS; M_1: plants grown from seeds that were treated with EMS; M_2, M_3: generations obtained by selfing from M_1; kanamycin-selection is performed on M_2 seeds, derived M_3 seeds are crossed to Landsberg *erecta* (L*er*) to generate mapping populations

target and silencer transgene in the homozygous state and uniformly showed resistance to kanamycin, thus meeting our criteria for "suppressor of silencing" mutants.

3.2 Target transgene expression and RNA-directed DNA methylation status in "suppressor of silencing" mutants

In order to test whether the kanamycin resistance in the mutant lines was due to reactivation of *NPTII* expression, NPTII protein levels in leaves were determined by a NPTII-specific ELISA (Fig. 4a). Four of the six analysed mutant lines (mut2-5, mut8-6, mut26-5 and mut29-8) showed a significant increase in NPTII protein compared to non-mutagenised plants containing the target transgene in the presence of the silencer, while two of the plant lines (mut19-7 and mut30-2) showed no detectable NPTII protein expression.

To see whether RdDM was affected in the mutant lines, DNA methylation of the *proNOS* in the target transgene construct was tested by a DNA methylation assay based on cytosine methylation-sensitive restriction enzymes and quantitative PCR (Fig. 4b). In agreement to prior analysis (Fischer et al. 2008), in all plants analysed no methylation was detected at the *Nco*I site outside of the region targeted by *proNOS* siRNAs derived from the silencer. In all plants that contained the silencer transgene, substantial symmetric DNA methylation was detected (*Psp*1406I site). In the four mutant lines with elevated NPTII protein levels (mut2-5, mut8-6, mut26-5 and mut29-8), we detected a reduction of asymmetric DNA methylation (*Nhe*I and *Alw*26I sites), while the amount of symmetric DNA methylation (*Psp*1406I) was comparable to non-mutagenised control plants containing target and silencer transgenes. The two mutant lines without detectable NPTII protein (mut19-7 and mut30-2) showed mostly unaltered DNA methylation.

To test for general hypomethylation in the identified mutant lines, we analysed the level of DNA methylation at *AtSN1*, an endogenous target of RdDM, by bisulfite sequencing (Fig. 4c). This confirmed the reduced level of DNA methylation at cytosines in asymmetric CHH context in lines mut2-5, mut8-6, mut26-5 and mut29-8. The *AtSN1* methylation in CHH context in mutant line mut19-7 was partially reduced, while no change was observed for mutant line mut30-2.

4 Discussion

Using a forward genetic approach based on a two component transgene system, we were able to identify six independent mutant lines displaying kanamycin resistance. In at least four (mut2-5, mut8-6, mut26-5 and mut29-8) of the six mutant lines, kanamycin resistance of the plants is due to a release of silencing of the *NPTII* reporter gene in the target transgene. The reactivation of NPTII expression was correlated with DNA hypomethylation at the reporter gene promoter. Methylation loss was most prominent for cytosines in an asymmetric context, while methylation in CG context was hardly affected. In the same four mutant lines, bisulfite sequencing of the *AtSN1* locus revealed a hypomethylation for cytosines in asymmetric context also for this endogenous target of RdDM. Thus, the mutations in these four lines most likely generate "loss of function" alleles of factors essential to RdDM. An interesting aspect is that these

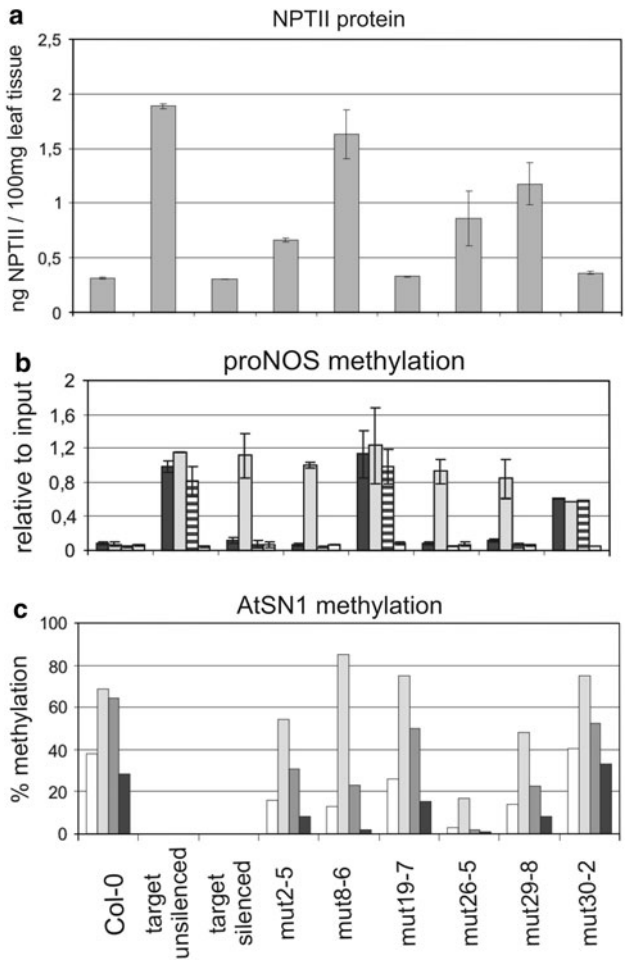


Fig. 4 Characterisation of candidate "suppressor of silencing" mutant lines. **a** NPTII protein levels in ng per 100 mg fresh weight determined by ELISA: Col-0 (non-transformed); target unsilenced (homozygous for target and an inactivated silencer variant); target silenced (homozygous for target and silencer); M_3 mutant plants from lines mut2-5; mut8-6; mut19-7; mut26-5; mut29-8; mut30-2; measurements were done for two independent plants. **b** Target *proNOS* methylation determined by quantitative PCR after cleavage of genomic DNA with methylation-sensitive restriction enzymes *Nhe*I (*black* asymmetric context: GCTAGC); *Psp*1406I (*grey* symmetric context: AACGTT); *Alw*26I (*black lines* asymmetric context: GTCTC, GAGAC) and *Nco*I (*white* control: CCATGG): target unsilenced (homozygous for target); target silenced (homozygous for target and silencer); M_3 mutant plants from lines mut2-5; mut8-6; mut19-7; mut26-5; mut29-8; mut30-2; measurements were done for two independent plants, except for mut30-2. **c** *AtSN1* DNA methylation region determined by bisulfite sequencing in percent of cytosines in total (*white*), CG context (*light grey*), CNG context (*dark grey*) and CHH context (*black*): Col-0 (non-mutagenised, $N = 12$); M_3 mutant plants from lines mut2-5 ($N = 12$); mut8-6 ($N = 10$); mut19-7 ($N = 12$); mut26-5 ($N = 22$); mut29-8 ($N = 12$); mut30-2 ($N = 9$) (N number of sequences analysed)

mutants show partial reactivation of reporter gene expression. Thus, although symmetric-context DNA methylation is persistent at the *proNOS*, it is solely not sufficient to grant full silencing. The inactivation of reporter gene expression seems to be an additive effect of different types of DNA methylation.

The line mut19-7 shows only a very weak reduction of reporter gene promoter methylation, but nevertheless a reduction by half of asymmetric context cytosine methylation in *AtSN1*. The mutation in mut19-7 might generate a "weak" allele of a factor with still some remaining gene function. Depending on the particular RdDM target, this remaining activity might be sufficient or not to sustain normal *de novo* methylation levels. Mutant line mut30-2 does neither show reactivation of reporter gene expression nor reduction of RdDM. It might contain a mutation conferring kanamycin resistance independent of transgene-based *NPTII* expression, e.g. by affecting a gene required for kanamycin uptake, possibly similar to a chloroplast transporter that has been described by Aufsatz et al. (2009).

The map-based cloning of mutations will be focused on the ones clearly affecting RdDM and will be performed with the help of CAPS and InDel markers in classical Col-0 X *Ler* mapping populations (Fig. 3). Our approach has a high potential to lead us to new components and further insights into the complex mechanism of DNA methylation mediated by small non-coding RNAs.

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Conflict of interest The authors declare that they have no conflict of interest.

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