

DNA hypomethylation reduces homologous pairing of inserted tandem repeat arrays in somatic nuclei of *Arabidopsis thaliana*

Koichi Watanabe^{1,†}, Ales Pecinka^{2,†}, Armin Meister², Ingo Schubert² and Eric Lam^{1,*}

¹Biotech Center for Agriculture and the Environment, Rutgers The State University of New Jersey, 59 Dudley Rd., Foran Hall, New Brunswick, NJ 08901, USA, and

²Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstrasse 3, D-06466 Gatersleben, Germany

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*For correspondence (fax +1 732 932 6535; e-mail lam@aesop.rutgers.edu).

†These authors contributed equally to the paper.

Summary

Fluorescent chromatin tagging makes possible tracking of specific loci *in vivo* and *in situ*. Loci tagged by the *lac* operator (*lacO*)/GFP-LacI/Nuclear Localization Signal (NLS) system show rapid motility and constrained chromatin dynamics in somatic nuclei of a transgenic line, designated EL702C, in *Arabidopsis thaliana*. The tagged loci associated with each other significantly more often than expected at random, due to homologous pairing of the *lacO* tandem repeat arrays. Furthermore, these arrays associated significantly more often than average euchromatic regions with heterochromatic chromocenters (CCs). We show now that the inserted *lacO* array in this transgenic line became strongly methylated at CG sites in the T3 generation, which can be reversed upon transfer into the mutant backgrounds of *decrease in DNA methylation 1* (*ddm1*) and *methyltransferase 1* (*met1*). Concomitantly, the tagged loci showed lower association frequencies as compared with the transgenics in wild-type background, which is correlated with a significant decrease in allelic and ectopic pairing of the *lacO* repeat arrays as visualized by fluorescence *in situ* hybridization. In contrast, the preferential association of the *lacO* arrays with heterochromatin, locus mobility in somatic nuclei and transcription of neighboring transgenes were not altered by reduced DNA methylation in *ddm1* and *met1* backgrounds. Our results show that repeat arrays can activate hypermethylation of the inserted locus that correlates with high frequencies of homologous pairing in somatic cells. In contrast, the preferential association of these inserted arrays with CCs in plant cells occurs through another mechanism.

Keywords: *Arabidopsis*, DNA methylation, somatic pairing, heterochromatin, tandem repeat arrays.

Introduction

Gene expression studies in relation to structural and spatial alterations of chromatin within the nucleus may help to understand the mechanisms that regulate cell-type-specific expression patterns during differentiation or in response to environmental stimuli. In interphase nuclei, each chromosome occupies a discrete space called 'chromosome territory' (reviewed by Cremer and Cremer, 2001; Dundr and Misteli, 2001). In chicken and primates, radial positioning places chromosome territories towards either the periphery or the interior of the nucleus depending on the size and gene density of the chromosomes (Boyle *et al.*, 2001; Sun *et al.*, 2000). Cultured chicken and human cell lines revealed

alterations in chromosome territory arrangement during differentiation (Kuroda *et al.*, 2004; Stadler *et al.*, 2004), suggesting that chromatin organization might be linked with gene expression of a particular chromosome.

A chromatin tagging system to analyze the dynamics of tagged chromatin in nuclei of living cells has been adapted to *Arabidopsis* (Kato and Lam, 2001). The vector of this system (EL702) comprises an inducible *GFP::lac::NLS* gene, the hygromycin phosphotransferase gene, the gene expressing the fusion protein of Glucocorticoid receptor-VP16 transcription activation domain-Gal4 DNA binding domain (GVG) and a tandem array of 256 *lac* operator (*lacO*) repeat

units. The transgenic line EL702C carries three copies of this transgene at two loci, 4.2 Mb apart from each other, on the top arm of chromosome 3. The proximal locus contains two transgenes in a tail-to-tail orientation (Kato and Lam, 2003). Within nuclei of the transgenic Arabidopsis line EL702C, we found that homologous pairing occurred more often between the *lacO* arrays than that between endogenous euchromatic regions (Pecinka *et al.*, 2005). Interactions between homologous DNA sequences in somatic cells might be involved in epigenetic regulation of gene expression responsible for phenomena such as transvection (reviewed in Duncan, 2002) or paramutation (for a review see Chandler and Stam, 2004). Introduction of a high copy number of transgenes in some cases mediates inactivation of the transgenes and of their endogenous homolog together with *de novo* DNA methylation of the transgene sequences and/or its association with heterochromatin (Csink and Henikoff, 1996; Davies *et al.*, 1997; Dorer and Henikoff, 1997; Garrick *et al.*, 1998; Park *et al.*, 1996). In the Arabidopsis DNA hypomethylation mutants *decrease in DNA methylation 1* (*ddm1*) and *methyltransferase 1* (*met1*), silenced transgenes (Jones *et al.*, 2001; Morel *et al.*, 2000) and endogenous genes (Kankel *et al.*, 2003; Kato *et al.*, 2003; Singer *et al.*, 2001; Soppe *et al.*, 2000; Xiao *et al.*, 2003) become reactivated by epigenetic regulation. Furthermore, dimethylation of histone H3 at lysine 9 (H3K9me2) at pericentromeric regions and the size of these heterochromatic CCs are both reduced in *ddm1* and *met1* mutant backgrounds as compared with those in wild-type (WT) nuclei (Johnson *et al.*, 2002; Soppe *et al.*, 2002; Tariq *et al.*, 2003). However, it remains unclear whether such chromatin modifications have an impact on the association of homologous sequences and whether chromatin

dynamics could be affected and contributes to the observed changes.

According to the DNA/DNA interaction model (Assaad *et al.*, 1993; Bender, 1998), homologously aligned repetitive sequences are proposed to be susceptible to *de novo* DNA methylation; however, this has not been proven experimentally in somatic plant nuclei. To understand better the relationship between DNA methylation, association between repetitive sequences and chromatin dynamics, we compared these properties between the transgenic loci of the EL702C line and adjacent euchromatic regions in WT Arabidopsis as well as in the hypomethylated genomic background of *ddm1* and *met1*.

Results

The lacO array is heavily methylated in the EL702C line

To compare the methylation status of the inserted repetitive sequences in EL702C plants between WT and mutant backgrounds, T3 EL702C plants were crossed with the DNA hypomethylation mutants *ddm1-2* and *met1-1* and the progeny generated as shown in Figure 1. The DNA methylation state of the *lacO* arrays in the starting T3 EL702C plants and the resultant F2 and F3 progeny from these crosses with various genotypes were analyzed by Southern hybridization with the restriction endonucleases *EcoRI* and *NarI*. Both enzymes can potentially cleave *lacO* repeat arrays into 315 bp fragments. However, *NarI* is sensitive to methylation at the CpG site within its recognition sequence (GGCGCC) while *EcoRI* is not. In T3 EL702C plants, *EcoRI* but not *NarI* could digest the *lacO* arrays of EL702C plants into small DNA fragments (Figure 2), indicating methylation of most of the

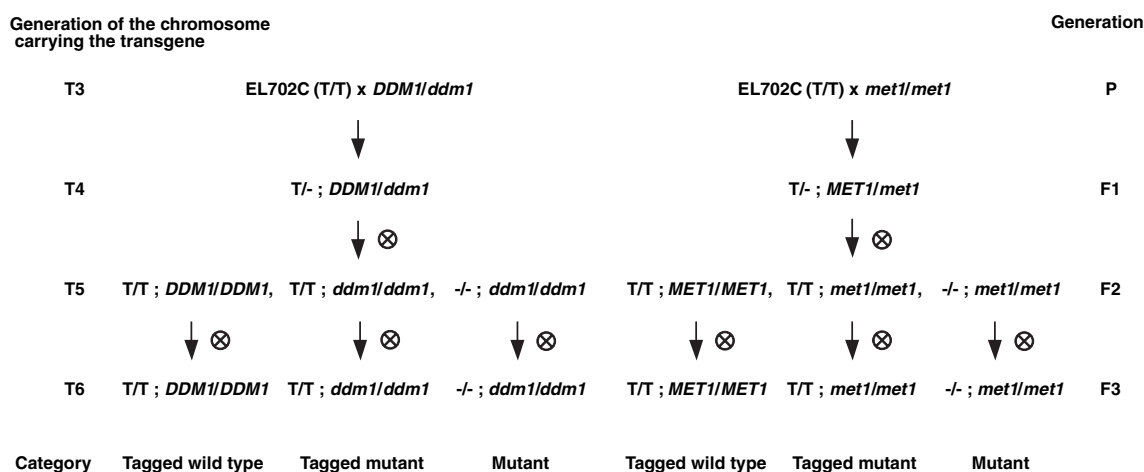


Figure 1. Pedigree of the plants used in this study.

The EL702 transgene was introduced into the mutant plants via crossing of an EL702C plant in the T3 generation with a *decrease in DNA methylation 1* (*DDM1*)/*ddm1* plant or a *methyltransferase 1* (*met1*)/*met1* plant. Plants with the indicated genotypes were identified by restriction fragment length polymorphisms (RFLPs). 'T' on the left side indicates the generation of individuals with the chromosome 3 carrying the EL702 transgenes after transformation has happened. 'T/T' designates the homozygous EL702C transgene loci on chromosome 3.

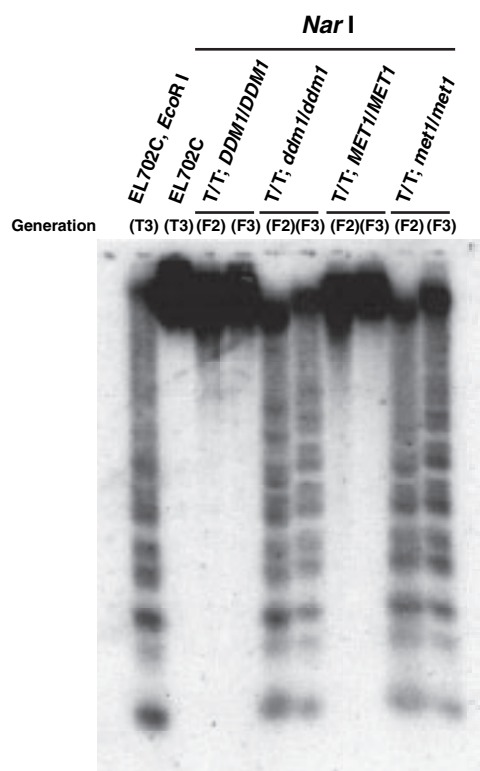


Figure 2. DNA methylation state of the *lacO* array. Genomic DNA samples were prepared from the EL702C plant in the T3 generation and from homozygous *Decrease in DNA Methylation 1* (*DDM1*), *ddm1*, *Methyltransferase 1* (*MET1*) and *met1* individuals in the F2 and F3 generations, digested with *EcoRI* (EL702C only) and *NarI*, and hybridized with the *lacO* sequences. 'T' = EL702C transgene loci on chromosome 3.

CpG sites in the *lacO* arrays. In contrast, the *lacO* arrays in F2 and F3 *ddm1* and *met1* mutant backgrounds ('tagged mutants': *ddm1* and *met1* harboring the transgene) became cleavable by *NarI*, while the *lacO* arrays in *DDM1* and *MET1* sibling plants ('tagged WT's': *DDM1* and *MET1* lines harboring the transgene) in F2 and F3 generations were not (Figure 2), suggesting that methylation at the inserted *lacO* repeat arrays was present in the parental EL702C plants, but became lost in *met1* or *ddm1* backgrounds.

Hypomethylation decreases in vivo association of the transgene loci but does not alter the expression of an array-associated gene

In the EL702C line, the transgene is inserted into two loci at the top arm of chromosome 3 (Kato and Lam, 2003). Because these loci are separated by sufficient length of intervening sequences (4.2 Mbp), binding of expressed GFP-LacI/NLS proteins to the arrays should result in visualization of individual spots. In guard cell nuclei of homozygous EL702C plants, which maintain a relatively constant 2C DNA composition, a maximum of four individual GFP spots might be

resolved. To examine the effects of DNA methylation on the association between *lacO* arrays in interphase nuclei of living plants, the number of GFP spots was counted in guard cell nuclei from F2 and F3 plants that are homozygous for the EL702C transgene positions in *DDM1*, *MET1*, *ddm1* and *met1* backgrounds. Significantly more nuclei with higher spot numbers were found in the tagged mutants ($P < 0.001$; Table 1). The frequency of nuclei with two or more GFP spots is $<30\%$ in the tagged WT plants in the F2 and F3 generations; it increases to $>60\%$ in the tagged mutants in the F3 generation. Apparently, DNA hypomethylation is correlated with a decrease in the association frequency of the tagged transgene *lacO* array. Interestingly, the effects of the *met1* mutation on GFP spot number significantly increased from F2 to F3 generation in contrast to that of the *ddm1* mutation ($P < 0.001$; χ^2 -test; Table 1). This observation suggests that the two genes affected in these mutants could modulate association of the inserted arrays via distinct mechanisms, even though both mutations lead to DNA hypomethylation.

For the same plants used for microscopy studies, the activities of the *GVG* and *GFP-LacI/NLS* transgenes were analyzed by northern hybridization after induction of the *GFP-LacI/NLS* gene with dexamethasone. Figure 3 demonstrates that the levels of *GFP-LacI/NLS* and *GVG* transcripts in the tagged *ddm1* and *met1* mutants are similar to that observed for the *DDM1* and *MET1* siblings. Because the promoter of the *GVG* gene is located next to the *lacO* array in the EL702 transgene, this result implies that the activity of the transgene depends neither on the degree of association of the tagged loci nor on the methylation level of the neighboring *lacO* array.

Homologous pairing of the lacO arrays is reduced in DNA hypomethylation mutants, but is still higher than for average euchromatic regions in wild-type nuclei

Microscopic observations with guard cells of live plants suggest that reduction of DNA methylation in the *ddm1* and *met1* backgrounds may cause a change in the interaction between the tagged chromosome loci. The decreased number of *ddm1* (F2/F3) and *met1* (F3) nuclei showing no GFP spot (in comparison with the WT situation, see Table 1) further suggests that reduced DNA methylation in these mutant backgrounds might turn the *lacO* repeats into more accessible targets for the LacI-GFP/NLS protein through subsequent changes in chromatin organization. This possible increase in LacI-GFP/NLS binding to the *lacO* repeats might also contribute to the increase in the percentage of nuclei with higher frequencies of GFP spots and to the apparent loss of the interaction between *lacO* repeat arrays. Previously, in homozygous transgenic EL702C nuclei, we observed allelic pairing significantly more often for the transgenic *lacO* arrays than for average euchromatic regions in WT nuclei. Furthermore, allelic pairing of *lacO* arrays

Table 1 Average frequencies (%) of nuclei with different numbers of GFP spots in wild-type (WT) and homozygous hypomethylation backgrounds

Strain (generation)	Average number of GFP spots per nucleus					Number of observed nuclei
	0	1	2	3	4	
<i>DDM1</i> (F2)	35.2 ± 1.59	41.7 ± 0.11	14.4 ± 0.91	4.94 ± 0.61	0.813 ± 0.21	120
<i>DDM1</i> (F3)	30.8 ± 2.59	40.8 ± 1.7	24.2 ± 0.76	3.33 ± 0.56	0.833 ± 0.21	120
<i>ddm1</i> (F2)	12.9 ± 0.69	32.5 ± 0.36	39.0 ± 0.19	10.6 ± 0.24	4.94 ± 0.37	123
<i>ddm1</i> (F3)	13.3 ± 1.52	22.5 ± 0.97	42.5 ± 0.37	19.2 ± 0.92	5.83 ± 1.17	120
<i>MET1</i> (F2)	54.6 ± 0.97	39.7 ± 1.14	5.69 ± 0.38	0	0	156
<i>MET1</i> (F3)	35.3 ± 0.68	46.3 ± 1.12	17.6 ± 1.08	0.833 ± 0.21	0	119
<i>met1</i> (F2)	40.1 ± 0.22	43.2 ± 0.88	15.6 ± 0.88	1.10 ± 0.25	0	147
<i>met1</i> (F3)	10.8 ± 0.92	27.5 ± 1.46	46.7 ± 1.38	14.2 ± 0.42	0	120

The values observed in living nuclei ($\pm$ confidence interval at a 95% confident level) were obtained for progenies from three independent F1 plants. The proportions of nuclei with two or more spots in F3 progenies of *ddm1* and *met1* mutants were significantly higher ($P < 0.001$; χ^2 -test) than observed for the corresponding WT with the transgene (*DDM1*, *MET1*).

enhanced the pairing frequency of endogenous regions flanking the transgene, thus altering the local chromatin arrangement within the tagged nuclei (Pecinka *et al.*, 2005). Thus we analyzed by fluorescence *in situ* hybridization (FISH) the effects of reduced DNA methylation on somatic pairing frequencies of *lacO* arrays and of their flanking regions represented by Bacterial Artificial Chromosomes (BACs) MGL6 and F18C1 (Figure 4a) in the *ddm1* and *met1* mutants harboring the transgenes ('tagged mutants'). The pairing of *lacO* arrays and of F18C1 and MGL6 insert regions, respectively, was compared: (i) between the 'tagged mutants' (*ddm1* and *met1* lines harbouring the transgene); (ii) between 'tagged mutants' and 'tagged WT's' (EL702C, *DDM1* and *MET1* lines harboring the transgene); and (iii) between the former two groups ('tagged mutants' and 'tagged WT's') and 'WT and mutants without transgene' (accession Columbia, *met1* and *ddm1*). All lines were studied in the homozygous condition and without dexamethasone treatment (i.e. in the absence of GFP-LacI/NLS proteins). Mutant lines and tagged *DDM1* and *MET1* lines were analyzed in F3 seedlings for the frequencies of homologous pairing using FISH. Flow-sorted 2C nuclei were isolated and probed with *lacO*, F18C1 and MGL6 sequences in order to discriminate between allelic (Figure 4b, top) and ectopic pairing (Figure 4b, middle) of the two *lacO* insertion loci. In addition, allelic pairing frequencies for the F18C1 and MGL6 loci were also quantified. The χ^2 -test revealed, in some cases, significant variation in *lacO* homologous pairing frequencies between individual genotypes within the group of 'tagged WT's'. No such variation was observed within the other groups (i.e. 'tagged mutants', 'WT and mutants without transgene'). To reduce the effects of experimental variation in the apparent pairing frequencies when we ascertain the effects that hypomethylation mutations may have on chromatin pairing, we also present the pooled values for individual lines into the three previously defined groups ('WT and hypomethylation mutants without transgene',

'tagged WT's' and 'tagged hypomethylation mutants'). Next, the mean pairing frequencies of *lacO*, F18C1 and MGL6 for these groups were compared (Table 2). Allelic pairing of *lacO* arrays was significantly more frequent ($P < 0.001$) in 'tagged WT' (30.4%) than in 'tagged mutant' nuclei (15.0%). A similar difference, although to a lesser extent, was observed also for ectopic pairing of *lacO* loci (36.1 versus 26.5%; $P < 0.001$). Because ectopic pairing for *lacO* arrays is more frequent than allelic pairing, intrachromosomal ectopic pairing might be more preferential than pairing between loci on separate chromosomes, in particular within the nuclei of tagged mutants. In spite of a significant reduction compared with 'tagged WT's', allelic pairing of *lacO* was still significantly more frequent within 'tagged mutant' nuclei (15.0%) than observed for the flanking sequences detected with BACs F18C1 and MGL6 within nuclei of 'WT and mutants without transgene' (approximately 5%; $P < 0.001$). The same is true when ectopic pairing of *lacO* loci in 'tagged mutant' nuclei (26.5%) is compared with ectopic association of F18C1 and MGL6 in the 'WT and mutants without transgene' (6.1%; $P < 0.001$). Allelic pairing and ectopic association of F18C1 and MGL6 occurred significantly more often in 'tagged WT' than in nuclei of 'WT and mutants without transgene' ($P < 0.001$). Within the nuclei of 'tagged mutants', allelic pairing of F18C1 and MGL6 was less frequent than in the 'tagged WT's' ($P < 0.001$) but still more frequent than in nuclei of 'WT and mutants without transgene'. The association frequency of F18C1 with MGL6 did not significantly differ between the 'tagged mutant' (12.6%) and 'tagged WT' nuclei (10.7%; $P > 0.05$) but occurred significantly more often in both cases than in nuclei of 'WT and mutants without transgene' (6.1%; $P < 0.001$).

These data show that, on the one hand, the decreased level of DNA methylation in *ddm1* and *met1* mutants often correlates with lower frequencies of allelic pairing of the *lacO* arrays and with a reduced 'dragging effect' exerted by transgene pairing on the flanking regions in the tagged lines.

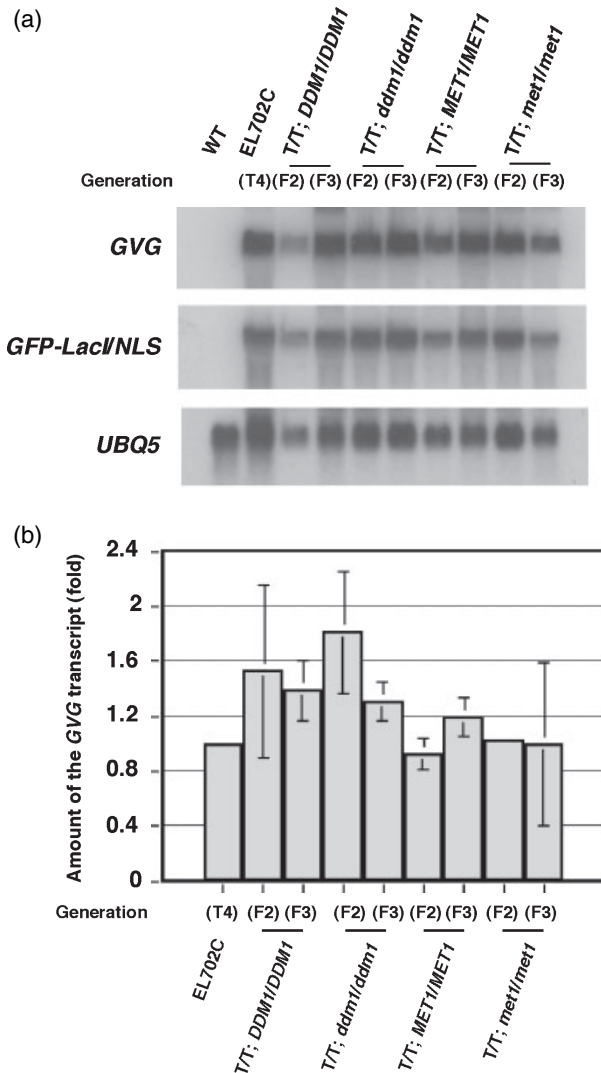


Figure 3. Expression analysis of the EL702 transgene ORFs by Northern hybridization.

(a) Total RNA samples were prepared from the leaves after induction of the *GFP-LacI/NLS* gene and hybridized with labeled *GVG*, *GFP-LacI/NLS*, or *UBQ5* (for control) probes and in (b) the level of *GVG* transcript in each line was compared with that of the homozygous EL702C plants (T4). The accumulation level of the *UBQ5* transcript of each sample was used for normalization. The hybridization experiment was repeated twice with similar results.

On the other hand, decreased DNA methylation has no obvious effect on the pairing of endogenous euchromatic regions because there were no significant differences as to the allelic pairing and ectopic association frequencies for F18C1 and MGL6 between 'WT and mutants without transgene' (for all comparisons $P > 0.05$).

Hypomethylation does not reduce the co-localization of lacO arrays with heterochromatic chromocenters

Repetitive DNA sequences make up approximately 15% of the *Arabidopsis* genome and are located primarily at

Nucleolus Organizer Regions (NORs) and (peri-)centromeres (*Arabidopsis* Genome Initiative, 2000) and form the intensely 4'-6-diamino-2-phenylindole (DAPI)-stained CCs of interphase nuclei (Fransz *et al.*, 2002). The *lacO* arrays of EL702C nuclei (located within euchromatin) co-localize with heterochromatic CCs approximately fourfold more often than endogenous euchromatic regions (Pecinka *et al.*, 2005). Here, we analyzed the association frequency of *lacO* arrays and of the flanking regions with CCs in *ddm1* and *met1* mutants by FISH with *lacO*, F18C1 and MGL6 sequences on flow-sorted 2C nuclei. Because CCs are often not easily distinguished by DAPI staining in flow-sorted nuclei, we applied FISH with 45S rDNA and the *Arabidopsis thaliana* 180-bp centromeric repeat (pAL) in order to distinguish CCs unambiguously (Fransz *et al.*, 2002; Martinez-Zapater *et al.*, 1986). There were some variations as to the co-localization frequency of *lacO* arrays with CCs between individual genotypes within the groups of 'tagged WT's and 'tagged mutants'. Nevertheless, the mean values for *lacO* co-localization with CCs revealed no significant differences when 'tagged WT' (29.2%) and 'tagged mutant' (36.7%) nuclei were compared ($P > 0.05$; Table 3). In all tested genotypes, *lacO* arrays co-localized with CCs significantly (twofold to sevenfold) more often than F18C1 and MGL6 sequences ($P < 0.001$). The frequency of co-localization of F18C1 and MGL6 with CCs (8.6–9.8%) did not significantly differ between any of the analyzed genotypes ($P > 0.05$). Thus, the reduced DNA and histone methylation (Johnson *et al.*, 2002; Tariq *et al.*, 2003) and the reduced size of CCs in *ddm1* and *met1* mutants (Soppe *et al.*, 2002) has no apparent impact on the high co-localization frequency of the tandem repetitive DNA sequences with CCs. This shows that DNA methylation at the inserted *lacO* arrays is not an important determinant for its frequent association with CCs.

The motility of GFP-tagged loci is not altered in hypomethylation mutants

A constrained movement of the tagged loci with a larger confinement area in 8C than in 2C nuclei of the EL702C line was previously reported (Kato and Lam, 2003). In *ddm1* and *met1* nuclei, pericentromeric sequences loop out from heterochromatin and acquire euchromatic features, thus reducing the size of CCs (Soppe *et al.*, 2002). To examine whether the decreased methylation of *lacO* arrays has an effect on their mobility in the interphase nucleus, the GFP-tagged loci were observed using time-lapse microscopy and the distances between GFP spots over time were analyzed in nuclei of homozygous EL702C plants in either the *ddm1* or *met1* mutant background. To characterize chromatin movement of the tagged loci in these mutant backgrounds, the mean square change in distance between two GFP-tagged loci (Δd^2) was plotted as a function of increasing time interval (Δt ; Figure 5). In all cases, we found a plateau was reached

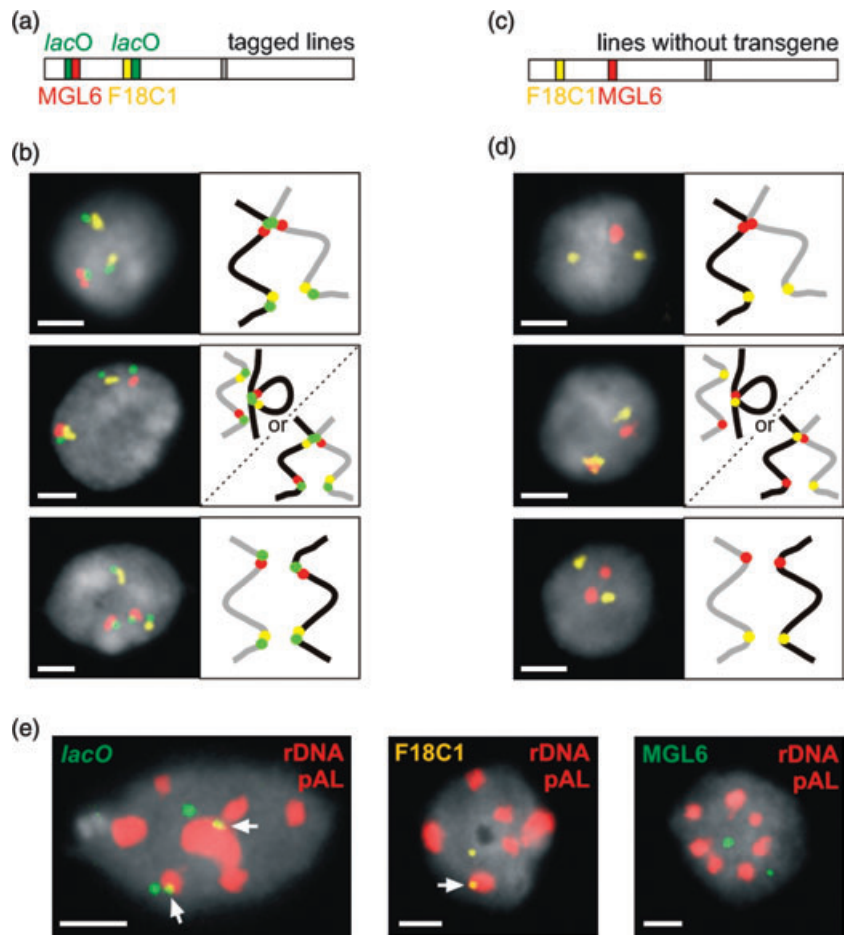


Figure 4. Pairing of the *lac* operator (*lacO*) loci, of the flanking regions F18C1 and MGL6 and their co-localization with heterochromatic chromocenters (CCs). (a, c) Scheme of chromosome 3 in the tagged lines (a) and in the lines without transgene (c). The inverse arrangement of F18C1 and MGL6 in (a) and (c) is due to a paracentric inversion between the transgene integration sites (Pecinka *et al.*, 2005). (b) Selected nuclei of the homozygous EL702C/*decrease in DNA methylation 1* (*ddm1*) line showing allelic pairing of the distal *lacO* locus (top), ectopic pairing of one distal and one proximal locus (middle) and separation of all loci (bottom). (d) Examples of homozygous *ddm1* nuclei without transgene showing homologous pairing of MGL6 (top), ectopic association of MGL6 and F18C1 (middle) and separation of both loci (bottom). (e) Nuclei of the homozygous EL702C/*ddm1* line after fluorescence *in situ* hybridization (FISH) with the 45S rDNA and centromeric repeat (pAL) probes to label the CCs, and the *lacO*, F18C1 and MGL probes, respectively. Selected nuclei showing: two (arrows) out of four *lacO* signals co-localized with CCs (left), one (arrow) out of two signals for F18C1 co-localized with a CC (middle) and both signals for MGL6 localized outside CCs (right). Bars, 2 μ m.

Table 2 Pairing frequency of inserted *lac* operator (*lacO*) arrays and/or of the flanking BAC insert regions in wild-type (WT) or hypomethylation backgrounds

Group	Genotype	<i>lacO</i>			F18C1		MGL6		F18C1 + MGL6	
		<i>n</i>	Allelic p. (%)	Ectopic p. (%)	<i>n</i>	Allelic p. (%)	<i>n</i>	Allelic p. (%)	<i>n</i>	Ectopic assoc. (%)
WT and mutants without transgene	Columbia WT ^a				306	4.6	306	3.9	612	6.0
	F3 -/- <i>ddm1/ddm1</i>				186	5.4	186	6.4	372	5.9
	F3 -/- <i>met1/met1</i>				186	4.3	186	5.4	372	6.4
	Σ				678	4.8	678	5.2	1356	6.1
Tagged WT	T/T EL702C ^a	248	33.9	26.6	124	14.5	124	9.7	248	9.3
	F3 T/T <i>DDM1/DDM1</i>	244	24.6	33.2	122	18.0	122	16.4	244	10.2
	F3 T/T <i>MET1/MET1</i>	248	32.8	48.4	124	16.4	124	18.0	248	12.7
	Σ	740	30.4	36.1	370	16.3	370	14.7	740	10.7
Tagged mutants	F3 T/T <i>ddm1/ddm1</i>	244	15.3	23.8	122	8.1	122	9.7	244	11.7
	F3 T/T <i>met1/met1</i>	256	14.8	29.3	128	6.2	128	7.8	256	13.6
	Σ	500	15.0	26.5	250	7.1	250	8.7	500	12.6

Tagged WT: transgenic repeats in WT background. Tagged mutants: transgenic repeats in hypomethylated backgrounds. p, pairing; F3, third generation; -, without transgene; T, transgene; *n*, number of loci analyzed by fluorescence *in situ* hybridization (FISH).

^aPrevious data from Pecinka *et al.* (2005).

after 3 to 4 min, which indicates that movement of the tagged loci is constrained. The height of the plateau reflects the confinement area of chromatin movement, and our results indicate that the nuclear space within which the tagged loci

can move is almost the same between WT and the hypomethylation mutant plants (Figure 5). Thus, the mobility of GFP-spots in mutant nuclei showed no significant difference from that of the EL702C construct in WT nuclei (*t*-test,

Table 3 Co-localization of the *lacO* operator (*lacO*) arrays and of the flanking BAC insert regions with heterochromatic chromocenters (CCs)

Group	Genotype	<i>lacO</i>		F18C1		MGL6	
		<i>n</i>	Co-localized with CCs (%)	<i>n</i>	Co-localized with CCs (%)	<i>n</i>	Co-localized with CCs (%)
WT and mutants without transgene	Columbia WT ^a			124	10.5	132	11.4
	F3 <i>-/- ddm1/ddm1</i>			186	9.7	186	9.1
	F3 <i>-/- met1/met1</i>			186	9.1	186	8.6
	Σ			496	9.8	504	9.7
Tagged WT	T/T EL702C ^a	164	36.6	82	8.5	82	11.0
	F3 T/T <i>DDM1/DDM1</i>	376	21.5	180	8.3	170	7.6
	F3 T/T <i>MET1/MET1</i>	372	29.6	184	9.1	182	8.6
	Σ	912	29.2	446	8.6	434	9.1
Tagged mutants	F3 T/T <i>ddm1/ddm1</i>	372	22.6	186	9.8	186	9.3
	F3 T/T <i>met1/met1</i>	372	50.8	186	8.1	180	8.9
	Σ	744	36.7	372	8.9	366	9.1

Tagged wild types (WTs): transgenic repeats in WT background. Tagged mutants: transgenic repeats in hypomethylated backgrounds. Co-localized with CCs: percentage of loci found to be co-localized with heterochromatic CCs (i.e. pericentromeres/NORs). F3, third generation; -, without transgene; T, transgene; *n*, number of loci analyzed by fluorescence *in situ* hybridization (FISH).

^aPrevious data from Pecinka *et al.* (2005).

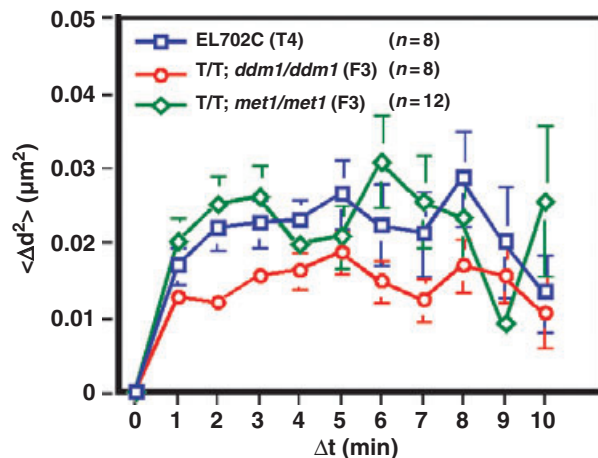


Figure 5. The dynamics of GFP-tagged chromosome loci in nuclei of the hypomethylation mutants.

The distance between two GFP spots per nucleus was monitored by time-lapse microscopy. The average value of mean square change of the distance $\langle \Delta d^2 \rangle$ (μm^2) was plotted against the time interval (Δt ; min; vertical bars represent the standard error).

$P > 0.05$). This indicates that the movement of the tagged loci is not significantly affected by the level of DNA methylation, at least in 2C guard cell nuclei of Arabidopsis.

Discussion

Our results show that the inserted *lacO* repeat arrays, heavily methylated in the WT background, are largely demethylated by the second generation in the *ddm1* or *met1* backgrounds. In parallel, the appearance of a higher proportion of nuclei with two or more GFP-fusion protein tagged spots indicates decreased association of tagged loci

in living nuclei with decreasing DNA methylation of the repeat arrays. Interestingly, whereas similar demethylation of the *lacO* arrays was detected in the F2 generation for *met1* and *ddm1* mutant lines, a higher number of GFP spots per nucleus was not apparent in the *met1* mutant until the F3 generation. Because MET1 is a Dnmt1-type DNA methyltransferase (Ronemus *et al.*, 1996), whereas DDM1 is a chromatin remodeling factor (Brzeski and Jerzmanowski, 2003), it seems possible that reduction of 5-methyl cytosine at CpG sites alone is not enough to release the association of allelic repeat arrays. In *ddm1* plants, the loss of chromatin remodeling activity of DDM1 may result in an aberrantly organized chromatin, and therefore reduction of DNA methylation and dissociation of the transgene loci may occur rapidly together. In *met1* plants, the reorganization of chromatin affecting the interaction between the transgene loci occurs only after the decrease in DNA methylation has occurred. This may explain the difference in behavior of the repeat arrays in the F2 and F3 generations with respect to the two types of mutations that affect DNA methylation.

The arrangement of FISH signals for *lacO* repeats and for the flanking regions shows that allelic and ectopic pairing of the *lacO* loci is significantly reduced in the F3 generation of both hypomethylation mutant backgrounds in comparison to the 'tagged WT's. Nevertheless, *lacO* pairing in these hypomethylation backgrounds still occurs significantly more often than at random. Random association of average euchromatic regions occurs typically in approximately 5% of WT or mutant nuclei without the transgene (Pecinka *et al.*, 2004, 2005). Therefore, it is likely that, for the lower than maximum number of GFP-spots in living WT nuclei, allelic and ectopic pairing of *lacO* arrays is primarily responsible. The remaining methylated repeat units in *ddm1* and *met1*

background (i.e. the *NarI* insensitive repeat arrays shown in the Southern blot of Figure 2) may explain why homologous pairing of the *lacO* loci is still significantly more frequent than pairing of the flanking regions in 'WT and mutants without transgene'. The flanking regions show an increased pairing frequency (as a consequence of a 'dragging' effect) reflecting the behavior of their neighboring *lacO* repeat arrays. Disruption of allelic and ectopic pairing of multiple homologous transgenes has also been observed in wheat after 5'-azacytidine treatment (Santos *et al.*, 2002) and therefore seems to be a general phenomenon, at least in plants.

Contrary to its effect on allelic association of transgene repeats, reduced methylation of the *lacO* repeat units does not seem to affect the transcriptional activity of the adjacent transgene open reading frames (ORFs) compared with the level observed in tagged WT plants. Also, reduced DNA methylation apparently has no significant impact on the motility of the tagged loci nor does it decrease the (non-homologous) high association frequency of *lacO* arrays with heterochromatic CCs.

In summary, our work demonstrates that inserted repetitive DNA sequences in the *Arabidopsis* genome lead to their methylation that depends on DDM1 and MET1. This methylation is correlated with a high frequency of allelic and ectopic pairing between these repetitive sequences, although other determining factors affected in *ddm1* but not in *met1* mutant background are likely contributing to this pairing as well. Because the motility of the inserted repeat loci is apparently not affected by their methylation status, the changes in association frequency of the repeat arrays in the hypomethylation mutant backgrounds is likely not the result of an alteration in the dynamic properties due to reduced DNA methylation of these inserted repeats. The inserted repetitive DNA sequences are also found to frequently associate with CCs, irrespective of their methylation status. This observation clearly indicates that there exist mechanisms, distinct from DNA methylation, through which repetitive sequences, such as the *lacO* tandem arrays, could be guided to CCs. Further studies with our transgenic system may help to uncover the mechanisms through which repetitive DNA may induce localized DNA methylation along with heightened frequencies of homologous association between these sequences. Studies with this transgenic system may also aid in the future characterization of factors involved in the formation of CCs.

Experimental procedures

Plant material

An EL702C plant (Kato and Lam, 2001) in T3 generation was crossed with a *DDM1/ddm1* plant and a *met1/met1* plant of *Arabidopsis thaliana* in the Columbia accession. The progeny from these crosses (Figure 1) were genotyped on the basis of polymorphic DNA

fragments. To identify the *ddm1-2* and the *met1-1* mutation, the amplified DNA fragments were digested and the restriction fragment length polymorphisms were detected on agarose gels after electrophoresis as described (Kankel *et al.*, 2003). To screen plants carrying the EL702 transgene at the centromere-distal site on chromosome 3, two PCR primers from outside the integration site (EL1202, 5'-GCCAAACGAGATCACATACATATCTTGAT-3'; EL1204, 5'-AGATTAGGAAAGCGTGAGAGTAAACCTTA-3') and a third one with homology to the T-DNA of the vector (EL1080, 5'-GGGATCCTGTACTCCACCAAAGAAGAAGAG-3') yielded a 1.4- and a 3.2-kb fragment from genomic DNA of plants carrying the transgene loci on chromosome 3 of the line EL702C. A DNA fragment of 2.1 kb is amplified with primers EL1202 and EL1204 from WT plants.

Microscopic observation of living nuclei

A Delta Vision restoration microscope system (Applied Precision, Issaquah, WA, USA) equipped with a TE200 microscope (Nikon, Tokyo, Japan) was used to observe live cell nuclei in true leaves of 2–3 week old plants. Forty images at 0.2 μm z-axis steps were collected using a Nikon PlanApo 60 $\times$ 1.2 NA water-immersion objective lens. The exposure time was 0.3 sec and appropriate filters (Chroma, Rockingham, VT, USA) were used. The images were deconvolved based on a set of point-spread function data.

The sample leaves were treated with 0.3 μM dexamethasone (DEX) solution on agar plates for 10–12 h to induce the expression of GFP-LacI/NLS fusion protein. After treatment, the leaves were placed between two coverslips under water for observation. The background-to-signal (free versus bound GFP fusion protein) ratio is important for detection of GFP spots at *lacO* arrays. GFP spots were defined as before (Kato and Lam, 2003). The stacked images of nuclei were analyzed for GFP spots by *SOFTWORX* software (Applied Precision, Mountain View, CA, USA) on an Octance Workstation (Silicon Graphics, CA, USA).

Measuring chromatin movement

A single 37-frame z stack, with a 0.2 μm z interval was collected every minute, for 10 min, with a scan time of 0.3 sec. The distance between two GFP spots was measured by *SOFTWORX* software. The step size was calculated for the time interval of 1 min and the mean square change in distance, ' Δd^2 ', was calculated for all time intervals according to the formula: $\Delta d^2 = [d(t) - d(t + \Delta t)]^2$ (Vazquez *et al.*, 2001). As values of the time interval increase, the number of data points used for calculating ' Δd^2 ' decreases and results in increasing errors in ' Δd^2 ' value (Vazquez *et al.*, 2001).

Southern and Northern hybridization

Genomic DNA was isolated from leaves by using Nucleon Phyto-Pure from Plant DNA Extraction Kit (Amersham, Piscataway, NJ, USA) as described by the manufacturer. Digested DNA samples were electrophoresed on agarose gels and blotted onto a nylon membrane (Zeta-Probe GT Genomic Tested Blotting Membrane; Bio-Rad, Hercules, CA, USA). Total RNA was prepared from leaves treated with 3.0 μM dexamethasone for 12 h by using the RNeasy plant mini kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions. RNA (10 μg per sample) was electrophoresed and transferred to a Hybond N⁺ nylon membrane (Amersham). Hybridization was performed in ExpressHyb hybridization solution

(Clontech, Mountain View, CA, USA) according to manufacturer's instructions. Hybridization probes were generated from cloned inserts of: *lacO* repeat, 40mer *lacO*/SK; *GVG* gene, pTA7002 (Aoyama and Chua, 1997); *GFP-LacI/NLS* gene, pEL700 (Kato and Lam, 2001); *ubiquitin 5 (UBQ5)* gene, pCH1-002. The intensities of hybridization signals on Northern blots were measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Preparation of nuclei, probe labeling and fluorescence in situ hybridization

Young leaves were fixed for 20 min in 4% formaldehyde in TRIS buffer (100 mM TRIS-HCl, pH 7, 5 mM MgCl₂, 85 mM NaCl, 0.1% Triton X100) under vacuum. After homogenization in TRIS buffer, suspension was filtered through a 35-µm nylon mesh and the nuclei were stained with DAPI (1 µg ml⁻¹). 2C nuclei were flow-sorted onto microscopic slides using an FACStar^{Plus} flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an Argon-ion laser (Coherent, Santa Clara, CA, USA) emitting UV light as described (Pecinka *et al.*, 2004).

The following DNA clones were used as probes: BAC MGL6 (GenBank accession no. AB022217), BAC F18C1 (accession no. AC011620), BAC T15P10 containing 45S rDNA sequence (accession no. AF167571), plasmid 128x *lacO*-SK (Kato and Lam, 2001) and plasmid pAL1 (Martinez-Zapater *et al.*, 1986). BAC and plasmid DNA was labeled by nick translation (Ward, 2002). The BAC DNA was precipitated and resuspended in 20 µl of hybridization buffer (50% formamide, 10% dextran sulphate, 2× SSC, 50 mM sodium phosphate, pH 7.0) per slide. After mounting the probe, the slides were placed on a heat block at 80°C for 2 min and then incubated in a moist chamber at 37°C for approximately 12 h. Post-hybridization washes and detection steps were as described (Schubert *et al.*, 2001). In brief, biotin-(2'-Deoxyuridine 5'-thiophosphate) (dUTP) was detected by avidin conjugated with Texas Red (1:1000; Vector Laboratories, Burlingame, CA, USA), goat-anti-avidin conjugated with biotin (1:200; Vector Laboratories) and again with avidin conjugated with Texas Red, digoxigenin-dUTP by mouse-anti-digoxigenin (1:250; Roche, Mannheim, Germany) and goat-anti-mouse conjugated with Alexa 488 (1:200; Molecular Probes, Eugene, OR, USA), 2,4-dinitrophenol-dUTP (DNP-dUTP) by rabbit-anti-DNP (1:400; Sigma, Saint Louis, MO, USA) and goat-anti-rabbit conjugated with Cy5 (1:100; Jackson Laboratories, West Grove, PA, USA), Cy3-dUTP was observed directly. Nuclei and chromosomes were counterstained with 1 µg ml⁻¹ of DAPI in Vectashield mounting medium (Vector Laboratories).

Microscopic evaluation and image processing

Fluorescence signals in flow-sorted 2C nuclei were analysed using an Axioplan 2 (Zeiss, Jena, Germany) epifluorescence microscope with a 100×/1.4 Zeiss plan apochromat objective. Images were acquired with METAVIEW (Universal Imaging) software and a cooled charge-coupled device camera (Spot 2e; Diagnostic Instruments, Sterling Heights, MI, USA) separately for each fluorochrome using the appropriate excitation and emission filters. The monochromatic images were pseudocoloured and merged using Adobe PHOTOSHOP 6.0 (Adobe Systems) software. A spatial overlap of compact spheric FISH signals of homologous and/or heterologous sequences was regarded as homologous pairing and heterologous association, respectively. Allelic versus ectopic pairing of transgenic loci was distinguished on the basis of FISH signals obtained from differently labeled BACs that contain sequences flanking the respective transgene insertion loci.

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