nature genetics

Dissecting *Arabidopsis thaliana* DICER function in small RNA processing, gene silencing and DNA methylation patterning

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Small RNAs have several important biological functions[1]. MicroRNAs (miRNAs) and *trans*-acting small interfering RNAs (tasiRNAs) regulate mRNA stability and translation, and siRNAs cause post-transcriptional gene silencing of transposons, viruses and transgenes and are important in both the establishment and maintenance of cytosine DNA methylation[2]. Here, we study the role of the four *Arabidopsis thaliana DICER-LIKE* genes (*DCL1–DCL4*) in these processes. Sequencing of small RNAs from a *dcl2 dcl3 dcl4* triple mutant showed markedly reduced tasiRNA and siRNA production and indicated that DCL1, in addition to its role as the major enzyme for processing miRNAs, has a previously unknown role in the production of small RNAs from endogenous inverted repeats. *DCL2*, *DCL3* and *DCL4* showed functional redundancy in siRNA and tasiRNA production and in the establishment and maintenance of DNA methylation. Our studies also suggest that asymmetric DNA methylation can be maintained by pathways that do not require siRNAs.

Small RNAs are processed by DICER endonucleases before their incorporation into effector complexes for use in various biological processes[1]. *Arabidopsis thaliana* DICER-LIKE enzymes (DCLs) act during miRNA metabolism (DCL1), viral resistance (DCL2), transcriptional silencing (DCL3) and post-transcriptional silencing and tasiRNA metabolism (DCL4)[3–8]. DCL specialization is also evidenced by production of distinct siRNA size classes by DCL1 (21 nt) and DCL3 (24 nt)[9,10]. Phylogenetic analysis shows that plant DCLs form a monophyletic and plant-specific group (**Fig. 1a**). Four distinct lineages are apparent (DCL1–DCL4), each containing sequences from both monocots and dicots[11]. Thus, *DCL* gene diversification occurred after the plant-animal split but before the divergence of monocots and dicots (at least 150 million years ago).

We sought to test the function of *DCL* genes using loss-of-function mutations. However, this analysis is complicated by the fact that null *DCL1* alleles are embryonic lethal, and by previous observations that DCL2, DCL3, and DCL4 have partially redundant functions[4,6,8]. We therefore used transfer-DNA (T-DNA) insertional mutations in *DCL2*, *DCL3* and *DCL4* to create all possible double mutants as well as the *dcl2-1 dcl3-1 dcl4-2* triple mutant (**Fig. 1b**). We analyzed these mutants to infer the function of the four *DCL* genes in miRNA, tasiRNA and siRNA metabolism and to examine their role in transgene silencing and RNA-directed DNA methylation.

All combinations of *dcl* mutations were viable and fertile (**Fig. 1c**). The *dcl4-2* mutant showed developmental phenotypes associated with defective tasiRNA production, including epinastic leaves[4–7] (**Fig. 1c**). The *dcl2-1 dcl3-1 dcl4-2* triple mutant showed more pronounced developmental defects than the *dcl4-2* single mutant and showed a slight delay in flowering under continuous light (**Table 1**).

To determine the effect of *dcl2-1 dcl3-1 dcl4-2* on small RNA metabolism, we compared small RNA populations of triple mutant plants with wild-type plants through the sequencing of approximately 11,000 small RNAs from each, using 454 sequencing technology (see Methods) (**Fig. 2**)[12]. The most common length of small RNAs in wild-type plants was 24 nt and 21 nt (**Fig. 2a**), and 22mers and 23mers also accumulated, although less frequently. In contrast, most small RNAs from *dcl2-1 dcl3-1 dcl4-2* were 21 nt in length, consistent with DCL3 generating 24-nt siRNA[3,10]. The majority of small RNAs remaining in *dcl2-1 dcl3-1 dcl4-2* were known miRNAs (67.5%); miRNAs made up a greater percentage of total small RNAs in the mutants than in the wild-type plants (20.9%) (**Supplementary Table 1** online). Most known miRNAs were present at wild-type levels in the *dcl2-1 dcl3-1 dcl4-2* data set, which was confirmed by small RNA blot experiments (**Fig. 2b,d**). Hence, our genetic evidence suggests that *DCL1* alone is sufficient for miRNA metabolism, consistent with a lack of miRNA-related phenotypes in *dcl2-1 dcl3-1 dcl4-2*.

In contrast to miRNAs, known tasiRNAs were absent from *dcl2-1 dcl3-1 dcl4-2* sequences (**Fig. 2c**). RNA blotting for tasiRNA255 uncovered multiple species of small RNAs, which were completely eliminated only in *dcl2-1 dcl3-1 dcl4-2* (**Fig. 2d**). However, the miRNAs (miR173, miR390)[13] that initiate the production of these

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Received 9 March; accepted 19 April; published online 14 May 2006; doi:10.1038/ng1804

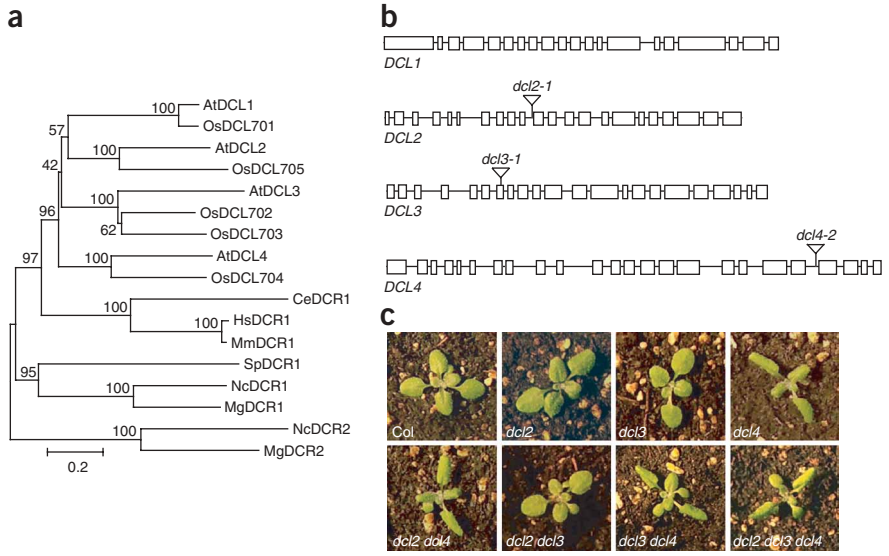

Figure 1 Generation of *Arabidopsis thaliana dcl* mutant backgrounds. (**a**) Neighbor joining tree showing the phylogenetic relationships between DICER-LIKE proteins. Species abbreviations are At (*Arabidopsis thaliana*), Os (*Oryza sativa*), Ce (*Caenorhabditis elegans*), Hs (*Homo sapiens*), Mm (*Mus musculus*), Sp (*Schizosaccharomyces pombe*), Nc (*Neurospora crassa*) and Mg (*Magnaporthe grisea*). (**b**) Schematic representation of *DCL* genes and position of T-DNA insertions. Boxes represent exons, and lines represent introns. T-DNA insertions are represented by triangles. (**c**) Phenotypes of plants carrying combinations of *dcl2-1*, *dcl3-1* and *dcl4-2* mutations.

tasiRNAs remained in *dcl2-1 dcl3-1 dcl4-2*, confirming previous observations that this is a separable step in the process[4,6].

We were surprised to find that roughly one-third of the small RNAs remaining in *dcl2-1 dcl3-1 dcl4-2* did not match previously described miRNAs, demonstrating an unexpected abundance and diversity of DCL1 products. Comparison of these small RNAs to those in wild-type plants uncovered several interesting features. First, similar to miRNAs, the remaining DCL1 products are predominantly 21 nt (**Fig. 2a**). Second, a much higher proportion of small RNAs accumulate as 'families' (exact sequences recovered more than once) in *dcl2-1 dcl3-1 dcl4-2* (41.9%) than accumulate in wild-type plants (9.1%), indicating that DCL1 has a tendency to cleave at defined sites (**Supplementary Table 1**). Third, although the relative abundance of small RNAs derived from dispersed or tandem repeats remained similar in *dcl2-1 dcl3-1 dcl4-2* and wild-type plants, those derived from inverted repeats were substantially more abundant in the triple mutant (**Fig. 3a**). In addition, a more detailed analysis of several randomly selected small RNAs from the triple mutant showed that, although they were not derived from annotated inverted repeats, in many cases their immediate flanking sequences could potentially fold into imperfect hairpin-like structures. Taken together, these results suggest that, reminiscent of miRNAs, DCL1 prefers substrates with hairpin-like structures and cleaves at defined sites that are 21 nt apart[8,10]. Many of the *dcl2-1 dcl3-1 dcl4-2* small RNAs might therefore be unidentified, recently evolved or evolving miRNA genes[14]. Analysis of siRNA accumulation patterns at the large inverted repeat *IR-71* showed peaks of abundance, the largest of which corresponds to 60-bp direct repeats (**Fig. 3b**, **Supplementary Fig. 1** online). This is similar to findings in *S. pombe* in which local repeat structure correlated with siRNA accumulation patterns[15].

To further dissect the role of *DCL2*, *DCL3* and *DCL4* in siRNA and tasiRNA production, we analyzed siRNA accumulation by RNA blot analysis in *dcl* single and double mutants at loci representing tandem repeats (*FWA* and *TR2558*), inverted repeats (*IR-71*), dispersed repeats (*COPIA1*, *COPIA2* and *AtSN1*), tasiRNA-producing loci (tasiRNA255) and single-copy sequences (*Cluster4*) (**Figs. 2d** and **3c**). All siRNA signals were reduced in *dcl3* and were virtually eliminated in *dcl2-1 dcl3-1 dcl4-2*. Furthermore, siRNAs and tasiRNAs showed complex patterns of siRNA accumulation, with at least three size classes (21 nt, 22 nt and 24 nt). These results, combined with previous findings[3,4,6,7,10], suggest that DCL1 and DCL4 act to process 21-nt small RNAs, DCL2 22-nt small RNAs and DCL3 24-nt small RNAs (**Figs. 2d** and **3c**).

At *COPIA2*, the siRNAs in *dcl3-1* were present as a ladder of single-nucleotide steps, reminiscent of that observed in *hen1* mutants[16,17]. Notably, in *dcl2-1 dcl3-1 dcl4-2*, the ladder shifted to a size of 40 nt or greater (**Fig. 3c**). This ladder may represent siRNAs end-modified by uridylation[16–18] or may represent the accumulation of unprocessed DCL substrate RNAs.

We used *dcl* mutant combinations to test the role of small RNAs in the control of DNA methylation. Cytosine methylation in *Arabidopsis thaliana* occurs in CG, CNG and CHH (asymmetric) sequence contexts[2]. CNG and asymmetric methylation are maintained by the DRM2 and CMT3 methyltransferases, and DRM2 controls *de novo* methylation in all sequence contexts[2,19,20]. Transformation-dependent *de novo* methylation and silencing of an *FWA* transgene has been shown to require RNA silencing genes such as RNA-DEPENDENT RNA POLYMERASE2 (*RDR2*) and *DCL3*, although the effects of *dcl3* in this assay were much weaker than those of *rdr2* (refs. 2,19,20). These RNA silencing genes were also required for maintenance of non-CG methylation, but again, *dcl3* showed weak effects[2,3,20].

We tested *dcl* mutant combinations with the *FWA* transformation assay. Transformation with an extra copy of *FWA* leads to *de novo* methylation of two direct repeats in the transgene and silencing of *FWA* expression[2,19–21]. Failure to silence causes *FWA* overexpression and a dominant late-flowering phenotype[2,19,20,21]. As previously reported, *dcl3-1* caused a partial defect in establishment of *FWA* silencing[20] (**Fig. 4** and **Table 1**). The double mutants *dcl2-1 dcl3-1*

Table 1 Average flowering-time of T_1 transformant populations

	Total leaf number ± s.e.m.	
Genotype	+ *FWA*	+ Empty vector
Wild-type Col	18.1 ± 0.31	13.3 ± 0.23
dcl2	19.0 ± 0.60	15.6 ± 0.25
dcl3	28.6 ± 0.82	14.7 ± 0.32
dcl4	19.0 ± 0.41	14.9 ± 0.34
dcl2 dcl3	29.5 ± 0.69	16.3 ± 0.36
dcl2 dcl4	20.3 ± 0.48	13.5 ± 0.29
dc3 dcl4	27.4 ± 1.00	13.2 ± 0.52
dcl2 dcl3 dcl4	46.3 ± 0.93	19.7 ± 0.36

Col: wild-type accession Columbia.

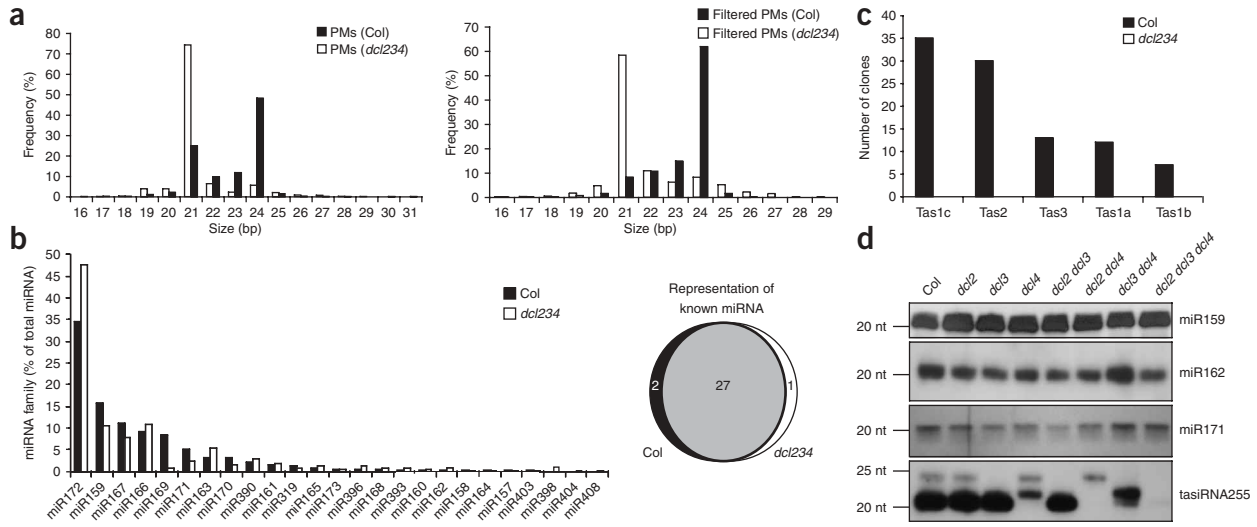

Figure 2 Accumulation of miRNAs and *trans*-acting siRNAs in *dcl2 dcl3 dcl4*. (**a**) Abundance of small RNA with perfect matches (PMs) to the genome according to size in Col and *dcl2-1 dcl3-1 dcl4-2* ('*dcl234*') before and after filtering out known miRNAs ('filtered PMs'). (**b**) Percentage of specific miRNAs in the total miRNA cloned population shown for each genotype. Venn diagram shows representation and overlap of known miRNA for Col and *dcl2-1 dcl3-1 dcl4-2*. (**c**) Number of clones of known tasiRNA sequenced from Col and *dcl2-1 dcl3-1 dcl4-2*. (**d**) RNA blots analyzing miRNA and tasiRNA accumulation in Col and *dcl* mutant backgrounds.

and *dcl3-1 dcl4-2* resembled *dcl3-1*; however, the *dcl2-1 dcl3-1 dcl4-2* triple mutant flowered significantly later (**Fig. 4** and **Table 1**). Thus, DCL2 and DCL4 act in a redundant manner, along with DCL3, in *FWA de novo* methylation.

We also analyzed the function of *DCL* genes and siRNAs in maintaining CNG and asymmetric methylation, by measuring DNA methylation at endogenous genes using bisulfite genomic sequencing[22]. We observed three patterns of methylation change in the *dcl* mutants. First, *MEA-ISR* shows reduced non-CG methylation in *dcl3-1* (ref. 20), and we found that remaining methylation was eliminated in *dcl2-1 dcl3-1 dcl4-2* (**Fig. 5a**, **Supplementary Table 2** and **Supplementary Fig. 2** online). Thus, at *MEA-ISR*, partially redundant functions of DCL2, DCL3 and DCL4 are responsible for directing all non-CG methylation. In contrast, *AtSN1* shows only a modest decrease in non-CG methylation in *dcl3-1* (ref. 3), and these levels were similar in the triple *dcl2-1 dcl3-1 dcl4-2* mutant (**Fig. 5b**, **Supplementary Table 2** and **Supplementary Fig. 2**). As RNA blot and 454 sequencing experiments did not detect AtSINE element siRNAs in *dcl2-1 dcl3-1 dcl4-2* (**Fig. 3b**,**c**), this strongly suggests that an siRNA-independent mechanism has a role in the maintenance of non-CG methylation at *AtSN1* (though we cannot rule out that siRNAs are still present at levels below detection). The maintenance mechanism is likely to involve targeting of the enzyme CMT3 by histone methylation[23,24], consistent with the total loss of non-CG methylation in *drm1 drm2 cmt3* (**Fig. 5b**, **Supplementary Table 2** and **Supplementary Fig. 2**). The third pattern was more complicated. At *IR-71*, non-CG methylation was unaffected in *dcl3-1* and only moderately reduced in *dcl2-1 dcl3-1 dcl4-2* (**Fig. 5c**, **Supplementary Table 2** and **Supplementary Fig. 2**). However, this

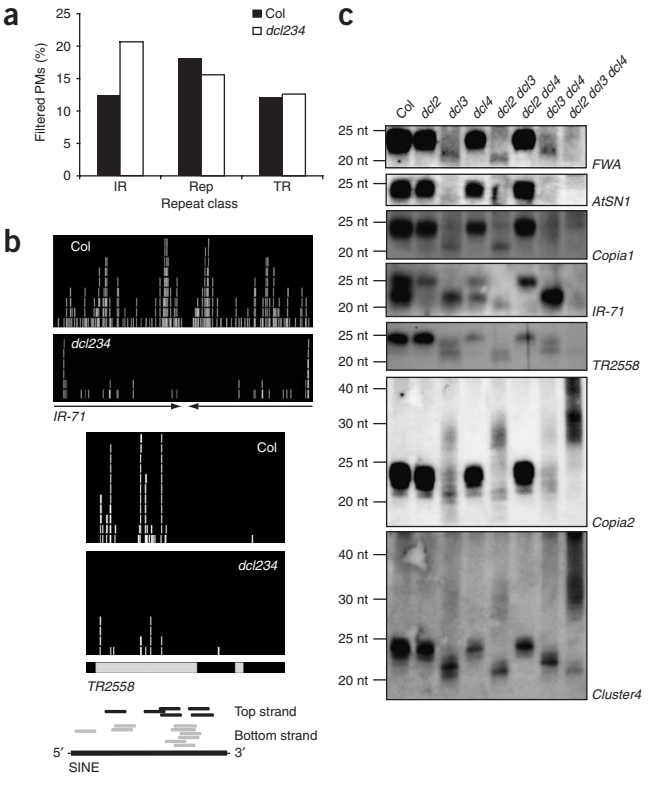

Figure 3 siRNA accumulation in *dcl* mutants. (**a**) Relative abundance of perfectly matching siRNAs deriving from local inverted repeats (IR), dispersed repeats (Rep) and local tandem repeats (TR) in Col and *dcl2-1 dcl3-1 dcl4-2*. (**b**) Graphical representation of cloned siRNA deriving from inverted repeat-71 (*IR-71*), tandem repeat 2558 (*TR2558*) and SINE elements in Col and *dcl2-1 dcl3-1 dcl4-2*. The positions of *IR-71* inverted repeat arms are shown underneath the graph as arrows. The positions of tandem repeats in *TR2558* are shown underneath the graph as the gray boxes. The SINE element represents a consensus for all 40 full-length elements in the genome. SINE element–derived siRNAs were not present in *dcl2-1 dcl3-1 dcl4-2*. (**c**) RNA blot analysis of repeat-derived siRNAs in *dcl* mutant backgrounds.

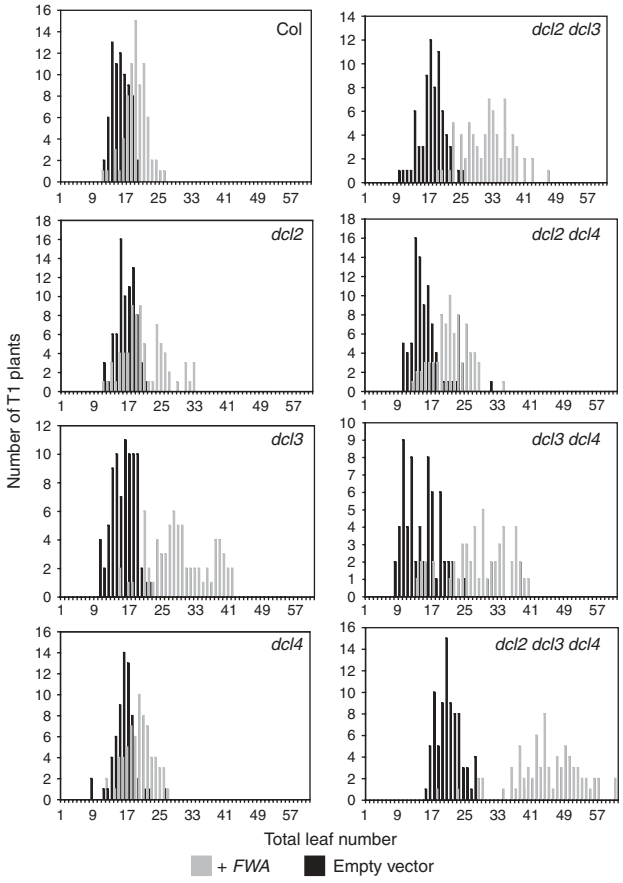

Figure 4 *FWA* silencing in *dcl* mutant backgrounds. Flowering-time distributions shown as total leaf number of T_1 populations transformed with an *FWA* transgene or empty vector. *X*-axes show total numbers of leaves of individual plants, and *y*-axes show the numbers of individual plants. For each genotype, individual plants transformed with the *FWA* transgene are represented as gray bars, and individual plants transformed with empty vector are represented as black bars. All *FWA* transformant populations flowered slightly later than the empty vector populations; this is likely to reflect incomplete silencing in the T_1 generation[19,20].

methylation could still be RNA directed, as 21-nt small RNAs were still present in *dcl2-1 dcl3-1 dcl4-2* (**Fig. 3b**). Notably, this locus also showed a low level of non-CG methylation in the *drm1 drm2 cmt3* triple mutant, suggesting the involvement of another DNA methyltransferase (**Fig. 5c**, **Supplementary Table 2** and **Supplementary Fig. 2**). We observed a similar phenomenon at two other loci, the *Ta3* retrotransposon (**Fig. 5d**) and the *AtCOPIA4* element (**Fig. 5e**). *Ta3* is of particular interest, as it shows no detectable siRNAs from our data set, or from one previously described[25]. Although Dnmt1 and MET1 methyltransferases have a strong preference for CG dinucleotides, they have some *in vitro* activity against non-CG cytosines[26,27], and hence MET1 is a candidate for an enzyme contributing to siRNA-independent non-CG methylation. Taken together, these results confirm the partially redundant function of DCL2, DCL3 and DCL4 in RNA-directed DNA methylation and also demonstrate unexpected complexity in the mechanisms controlling non-CG methylation via siRNA-directed and non–siRNA-directed pathways and through an uncharacterized non-CG methylation activity.

METHODS

Plant materials. The *dcl2-1* (ref. 3), *dcl3-1* (ref. 3) and *dcl4-2* (ref. 4) T-DNA insertions have been described. The *dcl4-2* allele was obtained from GABI-Kat.

Phylogenetic tree construction. Multiple sequence alignment was performed using the European Bioinformatics Institute CLUSTALW server. The phylogenetic tree was constructed using the MEGA2 program with the neighbor-joining method. Bootstrap values were calculated with 1,000 replicates.

Small RNA cloning and 454 sequencing. Gel-purified small RNA molecules (20–30 nt) were ligated sequentially to 5′ and 3′ RNA oligonucleotide adapters (Dharmacon Research) using T4 RNA ligase (Ambion). The 5′ RNA adapter possessed 5′ and 3′ hydroxyl groups. The 3′ RNA adapter possessed a 5′ monophosphate and a 3′ inverted deoxythymidine (idT) that prevented self-ligation. The products were gel purified after each ligation. The final ligation product then was used as a template in a reverse transcription reaction using the primer JP4541 and Superscript II reverse transcriptase (Invitrogen). This was followed by PCR using the primer set JP4542 and JP4543 and Taq DNA polymerase (Invitrogen). Conditions for PCR were 18 cycles of 94 °C for 40 s, 58 °C for 40 s and 72 °C for 40 s. PCR products were cleaned by phenol-chloroform extraction, ethanol precipitated, gel purified and sequenced by 454 Life Sciences. Adaptor and primer sequences are listed in **Supplementary Table 3** online.

Bioinformatic analysis of cloned small RNAs. Sequences derived from the cloning adaptors and those containing ambiguous residues were removed from the raw 454 reads, and the remaining insert sequences were used as queries in BLAST searches against the entire *A. thaliana* genome. The perfect match sequences cloned from the wild-type ecotype Col and *dcl2-1 dcl3-1 dcl4-2* are listed in **Supplementary Table 4** and **Supplementary Table 5** online,

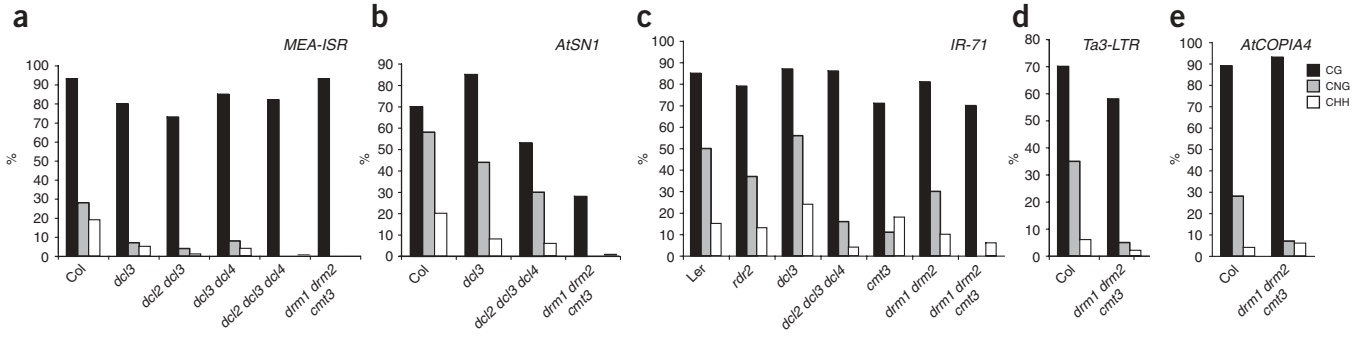

Figure 5 Maintenance of cytosine methylation in *dcl* mutants. Bisulfite sequence data for methylation at (**a**) *MEA-ISR*, (**b**) *AtSN1*, (**c**) *IR-71*, (**d**) *Ta3-LTR* and (**e**) *AtCOPIA4* in DCL and DNA methyltransferase–deficient backgrounds. Methylation at CG, CNG and CHH contexts are represented by black, gray and white bars, respectively.

respectively. Only sequences that perfectly matched the genomic sequence over their entire length were analyzed further. Next, sequences matching tRNAs, rRNAs, small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) that could potentially represent degradation products of these abundant RNA species were discarded. miRNAs and tasiRNAs were identified by comparing the remaining 454 sequences against miRNA and tasiRNA precursor genes. Tandem repeats and inverted repeats were identified using the programs Tandem Repeat Finder and Inverted Repeat Finder, respectively. Dispersed repeats were identified using the RepeatMasker program, using a dispersed repeat library downloaded from RepBase.

Small RNA blotting and hybridization. Small RNAs were extracted, blotted and probed as previously reported[28]. Blots were hybridized with end-labeled oligonucleotide probes[29]. Several probes were LNA modified (Proligo) to increase sensitivity. Probe sequences are listed in **Supplementary Table 3**.

***FWA* transformation and flowering-time analysis.** Plants were transformed with *FWA* as reported[19,20]. Hygromycin-resistant T_1 seedlings were transferred to soil under continuous light and assayed for flowering time by leaf-counting.

Bisulfite analysis. We performed sodium bisulfite sequencing as previously reported[22]. The primers used for *AtSN1* were JP1821 (forward primer) and JP1822 (reverse primer) and *MEA-ISR* were JP1027 (forward primer) and JP1026 (reverse primer). The primers used to amplify *IR-71* were JP2509 (forward primer) and JP2510 (reverse primer), which are flanked by *Eco*RI sites for use in cloning. The primers used to amplify *Ta3-LTR* were JP1615 (forward primer) and JP1616 (reverse primer). The primers used to amplify *AtCOPIA4* were JP3102 (forward primer) and JP3101 (reverse primer). All primer sequences are listed in **Supplementary Table 3**.

Accession numbers. GenBank: *Arabidopsis thaliana*: *DCL1*, At1g01040; NP_171612; *DCL2*, At3g03300; NP_566199; *DCL3*, At3g43920, NP_189978; *DCL4*, At5g20320, NP_197532; *RDR2*, At4g11130; *CMT3*, At1g69770; *DRM1*, At5g15380; *DRM2*, At5g14620. *Oryza sativa*: OsDCL701, NP_912466; OsDCL702, XP_463595; OsDCL703, NP_922059; OsDCL704, Os4g43050; OsDCL705, Os03g38740. *Caenorhabditis elegans*: CeDCR1, NP_498761. *Homo sapiens*: HsDCR1, NP_803187. *Mus musculus*: MmDCR1, NP_683750. *Schizosaccharomyces pombe*: SpDCR1, Q09884. *Neurospora crassa*: NcDCR1, XP_961898; NcDCR2, XP_963538. *Magnaporthe grisea*. MgDCR1, MG01541.4; MgDCR2, MG07167.4.

URLs. CLUSTALW: http://www.ebi.ac.uk/clustalw/; Tandem Repeat Finder: http://tandem.bu.edu/home.html; RepBase: http://www.girinst.org.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank S. Chan for the *dcl2 dcl3* double mutant, J. Carrington for drawing our attention to the *IR-71* locus and members of the Jacobsen laboratory for critical reading of the manuscript. I.R.H. was supported by a European Molecular Biology Organization (EMBO) Long Term Postdoctoral Fellowship. S.E.J. is an investigator of the Howard Hughes Medical Institute. This work was supported by US National Science Foundation grants 0439186 and 0548569 to P.J.G. and B.C.M., and NIH grant GM60398 and funds from the Howard Hughes Medical Institute to S.E.J.

AUTHORS' CONTRIBUTIONS

C.L. purified and cloned small RNAs in the laboratories of B.C.M. and P.J.G. X.Z. performed bioinformatics analysis of the cloned small RNAs. L.J. provided *AtCOPIA4* and *Ta3-LTR* DNA methylation data. I.R.H. performed the remainder of the experiments. I.R.H. and S.E.J. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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