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Interplay between Two Epigenetic Marks: DNA Methylation and Histone H3 Lysine 9 Methylation

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Summary

Background: The heterochromatin of many eukaryotes is marked by both DNA methylation and histone H3 lysine 9 (H3-K9) methylation, though the exact relationship between these epigenetic modifications is unknown. In Neurospora, H3-K9 methylation is required for the maintenance of all known DNA methylation. In *Arabidopsis*, H3-K9 methylation directs some of the CpNpG and asymmetric methylation. However, it is not known in any organism whether DNA methylation may also direct histone H3 methylation.
Results: Using chromatin immunoprecipitation (ChIP) assays, we show that *Arabidopsis* heterochromatin is associated with H3-K9 methylation. This histone methylation is dependent on the *KRYPTONITE* and *DDM1* genes (*SU[VAR]3-9* and *SWI2/SNF2* homologs, respectively). We also find that a decrease in DNA methylation does not directly cause a loss of H3-K9 methylation. Instead, a decrease in H3-K9 methylation is only seen at loci where transcription is derepressed.
Conclusions: We conclude that DNA methylation does not control the methylation of histone H3-K9. We propose that loss of H3-K9 methylation is due to transcriptional reactivation, coupled with deposition of unmethylated nucleosomes. These findings are consistent with recent observations of DNA replication-independent deposition of histone H3.3 in *Drosophila*. Our results also suggest that, in *Arabidopsis*, DNA methylation is sufficient for gene silencing, but H3-K9 methylation is not.

Introduction

Heterochromatic regions of the genome, located primarily in centromeres and telomeres, are generally characterized by increased chromatin condensation and decreased access to regulatory proteins [1]. Many repetitive genes, transposable elements, imprinted genes, and transgenes are silenced in a sequence-independent manner and have some or all of the characteristics of heterochromatin [2]. Heterochromatin is stably inherited and thus must contain one or more epigenetic marks to direct its maintenance during cell division [1]. Two such marks have been under intense scrutiny: DNA methylation [3] and histone H3-K9 methylation [4].

DNA methylation is found most often at symmetrical CpG sites, but it is also found at asymmetric sites and, in plants, at CpNpG sites. Once established, DNA methylation is inherited through mitosis, and often through meiosis, and this provides an effective epigenetic mark [5]. H3-K9 methylation is also associated with heterochromatin and provides a binding site for the heterochromatin protein 1 (HP1) [6–9]. H3-K9 methylation is carried out by the SU(VAR)3-9 class of proteins [8, 10] and is correlated with silenced genes in mammals [11], chickens [12], *Schizosaccharomyces pombe* [8], and *Neurospora crassa* [13]. Several lines of evidence suggest that H3-K9 methylation is an early event in the formation of heterochromatin: in humans, H3-K9 methylation has been shown to mark the X chromosome shortly after coating with Xist RNA and prior to gene inactivation [14]. This is followed much later by DNA methylation [15]. In Neurospora, it has been shown that all DNA methylation is dependent on H3-K9 methylation, suggesting that methylation of H3-K9 occurs prior to DNA methylation [13]. Similarly, in *Arabidopsis*, CpNpG methylation is partially dependent on H3-K9 methylation [16]. It has been proposed that these two epigenetic marks may signal to one another to ensure propagation of the silenced state [1]. However, it is still not known whether DNA methylation can actually direct H3-K9 methylation or whether DNA methylation can act independently to silence gene expression in the absence of H3-K9 methylation.

Arabidopsis serves as an ideal system in which to examine these questions, as many viable mutants that affect DNA methylation have been isolated. One of the first mutations isolated was in the *SWI2/SNF2* homolog, *DDM1* (decrease in DNA methylation), encoding a putative chromatin remodeling protein [17, 18]. Mutations in this gene result in the loss of approximately 70% of total DNA methylation. More recently, loss-of-function mutations in a *SU(VAR)*3-9 homolog, *KRYPTONITE* (*KYP*), were isolated in a suppressor screen for reactivation of hypermethylated and silenced *superman* (*sup*) alleles [16]. The KYP protein was shown to methylate H3-K9 in vitro, and *kyp* mutants were found to reduce the overall levels of CpNpG methylation in vivo. Two mutations in DNA methyltransferases are also available: *met1*, a CpG methyltransferase that is homologous to mammalian *Dnmt1* [19], and *cmt3*, a chromomethyltransferase that is important for CpNpG and asymmetric methylation [20, 21].

In this paper, we use chromatin immunoprecipitation (ChIP) assays to show that methylation of histone H3-K9 is preferentially localized to heterochromatin in plants. We also show that a mutation in the histone methyltransferase gene, *KYP*, reduces methylation of H3-K9 at all loci tested: the centromeric 180-bp repeats, the *Ta3* and *Ta2* retrotransposons, and the hypermethylated *SUP* gene. Histone H3-K9 methylation is also reduced in lines carrying a mutation in the *SWI2/SNF2* homolog, *DDM1*. Finally, we find that the reduction of DNA methylation itself does not lead to a reduction in

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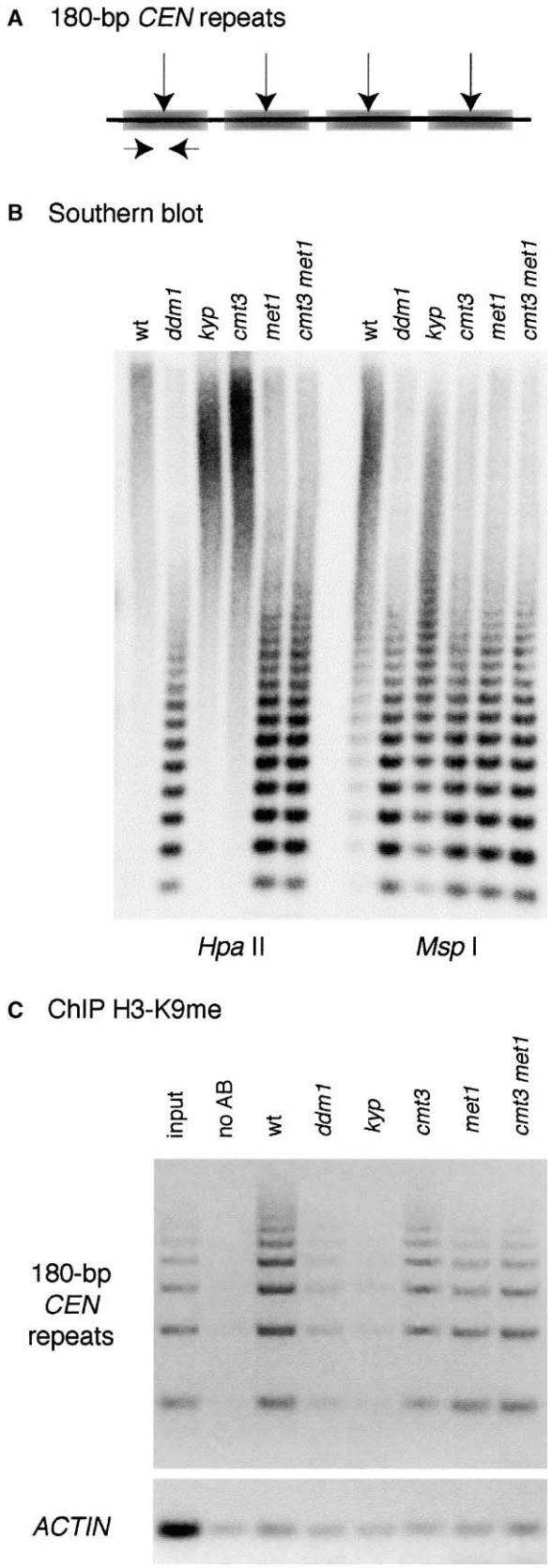

H3-K9 methylation. However, when coupled with an increase in transcription, a loss of methylated H3-K9 is observed.

Results and Discussion

DNA Methylation at the Centromeric 180-bp Repeats

We have analyzed the centromeric 180-bp repeats [22–24] in the four DNA methylation-deficient lines described above and in a newly isolated *cmt3 met1* double mutant (see the Experimental Procedures). The centromeric repeats are found as tandem arrays of related sequences that span the core centromeres of all five chromosomes. They have been shown to be heterochromatic based on decreased recombination frequencies, increased condensation, and high levels of CpG and CpNpG methylation [24, 25]. Using reverse transcription PCR (RT-PCR) analysis, we did not detect transcription of centromeric repeat sequences in wild-type or in any of the DNA methylation-deficient mutant lines in poly-A RNA, and we only detected very low levels in total RNA preparations (data not shown). Therefore, the centromeric repeats serve as excellent loci in which to study the relationship between histone methylation and DNA methylation without the complication of transcriptional effects.

As diagrammed in Figure 1A, one HpaII/MspI site (5′-CCGG-3′) exists within most repeat elements. HpaII is inhibited by the methylation of either C, allowing for detection of CpG and CpNpG methylation, whereas MspI is only inhibited by the methylation of the first C, allowing for detection specifically of CpNpG methylation. As shown in Figure 1B, the wild-type strain (wt) is highly methylated at CpG sites and moderately methylated at CpNpG sites. This is consistent with the 71% CpG methylation and 38% CpNpG methylation found by bisulfite sequencing [20]. Lines homozygous for either *cmt3* or *kyp* have no effect on CpG methylation but reduce CpNpG methylation, with *cmt3* removing essentially all CpNpG methylation and *kyp* causing a partial reduction (Figure 1B; also see [16]). The *ddm1*, *met1*, and *cmt3 met1* mutants eliminate almost all CpG and CpNpG methylation (Figure 1B; also see [17] and [26]). These mutant lines, therefore, provided us with a range of DNA methylation phenotypes at the centromeric repeats.

Figure 1. Analysis of the Centromeric 180-bp Repeats

(A) A schematic diagram of 180-bp centromeric repeats. The arrow above each repeat indicates a single HpaII/MspI restriction site. The arrows below the repeats indicate primers that bind to the repetitive DNA generating a PCR ladder.

(B) DNA from each of the indicated lines was digested with either HpaII or MspI and was probed with the 180-bp centromeric repeat probe.

(C) ChIP analysis of mutant lines was performed with dimethyl-lysine 9 histone H3 (H3-K9me) antibodies. Primers specific for either *ACTIN* (lower panel) or 180-bp repeats were used. PCR reactions (180 bp) were stopped after 23 cycles, while *ACTIN* was allowed to go to 36 cycles. "No AB" refers to the no antibody control.

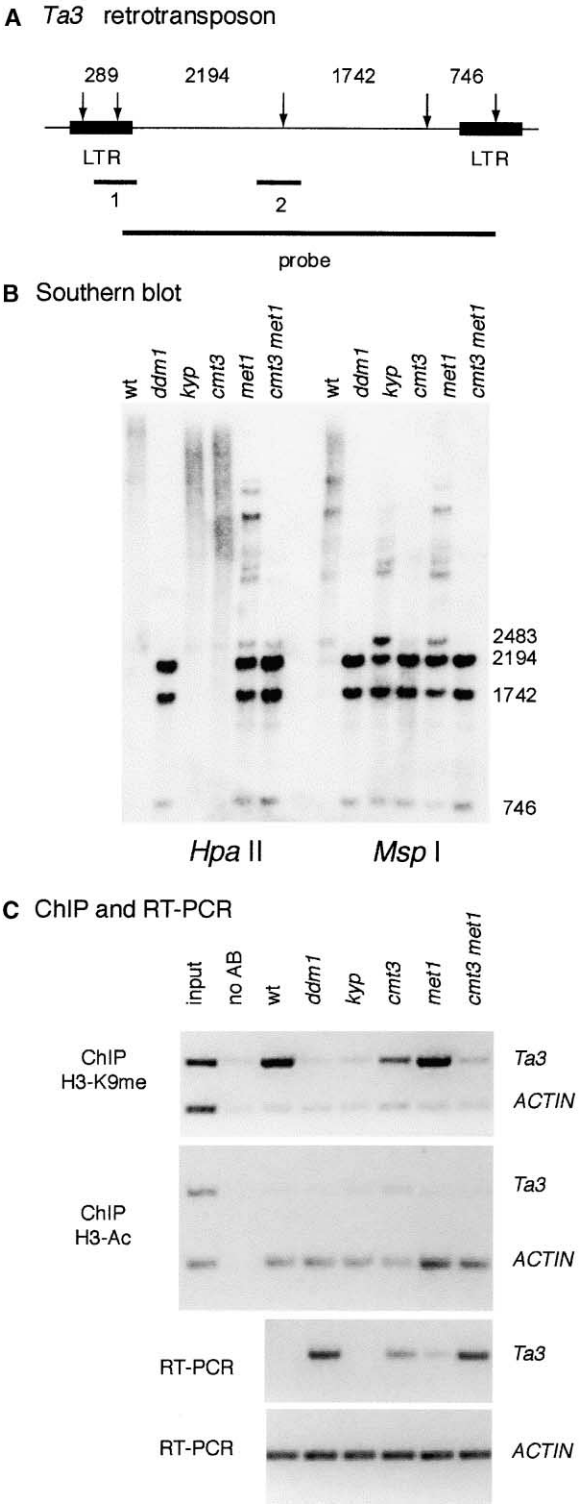

Figure 2. Analysis of the *Ta3* Retrotransposon

(A) A schematic diagram of *Ta3*. The arrows indicate HpaII/MspI restriction sites, with the sizes of fragments shown in bp. The *Ta3* probe spanned the 2194-, 1742-, and 746-bp fragments. Two regions were amplified for ChIP analysis, the left LTR (region 1) and the middle portion (region 2).

(B) A Southern blot comparing DNA methylation at *Ta3* for each of the mutant lines.

(C) Results of ChIP assays using either dimethylated H3-K9 antibodies or acetylated H3 (H3-Ac) antibodies, and results of RT-PCR from the same mutant lines. ChIP PCR reactions were stopped after 34 cycles.

Histone H3-K9 Methylation Marks Heterochromatin Independent of DNA Methylation Levels

To address the relationship between DNA methylation and histone modifications in heterochromatin, we used chromatin immunoprecipitation (ChIP) assays. DNA precipitated with dimethylated H3-K9 antibodies in the different *Arabidopsis* mutants was first normalized by using a primer pair specific for the 5′ end of *ACTIN 2/7* (a constitutively expressed gene assumed to be euchromatic, [27]), as shown in the lower panel of Figure 1C. Next, using a primer pair specific for the centromeric repeat (which generates a ladder due to the multiple sites to which the primers can bind), we found a strong enrichment of H3-K9 methylation over the no antibody control in the wild-type strain (Figure 1C). This result shows that H3-K9 methylation marks heterochromatin in plants as it does in fungi and animals. Furthermore, much of this H3-K9 methylation is dependent on the *KRYPTONITE* histone methyltransferase gene, as *kyp* mutant lines show greatly reduced levels of H3-K9 methylation (Figure 1C).

Examination of the centromeric repeats in the DNA methyltransferase mutants (*cmt3*, *met1*, and the *cmt3 met1* double mutant) revealed little effect on the level of H3-K9 methylation. This suggests that a decrease in either CpNpG methylation or CpG methylation or both does not reduce H3-K9 methylation. Hence, while it is possible that residual levels of DNA methylation remaining in *met1 cmt3* mutants are sufficient to maintain H3-K9 methylation, our data do not support the hypothesis that a direct feedback loop exists between DNA methylation and H3-K9 methylation.

Finally, we examined the *ddm1* mutant line, which reduces both CpG and CpNpG methylation at the centromeric repeats (Figure 1B). We found that the *ddm1* mutation caused a significant reduction in the amount of H3-K9 methylation (Figure 1C). Since DDM1 shows sequence similarity to SWI2/SNF2 proteins, this suggests that chromatin remodeling may be critical for the maintenance of H3-K9 methylation. This result also raises the question of whether DDM1 might have a primary role in maintaining H3-K9 methylation, a primary role in maintaining DNA methylation, or is independently affecting both processes. If DDM1 only aids in the methylation of H3-K9, then loss-of-function mutants in *ddm1* should mimic the *kyp* mutants. However, *kyp* has a very different phenotype than *ddm1* since *kyp* eliminates most H3-K9 methylation but only has an intermediate effect on CpNpG methylation and has no effect on CpG methylation. On the other hand, if DDM1 is only necessary for DNA methylation, *ddm1* alleles should mimic the *cmt3 met1* double mutant. However, we found that *cmt3 met1* double mutants have little effect on H3-K9 methylation but drastically reduce DNA methylation. Thus, these data suggest that DDM1 plays an independent role in both histone methylation and DNA methyla-

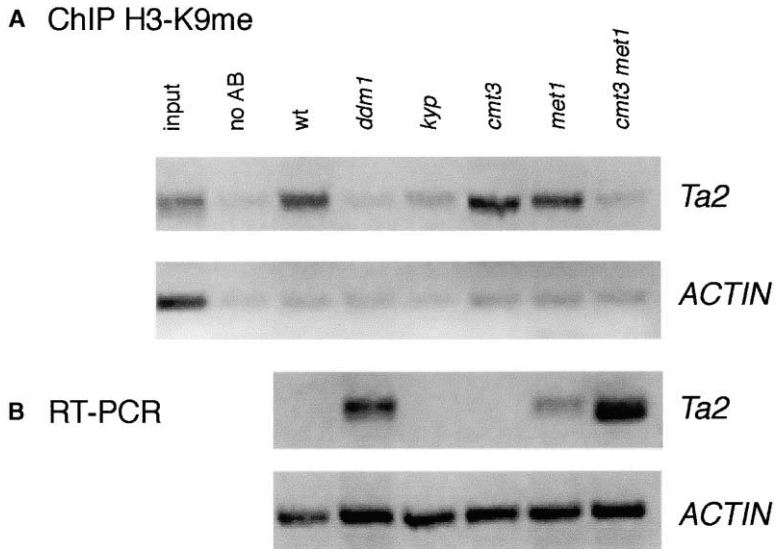

Figure 3. Analysis of the *Ta2* Retrotransposon

(A) Results of ChIP assays using dimethylated H3-K9 antibodies and amplifying *Ta2* and *ACTIN* in a multiplex reaction.

(B) Results of RT-PCR from the same mutant lines.

tion, consistent with a model in which DDM1 unwinds higher-order chromatin structures to allow both DNA methyltransferases and histone methyltransferases access to chromatin [18, 28].

DNA Methylation at *Ta3*

One of the known functions of heterochromatin is in the silencing of transposable elements [29]. To address the relationship between histone and DNA methylation at retrotransposons, we studied the *Ta3* sequence. This is a single copy, *copia*-like retrotransposon located in the pericentromeric region of chromosome 1 [30]. We were particularly interested in this retrotransposon because it is highly methylated and silenced in *Arabidopsis* and yet is transcribed in lines homozygous for mutations in the DNA methyltransferase gene *CMT3* [20].

We first analyzed DNA methylation by Southern blots, as shown in Figure 2B. The wild-type strain is highly methylated at both the CpG and CpNpG sites, as indicated by the high molecular weight bands (complete digestion should yield only the 746-, 1742-, and 2194-bp fragments; Figure 2A). As has been shown previously, lines carrying either *cmt3* or *kyp* have no effect on CpG methylation but reduce CpNpG methylation, with *cmt3* having a more prominent effect [16]. Both *ddm1* (also see [31]) and *cmt3 met1* eliminate almost all CpG and CpNpG methylation, whereas the *met1* single mutant eliminates almost all CpG methylation but has an intermediate effect on CpNpG methylation.

Of particular interest is the band located at 2483 bp due to methylation of the HpaII/MspI site in the left LTR, which appears to correlate with transcriptional silencing (see below). This site is located close to the predicted promoter for *Ta3* and is a major band in the *kyp* and *met1* digests. The methylation at this site is mostly due to CpNpG methylation since the *met1* strain gives similar patterns in both the HpaII (inhibited by CpG and CpNpG methylation) and MspI (inhibited by only CpNpG methylation) digests. Mutation of *cmt3*, either as a single or double mutant, completely eliminates this band in the MspI digests, as does mutation of *ddm1*.

Histone H3-K9 Methylation at *Ta3* Is Reduced in Lines Transcriptionally Derepressed by Loss of DNA Methylation

Figure 2C shows the results of both ChIP analyses as well as RT-PCR assays to examine transcriptional reactivation of *Ta3* in the various mutant lines. We found that H3-K9me antibodies preferentially precipitated *Ta3* in the wild-type strain in a multiplex PCR reaction (13 $\pm$ 1.8-fold enrichment [mean $\pm$ standard error] of *Ta3* compared to *ACTIN*), whereas antibodies to acetylated H3 preferentially precipitated *ACTIN* DNA (15 $\pm$ 2.5-fold enrichment). In the *ddm1* and *kyp* mutant lines, H3-K9 methylation is reduced to background levels, which is consistent with the results at the centromeric repeat sequences. RT-PCR revealed that transcripts were not detected in wild-type plants and were only detected upon many additional PCR cycles in *kyp* lines (Figure 2C). *ddm1*, however, derepressed *Ta3* transcription to an easily detectable level. The lack of strong reactivation in the *kyp* line compared to *ddm1* suggests that, although H3-K9 methylation is greatly reduced, this is not enough to significantly reactivate gene expression. In other words, reduction of H3-K9 methylation without a substantial reduction of DNA methylation does not derepress gene silencing of *Ta3*.

ChIP analysis of the DNA methyltransferase mutant lines reveals results that are significantly different than those observed at the centromeric repeats. The *cmt3* line causes a loss of H3-K9 methylation to approximately 37% of the wild-type levels (4.8 $\pm$ 1.0-fold enrichment compared to 13-fold enrichment; Figure 2C). The *met1* strain, on the other hand, has little effect on H3-K9 methylation (14 $\pm$ 1.5-fold enrichment). In the *cmt3 met1* line, the level of H3-K9 methylation is greatly reduced, measuring just above background levels (2.1 $\pm$ 0.17-fold enrichment). In all lines, acetylation of H3 remained the same as the wild-type strain. All of the ChIP analyses were repeated with primers specific for the left LTR of *Ta3*, and the results were very similar to those shown (data not shown). These data indicate that, at the *Ta3* locus, histone H3-K9 methylation is affected by DNA

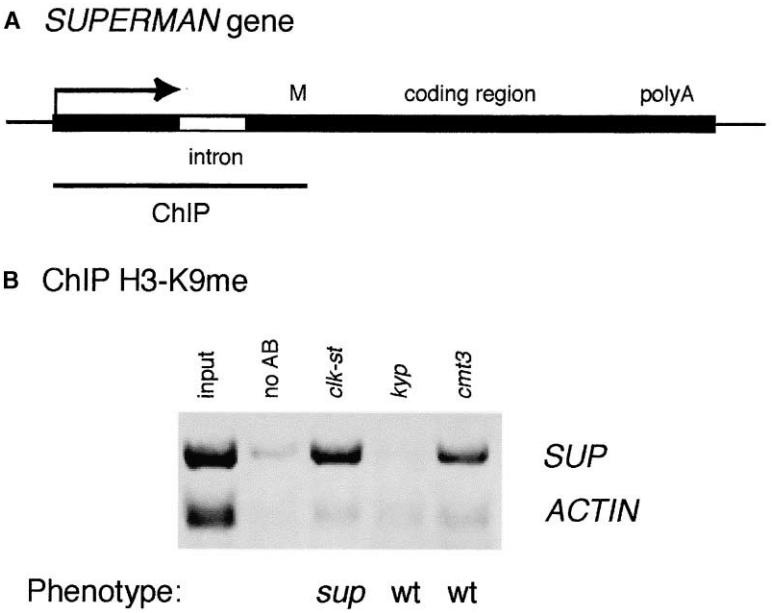

Figure 4. Analysis of the *SUPERMAN* Gene

(A) DNA from the 5′ portion of the *SUP* gene was amplified from ChIP preparations. This region contains an intron and the start of the coding region (M: initiating methionine).

(B) ChIP using dimethylated H3-K9 antibodies. *SUP* and *ACTIN* were amplified in a multiplex PCR reaction.

methylation levels. However, this seems unlikely to be a direct relationship, as it was not observed at the centromeric repeats.

Instead, we found a correlation between the loss of H3-K9 methylation and reactivation of transcription (Figure 2C). RT-PCR revealed that *cmt3* activates *Ta3* transcription to approximately 1/3 the level observed in *ddm1* lines, which indicates that loss of CpNpG methylation was able to derepress this retrotransposon (also see [20]). The *met1* line had a weaker effect on reactivation of *Ta3* and yielded approximately 1/10 the levels observed in *ddm1*. This suggests that CpNpG methylation plays a more important role in silencing *Ta3* than CpG methylation, which may be due to the location of key CpNpG sites in the promoter region (for instance, the band at 2483, see Figure 2B). The *cmt3 met1* double mutant reactivated transcription better than either single mutant (90% *ddm1* levels, Figure 2C). This indicates that, while CpNpG methylation is primarily responsible for gene silencing, CpG methylation plays some role. Comparison of the *Ta3* RNA levels for each of the lines with the amount of H3-K9 methylation at *Ta3* reveals an inverse relationship between transcription and H3-K9 methylation. These observations suggest that a loss of DNA methylation leads to a loss of H3-K9 methylation only when coupled with transcription.

The *Ta2* Retrotransposon Also Reveals an Inverse Relationship between Transcriptional Activity and Histone H3-K9 Methylation

A related retrotransposon, *Ta2*, also resides in the pericentromeric region of chromosome 1 [30]. This retrotransposon is similar to *Ta3* (75% nucleic acid identity); however, it is missing its left LTR. We examined both the methylation status of H3-K9 using ChIP assays and the activation of transcription using RT-PCR. As shown in Figure 3A, this retrotransposon contains methylated H3-K9 in the wild-type strain, but H3-K9 methylation is greatly reduced in both *ddm1* and *kyp* mutant lines. In the DNA methylation mutants *cmt3* and *met1*, H3-K9 methylation appears at about the same level as the wild-type strain, but, in the *cmt3 met1* double mutant, only background levels are observed. These results correlate well with the RT-PCR results (Figure 3B). In the wild-type strain, *Ta2* is not transcribed, whereas, in *ddm1* and *cmt3 met1*, significant levels of transcription are observed. *kyp* and *cmt3* mutant lines do not activate transcription at this retrotransposon, and *met1* gives only a weak reactivation. Thus, as was observed at *Ta3*, a decrease in H3-K9 methylation is only observed in the DNA methylation-deficient lines when significant levels of transcription are induced.

DNA Methylation Loss at the *SUP* Locus Does Not Cause Loss of H3-K9 Methylation

Another example of gene silencing exists in *Arabidopsis*: genes that are highly methylated and silenced but reside in the midst of euchromatin. For instance, hypermethylated epigenetic alleles have been isolated at the *SUP* locus as a result of mutagenesis and in both *ddm1* and *met1* inbred lines (*clark kent* [*clk*] alleles; [32, 33]). The *SUP* gene encodes a transcription factor that is involved in floral development, whose silencing leads to a floral phenotype that includes the production of extra stamens. Release from gene silencing of the *clk* alleles can be followed by the floral phenotype (six stamens in the wild-type versus eight or more stamens in *clk*). However, since the *SUP* gene is only transiently expressed in a few tissues of the developing flower [34], only a very small fraction of the tissue harvested for ChIP assays (whole shoots) are actively transcribing *SUP* even when released from silencing (see the Experimental Procedures). Therefore, like the centromeric repeat sequences, the chromatin examined by ChIP assays at the *SUP* locus is for the most part undisturbed by transcription.

We examined the *kyp* and *cmt3* mutant lines at the *SUP* locus. These mutants were isolated as suppressors

of the hypermethylated *sup* mutant in a line known as *clk-st* (*clark kent-stable*). In this line, hypermethylation is reinforced by the presence of an inverted repeat of the *SUP* gene, leading to a stabilized phenotype but also introducing four extra copies of the *SUP* gene [35]. As *kyp* and *cmt3* were isolated in a *clk-st* background, they each contain six copies of the *SUP* gene and can be directly compared using ChIP assays (as these are copy number-sensitive assays).

Figure 4 shows that H3-K9 methylation is present in much higher concentrations at *SUP* compared to *ACTIN* in the *clk-st* line. In the *kyp* line, we are unable to detect H3-K9 methylation above background levels. However, in *cmt3*, H3-K9 methylation is present at close to the same level as in *clk-st*. Therefore, although *cmt3* causes a dramatic loss of DNA methylation and derepresses *SUP* gene silencing, little loss in H3-K9 methylation is observed. These results suggest that DNA methylation, not H3-K9 methylation, is primarily responsible for gene silencing at *SUP*. These results are also consistent with a model in which transcription is required to reduce methylated histone H3 from previously silenced regions following loss of DNA methylation.

Conclusions

We have shown that *Arabidopsis* heterochromatin is characterized not only by DNA methylation, but also by histone H3-K9 methylation. Thus, plants, like animals and fungi, show high levels of H3-K9 methylation in heterochromatin and low levels in euchromatin, and this finding suggests conservation of this phenomenon amongst the major eukaryotic groups. The loss of this methylation in the *kyp* mutant suggests that the KRYP TONITE lysine 9-specific histone H3 methyltransferase is the major enzyme maintaining H3-K9 methylation at heterochromatin. Furthermore, we find that mutation of the *SWI2/SNF2*-related *DDM1* gene is as effective in universally reducing histone H3-K9 methylation as is the *kyp* mutant. This suggests that chromatin remodeling is required not only for maintenance of DNA methylation, but also for the maintenance of histone methylation.

To study whether DNA methylation might affect H3-K9 methylation, we analyzed DNA methylation, H3-K9 methylation, and RNA expression levels in several mutant backgrounds and at several discrete loci. We found that, while the loss of histone methylation causes the partial loss of DNA methylation, the reverse is not necessarily true. These results confirm earlier observations from both Neurospora [13] and *Arabidopsis* [16] that DNA methylation acts downstream of histone methylation. This relationship is also supported by our finding that the loss of histone H3-K9 methylation is not sufficient to reactivate expression of the *Ta3* and *Ta2* retrotransposon sequences, but loss of DNA methylation is sufficient.

We did not observe a correlation between the loss of DNA methylation and the loss of H3-K9 methylation. Instead, we found an inverse relationship between the levels of transcriptional reactivation and the levels of H3-K9 methylation. For instance, the nontranscribed centromeric repeat sequences and the *SUP* locus retained high levels of H3-K9 methylation despite major losses of DNA methylation. However, at the *Ta3* and *Ta2* retrotransposons, we observed that H3-K9 methylation is lost in direct proportion to the level of gene expression observed in the various methylation mutants.

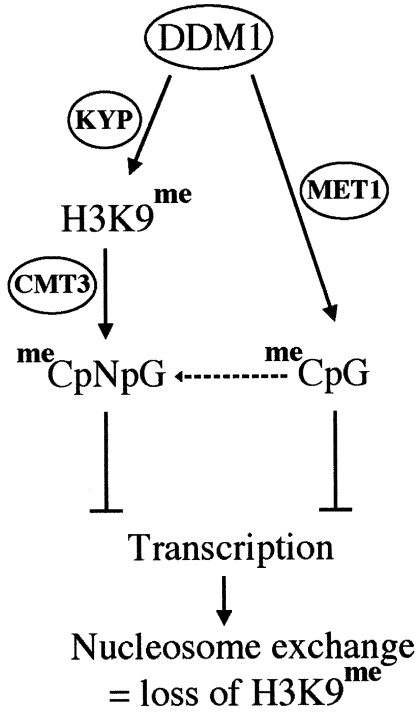

Figure 5. Model for the Relationship between Histone H3-K9 Methylation and DNA Methylation

DDM1 is required for both KYP-dependent H3-K9 methylation and MET1-dependent DNA methylation. CpNpG methylation is controlled by CMT3 through H3-K9 methylation. CpNpG methylation is also reduced in *met1* mutant plants (dashed line), but this seems to be an indirect effect, as MET1 does not appear to methylate CpNpG sites [41]. Derepression of transcriptional silencing due to loss of DNA methylation causes a decrease in H3-K9 methylation levels, possibly through nucleosome exchange.

How can we explain this transcription-coupled loss of H3-K9 methylation? Recent observations from Ahmad and Henikoff [36] provide an attractive model. They have found that the histone H3.3 variant in *Drosophila* is deposited in a DNA replication-independent manner at chromatin regions that are actively transcribed. Further, they have observed an anti-correlation of the presence of H3-K9 methylation and deposition of H3.3 that suggests that transcription reduces H3-K9 methylation by replacing the H3 histones. This explanation fits well with the idea that lysine methylation may be irreversible (no demethylase has been found) [37], such that replacement of methylated nucleosomes with unmethylated ones may be the only way to reduce H3-K9 methylation. Therefore, we propose that reduced DNA methylation reactivates transcription, which facilitates the replacement of nucleosomes, thereby reducing the H3-K9 methylation at transcribed loci (Figure 5). This process most likely involves chromatin remodeling factors that function during transcription [38]. It remains to be seen if reactivation of silenced genes in *Arabidopsis* is coupled with the deposition of histone H3 variants.

In summary, our results suggest that DNA methylation acts downstream of H3-K9 methylation and can cause gene silencing in the absence of H3-K9 methylation. However, DNA methylation can influence the levels of H3-K9 methylation possibly through the activation of transcription.

Experimental Procedures

Plant Strains

Two strains were used as wild-type controls in this study: Landsberg *erecta* (L*er*) for *Ta3*, *Ta2*, and the centromeric repeat studies, and *clk-st* for *SUP* ChIP assays [20]. The *ddm1-2 C4 37* line was introgressed into L*er* five times and was selfed seven times [32]. Similarly, the *ddm2-1 170-1A* line (referred to as *met1*) was introgressed into L*er* and selfed five times [32]. The *Ler* introgressed *ddm1* and *ddm2* strains were a kind gift from Eric Richards. The *kyp-2* line was described in Jackson et al. [39], and the *cmt3-7* line was described in Lindroth et al. [21]. The *cmt3 met1* double mutant was isolated by first back crossing homozygous *clk-st cmt3-7/cmt3-7* three times to L*er* (to eliminate *clk-st*), back crossing the *met1* strain five times to L*er*, and then crossing a *cmt3/CMT3* plant with a *met1/MET1* plant and isolating a double heterozygote. This plant was then selfed, and *cmt3/cmt3 met1/met1* homozygotes were identified by PCR genotyping. Tissue for ChIP assays and DNA and RNA preparations was isolated from F3 plants (double homozygotes were selfed one time).

ChIP Assays

The ChIP protocol described here is a variation on previously published protocols [39, 40] that eliminates any nuclei preps or gradient steps. Three- to four-week-old plants were harvested and immersed in buffer A (0.4 M sucrose, 10 mM Tris [pH 8], 1 mM EDTA, 1 mM PMSF, 1% formaldehyde) under vacuum for 10 min. Glycine was added to a final concentration of 0.1 M, and incubation was continued for an additional 5 min. The leaves were then washed and frozen with liquid nitrogen. Approximately 0.3 g of leaves was ground for each immunoprecipitation and was resuspended in 1 ml lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 mM Na butyrate, 1 μg/ml aprotinin, 1 μg/ml pepstatin A). DNA was sheared by sonication to approximately 500–1000-bp fragments. After centrifugation (10 min at 13,000 rpm), the supernatents were precleared with 60 μl salmon sperm (SS) DNA/Protein A agarose for 60 min at 4°C. After 2 min of centrifugation at 3,000 rpm, the supernatent was transferred to a siliconized tube, and 10 μl of the appropriate antibody was added (anti-dimethyl-histone H3 [Lys9] #07-212 and anti-acetyl-histone H3 #06-599 from Upstate Biotechnology). After incubation overnight with rotation, 60 μl SS DNA/Protein A agarose was added and incubation continued for 2 hr. The agarose beads were then washed with 1 ml of each of the following: 2× lysis buffer, 1× LNDET (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris [pH 8]; [39]), and 3× TE (10 mM Tris-HCl [pH 8], 1 mM EDTA). The immunocomplexes were eluted from the beads with 300 μl 1% SDS, 0.1 M $NaHCO_3$. A total of 12 μl 5 M NaCl was then added to each tube, and crosslinks were reversed by incubation at 65°C for 5–6 hr. Residual protein was degraded by the addition of 20 μg Prot K (in 10 mM EDTA and 40 mM Tris [pH 8]) at 45°C for 1 hr, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Pellets were washed with 70% EtOH and resuspended in 75 μl TE. Approximately 1–2 μl was used for PCR. Each of the immunoprecipitations was performed at least three independent times, and control precipitations with other antibodies were done at the same time (e.g., anti-dimethylated-lys4-histone H3, anti-diacetylated-lys9,14-histone H3, anti-tetra-acetylated histone H4, data not shown).

ChIP PCR

Quantitative PCR was used to determine the amounts of genomic DNA immunoprecipitated in the ChIP experiments. The primer pairs used were as follows: 180-bp repeats (JP1623: 5′-ACCATCAAAGC CTTGAGAAGCA-3′ and JP1624: 5′-CCGTATGAGTCTTTGTCTTTG TATCTTCT-3′); middle of *Ta3* (JP1565: 5′-GATTCTTACTGTAAA GAACATGGCATTGAGAGA-3′ and JP1566: 5′-TCCAAATTTCCTG AGGTGCTTGTAACC-3′); LTR of *Ta3* (JP1617: 5′-TAGGGTTCTTAG TTGATCTTGTATTGAGCTC-3′ and JP1618: 5′-TTTGCTCTCAAACT CTCAATTGAAGTTT-3′); *Ta2* (JP1725: 5′-AAACGATGCGTTGGGA TAGGTC-3′ and JP1726: 5′-ATACTCTCCACTTCCCGTTTTTCTTT TTA-3′); *ACTIN 2/7* 5′ end primers (JP1595: 5′-CGTTTCGCTTTCC TTAGTGTTAGCT-3′ and JP1596: 5′-AGCGAACGGATCTAGAGACT CACCTTG -3′); *SUP* 5′ end primers (JP1559: 5′-GATGGGGATTTGA TAATGCGTCCAAGAA-3′ and JP1560: 5′-TTCTTGCACGGCCATA GAAGCTGTTCCTCAA-3′). The amount of immunoprecipitate used in each assay was determined empirically such that an equal amount of *ACTIN* was amplified. All PCR reactions were done in 50 μl, starting with 5 min at 96°C, followed by 23–36 cycles (depending on the region being amplified) of 94°C (15 s), 60°C (30 s), and 72°C (1 min). Aliquots of the PCR reaction were removed after various numbers of cycles and were resolved by electrophoresis on a 3% agarose gel. Images were captured with the Kodak Digital Science System, and quantitation was performed with ImageQuant software (Amersham).

Reverse Transcription-PCR

Total RNA from *Arabidopsis* leaves and inflorescences from 4-week-old plants was isolated with Tri Reagent (Molecular Research Center) and was treated with RNase-free DNase (Promega). RNA was recovered by CHROMA SPIN-100 Columns (CLONTECH) and ethanol precipitation. A total of 500 ng total RNA was mixed with 800 ng Oligo-(dT) 15 primer (Roche) and 1 μl dNTPs (10 mM each) in a total volume of 17.5 μl, heated at 65°C for 10 min, and cooled in ice. Reverse transcription (RT) was performed in a total volume of 25 μl containing the RNA, primer, and dNTPs, 5 μl 5× RT buffer, 2.5 μl 0.1M DTT, and 1 μl SuperScript II RT (Invitrogen) at 50°C for 1 hr. Equal amounts of RT products were used to perform PCR as described above. For the *ACTIN* gene, reactions proceeded for 28–32 cycles, with the following primer pairs: JP1564 (5′-GGTTG TGTCAAGAAGTCTTGTGTACTTTAGTTTTA-3′) and JP771 (5′-ATA GCTGCATTGTCACCCGA-3′). The primer pairs for *Ta3* were JP1565 and JP1566, and, for *Ta2*, they were JP1725 and JP1726 (see above), and reactions proceeded for 37 and 39 cycles. Control reactions without reverse transcription were used to assess the presence of any contaminating DNA.

Southern Blot

The 180-bp centromeric repeat probe was kindly provided by Eric Richards and is described in [17]. The *Ta3* probe was generated by amplifying genomic DNA corresponding to positions 83448–88130 in BAC clone F28L22 (GenBank accession AC007505).

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Note Added in Proof

Gendrel et al. (Gendrel, A.-V., Lippman, Z., Yordan C., Colot, V., and Martienssen, R. [2002]. Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*. Published online June 20, 2002. 10.1126/Science.1074950) have also found that H3-K9 methylation depends on *DDM1*.