In plants, post-transcriptional gene silencing (PTGS) is mediated by DICER-LIKE 1 (DCL1)-dependent microRNAs (miRNAs), which also trigger 21-nucleotide secondary short interfering RNAs (siRNAs) via RNA-DEPENDENT RNA POLYMERASE 6 (RDR6), DCL4 and ARGONAUTE 1 (AGO1)1–3, whereas transcriptional gene silencing (TGS) of transposons is mediated by 24-nucleotide heterochromatic (het)siRNAs, RDR2, DCL3 and AGO4 (ref. 4). Transposons can also give rise to abundant 21-nucleotide ‘epigenetically activated’ small interfering RNAs (easiRNAs) in DECREASED DNA METHYLATION 1 (ddm1) and DNA METHYLTRANSFERASE 1 (met1) mutants, as well as in the vegetative nucleus of pollen grains5 and in dedifferentiated plant cell cultures6. Here we show that easiRNAs in Arabidopsis thaliana resemble secondary siRNAs, in that thousands of transposon transcripts are specifically targeted by more than 50 miRNAs for cleavage and processing by RDR6. Loss of RDR6, DCL4 or DCL1 in ddm1 background results in loss of 21-nucleotide easiRNAs and severe infertility, but 24-nucleotide het-siRNAs are partially restored, supporting an antagonistic relationship between PTGS and TGS. Thus miRNA-directed easiRNA biogenesis is a latent mechanism that specifically targets transposon transcripts, but only when they are epigenetically reactivated during reprogramming of the germ line. This ancient recognition mechanism may have been retained both by transposons to evade long-term heterochromatic silencing and by their hosts for genome defence.

Twenty-one-nucleotide epigenetically activated small RNAs, which we have termed easiRNAs, were previously found to accumulate from the 3’ untranslated region (UTR) of ATHILA retrotransposons in wild-type pollen and ddmi1 inflorescence7, and to depend on RDR6, DCL4 and AGO1 (refs 7, 8). To assess the origin of easiRNAs, we performed small RNA sequencing from inflorescence tissue, and we found that they accumulated in ddm1, but not in wild type (Columbia-0 (Col-0)) nor in ddm1 rdr6 double mutants5–8 (Fig. 1 and Extended Data Fig. 1a). Twenty-one-nucleotide easiRNAs in ddm1 mostly originated from ATGYPSY long terminal repeat (LTR) retroelements (Supplementary Table 1) located in pericentromeric regions, especially the high copy but defective ATHILA retrotransposons, which are integrated into pericentromeric satellite repeats (Fig. 1). However, easiRNAs also arose from ATCOPIA families found in euchromatic regions, such as ATCOPIA93 (also known as EVADÉ7) (Supplementary Table 1), and from specific VANDAL (also known as MuDR), HAT and CACTA elements (such as ATENSPM6), some of which are present in much lower copy numbers and known to transpose in ddmi1 (ref. 10). This raised the important question as to how specificity was conferred to these transposable elements (TEs) such that they gave rise to easiRNAs, when other transposons and genes did not. Genetically, easiRNAs resemble 21-nucleotide trans-acting (ta)siRNAs and other secondary siRNAs, which are derived from non-coding RNAs and messenger RNAs, respectively, and are triggered by miRNAs bound by AGO7 (ref. 11) and AGO1 (refs 2, 3, 12, 13). miRNAs were thus excellent candidates to confer transposon specificity.

miRNA biogenesis in Arabidopsis depends largely on DCL1, such that miRNAs are greatly reduced in sterile dcl1-9 plants and partly reduced in the weaker fertile allele dcl1-11 (ref. 14). We were unable to recover ddm1 dcl1-9 double mutants, but easiRNA levels were reduced in ddm1 dcl1-11, consistent with a role for miRNA in targeting and easiRNA biogenesis (Fig. 1 and Extended Data Fig. 1b). We identified miRNAs in our small RNA sequencing libraries from Col-0, ddm1 and ddm1 rdr6 inflorescence tissue. In addition to using a miRNA identification algorithm15, miRNAs were distinguishable from other 21-nucleotide siRNAs that are RDR6 dependent by comparing their abundance in ddm1 and ddm1 rdr6 small RNA sequencing libraries. We identified several known miRNAs, validating our algorithm (Extended Data Fig. 10) and genome-wide target prediction revealed that 3,662 TEs are potentially targeted by these miRNAs (Supplementary Table 3). To determine whether miRNAs mediate targeting and cleavage of TE transcripts, we sequenced cleavage sites genome wide by parallel analysis of RNA ends (PARE)11. To assess specificity, 5’ RNA ends from a 5-nucleotide window surrounding each predicted miRNA target site were compared to those from a larger 30-nucleotide window (Supplementary Table 3; for more specific 1-nucleotide and 3-nucleotide windows, refer to Supplementary Table 9). Significant enrichment of RNA ends at or near the target site was strong evidence for cleavage of the transposon transcripts guided by miRNA.

Approximately half of the 3,662 predicted TE targets showed evidence of miRNA-guided cleavage (Supplementary Table 3), and were targeted by more than 50 distinct miRNAs (Extended Data Fig. 10), although some TEs had only one cleavage product in the target window (such as ATCOPIA93, which is predicted to be cleaved by miR-833). In some cases multiple miRNAs targeted single TEs (Extended Data Fig. 10), reminiscent of the ‘two-hit’ model for tasiRNA biogenesis16. In other cases, TEs were targeted by longer forms of miRNA (22 nucleotides), which are thought to promote secondary siRNA biogenesis16,17. Specifically, 1,733 TEs were predicted to be miRNA targets and generated easiRNAs (at least ten reads). Of these, 1,247 were detectably cleaved by PARE sequencing. An additional 1,929 TEs were predicted to be targeted by miRNAs but did not generate easiRNAs, and of these 442 were detectably cleaved by PARE-seq. Thus, more than half of the TEs targeted by miRNAs also generated easiRNAs in inflorescence tissue from ddm1. However, phasing of easiRNAs, typical of other secondary siRNAs, was not detected, probably due to the repetitive nature of the TE targets and their targeting by multiple miRNAs (data not shown).

Interestingly, the miRNAs found to target TEs were mostly known miRNAs (Extended Data Fig. 10), such as miR-156, miR-159, miR-172 and miR-859, which also generate secondary siRNAs from miRNA targets1,2. Many of these miRNAs were predicted to target ATHILA elements (Extended Data Fig. 1), generating abundant easiRNA corresponding to ATHILA OPEN READING FRAME 1 (ORTF), also known as TRANSCRIPTIONALLY SILENT INFORMATION (TSI)

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miRNAs trigger RDR6-dependent 21-nucleotide easiRNA biogenesis from reactivated transposons. Whole-genome representation illustrating miRNAs triggering widespread easiRNA biogenesis at transposons in Arabidopsis. Outermost to innermost tracks depicting: miRNA abundance in ddm1-2 (histogram); Arabidopsis chromosome (Chr) 1–5 pericentromeric region (ideogram); transposon density (heat map: low density, blue; high density, red); gene density (heat map); retrotransposon-derived 21-nucleotide easiRNAs (dark blue, unique; light red, multiple mapping) and DNA transposon-derived 21-nucleotide small RNAs (dark blue, unique; light blue, multiple mapping) in the order Col-0, ddm1-2, ddm1-2 rdr6-15 and ddm1-2 dcl1-11 (histograms); miRNAs targeting transposons (connectors); miR-859a (Chr 1, red), miR-390a (Chr 2, blue), miR-172d (Chr 3, red), eamiR-R from ATHILAIV (Chr 4, grey) and miR-172e (Chr 5, blue). Transposons are post-transcriptionally targeted by 50 known miRNAs and newly discovered eamiRNAs (Extended Data Fig. 10), giving rise to abundant RDR6-dependent 21-nucleotide easiRNAs from transposons in Arabidopsis. (See also Extended Data Fig. 1 and Supplementary Tables 1, 3.)

ATHILA ORF1 contains a predicted target site for miR-859, and PARE confirmed cleavage at this site (Fig. 2c and Supplementary Table 3). We further validated miR-859-directed cleavage by modified 5′-RNA-ligase-mediated rapid amplification of cDNA ends polymerase chain reaction (5′ RLM RACE PCR) (Fig. 2e).

Despite cleavage of many TE families by multiple miRNAs (Extended Data Fig. 10), some did not generate easiRNAs. This was especially true of CACTA and ATCOPIA elements, for which most cleavage events were non-productive. For example, ATCOPIA43 elements were targeted by miR-390, which targets non-coding RNA for tasiRNA production by the two-hit model16, but which did not generate easiRNAs from ATCOPIA43 in ddm1 (Fig. 2b). Instead, PARE detected uncapped degradation products from ATCOPIA43, indicating extensive secondary RNA decay (Fig. 2d) after miRNA cleavage (Fig. 2f), and similar mRNA decay patterns were found at many genes targeted by miRNA (Extended Data Fig. 2). In general, easiRNA-producing TEs were intact ATENSPM2, ATENSPM5, ATENSPM6, ATCOPIA43 and ATCOPIA28 elements, whereas those that did not generate easiRNAs were non-autonomous elements (for example, ATENSPM1A) interrupted by insertion of other TEs or otherwise truncated, and were subject to RNA decay. Occasionally, TEs were found to produce easiRNAs and were predicted to be targeted by miRNAs, but cleavage could not be detected by PARE, which may be due to the underrepresentation of some miRNAs in inflorescence tissue (Supplementary Table 2). For example, ATCOPIA93 produces abundant easiRNAs from the GAG gene, which is predicted to be targeted by several known miRNAs and eamiRNAs (Extended Data Fig. 3a), yet only miR-833 shows evidence of cleavage by PARE, and this did not pass our cut-off for miRNA secondary cleavage site.

Two new classes of miRNAs were found in ddm1, namely those encoded by transposons and those for which miRNA precursor genes are methylated. We have labelled these miRNAs epigenetically activated (ea)miRNAs, as, like easiRNAs, they are abundant in pollen and ddm1 (Extended Data Figs 4, 10 and Supplementary Table 2). We also identified new miRNA isomers, changed from 21-nucleotide to 22- and 24-nucleotide sequence variants, originating from known miRNA precursors (Supplementary Table 2). Twenty-two-nucleotide isoforms promote secondary siRNA biogenesis3,17, whereas 24-nucleotide isoforms promote DNA methylation9,10. The newly identified eamiR-2
originates from an immature precursor sequence within an ATAILA4 retroelement and is abundant in ddm1 only (Supplementary Table 2). PARE analysis of ddm1 confirmed release of the eamiRNA from its own ATAILA4 precursor (Supplementary Table 3), and cleavage of other ATAILA4 elements in trans (Fig. 1). However, this TE-derived eamiRNA does not seem to direct easiRNA biogenesis from its own precursor (Supplementary Table 1). Thus, the release of TE-producing eamiRNAs by DICER does not trigger eamiRNA biogenesis per se, but only when the miRNA targets TE transcripts via AGO1 (ref. 7).

To test the requirement for miRNA in eamiRNA biogenesis, we used miR-845b, a 22-nucleotide miRNA predicted to target several retroelements (Supplementary Table 3), but only in pollen where it is specifically expressed. We fused a green fluorescent protein (GFP) reporter gene to a ubiquitously expressed promoter, and to a 300 base pair (bp) region of ATG1 that included the predicted miR-845b target site (Fig. 3a). Sequencing small RNA from pollen revealed novel 21-nucleotide eamiRNAs surrounding the miR-845b target site (Fig. 3a), which were not found in pollen with constructs in which the miRNA target site was deleted (Fig. 3b). We confirmed by modified 5’ RLM RACE PCR that miR-845b-directed cleavage products from GFP-ATG1 transcripts accumulate exclusively in pollen (Fig. 3c). Thus, 21-nucleotide eamiRNA biogenesis at TEs depends on targeting by miRNA.

Twenty-four-nucleotide hetsiRNAs guide asymmetric CHH methylation at TEs and are RDR2 dependent1. We profiled 24-nucleotide hetsiRNAs in Col-0, ddm1, rdr6, ddm1 rdr6 and ddm1 dcl1-11 (Supplementary Table 1 and Fig. 4). In Col-0, 24-nucleotide hetsiRNAs target LTR retrotransposons and most DNA transposons (Extended Data Fig. 5). In ddm1, we found a slight increase in multiple mapping 24-nucleotide hetsiRNAs from ATGYPSY retrotransposons that were saturated by eamiRNA biogenesis (Supplementary Table 1), but an overall general loss from DNA transposons and other individual retrotransposons, such that less than half of these retained 24-nucleotide siRNA in ddm1 (Extended Data Fig. 5). Many of these TEs gained 24-nucleotide hetsiRNAs in ddm1 rdr6 and ddm1 dcl1-11 (Supplementary Table 1 and Extended Data Fig. 5) and, furthermore, hundreds of TEs that did not have detectable hetsiRNAs in Col-0 or ddm1 gained them in ddm1 rdr6 (Fig. 4a and Extended Data Fig. 6). Thus, miRNA targeting and eamiRNA biogenesis might inhibit 24-nucleotide hetsiRNA production from transposons in ddm1. We found that almost all TEs cleaved by miRNA either gained 21-nucleotide eamiRNAs in ddm1, lost 24-nucleotide hetsiRNAs in ddm1, or gained 24-nucleotide hetsiRNAs in ddm1 rdr6 (Fig. 4b, c and Extended Data Fig. 6). This enrichment was even greater for those TEs targeted by more than one miRNA (Extended Data Fig. 6c).

Thus, miRNA cleavage seems to inhibit 24-nucleotide siRNA biogenesis in a manner that depends on RDR6, whether or not eamiRNAs accumulate (Fig. 4b and Supplementary Table 3). This response was observed when miRNA cleavage sites were on the sense (miRNA) or occasionally on the antisense strand (Supplementary Table 3). It is possible, therefore, that miRNA-directed cleavage of antisense Pol IV, or Pol V transcripts, and processing by RDR6 rather than RDR2, might prevent the formation of 24-nucleotide siRNAs without necessarily generating 21-nucleotide eamiRNAs (that are Pol II dependent). Thus, RDR6 partially antagonizes RDR2 activity, inhibiting 24-nucleotide hetsiRNA biogenesis at TEs. Likewise, RDR2 partially antagonizes RDR6 activity at transgenes subject to PTGS34.

Symmetric CG and CHG methylation contexts are maintained by DNA methyltransferases and histone modifications, whereas CHH methylation is associated with 24-nucleotide hetsiRNA-guided RNA-directed DNA methylation (RdDM)4. Methylation mediates transcriptional silencing of TE promoters31, found near the terminal inverted repeats (TIR) of most DNA transposons and in the LTR of retrotransposons, as well as internally in the case of the ATHILA ORF1 and some DNA transposons. As expected, whole-genome sequencing of bisulphite-treated DNA from inflorescence tissue revealed global loss of DNA methylation at TEs and are RDR2 dependent1. We profiled 24-nucleotide hetsiRNAs in Col-0, ddm1, rdr6, ddm1 rdr6 and ddm1 dcl1-11 (Supplementary Table 1 and Fig. 4). In Col-0, 24-nucleotide hetsiRNAs target LTR retrotransposons and most DNA transposons (Extended Data Fig. 5). In ddm1, we found a slight increase in multiple mapping 24-nucleotide hetsiRNAs from ATGYPSY retrotransposons that were saturated by eamiRNA biogenesis (Supplementary Table 1), but an overall general loss from DNA transposons and other individual retrotransposons, such that less than half of these retained 24-nucleotide siRNA in ddm1 (Extended Data Fig. 5). Many of these TEs gained 24-nucleotide hetsiRNAs in ddm1 rdr6 and ddm1 dcl1-11 (Supplementary Table 1 and Extended Data Fig. 5) and, furthermore, hundreds of TEs that did not have detectable hetsiRNAs in Col-0 or ddm1 gained them in ddm1 rdr6 (Fig. 4a and Extended Data Fig. 6). Thus, miRNA targeting and eamiRNA biogenesis might inhibit 24-nucleotide hetsiRNA production from transposons in ddm1. We found that almost all TEs cleaved by miRNA either gained 21-nucleotide eamiRNAs in ddm1, lost 24-nucleotide hetsiRNAs in ddm1, or gained 24-nucleotide hetsiRNAs in ddm1 rdr6 (Fig. 4b, c and Extended Data Fig. 6). This enrichment was even greater for those TEs targeted by more than one miRNA (Extended Data Fig. 6c).

Thus, miRNA cleavage seems to inhibit 24-nucleotide siRNA biogenesis in a manner that depends on RDR6, whether or not eamiRNAs accumulate (Fig. 4b and Supplementary Table 3). This response was observed when miRNA cleavage sites were on the sense (miRNA) or occasionally on the antisense strand (Supplementary Table 3). It is possible, therefore, that miRNA-directed cleavage of antisense Pol IV, or Pol V transcripts, and processing by RDR6 rather than RDR2, might prevent the formation of 24-nucleotide siRNAs without necessarily generating 21-nucleotide eamiRNAs (that are Pol II dependent). Thus, RDR6 partially antagonizes RDR2 activity, inhibiting 24-nucleotide hetsiRNA biogenesis at TEs. Likewise, RDR2 partially antagonizes RDR6 activity at transgenes subject to PTGS34.

Figure 4 Transposons targeted by miRNA gain hetsiRNA when the eamiRNA pathway is lost. a, Overlap of transposons targeted by hetsiRNAs in Col-0, ddm1-2 and ddm1-2 rdr6-15. b, Overlap of transposons targeted by miRNAs that have lost hetsiRNAs in ddm1-2, gained hetsiRNAs in ddm1-2 rdr6-15, and those whose transcripts undergo productive miRNA cleavage resulting in eamiRNA biogenesis in ddm1-2. c, d, Twenty-one-nucleotide eamiRNAs, 24-nucleotide hetsiRNAs and PARE degradome reads from eamiRNA-generating ATAILA (AT3G32118) (c) in comparison with non-eamiRNA generating ATENSPM10 (AT4G23143) (d). Individual reads: sense, red; antisense, blue. Track order: 1, Col-0; 2, ddm1-2; 3, rdr6-15; 4, ddm1-2 rdr6-15.
methylation from all classes of transposons in *ddm1* when compared to wild type (Extended Data Fig. 7a, b). As previously reported, most of this loss occurred in the symmetric CG and CHG contexts, rather than the asymmetric CHH context, consistent with retention of 24-nucleotide hetsiRNAs27 (Supplementary Table 4). By using whole-genome bisulphite sequencing, we measured levels of DNA methylation in *ddm1* *rdr6* F3 progeny from two independent double mutants relative to *ddm1* (Extended Data Fig. 7a). A modest difference in DNA methylation is expected due to inbreeding of the *ddm1* parents23, and was observed in one *ddm1* *rdr6* replicate; however, methylation at TEs substantially increased in the other replicate (Extended Data Fig. 7e, f and Supplementary Table 4). These results suggest that elevated levels of 24-nucleotide hetsiRNAs could only guide remethylation of TEs stochastically in *ddm1* *rdr6* (Extended Data Fig. 7g, h), probably reflecting the epigenetic inheritance of unmethylated and methylated TEs from the *ddm1* and *rdr6* parents, respectively, as previously observed24.

To assess whether the loss of the easiRNA pathway leads to a further outburst of transposon reactivation in *ddm1* *rdr6*, we used Affymetrix *ATH1* microarrays to analyse the transcriptome. A few hundred representative TEs are included on Affymetrix *ATH1* microarrays, and can be used to assess TE reactivation2. We found that most of these TE transcripts were abundantly and similarly expressed in both *ddm1* and *ddm1* *rdr6* (Supplementary Table 5 and Extended Data Fig. 8), indicating that easiRNA biogenesis does not reduce TE transcript levels in *ddm1* mutants. In fact, consistent with our findings of sporadic TE methylation and gain of hetsiRNAs in *ddm1* *rdr6*, several TEs had reduced expression in *ddm1* *rdr6*, including *ATHILA* and *ATCOPIA* elements, as well as TEs of the MuDR superfamily (Supplementary Table 5). Therefore, the loss of both *DDM1* and *RDR6* does not enhance transcriptional reactivation of TEs, but instead, some TEs become transcriptionally repressed in *ddm1* *rdr6* (Extended Data Fig. 8 and Supplementary Table 5) and an elevation in methylation level is observed at least for some of these TEs, for example, *ATCOPIA22* and *ARNOLD3*, in *ddm1* *rdr6* (Supplementary Table 4).

We have found that loss of TE methylation results in epigenetic activation and consequent transcription, whereby transposon mRNA become preferentially targeted by more than 50 miRNAs bound to AGO1. Productive cleavage of transposon transcripts usually engages *RDR6*, allowing *DCL4* to generate 21-nucleotide easiRNAs in a PTGS mechanism (Extended Data Fig. 9). About half of these transposons belong to the LTR/ATHILA element family (Extended Data Fig. 10), which occur in high copy number at pericentromeric repeats and are targeted by multiple miRNAs (Extended Data Fig. 3c and Fig. 2). Several known miRNA genes—for example, *MIR843*—are methylated, and only target transposon transcripts when unmethylated and expressed: in pollen and in *ddm1* mutants (Supplementary Table 2 and Extended Data Fig. 4). Other miRNAs are developmentally regulated—for example, miR-156, which is required to maintain the juvenile phase of plant development, and miR-172, which is required for the transition to the adult phase25—accounting perhaps for the tissue-specific silencing of TEs during different phases of plant development26.

TEs are targeted by the same conserved miRNA (miR-845) in rice and *Arabidopsis*, leading to the idea that they may have retained miRNA-binding sites despite selection against them. Furthermore, some of the newly identified miRNAs are themselves encoded by TEs (Extended Data Fig. 10). It is unclear why transposons would retain miRNA sites that could potentially silence them post-transcriptionally. One explanation lies in the preferential processing of targeted TE transcripts via *RDR6*, which seems to act antagonistically with *RDR2*, preventing 24-nucleotide hetsiRNA biogenesis30 (Extended Data Fig. 9). We found a notable overlap between TEs that produced easiRNAs in *ddm1* and those that gained 24-nucleotide hetsiRNAs in *ddm1* *rdr6* (Fig. 4b and Extended Data Fig. 6). Twenty-four-nucleotide hetsiRNAs promote RdDM, and it is likely, therefore, that TEs tolerate developmentally regulated PTGS by miRNA and easiRNAs to avoid long-term TGS by DNA methylation. Given the probable evolution of miRNA precursors from TEs and other inverted repeats31, miRNAs may have arisen in ancient eukaryotes to target retrotransposons and other TEs rather than genes.

*ddm1* mutants are remarkably normal, despite the heritable loss of heterochromatin. By contrast, *ddm1* *rdr6*, *ddm1* *dcl4* and *ddm1* *dcl1* have a wide range of developmental phenotypes and heritable epigenetic defects, including widespread infertility in subsequent generations (K.M.C. and R.A.M., unpublished observations). We therefore postulate that the function of the easiRNA biogenesis pathway within the host is to protect the genome from TE-mediated epigenomic instability. Consistent with this idea, easiRNAs specifically target the *ATHILA* *ORF1* gene, thought to be involved in retrovirus-like particle formation, as well as the *ATCOPIA* integrase gene and the *CACTA* transposase gene, required for transposition (Extended Data Fig. 3b and Fig. 4c). *RDR6*-directed PTGS could be dangerous to the host by presenting an opportunity to the TE for evasion and transposition, whereas *RDR2*-directed TGS is a safer silencing strategy not requiring transcription. An important developmental stage during which PTGS is deployed is in the pollen grain, when pericentromeric retrotransposons, and some DNA transposons, lose CHH methylation and RdDM in the male germ line27. Many of these same TEs accumulate easiRNAs in sperm, which are generated by TE activation in the companion vegetative nucleus, accompanied by the loss of DDM1 (ref. 5). Transient loss of RdDM in sperm is restored after fertilization by maternal 24-nucleotide hetsiRNAs27,28 allowing an opportunity to distinguish ‘self’ from ‘non-self’ pollen on fertilization, according to whether the transposon load is sufficiently foreign not to be recognized by maternal small RNAs. PTGS, mediated by easiRNAs, would provide a backup silencing mechanism during this critical but potentially dangerous window. A similar secondary siRNA transposon control mechanism, triggered by PIWI-interacting RNA (piRNA) rather than miRNA, has recently been described in *Caenorhabditis elegans* and may have similar roles29,30.

**METHODS SUMMARY**

Genomic DNA and total RNA were isolated from inflorescence tissue (stage 9–11) collected from Col-0 wild type and loss of function mutants: *ddm1-2*, *rdr6-15*, *ddm1-2*rdr6-15 and *ddm1-2*dcl1-11 in a Col-0 background, grown under standard long-day growth conditions. Small RNA libraries were sequenced on Illumina HiSeq2000 using paired-end reads for 50 bases and mapped using Bowtie from the open-source Tuxedo suite, allowing for both unique (U) and multiple (M) mapping reads. Bisulphite sequencing (BIS-seq) library construction and bisulphite conversion were performed as previously described. Col-0, *ddm1-2*, *rdr6-15* and *ddm1-2*rdr6-15 replicate BIS-seq libraries were sequenced on Illumina HiSeq2000 using paired-end reads for 50 cycles, and mapped using Bismark. Our miRNA prediction algorithm was used to identify miRNAs that target TEs, miRNA purification (Dynabeads), 5’ adapter ligation (Invitrogen) and cDNA purification (AMPure XP) for PARE were performed according to the manufacturer’s instructions, followed by PCR amplification and PAGE purification. 5’ RLM RACE was performed according to the manufacturer’s instructions (Ambion). Sequencing and mapping information for each library described in this study is given in Supplementary Table 8.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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METHODS

Biological plant materials, DNA and RNA isolation. Genomic DNA and total RNA were isolated from inflorescence tissue collected from Col-0 wild type and loss of function mutants: ddm1-2, rdr6-15, ddm1-2 rdr6-15 and ddm1-2 dcl-11 in a Col-0 background, grown under standard long-day growth conditions. The ddm1-2 line used in this study and for the genetic cross with rdr6-15, was in the fifth generation of inbreeding. Double heterozygous lines were selected and selfed, and second generation ddm1-2 rdr6-15 mutants were isolated for sequencing in this study. DNA was isolated from pooled inflorescence tissue (n = 3) collected by phenol/chloroform extraction. Total RNA was isolated from pooled inflorescence tissue (n = 3) and TRIzol (Invitrogen) extraction was carried out following the manufacturer’s instructions.

Small RNA sequencing library construction and analysis. TruSeq small RNA sequencing libraries were made from pooled inflorescence tissue between stages 9 and 11 (unopened or just opened flowers) (n = 3) following the manufacturer’s instructions and sequenced on Illumina HiSeq. NEBNext multiplex small RNA libraries were made from pooled inflorescence tissue (n = 3) following the manufacturer’s instructions and sequenced on Illumina MiSeq. Mapping small RNA sequencing raw data was performed using Bowtie from the open-source Tuxedo suite, allowing for both unique (U) and multiple (M) mapping reads, normalized by reads per million (RPM). Library read count is given in Supplementary Table 1.

Whole-genome bisulphite sequencing library construction and analysis. Library construction and bisulphite conversion were carried out essentially as described previously31. For each library, 1–5 μg of genomic DNA was sheared using a Covaris S220 Adaptive Focused Acoustics ultrasonic. Libraries were constructed following standard protocol using the NEB Next DNA Sample Prep Master Mix Set 1 (NEB E6040) and Illumina-compatible paired-end adaptors, in which all cytosines were methylated. Each library was treated with sodium bisulphite using an EZ DNA Methylation-Gold Kit (Zymo Research D5005) according to the protocol provided by the manufacturer. For each purified library, 5–10 ng of purified library was amplified using the Expand High Fidelity PLUS PCR system (Roche 03300242001), which is capable of efficiently amplifying uracil-containing templates. Fifty-microtitre reactions contained 200 μM each of dNTP, 1 μM primer, 2.5 mM MgCl2 and 2.5 U Expand HiFi enzyme, and were performed according to the manufacturer’s instructions for 18 cycles. Amplified libraries were run on 2% MetaPhor agarose (Lonza 50108) gel. Fragments of 220–350 bp were excised from the gel and purified using the QIAquick PCR Purification kit (Qiagen 28104). DNA concentrations were quantified on Bioanalyzer (Agilent), diluted to 10 nM and loaded on flow cells to generate clusters. Libraries were sequenced on Illumina GAII or HiSeq2000 machines using the paired-end 50 cycles protocol. Mapping of bisulphite sequencing (BIS-seq) libraries was performed using Bismark32. Library read count is given in Supplementary Table 8. For statistical significance we performed the $W_7$ goodness of fit test, calculating the $P$ value for the observed value given the expected value. If we assume PARE tags fall randomly on all the positions in the long window (31 nucleotides) then the expected frequency for PARE reads falling into the small window (5 nucleotides) would be 5/31; therefore, the expected frequency would be 26/31 for other regions. For ATGRF8, the target of miR-396, in Col-0 we observe 603 PARE tags in the small window and 614 PARE tags in the long window (614 – 603 = 11 in the other region); the $W_7$ for the given the conditional probabilities ($\chi^2$, 11, $\chi^2 = 3057.857$, degrees of freedom = 1, $P$ value = $2.2 \times 10^{-14}$ (Supplementary Table 3). For specific miRNA targeting, the sum of abundance of PARE tags matching to (1) a small window ($W_5$) of 5 nucleotides (cleavage site ± 2 nucleotides), and (2) a long window ($W_I$) of 31 nucleotides (cleavage site ± 15 nucleotides) was calculated. Cleavage sites were filtered to retain those for which $W_I/W_{5} > 0.5$ in the PARE-seq libraries; sites failing these criteria were considered background mRNA cleavage levels. Some chloroplast contamination can account for the distribution of reads across multiple loci in our libraries. However, when comparing with the normalization basis, and the mapping of PARE tags to multiple loci, these reads appear to be of chloroplast mRNA in origin.

The miRNA target lists were filtered by combining both the miRNA target penalty score ($\sigma$) with the PARE-seq data filters of $W_5/W_{I} > 0.3$ and $W_{5} > 0.3$ and $W_{I} > 2$ (relaxed). Library information is given in Supplementary Table 8. For statistical significance we performed the $W_7$ goodness of fit test, calculating the $P$ value for the observed value given the expected value. If we assume PARE tags fall randomly on all the positions in the long window (31 nucleotides) then the expected frequency for PARE reads falling into the small window (5 nucleotides) would be 5/31; therefore, the expected frequency would be 26/31 for other regions. For ATGRF8, the target of miR-396, in Col-0 we observe 603 PARE tags in the small window and 614 PARE tags in the long window (614 – 603 = 11 in the other region); the $W_7$ for the given the conditional probabilities ($\chi^2$, 11, $\chi^2 = 3057.857$, degrees of freedom = 1, $P$ value = $2.2 \times 10^{-14}$ (Supplementary Table 3). For specific miRNA targeting, the sum of abundance of PARE tags matching to (1) a small window ($W_5$) of 5 nucleotides (cleavage site ± 2 nucleotides), and (2) a long window ($W_I$) of 31 nucleotides (cleavage site ± 1 nucleotide) was calculated (Supplementary Table 9).

5’ RLM RACE PCR. 5’ RLM RACE was used to map the cleavage of miRNA using FirstChoice (Ambion) following the manufacturer’s instructions. 5’ RLM RACE adaptor ligation was followed by RT–PCR, then by two PCRs (outer and inner), cloning and sequencing.

5’ RLM RACE adaptor: 5’-GCUGAUGCGAGAUGAAACAGCUGUUUGCCGCUUUGAUGAAA-3’. 5’ RLM RACE outer primer: 5’-GCTGATGCGAGTGAATGACACTG-3’. 5’ RLM RACE inner primer: 5’-CGCGATCGAACACTGCGTTTGCGGTTTGAATTC-3’.

31. Regulski, M. et al. The maize methylome influences mRNA splice sites and reveals widespread paramutation-line switches guided by small RNA. Genome Biol. 23, 1651–1662 (2013).
32. Krueger, F. & Andrews, S. R. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics 27, 1571–1572 (2011).
33. Zhai, J. et al. MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. Genes Dev. 25, 2540–2553 (2011).
Extended Data Figure 1 | Twenty-one-nucleotide easiRNAs originate from transposons in *ddm1* and are miRNA and RDR6 dependent. 

**a**, Eighteen-nucleotide to 26-nucleotide small RNA abundance in Col-0, *ddm1-2* and *ddm1-2 rdr6-15*. Normalized reads per million (RPM). nt, nucleotides.

**b**, Eighteen-nucleotide to 26-nucleotide small RNA abundance in Col-0, *ddm1-2* and *ddm1-2 dcl1-11*. Normalized reads per million (RPM).
Extended Data Figure 2 | miRNA target genes and transposons that do not promote tasiRNA nor easiRNA, respectively, have degradation covering the entire region. a-f, Read pattern distribution of 21-nucleotide unique reads (represented as a histogram of read density (grey bars)) and PARE signatures at TAS2 (AT2G39681) (a), RCC (AT3G02300) and SEP2 (AT3G02310) (b), MEE58 (AT4G13940) (c), and transposons ATENSPM6 (AT2G06720) (d), ATLINE1_4 (AT2G15540) (e) and ATCOPIA43 (AT3G0410) (f), in track order Col-0, ddm1-2, rdr6-15, ddm1-2 rdr6-15 and ddm1-2 dcl1-11.
Extended Data Figure 3 | Relationship between DNA methylation, easiRNA and hetsiRNA at transposons that miRNAs are predicted to target.

a–c, DNA methylation, by CG (red), CHG (blue) and CHH (+ strand, − strand) context (green) and cytosine context (scale: 1 = methylated cytosine; 0 = unmethylated cytosine) at ATCOPIA93 (AT5G17125) (a), the ATMUS (AT4G08680) DNA transposon and the surrounding region (b) and the ATHILA6A (AT4TE15030) retrotransposon and the surrounding region (c). Twenty-one-nucleotide and 24-nucleotide siRNAs, represented as a histogram of read density (grey bars). sRNA reads: unique (U), mapping to one location of the genome; and multiple (M), mapping to more than one location of the genome. PARE read density. Track order for panels Col-0 (1), ddm1-2 (2), rdr6-15 (3) and ddm1-2 rdr6-15 (4).
Extended Data Figure 4 | eamiRNA immature precursor sequence and predicted structure. eamiRNA immature precursor sequences have methylated cytosines (asterisks) in Col-0 that are unmethylated in ddm1-2. Mature miRNAs are underlined, and the putative stem-loop structures of the precursors are illustrated.
Extended Data Figure 5 | Twenty-four-nucleotide hetsiRNAs at transposons in Col-0 are lost in ddm1 and gained in ddm1 rdr6 and ddm1 dcl1. Twenty-four-nucleotide hetsiRNAs by transposon class in Col-0, ddm1-2, rdr6-15, ddm1-2 rdr6-15 and ddm1-2 dcl1-11. Normalized reads per million (RPM).
Extended Data Figure 6 | Overlap of TEs that undergo easiRNA biogenesis, hetsiRNA loss and miRNAs targeting.  a–d, Individual transposons were grouped depending on small RNA abundance in each genotype.  a, TEs that lose 24-nucleotide hetsiRNAs in ddm1-2 and those that gain 21-nucleotide easiRNAs in ddm1-2 overlap with those that gain 24-nucleotide hetsiRNAs in ddm1-2 rdr6-15.  b, TEs that are targeted and cleaved by miRNAs overlap with those that gain 21-nucleotide easiRNAs in ddm1-2 and 24-nucleotide hetsiRNAs in ddm1-2 rdr6-15.  c, TEs that are targeted and cleaved by two or more miRNAs overlap with those that gain 21-nucleotide easiRNAs in ddm1-2 and those that gain 24-nucleotide hetsiRNAs in ddm1-5 rdr6-15.  d, TEs that are predicted to be targeted by miRNAs, but without supporting PARE cleavage data, also overlap with those that gain 21-nucleotide easiRNAs in ddm1-2 and 24-nucleotide hetsiRNAs in ddm1-2 rdr6-15.
Extended Data Figure 7 | Loss of methylation at transposons in ddm1 is partially restored in ddm1 rdr6. a, Transposon methylation in Col-0, ddm1-2, rdr6-15 and ddm1-2 rdr6-15 replicates. Scale: 1 = methylated cytosine; 0 = unmethylated cytosine. b–f, Total DNA methylation at transposons, grouped by superfamily in Col-0 (b), ddm1-2 (c), rdr6-15 (d) and ddm1-2 rdr6-15 (e, f) replicates. 1, LTR retrotransposon ATGYPSY; 2, LTR retrotransposon ATCOPIA; 3, non-LTR retrotransposons ATLINE; 4, non-LTR retrotransposon TSCL; 5, TIR DNA transposon MUDR; 6, non-TIR DNA transposon MuDR; 7, DNA transposon ENSPM; 8, DNA transposon HELITRON; 9, other repeats. Total methylation by total converted cytosine to thymine and non-converted cytosine counts (at least ten reads per cytosine). Scale: 0 = unmethylated; 1 = methylated. Boxplots indicate median, range and standard deviations (box). g, h, DNA methylation by CG (red), CHG (blue) and CHH 1/2 context (green) at ATHILA ORF1 (AT2G10280) (g) and ATCOPIA43 (AT1G36040) (h). Track order: 1, Col-0; 2, ddm1-2; 3, rdr6-15; 4, ddm1-2 rdr6-15.
Extended Data Figure 8 | Transposon transcript abundance in ddm1-2 and ddm1 rdr6. a–c, ATH1 Affymetrix microarray expression (log2 signal intensity) in Col-0 in comparison with ddm1-2, rdr6-15 and ddm1-2 rdr6-15. d, TEs upregulated in ddm1-2 were not further upregulated in ddm1-2 rdr6-15. Red indicates transposons, black indicates genes.
**Extended Data Figure 9 | miRNA-directed easiRNA biogenesis from activated transposons.** When TEs are epigenetically activated, through the loss of DNA methylation and/or heterochromatin, transposon mRNA transcripts become preferentially targeted by miRNAs (DCL1 dependent) bound by AGO1. Productive cleavage of transposon transcripts engages RDR6 and DCL4, which generate 21-nucleotide easiRNAs from transposon ORFs—in a PTGS mechanism—that are then loaded into AGO1 and, thus, prevents engagement of RDR2 and RdDM. This antagonism accounts for the retention of miRNA binding sites by transposons to evade long-term heritable silencing, elicited by DNA methylation via RDR2. This model also accounts for the retention of the miRNA-directed mechanism by the host organism, in order to generate easiRNAs to silence TEs when they are epigenetically reprogrammed in the germ line.
Extended Data Figure 10 | Arabidopsis miRNAs target transposons.

Known Arabidopsis miRNAs and novel eamiRNAs that arise in ddm1-2 are predicted to target transposon transcripts and confirmed to cleave transposon transcripts by PARE (Supplementary Table 3 and Methods). eamiRNAs, some known to be developmentally regulated, and TE-derived eamiRNAs that target specific transposon families. Transposons are identified by EVRY TE identifier (Supplementary Table 2; for further annotation refer to TAIR using ORF identification number). Transposon transcripts giving rise to 21-nucleotide easiRNAs (bold); those that are targeted by multiple miRNA (asterisks); and those miRNAs that target multiple transposons of the same family (italics) are highlighted.