DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in *Arabidopsis*

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In plants, transgenes can be silenced at both the transcriptional [1] and post-transcriptional levels [2]. Methylation of the transgene promoter correlates with transcriptional gene silencing (TGS) [3] whereas methylation of the coding sequence is associated with post-transcriptional gene silencing (PTGS) [4]. In animals, TGS requires methylation and changes in chromatin conformation [5]. The involvement of methylation during PTGS in plants is unclear and organisms with non-methylated genomes such as *Caenorhabditis elegans* or *Drosophila* can display RNA interference (RNAi), a silencing process mechanistically related to PTGS [6]. Here, we crossed *Arabidopsis* mutants impaired in a SWI2/SNF2 chromatin component (*ddm1* [7]) or in the major DNA methyltransferase (*met1* [8] and E. Richards, personal communication) with transgenic lines in which a reporter consisting of the cauliflower mosaic virus 35S promoter fused to the β-glucuronidase (GUS) gene (35S–GUS) was silenced by TGS or PTGS. We observed an efficient release of 35S–GUS TGS by both the *ddm1* and *met1* mutations and stochastic release of 35S–GUS PTGS by these two mutations during development. These results show that DNA methylation and chromatin structure are common regulators of TGS and PTGS.

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Results and discussion

The *ddm1* and *met1* mutations release TGS

The *Arabidopsis ddm1* mutant (decrease in DNA methylation) can release TGS and methylation of various transcriptionally silenced loci, including the transgene locus A and the endogenous transcriptionally silent information (TSI) loci [9–12], demonstrating that chromatin structure is crucial for TGS. Both methylation and silencing of TSI elements are also released in the *met1* mutant (previously named *ddm2*; [8] and E. Richards, personal communication) and in *Arabidopsis* plants containing an antisense transgene directed against the *MET1* gene (*asMET1* plants) [12]. However, methylation but not TGS of the transgenic locus A is released in *asMET1* plants [9], raising the question of the role of methylation during TGS. To further evaluate the role of transgene methylation on TGS, we tested the effect of the *ddm1* and *met1* mutations on the transcriptionally silenced 35S–GUS transgene of line 6b5 (Figure 1, left column). After crossing line 6b5 with the *ddm1* and *met1* mutants and allowing the F1 progeny to self-fertilize, GUS activity was first measured in randomly selected F2 progenies. We observed that 18 plants out of 100 in the cross with *ddm1*, and 17 plants out of 100 in the cross with *met1*, showed high GUS activity (the [GUS+] phenotype). This is the expected ratio (3/16; $p < 0.05$) for a recessive releasing effect of the *ddm1* and *met1* mutations on TGS. To further confirm these releasing effects, double homozygous F3 plants (*ddm1*/*ddm1* 6b5/6b5 and *met1*/*met1* 6b5/6b5) were selected (Figure 1a). Analysis of GUS activity in the F3 progenies confirmed that the *ddm1* and *met1* mutations inhibited TGS of all plants carrying the GUS transgenic locus of line 6b5 (Figure 1d). Methylation analysis showed that this inhibition of TGS correlated with reduced methylation of the locus 6b5 (Figure 1b,c).

The effect of the *ddm1* mutation on methylation (Figure 1b,c) and GUS activity (Figure 1d) of the locus 6b5 was stronger than the effect of the *met1* mutation. This is in accordance with the fact that *DDM1* and *MET1* exhibit differential effects [9]. Indeed, TGS of 6b5 and TSI loci is released in *ddm1* and *met1* mutants and in *asMET1* plants, whereas the locus A is reactivated in *ddm1* mutants but not in *asMET1* plants [9]. These results suggest that the requirement for methylation in TGS may vary depending on the structure of the transgenic locus and/or its location in the genome. Current models in vertebrates propose that transcription is not blocked by methylation *per se*, but rather by the formation of particular chromatin that assembles on methylated DNA [5]. This model could account for TGS at the 6b5 and TSI loci but not for TGS at locus A. Therefore, two modes of TGS may exist in plants: one for which MET1 activity is required (occurring at the 6b5 and TSI loci) and one for which MET1 activity is dispensable (occurring at locus A). The fact that MET1 activity could be dispensable is in accordance with the release of TGS at locus A in the *mom1* mutant, which does not modify methylation [13].

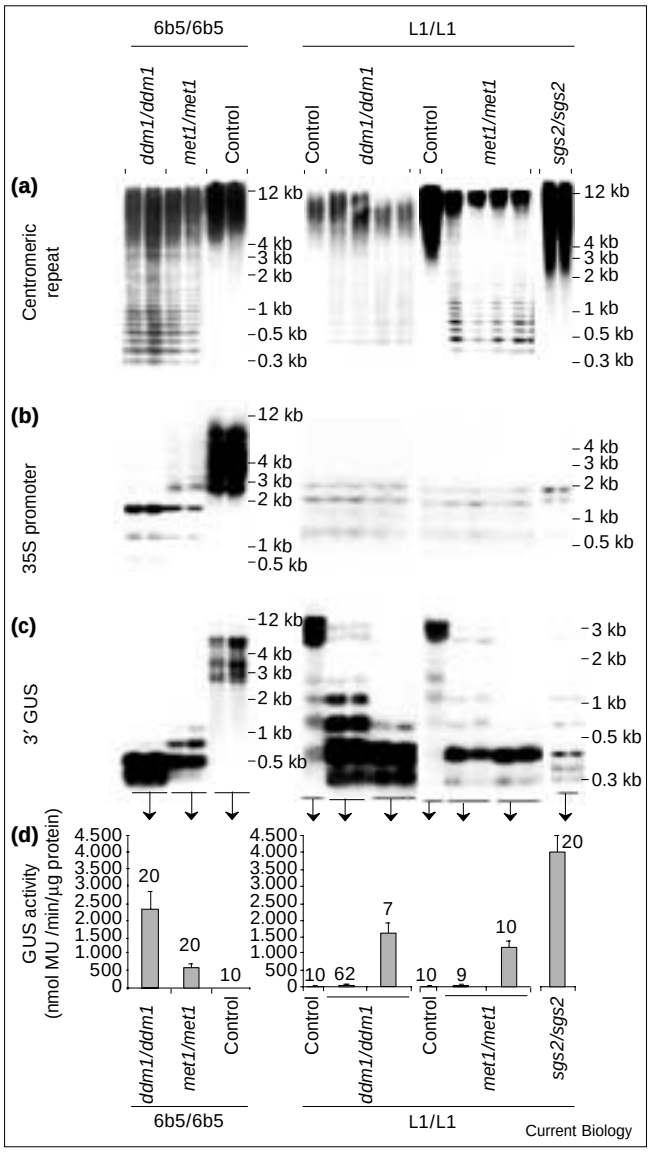


Figure 1

Inhibition of TGS (line 6b5, left panels) and PTGS (line L1, right panels) by the *met1* and *ddm1* mutations. One or two representative plants of each genotype (*met1*, *ddm1* or wild-type siblings, indicated as control) exhibiting GUS activity ([GUS+] phenotype) or no GUS activity ([GUS–] phenotype) are shown. (a) The *ddm1* and *met1* mutants showed extensive demethylation of the genome, characterized by reduced methylation of the 180 bp centromeric repeats. Inhibition of TGS correlated with reduced methylation of (b) the 35S promoter and (c) the coding sequence of the transgene. Inhibition of PTGS correlated with reduced methylation of the coding sequence of the transgene only. (d) GUS activity was monitored in F3 plants for *met1/met1* L1/L1, *ddm1/ddm1* 6b5/6b5, *met1/met1* 6b5/6b5 and in F4 plants for *ddm1/ddm1* L1/L1. The numbers above the bars represent the number of plants tested. The *sgs2-1* mutant, which is mutated in an RNA-dependent RNA polymerase [15], has been included as an example of an *sgs* mutation that releases PTGS and leads to low methylation of the 35S–GUS transgene. MU, 4-methylumbelliferone.

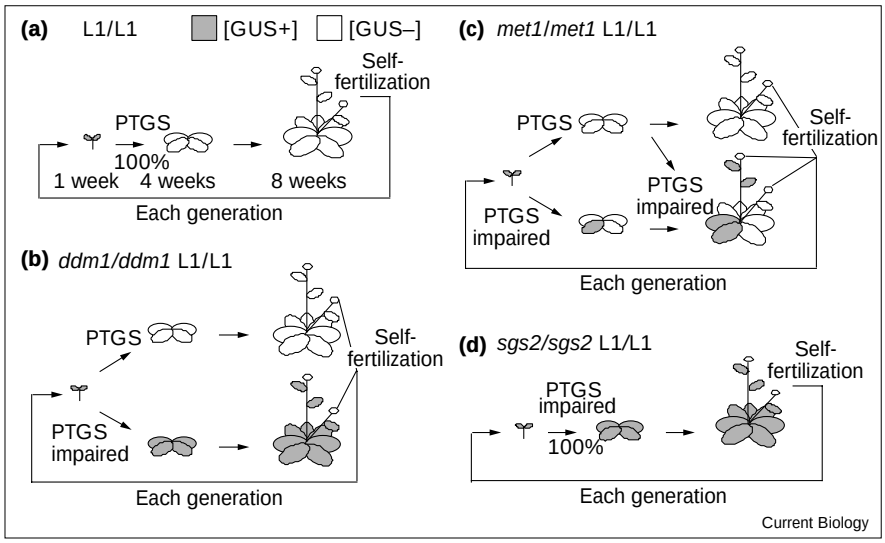
The *met1* mutation impairs maintenance of PTGS in developing tissues

In contrast to TGS, PTGS is characterized by transcription in the nucleus of the silenced transgene followed by specific RNA degradation [2]. Like TGS, methylation is also associated with PTGS but only in the transcribed sequence [4]. Moreover, methylation of transgenes is reduced in *sgs1*, *sgs2* and *sgs3 Arabidopsis* mutants deficient in PTGS [14,15] (see also Figures 1c and 2d), and recent pharmacological experiments suggest that methylation is required for PTGS [16]. To assess directly the possible links between methylation, chromatin structure and PTGS, we crossed the *met1* and *ddm1* mutants with the post-transcriptionally silenced L1 line (Figure 1, right columns), which triggers PTGS of a 35S–GUS transgene early in development with 100% efficiency [15] (Figure 2a). Of the nine *met1/met1* L1/L1 F2 plants isolated, one exhibited early transgene reactivation, as measured by high GUS activity, whereas all 11 *MET1/MET1* L1/L1 F2 control plants generated from the same cross were silenced. As plants developed further, the proportion of plants exhibiting PTGS release increased; five out of the eight [GUS–] *met1/met1* L1/L1 plants now had high GUS activity in some or all of the newly developed leaves (Figure 2c). The ability to inhibit PTGS in a fraction of the population was transmitted through meiosis. Indeed, like in the F2 generation, a stochastic inhibition of PTGS was observed in the F3 generation whether the plants were derived from [GUS+] or [GUS–] F2 *met1/met1* L1/L1 plants (Table 1). In these F3 plants, the percentage of [GUS+] plants also increased during plant development (Figure 3). Sectors with high GUS activity displayed reduced methylation of the GUS coding sequence whereas, in the silenced tissues, methylation of the GUS transgene was high (Figure 1c). The appearance of [GUS+] sectors in F2 and F3 plants, which had triggered PTGS earlier in development (Figures 2c and 3), suggests that transgene PTGS maintenance, rather than triggering, is impaired by the *met1* mutation. Thus, the maintenance DNA methyltransferase I activity encoded by the *MET1* gene seems to be required to maintain silencing of the GUS transgene throughout plant development, at each generation. The effect of methylation on PTGS maintenance may occur either by maintaining inactivation across cell divisions or by allowing, in new tissues, the perception of the PTGS systemic signal originating from silenced cells [17].

The *ddm1* mutation impairs PTGS early in development

None of the 12 isolated *ddm1/ddm1* L1/L1 F2 plants displayed GUS activity. However, inhibition of PTGS was observed in 7 out of 80, and 5 out of 80 F3 plants derived from two independent [GUS–] *ddm1/ddm1* L1/L1 F2 plants tested (Table 1), suggesting that the absence of [GUS+] plants in the F2 generation could be due to the small number of plants analyzed. As in *met1/met1* L1/L1 plants, the ability to inhibit PTGS in a fraction of the population

Figure 2



Evolution of GUS activity during development in (a) L1/L1 wild-type siblings, and the (b) *ddm1/ddm1* L1/L1, (c) *met1/met1* L1/L1 and (d) *sgs2/sgs2* L1/L1 lines. PTGS occurs with 100% efficiency in all tissues of the L1/L1 wild-type siblings whereas it never occurs in *sgs2/sgs2* L1/L1 plants [15]. PTGS was abolished in a fraction of plants of the *ddm1/ddm1* L1/L1 and *met1/met1* L1/L1 genotypes at each generation. PTGS was abolished in all tissues in *ddm1/ddm1* L1/L1 plants whereas it was abolished in sectors of *met1/met1* L1/L1 plants.

was transmitted through meiosis in *ddm1/ddm1* L1/L1 plants. Indeed, PTGS was inhibited only in a fraction of plants of the F4 and F5 generations whether they were derived from [GUS+] or [GUS–] F3 and F4 plants (Table 1). In contrast to the results obtained with the *met1* mutation, the percentage of plants exhibiting high GUS activity did not increase during development, and plants that were [GUS–] 10 days after germination remained [GUS–]. Moreover, the [GUS+] plants exhibited GUS activity in all tissues examined (Figure 2b) and throughout development (Figure 3). As with the *met1* mutation, high GUS activity correlated with reduced methylation of the GUS coding sequence in *ddm1/ddm1* L1/L1 plants (Figure 1d). Two hypotheses could account for PTGS inhibition in the whole plant. The *ddm1* mutation could be acting early during development, before PTGS is triggered. Alternatively, the *ddm1* mutation could be impairing the establishment rather than the maintenance of PTGS.

Our results show that PTGS, although often assumed to be a cytoplasmic phenomenon, can be affected by mutations acting at the DNA level. The effect of the *ddm1* and *met1* mutations is unlikely to be due to an epigenetic modification of *SGS* genes controlling PTGS in plants [15]. Indeed, PTGS inhibition by *ddm1* or *met1* was not associated with hypersusceptibility to cucumber mosaic virus, whether the inoculated plant was [GUS–] or [GUS+] (data not shown), a phenomenon so far associated with all *sgs* mutants ([15]; C.B., J-B.M. and H.V., unpublished work). Although we cannot rule out the possibility that *ddm1* and *met1* mutations provoke an epimutation in a gene required for PTGS but not for virus resistance, our results suggest that methylation, and more generally epigenetic modifications affecting the transgene itself, are components of the PTGS pathway in plants. In conclusion, although TGS and PTGS have so far been considered as different classes

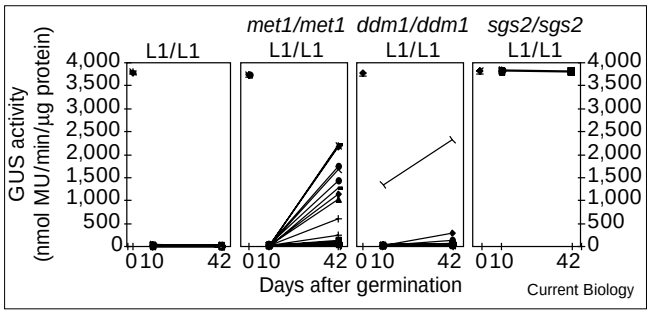
Table 1

Frequencies of inhibition of PTGS by the *ddm1* and *met1* mutations.

Genotype of parent	Progeny generation	[GUS]	Number of plants tested	Number of [GUS+] plants	Percentage of [GUS+] plants
ddm1/ddm1 L1/L1					
	F2	–	80	7	9
	F2	–	80	5	6
	F3	+	26	4	15
	F3	+	15	3	20
	F3	–	26	2	8
	F4	+	10	8	80
	F4	+	40	1	3
	F4	+	30	10	33
	F4	+	20	6	30
	F4	+	35	1	3
	F4	+	40	18	45
	F4	–	36	2	6
	F4	–	20	3	2
met1/met1 L1/L1					
	F2	+	20	5	25
	F2	+	20	0	0
	F2	+	20	1	0.5
	F2	+	24	13	54
	F2	+	24	2	8
	F2	–	20	6	30
	F2	–	20	0	0
	F2	–	25	2	8

Seeds were harvested from [GUS+] or [GUS–] F2, F3 or F4 parents. The number of [GUS+] and [GUS–] plants in the self-progeny was scored after eight weeks of growth. The *met1/met1* plants with [GUS+] sectors are scored as [GUS+] plants.

Figure 3



PTGS evolution in the *ddm1*, *met1*, *sgs2* or wild-type backgrounds during development. GUS activity was monitored on the same individual plants at the two-cotyledon stage (10 days) and flowering stage (42 days). GUS activity was also tested on individual seeds (40 tested for each). In contrast to the 6b5 line, all seeds from L1 lines (wild type, *ddm1/ddm1, met1/met1* and *sgs2/sgs2*) were [GUS+], suggesting that silencing, when it occurs in the *met1* and *ddm1* backgrounds, is post-transcriptional and not due to a block of transcription. Because the seed assay is destructive, the corresponding activities could not be assigned to individual plants and are shown as isolated dots. The graph represents one typical experiment in which 20 plants of each genotype were analyzed.

of phenomena [3], our results establish that, in plants, these gene-silencing processes share common effectors — methylation and chromatin structure — and that *DDM1* and *MET1* are general regulators of transgene silencing. Whether such DNA epigenetic modifications might be required during RNAi needs to be investigated, in particular, in vertebrates in which genomic methylation is found.

Materials and methods

Strains and isolation of the double homozygous lines

Lines L1 and 6b5 were obtained by transformation of wild-type *Arabidopsis* plants of the Columbia ecotype with a T-DNA composed of a GUS reporter gene driven by the 35S promoter of the cauliflower mosaic virus and an *NptII* gene conferring resistance to kanamycin [14]. Line L1 harbors one transgenic locus composed of a direct tandem repeat of the T-DNA [14] whereas line 6b5 harbors one transgenic locus with more than two copies of the T-DNA (T. Elmayan, P.M. and H.V., unpublished work). Run-on experiments showed that silencing occurs at the post-transcriptional level in line L1 [14] whereas it occurs at the transcriptional level in line 6b5 (P.M. and H.V., unpublished data). The *ddm1* and *met1* lines were crossed with the homozygous L1 or 6b5 line and double heterozygous F1 progenies were allowed to self-fertilize. Identification of the homozygous *ddm1* and *met1* genotypes in the F2 progenies was done by scoring methylation of *Hpa*II sites within the centromeric 180 bp repeats as described in [13]. The selected plants were allowed to self-fertilize and the F3 was sown on kanamycin-containing medium [14]. The double homozygous lines were identified as giving 100% of kanamycin-resistant F3 plants.

Transgene expression and methylation analysis

Measurement of GUS activity (in nanomoles of 4-methylumbelliferone (MU) per min per µg total protein), genomic DNA extraction and gel blot analyses were performed as described in [15]. Methylation was monitored by Southern blotting using the methylation-sensitive enzyme *Hpa*II whose cleavage is blocked by methylation at either cytosine residue of the CCGG sites.

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References

1. Wolffe A, Matzke M: Epigenetic regulation through repression. *Science* 1999, 286:481-486.
2. Fagard M, Vaucheret H: (Trans)gene silencing in plants: how many mechanisms? *Annu Rev Plant Physiol Plant Mol Biol* 2000, 51:167-194.
3. Razin A: CpG methylation, chromatin structure and gene silencing – a three-way connection. *EMBO J* 1998, 17:4905-4908.
4. Baulcombe DC: RNA as a target and a initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol Biol* 1996, 32:79-88.
5. Kass SU, Landsberger N, Wolffe AP: DNA methylation directs a time-dependent repression of transcription initiation. *Curr Biol* 1997, 7:157-165.
6. Bosher J, Labouesse M: RNA interference: genetic wand and genetic watchdog. *Nat Cell Biol* 2000, 2:E31-E36.
7. Jeddeloh J, Stokes T, Richards E: Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nat Genet* 1999, 22:94-97.
8. Finnegan J, Dennis E: Isolation and identification by sequence homology of a putative cytosine methyltransferase from ***Arabidopsis thaliana***. *Nucleic Acids Res* 1993, 21:2383-2388.
9. Mittelsten Scheid O, Afsar K, Paszkowski J: Release of epigenetic gene silencing by ***trans***-acting mutations in ***Arabidopsis***. *Proc Natl Acad Sci USA* 1998, 95:632-637.
10. Bender J, Fink G: Epigenetic control of an endogenous gene family is revealed by a novel blue fluorescent mutant of ***Arabidopsis***. *Cell* 1995, 83:725-734.
11. Hirochika H, Okamoto H, Kakutani T: Silencing of retrotransposons in ***Arabidopsis*** and reactivation by the ***ddm1*** mutation. *Plant Cell* 2000, 12:357-369.
12. Steimer A, Amedeo P, Afsar K, Fransz P, Mittelsten Scheid O, Paskowski J: Endogenous targets of transcriptional gene silencing in ***Arabidopsis***. *Plant Cell* 2000, 12:1165-1178.
13. Amedeo P, Habu Y, Afsar K, Mittelsten Scheid O, Paskowski J: Disruption of the plant gene ***MOM*** releases transcriptional gene silencing of methylated genes. *Nature* 2000, 405:203-206.
14. Elmayan T, Balzergue S, Beon F, Bourdon V, Daubremet J, Guenet Y, *et al.*: ***Arabidopsis*** mutants impaired in cosuppression. *Plant Cell* 1998, 10:1747-1758.
15. Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, *et al.*: ***Arabidopsis SGS2*** and ***SGS3*** genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* 2000, 101:533-542.
16. Kovarik A, Van Houdt H, Holy A, Depicker A: Drug-induced hypomethylation of a posttranscriptionally silenced transgene locus of tobacco leads to partial release of silencing. *FEBS Lett* 2000, 467:47-51.
17. Palauqui JC, Elmayan T, Pollien JM, Vaucheret H: Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J* 1997, 16:4738-4745.
18. Vongs A, Kakutani T, Martienssen R, Richards E: ***Arabidopsis thaliana*** DNA methylation mutants. *Science* 1993, 260:1926-1928.