Distinct Mechanisms Determine Transposon Inheritance and Methylation via Small Interfering RNA and Histone Modification

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Heritable, but reversible, changes in transposable element activity were first observed in maize by Barbara McClintock in the 1950s. More recently, transposon silencing has been associated with DNA methylation, histone H3 lysine-9 methylation (H3mK9), and RNA interference (RNAi). Using a genetic approach, we have investigated the role of these modifications in the epigenetic regulation and inheritance of six *Arabidopsis* transposons. Silencing of most of the transposons is relieved in DNA methyltransferase (*met1*), chromatin remodeling ATPase (*ddm1*), and histone modification (*sil1*) mutants. In contrast, only a small subset of the transposons require the H3mK9 methyltransferase *KRYPTONITE*, the RNAi gene *ARGONAUTE1*, and the CXG methyltransferase *CHROMOMETHYLASE3*. In crosses to wild-type plants, epigenetic inheritance of active transposons varied from mutant to mutant, indicating these genes differ in their ability to silence transposons. According to their pattern of transposon regulation, the mutants can be divided into two groups, which suggests that there are distinct, but interacting, complexes or pathways involved in transposon silencing. Furthermore, different transposons tend to be susceptible to different forms of epigenetic regulation.

Introduction

Transposable elements are classical models for epigenetic inheritance: silent transposons can be activated and then inherited in the active state (McClintock 1965). This inheritance can be transient, in the case of "presetting," or it can be more permanent, with cycles of activation and silencing lasting for several generations (McClintock 1965). The molecular mechanisms underlying the inheritance of epigenetically activated transposons remain obscure, although DNA methylation has been implicated in maize (Chandler and Walbot 1986; Banks et al. 1988; Martienssen and Baron 1994). DNA methylation can be inherited epigenetically following DNA replication, because hemi-methylated DNA is a substrate for the DNA methyltransferase *Dnmt1* (Martienssen and Colot 2001).

In addition to DNA methylation, transposons are also subject to histone deacetylation, histone H3 lysine-9 methylation (H3mK9), and RNA interference (RNAi) (Rea et al. 2000; Gendrel et al. 2002; Johnson et al. 2002; Schotta et al. 2002). These chromatin modifications are interrelated (Martienssen and Colot 2001; Selker 2002; Sleutels and Barlow 2002). For example, in *Neurospora* and *Arabidopsis*, DNA methylation can be triggered by H3mK9 (Tamaru and Selker 2001; Jackson et al. 2002; Malagnac et al. 2002) and vice versa (Johnson et al. 2002; Soppe et al. 2002; Tariq et al. 2003). In mammals, methyl CpG-binding proteins recruit histone deacetylase (HDAC) and histone H3 lysine-9 methyltransferase (HMT) activity (Nan et al. 1998; Fuks et al. 2003). Additionally, the mammalian maintenance DNA methyltransferase, Dnmt1, interacts directly with HDACs (Fuks et al. 2000). Finally, in the fission yeast *Schizosaccharomyces pombe*, the RNAi machinery somehow guides the association of H3mK9 with centromeric repeats (Volpe et al. 2002, 2003).

In mammals and *S. pombe*, however, there are some limitations to the study of epigenetic regulation. For example, DNA methylation has not been reported in fission yeast, but in the mouse, it is essential (Li et al. 1992; Okano et al. 1999). In contrast, DNA methylation mutants are viable and fertile in *Neurospora* and *Arabidopsis*, which permits genetic analysis (Martienssen and Colot 2001), and a variety of genes involved in epigenetic regulation have been identified in both organisms.

To explore further the interrelationships between epigenetic pathways, we have used several *Arabidopsis* mutants that affect DNA methylation, H3mK9, and RNAi and that in some cases have been implicated in the epigenetic regulation of transposons. For example, the chromatin remodeling ATPase *DDM1* (open reading frame [ORF] At5g66750) (Jeddeloh et al. 1999; Brzeski and Jerzmanowski 2003; Vongs et al. 1993), the *Dnmt1* homolog *MET1* (At5g49160) (Kankel et al. 2003), and the HDAC *HDA6* (At5g63110) (Murfett et al. 2001; Aufsatz et al. 2002) all affect silencing and DNA methylation. Further,

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Abbreviations: *AGO1*, *ARGONAUTE1*; *AGO4*, *ARGONAUTE4*; ChIP, chromatin immunoprecipitation; *CMT3*, *CHROMOMETHYLASE3*; *DCL1*, *DICER-LIKE1*; *DDM1*, deficient in DNA methylation 1; H3mK9, histone H3 lysine-9 methylation; H4K16, histone H4 at lysine-16; HDAC, histone deacetylase; HMT, histone H3 lysine-9 methyltransferase; K4, dimethyl lysine-4; K9, dimethyl lysine-9; *KYP*, *KRYPTONITE*; *Ler*, Landsberg *erecta*; LTR, long terminal repeat; miRNA, microRNA; NoRC, nucleolar chromatin remodeling complex; ORF, open reading frame; RNAi, RNA interference; RT–PCR, PCR amplifying reverse-transcribed cDNA; siRNA, small interfering RNA; Snf2h, SWI/SNF chromatin remodeling ATPase; TIR, terminal inverted repeat; WT, wild-type

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silencing of the *TA3*/*ATCOPIA44* retrotransposon (At1g37110) requires the DNA methyltransferase *CHROMOMETHYLASE3* (*CMT3*) (At1g69770), and the HMT *KRYPTONITE* (*KYP*)/*SUVH4* (At5g13960) (Bartee et al. 2001; Lindroth et al. 2001; Jackson et al. 2002; Malagnac et al. 2002). In our studies we have also used *sil1*, which is now known to be an allele of *hda6* (H. Vaucheret, O. Mittelsten-Scheid, and I. Furner, personal communication).

The mutants *cmt3* and *kyp/suvh4* were isolated as mutants that relieved silencing imposed by long inverted repeats of the *PAI* and *SUP* genes. A third mutant in this pathway, *argonaute4* (*ago4*) (Zilberman et al. 2003) is related to *ARGONAUTE1* (*AGO1*) (At1g48410), which is required for RNAi in plants, fungi, and animals (Fagard et al. 2000; Morel et al. 2002; Williams and Rubin 2002). *TA3* was unaffected in *ago4–1*, but five of nine non-CG cytosines lost methylation in the *MULE* DNA transposon *AtMu1*, although transcripts did not accumulate (Zilberman et al. 2003). In *ddm1*, loss of DNA methylation is accompanied by loss of H3mK9 and gain of H3mK4, which is correlated with transcriptional reactivation of transposons (Gendrel et al. 2002). Further, unmethylated centromeric repeats are inherited from *ddm1* homozygotes (Vongs et al. 1993; Kakutani et al. 1999). This led to the suggestion that histone modification was responsible for DNA methylation, which could not be restored when histone modification was lost (Gendrel et al. 2002). However, unmethylated centromeric repeats are also inherited from *met1* homozygotes (Kankel et al. 2003), and *met1* gametophytes (Saze et al. 2003) and the *copia*-like elements *TA3* and *TA2* lose H3mK9 in *cmt3 met1* double mutants (Johnson et al. 2002). This led to the suggestion that DNA methylation might be responsible for H3mK9, rather than the other way around (Gendrel et al. 2002; Richards 2002; Soppe et al. 2002; Tariq et al. 2003). Although it is clear that epigenetic mechanisms interact, the nature of those interactions is currently uncertain.

To explore these relationships further, we have investigated the molecular basis for epigenetic inheritance in a representative group of transposons by backcrossing mutants in DNA methylation, chromatin remodeling, and histone modification to wild-type plants and characterizing transposon chromatin modifications. Our results indicate that the mutants fall into two groups, which might reflect the existence of separate complexes or pathways responsible for the silencing of different classes of transposons. Neither loss of DNA methylation nor loss of H3mK9 can fully account for the inheritance of active transposons. Rather, the loss of small interfering RNA (siRNA) may also play an important role.

Results

Transposons Are Differentially Silenced by Chromatin Modification

We selected five class I retrotransposons and one class II DNA transposon to assess silencing in the *Arabidopsis* ecotype Landsberg *erecta* (L*er*) (*WT*) (Figure 1): the non-long terminal repeat (LTR) retrotransposon *ATLINE1-4* (At2g01840); the gypsy-class LTR retroelements *ATLANTYS2-1* (located between At4g03760 and At4g03770), *ATLANTYS2-2* (located between At3g43680 and At3g43690), and *ATGP1* (At4g03650); the *copia*-like element *ATCOPIA4/COPIA-LIKE23* (At4g16870); and the *MULE* DNA transposon *AtMu1* (At4g08680) (Singer et al. 2001). *ATLANTYS2-1* and *ATLANTYS2-2* were assayed with the same primer pair. In order to assess both activation and inheritance, mutants were backcrossed to *WT*, and F1 seed was planted and used in each assay alongside samples from selfed mutant and *WT* parents (Figure 1). By assessing transcript accumulation and association with methylated histone H3 as well as methylated DNA in backcrossed plants heterozygous for each mutation, we could determine whether each transposon remained silent ("cryptic"), was reversibly activated, or was heritably activated ("preset") in each mutant.

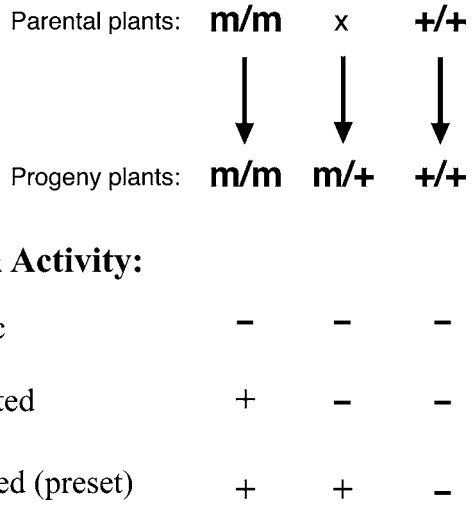

Figure 1. Inheritance of Transposon Activity

Pollen from homozygous mutant plants (m/m) was crossed onto *WT* (+/+) to generate backcrossed BC heterozygous seed (m/+). The parents were also self-pollinated as a control. Each class of progeny was then tested for expression of transposon mRNA, loss of DNA methylation, and changes in histone H3 methylation. Accumulation of transposon mRNA (+) or lack thereof (–) in each progeny genotype was used to determine whether the elements were silent ("cryptic"), reversibly activated, or heritably activated ("preset").
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In *WT*, transcripts were low or undetectable by PCR amplifying reverse-transcribed cDNA (RT–PCR), and these loci were associated with elevated levels of H3mK9 and reduced levels of H3mK4 according to chromatin immunoprecipitation (ChIP) analysis. The transposons were also heavily methylated when assayed by modified cytosine restriction McrBC digestion, which cuts DNA at methylated cytosine residues, preventing PCR amplification (Figure 2C), or by DNA gel blot analysis using HpaII and MspI, which are sensitive to both CG and CNG methylation and to CNG methylation alone, respectively (Figure 3). Transcripts, unmethylated DNA, and H3mK4 could be detected in the mutants (see Figure 2) and were indicative of the inheritance of activated transposons in backcrossed plants in all cases except *ATGP1*, which had substantial levels of H3mK4 in *WT* plants. Methylated DNA and H3mK9 were also measured, but could not be used to assess inheritance, as these were also inherited from silent elements in the *WT* parent.

Transcripts from all six transposons accumulated in *ddm1*, accompanied by loss of DNA methylation and H3mK9 and gain of H3mK4 (see Figure 2). Following backcrosses, each of the six transposons remained hypomethylated in *ddm1*/+ plants. They were associated with H3mK4, and transcripts

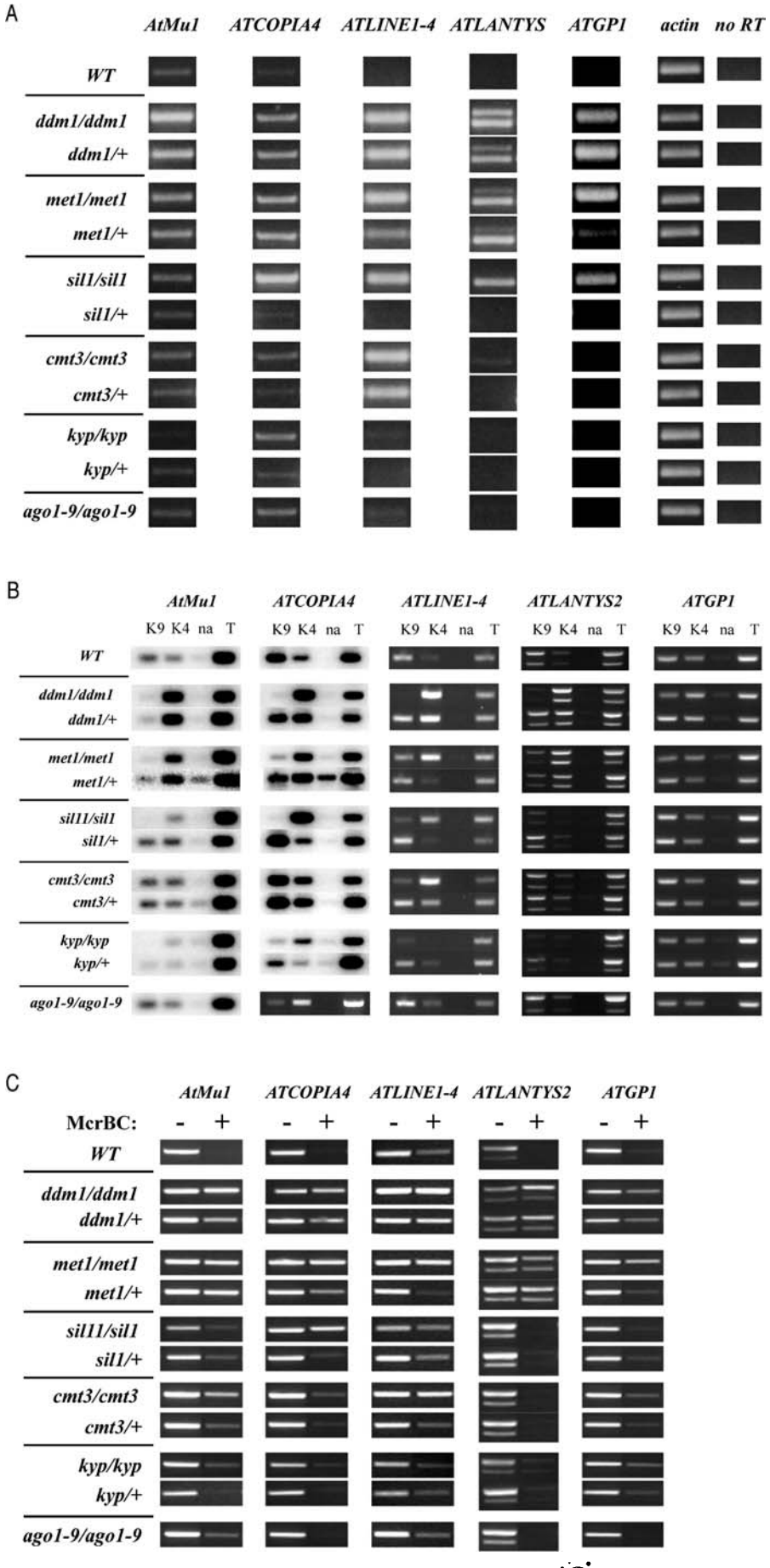

Figure 2. Inheritance of Transposon Modification

Reverse-transcribed cDNA (A), ChIP (B), and McrBC-digested genomic DNA (C) were amplified by PCR using primers from five retroelements and one DNA transposon in mutant (m/m) and backcrossed plants (m/+). Primers corresponded to transcribed ORFs for each element except for *AtMu1* ChIP, which was done on the terminal inverted repeat (TIR). For *ATLANTYS2*, the larger product is *ATLANTYS2-1* and smaller product is *ATLANTYS2-2*. Input RNA was normalized for each genotype using actin primers.

(A) Mock RT–PCR was performed without reverse transcriptase (–RT) using primers specific for the Cen180 repeat, which can detect trace amounts of contaminating DNA due to its high-copy number.

(B) ChIP was performed with antibodies recognizing dimethyl lysine-9 (K9) and dimethyl lysine-4 (K4) of histone H3 along with no antibody (na) and total (T) DNA controls. ChIP analysis for *AtMu1* and *ATCOPIA4* was performed using reduced cycles of PCR and Southern blotting (see Materials and Methods).

(C) McrPCR was carried out on untreated (–) and McrBC-treated (+) DNA (see Materials and Methods).

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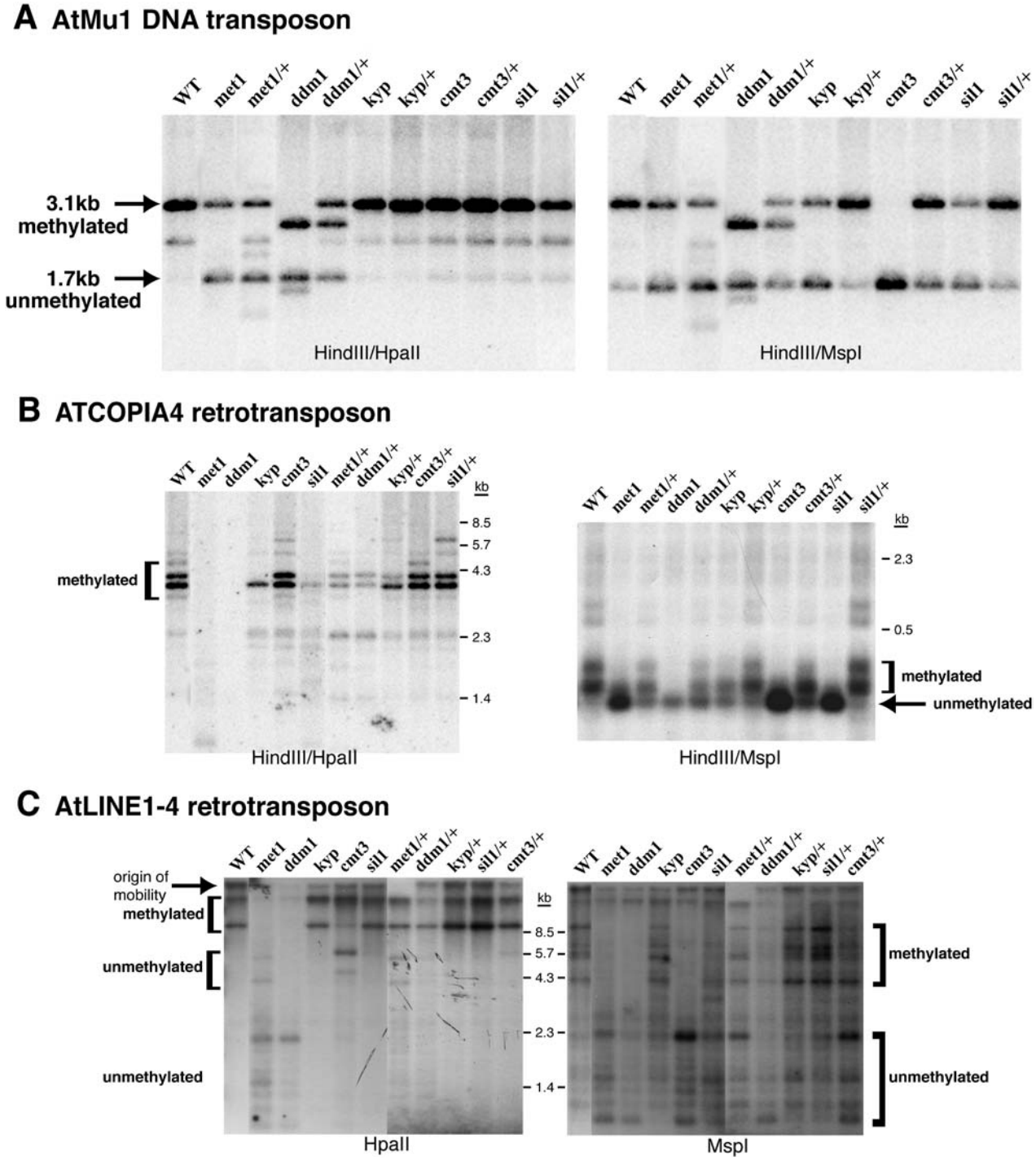

Figure 3. Southern Blot Analysis

(A and B) Genomic DNAs prepared from 4-wk-old plants of the indicated mutant and backcrossed (m/+) genotypes were digested with either HindIII and HpaII (left) or HindIII and MspI (right) and used for Southern blot analysis with a probe specific to the DNA transposons *AtMu1* and the retrotransposon *ATCOPIA4*. The L*er* genotype is shown. DNA methylation loss for each element within the mutants and their backcrosses is indicated by loss of band intensity relative to *WT* as indicated by the arrows or brackets.

(C) Genomic DNAs from the same genotypes in (A) and (B) were digested with either HpaII (left) or MspI (right) and used for Southern blot analysis with a probe specific to the *ATLINE1-4* element. The probe corresponds to a region flanked on both sides by more than five HpaII/MspI sites within 6 kb. Thus, fragment sizes generated upon digestion of the genotypes tested varied owing to a number of potential methylation changes. The fragments within the brackets depict significant changes in methylation between the genotypes.

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could still be detected. All six elements were also activated in *met1*, but *ATLINE1-4* and *ATGP1* were partially or completely resilenced in *met1*/+ backcrosses, respectively, and *ATLINE1-4* did not retain H3mK4 (see Figure 2). Interestingly, *ATLINE1-4* retained H3mK9 in *met1* (see Figure 2B), although it was hypomethylated (Figure 3). In *sil1*, transcripts from five of the six elements also accumulated. Three of these elements, *AtMu1*, *ATCOPIA4*, and *ATLINE1-4*, lost DNA methylation along with H3mK9 (see Figure 2; Figure 3), and two of them, *ATCOPIA4* and *ATLINE1-4*, gained H3mK4 (*AtMu1* already had substantial levels in *WT*). Histone H3 and DNA methylation were unchanged in the high-copy *ATLANTYS2* and *ATGP1* elements, perhaps because only a subset of elements was transcriptionally activated. In backcrossed *sil1*/+ plants, transcripts, DNA hypomethylation, and H3mK4 were restored to *WT* levels, unlike in *ddm1*/+ and *met1*/+, indicating these changes were reversible and not "preset." Thus, *SIL1* can silence transposons de novo when introduced in backcrossed plants (see Figure 1), unlike *DDM1* and *MET1*. This was unexpected, as the molecular changes observed in *ddm1*, *met1*, and *sil1* were comparable.

kyp and *cmt3* had much weaker effects on transposon activation, despite widespread loss of H3mK9 in *kyp*, and on CNG methylation in *cmt3* (see Figure 2; Figure 3). Specifically, in *cmt3*, *ATLINE1-4* was heritably activated and *ATCOPIA4* accumulated low levels of transcript. In *kyp*, only *ATCOPIA4* was activated and associated with high levels of H3mK4. CNG methylation was lost and not restored in *kyp*/+, as in *cmt3*/+ (Figure 3), although sensitivity to McrBC was unaffected, presumably due to methylation of non-CNG sequences (see Figure 2). The gypsy-class elements *ATLANTYS2* and *ATGP1* remained silent in both mutants. Thus, while loss of CG and CNG methylation, loss of H3mK9, and gain of H3mK4 accompany transposon activation, none of these can reliably predict their subsequent inheritance.

The Role of RNAi

In *Caenorhabditis elegans* and *Drosophila*, which lack DNA methylation, transposon silencing is maintained in the germline by RNAi (Plasterk and Ketting 2000; Aravin et al. 2001), and we examined whether RNAi impacts transposon silencing in *Arabidopsis* using a strong allele of *ago1*, *ago1-9* (C. Kidner and R. Martienssen, unpublished data). Strong and weak alleles of *ago1* are defective in transgene silencing and methylation (Fagard et al. 2000; Morel et al. 2002), they have strong developmental phenotypes, and they are sterile in L*er* (Fagard et al. 2000). In *ago1-9,* only *ATCOPIA4* was activated, accompanied by loss of H3mK9 and gain of H3mK4, but DNA methylation was unaffected (see Figure 2; data not shown). *ATCOPIA4* is located in a disease-resistance gene cluster on the long arm of Chromosome 4 that undergoes frequent epimutation in *ddm1* inbred strains (Stokes and Richards 2002). The DNA transposon *AtMu1* was weakly transcribed in *WT* plants (Singer et al. 2001), making its activation in *ago1*, *cmt3*, and *kyp* difficult to detect. However, DNA methylation was lost from *AtMu1* in each of these three mutants.

Thus, *ago1* resembles *kyp*, in having relatively minor effects on transposon silencing. One explanation is genetic redundancy. There are ten *AGO* genes in the *Arabidopsis* genome, and a mutant allele of *ago4* also has a modest impact on *AtMu1* methylation (Zilberman et al. 2003). Redundancy cannot be the entire explanation, however, because we found other similarities between *ago1*, *cmt3*, and *kyp*. Using primers from 24 retrotransposons and 18 DNA transposons from the heterochromatic knob (Gendrel et al. 2002), we found that almost all of them remained silent in *ago1* and *cmt3* (data not shown). However, *ATENSPM5* (At4g03910) was weakly activated in *ago1* and behaved exactly like *ATCOPIA4* in the other mutants (data not shown). In contrast, more than half of the transposons in the knob were strongly activated in both *ddm1* and *met1* (Gendrel et al. 2002; Tariq et al. 2003). This indicated that silencing mediated by *AGO1*, *KYP*, and *CMT3* is distinct from silencing mediated by *DDM1* and *MET1*.

We looked for siRNA in each of the mutants (Figure 4). Long siRNA (25 nt) is a hallmark of transposons targeted by RNAi (Llave et al. 2002) and is presumably the product of a *DICER*-like (*DCL*) enzyme specialized for this purpose (Hamilton et al. 2002). As a control, a 21 nt microRNA (miRNA) derived from hairpin precursors (Rhoades et al. 2002) accumulated to normal levels in all genotypes examined. miRNA is the product of *DICER-LIKE1* (*DCL1*) (At1g01040), and *dcl1-9* mutants (Jacobsen et al. 1999) had no effect on any of the transposons tested (data not shown). While we could not detect siRNA corresponding to *ATCOPIA4*, *ATLINE1-4*, or *ATLANTYS2*, 25 nt siRNAs corresponding to *AtMu1* and *ATGP1*, as well as the short interspersed nuclear retroelement *AtSN1* (Hamilton et al. 2002), accumulated in *WT* plants. These siRNAs accumulated to normal levels in *sil1*, *kyp*, and *cmt3*, but *AtMu1* and *AtSN1* were absent or nearly so in *met1* and *ddm1* (Figure 4; data not shown). In contrast, siRNA from the LTR and coding sequence of *ATGP1* was normal in *met1* and *ddm1* (Figure 4; data not shown). siRNA in *ago1* had the opposite pattern: transposon siRNA accumulated to normal levels except for *ATGP1*, which had reduced levels (Figure 4). This indicates a role for *MET1* and *DDM1* in siRNA accumulation and a role for siRNA in epigenetic inheritance.

Discussion

Two Distinct Mechanisms Silence Transposons

Each of the mutants described here has been previously shown to impact transposon methylation, transcription, and H3mK9 accumulation (Gendrel et al. 2002; Johnson et al. 2002; Tariq et al. 2003). However, different transposons were used in each case, and inheritance of activated transposons was not tested. For example, *TA3* (*ATCOPIA44*) is activated in *cmt3* (Johnson et al. 2002). *TA3* is closely related to *ATCOPIA4* (*copia* superfamily 6), which we show is also affected, but gypsy-class retrotransposons are not affected at all, and class II DNA transposons are only weakly affected. In another example, *ATLANTYS2* and *ATENSPM2* (as well as the defective *ATCOPIA* and *VANDAL* elements Ta2 and At4g03870) were shown to lose H3mK9 in a null allele of *met1*, leading to the conclusion that CG methylation is required for HMT activity (Tariq et al. 2003). Here we demonstrate that, while *ATLANTYS2-2*, *AtMu1*, and *ATCOPIA4* do indeed lose H3mK9 in *met1-1*, *ATLANTYS2-1*, *ATLINE1-4*, and *ATGP1* do not lose H3mK9, despite loss of CG methylation.

We have taken a genetic approach to dissecting transposon regulation. By examining representative transposons of each class in each mutant, we demonstrate first that transposons differ in their regulation. Next, we show that the mutants can

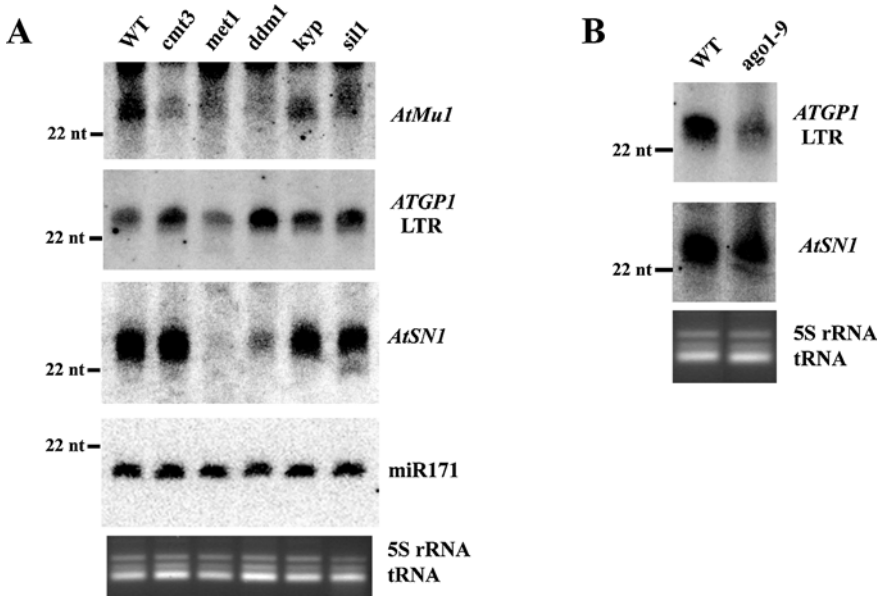

Figure 4. siRNA Differentially Accumulates in Chromatin Mutants

siRNA Northern blots were hybridized with sense RNA probes for each of the transposons indicated in (A) and (B) in order to detect 25 nt antisense siRNA from each of the sequences tested. *AtMu1* is single copy so that autoradiographic exposure was increased substantially. A 22 oligonucleotide size marker is indicated, and the 21 nt miRNA *miR-171* was used as a loading control. It is unaffected in the mutants tested.

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be grouped according to their pattern of transposon regulation, revealing two distinct mechanisms of transposon silencing (Figure 5A). *kyp* resembles *ago1-9* in that it is only required to silence a subset of transposons, even though *kyp* results in widespread loss of H3mK9. *met1* and *ddm1* resemble each other and *sil1* more closely than *cmt3*, *ago1*, and *kyp*. In *sil1*, H3mK9 is lost, but unlike in *kyp*, most of the elements are derepressed. *SIL1* encodes the HDAC HDA6 (H. Vaucheret, O. Mittelsten-Scheid, and I. Furner, personal communication), which has been implicated in posttranscriptional gene silencing (Murfett et al. 2001) as well as in RNA-directed DNA methylation (Aufsatz et al. 2002).

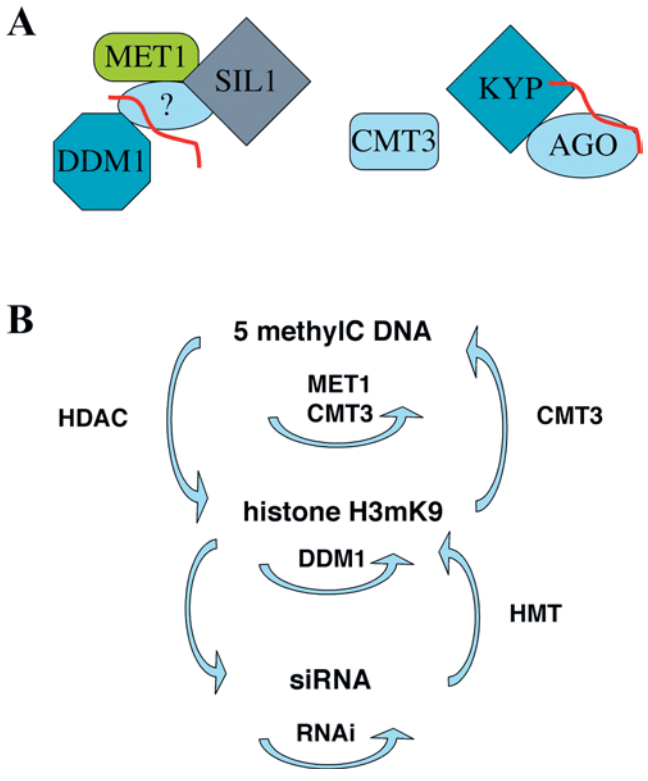

Figure 5. Model of Transposon Silencing Complexes

(A) DDM1, MET1, and SIL1 are all required for transposon silencing and may interact. MET1 and DDM1 are also required for siRNA accumulation (shown in red). AGO and KYP have similar effects on transposon activation and may also interact. They impact DNA methylation via CMT3 (Cao and Jacobsen 2002; Jackson et al. 2002). (B) siRNA, histone H3 methylation, and DNA methylation interact to silence transposons. Silencing is maintained by the MET1/DDM1/SIL1 complex. A possible network is shown.

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There are two formal explanations when mutants in different genes have similar phenotypes. The first is that the gene products interact in a complex, so that removal of any one will disrupt the function of the others. The second explanation is that the genes interact in a pathway, so that one is upstream of the other. We propose a model taking both of these possibilities into account (Figure 5B). MET1, DDM1, and SIL1 may act together in a complex, accounting for loss of histone modification in *met1* mutants and loss of DNA methylation in *ddm1* and *sil1*. This is also consistent with gain of H4K16 acetylation in *ddm1* chromocenters (Soppe et al. 2002). In contrast, *KYP* and *AGO1* affect only a subset of transposons and may interact in a separate complex (Figure 5A). Their effects on DNA methylation are mediated by CMT3, which utilizes H3mK9 as a guide (Cao and Jacobsen 2002; Jackson et al. 2002). There are precedents for each complex. The human nucleolar chromatin remodeling complex, NoRC, includes a SWI/SNF chromatin remodeling ATPase (Snf2h) as well as the RNA-binding protein TIP-5, the DNA methyltransferase Dnmt1, and HDAC1 (Santoro et al. 2002). DDM1 is strongly required for rDNA methylation, supporting this idea (Vongs et al. 1993). In *S. pombe*, *ago1*$^{+}$ and the HMT *clr4*$^{+}$ each effect H3mK9 as well as RNAi, indicating their products may also interact (Volpe et al. 2002; Schramke and Allshire 2003).

Transposon Silencing Complexes Interact via siRNA and Histone Modification

Although the mutants fall into separate groups, the *ATCOPIA4* and *ATENSPM5* transposons silenced by *KYP*, *AGO1*, and *CMT3* are also silenced by *DDM1*. Therefore, the

two complexes act in a common pathway. One common intermediate is siRNA. There are ten *AGO*-like genes in *Arabidopsis*, so different transposons may utilize different KYP/AGO complexes. These complexes presumably interact with siRNA (Caudy et al. 2002). siRNA is stabilized by DDM1/MET1. If siRNAs were shared between the two complexes, this would account for the complementary accumulation of siRNA in *ago1* and *met1*, in that siRNAs that accumulate in *met1* fail to accumulate in *ago1* and vice versa (see Figure 4).

Another common intermediate is histone H3 modification. H3mK9 by KYP may depend on deacetylation by SIL1, accounting for the observation that H3mK9 depends on both complexes. These changes in histone modification impact CXG methylation indirectly via CMT3 (Cao and Jacobsen 2002; Jackson et al. 2002). However, while both *sil1* and *kyp* impact H3mK9, only *sil1* has a major effect on transposon activation. The MET1/DDM1/SIL1 complex can maintain silencing in the absence of KYP, but KYP cannot maintain silencing in the absence of DDM1, MET1, or SIL1. The most likely explanation is that DDM1 and MET1 influence histone modification through SIL1 (Figure 5) rather than directly via KYP, as previously proposed (Johnson et al. 2002). These results implicate the gain of H3mK4, rather than the loss of H3mK9, as being important for transposon activation. It is possible, therefore, that H3mK4 is specifically excluded by DDM1 remodeling and that loss of H3mK9 in *ddm1* mutants is indirect (Gendrel et al. 2002).

Silencing of Active Transposons via siRNA

Active retrotransposons are epigenetically inherited from the methyltransferase mutants *met1* and *cmt3*. An attractive mechanism accounting for this inheritance is that loss of DNA methylation cannot be restored by maintenance methyltransferase (Tariq et al. 2003). However, the loss of DNA methylation in *sil1* is comparable to *cmt3* and *met1*, and yet active transposons are readily silenced in *sil1*/+ backcrosses. One difference between these mutants is that *met1* does not accumulate siRNA corresponding to *AtSN1* or *AtMu1*, resembling in this respect the silencing mutants *ago4* and *sde4* (Hamilton et al. 2002; Zilberman et al. 2003). siRNA accumulates normally in *sil1*. Loss of siRNA is not due to silencing of these transposons, as *AtMu1* is activated in *sil1*, *ddm1*, and *met1*. In contrast, *ATGP1* siRNA levels are unaffected and *ATGP1* is silenced in *met1*/+. Further, the only elements that retained H3mK9 in *met1* (*ATLANTYS2-1*, *ATLINE1-4*, and *ATGP1*) exhibited at least some resilencing in *met1*/+.

Thus, *MET1* may require siRNA for silencing de novo. CMT3 may also require siRNA: *ATLINE1-4* was not silenced when *cmt3* was backcrossed to *WT*, but *PAI2* and *SUP* genes activated in *cmt3* could be silenced by complementation with *CMT3* transgenes (Bartee et al. 2001; Lindroth et al. 2001). Complementation was in the presence of an inverted repeat, which could provide siRNA *in trans*. We have not been able to detect siRNA from *ATLINE1-4*. If siRNA guides silencing by *MET1*, it would have to act *in cis*, as it is provided from the *WT* parent in *met1*/+ backcrossed plants. siRNA contributed *in trans* might eventually reestablish silencing in subsequent generations, resembling the presetting and cycling of transposon activity in maize. Such long-term consequences of silencing deserve further investigation.

Materials and Methods

Plant material. All plants were of the Landsberg *erecta* (L*er*) ecotype and grown in a greenhouse under long days. *ddm1-2* and *met1-1* were introgressed into L*er* from Columbia by backcrossing five to eight times and inbreeding by single-seed descent for two (*met1-1*) or three (*ddm1-2*) generations (Singer et al. 2001; Kankel et al. 2003). *cmt3-m5662* is a *DsE* enhancer trap insertion in the 16th exon (ET5662; http://genetrap.cshl.org), which blocks *CMT3* transcription (data not shown), and was inbred for two generations. *sil1* (Furner et al. 1998), *dcl1-9* (Jacobsen et al. 1999), *ago1-9* (C. Kidner and R. Martienssen, unpublished data), and *kyp-2* (Jackson et al. 2002) were as previously described. Backcrosses onto L*er*, serving as females, were performed with mutant pollen, and progeny were pooled for analysis.

Expression analysis. Total RNA was extracted with Trizol reagent (Life Technologies, Carlsbad, California, United States) from 4-wk-old plants. Contaminating DNA was removed with RNase-free DNase (RQ1-DNase; Promega, Madison, Wisconsin, United States), and reactions were performed in 25 μl using 100 ng of RNA and the Qiagen (Valencia, California, United States) One-Step RT–PCR kit. Input RNA was normalized for each genotype using actin primers and dilutions of wild-type RNA (Figure2A). Mock RT–PCR was performed without reverse transcriptase using primers specific for the Cen180 repeat, which can detect trace amounts of contaminating DNA due to its high-copy number. RT–PCR conditions were as follows: +RT: 50°C for 30 min, 95°C for 15 min, 35 times (94°C for 30 s, 60°C for 30 s, 72°C for 2 min), 72°C for 10 min; –RT: 4°C for 30 min, 95°C for 15 min, 35 times (94°C for 30 s, 60°C for 30 s, 72°C for 2 min), 72°C for 10 min. The amplified DNA was visualized on a 2.0% agarose gel stained with ethidium bromide. *AtMu1* is weakly expressed in *WT* (Singer et al. 2001), and the highest level detected is shown in Figure 2A. In multiple replicates, *AtMu1* was consistently up-regulated in *sil1*.

siRNA was purified by clearing larger transcripts with PEG precipitation and was detected using 15% polyacrylamide gel blots as described (Dalmay et al. 2000). RNA—30 μg (*ATSINE*, *miR-171*, *ATGP1*) or 60 μg (*AtMu1*)—was loaded per lane and RNA gels were transferred onto Hybond N^+ (Amersham, Little Chalfont, United Kingdom) nitrocellulose membranes. Riboprobe templates were generated by PCR from genomic DNA using primers with a T3 promoter sequence (AATTAACCCTCACTAAAGGGAGA). Sense riboprobes were generated by in vitro transcribing of each DNA template with an Ambion (Austin, Texas, United States) Maxiscript T3 in vitro transcription kit. miRNA probes were prepared by end-labeling antisense oligonucleotides using T4 polynucleotide kinase (New England Biolabs, Beverly, Massachusetts, United States). An end-labeled 22 nt RNA was used as a size marker and its position is indicated in Figure 4. *AtMu1* siRNA analysis was repeated in two independent experiments to verify results from this single-copy element where only *met1* and *ddm1* exhibited loss of siRNA. All other sequences tested were multicopy. Therefore, our detection of siRNAs reflects the entire transposon population.

Chromatin immunoprecipitation. ChIP was carried out as described elsewhere (Gendrel et al. 2002) using 4-wk-old soil-grown plants and histone H3 anti-dimethyl lysine-9 or anti-dimethyl lysine-4 antibodies (Upstate Technologies, Avon, New York, United States). Precipitated DNA was resuspended in 100 μl for PCR analysis. An equal amount of chromatin was mock-precipitated without antibody, while a small aliquot of sonicated chromatin was reverse cross-linked, resuspended in 100 μl, diluted, and used as the total input DNA control. PCRs were performed in 25 μl with 1 μl of immunoprecipitated DNA. PCR conditions were as follows: 94°C for 3 min, 35 times (94°C for 20 s, 60°C for 30 s, 72°C for 1.5 min), 72°C for 10 min. The amplified DNA was visualized on 2% agarose gels stained with ethidium bromide. For *AtMu1* and *ATCOPIA4*, three different cycle numbers were compared (19, 21, and 23 cycles) by PCR and analyzed by Southern blots. Samples from each genotype were normalized to each other by amplifying dilutions of total input DNA with each of the primer pairs. Qualitative data were then obtained by comparing amplification with each set of primer pairs within the same ChIP extraction, which served as internal controls. In this way, control primers such as actin, whose association with lysine-9 is unclear, could be avoided. In all cases, mock precipitation with no antibody yielded little or no product.

Primers and PCR. Primers for RT–PCR, McrPCR, Southern blot probes, and riboprobes were selected using Primer3 (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) and BLASTN. Primer sequences are available upon request. All primer pairs were predicted to amplify a single product in the *Arabidopsis* genome except for *ATLANTYS2* and *ATGP1*. *ATGP1* is also highly repetitive and therefore multiple elements are detected by PCR. DNA

methylation was assessed by PCR amplification of DNA that had been pretreated with McrBC, a methylation-dependent restriction enzyme that restricts purine-C_{methyl} half-sites separated by 80 bp up to 3 kb (New England Biolabs). Successful amplification after digestion indicates lack of methylation. Genomic DNA (2 μg) from each genotype was digested for 0 min, 25 min, and 8 h, followed by heat inactivation. Template (60 ng) was then amplified using the PCR for 24 cycles, as described elsewhere (Rabinowicz et al. 2003).

Informatics. Transposons were annotated according to TIGR v3 (with supplementary information from RepBase), with the corresponding open reading frame (ORF) designations: *ATLINE1-4* (At2g01840), *ATLANTYS2-1/Cinful-1* (At4g03760–At4g03770) and *ATLANTYS2-2/Cinful-2* (At3g43680–At3g43690), *ATGP1* (At4g03650), *ATCOPIA4/COPIA-LIKE23* (At4g16870), *AtMu1* (At4g08680), *TA3/ATCOPIA44* (At1g37110), and *ATENSPM5* (At4g03910).

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Author Contributions. ZL, BM, and RM conceived and designed the experiments. ZL, BM, and CY performed the experiments. ZL, BM, and CY analyzed the data. ZL, BM, and TS contributed reagents/materials/analysis tools. ZL, BM, and RM wrote the paper. ∎

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