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RNA Silencing Genes Control de Novo DNA Methylation

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Cytosine DNA methylation silences harmful DNAs such as transposons and retroviruses (1). Maintenance DNA methyltransferases propagate pre-existing DNA methylation in the CG sequence context by methylating hemi-methylated sites after DNA replication. Much less is understood about how invasive DNAs are initially recognized and how de novo DNA methyltransferases of the DNMT3 family (DRM1 and DRM2 in the plant *Arabidopsis thaliana*) are directed to unmethylated loci to initiate gene silencing.

Transformation of *Arabidopsis* with the *FWA* gene is an assay for de novo DNA methylation and the establishment of gene silencing (2). Endogenous *FWA* is heritably silenced in most tissues by DNA methylation of two direct repeat promoter sequences (3). In epigenetic hypomethylated *fwa* alleles, ectopic *FWA* overexpression causes late flowering. Wild-type plants transformed with *FWA* efficiently silence the transgene and flower normally, whereas *drm1 drm2* double mutants cannot initiate DNA methylation and silencing and therefore flower late (2). De novo DNA methylation of *FWA* requires a transient signal generated during *Agrobacterium*-mediated transformation, because an unmethylated *FWA* transgene initially introduced into a *drm1 drm2* mutant is not methylated de novo when crossed with wild-type *DRM1 DRM2*. This may reflect the role of gene silencing as a defense against invasive DNA; the *FWA* direct repeats might mimic terminal direct repeats found in retroviruses and long terminal repeat (LTR) retrotransposons.

We used the *FWA* transformation assay to test de novo DNA methylation in a variety of silencing mutants (Fig. 1). Notably, *kyp* and *cmt3*, which affect gene silencing at the *SUPERMAN* locus, did not affect the initiation of *FWA* silencing. However, four mutants—*rna dependent rna polymerase2* (*rdr2*, At4 g11130), *dicer-like3* (*dcl3*), *silencing defective4* (*sde4*), and *argonaute4* (*ago4*)—could not initiate *FWA* silencing and flowered late. These mutants also failed to establish DNA methylation of *FWA* transgenes as assayed by bisulfite genomic sequencing and Southern blotting (Fig. 1; fig. S1). However, like *drm1 drm2*, each mutant in the absence of the transgene flowered normally and maintained preexisting CG methylation at the endogenous *FWA* locus, showing that these mutants block the establishment but not the maintenance of CG methylation and gene silencing. Interestingly, *RDR2*, *DCL3*, and *AGO4* are homologous to proteins involved in RNA silencing, a conserved genome defense mechanism that uses short, 21- to 25-nucleotide silencing-induced RNAs (siRNAs) to direct posttranscriptional messenger RNA (mRNA) destruction and also to cause chromatin-level gene silencing (4). siRNAs are processed from double-stranded RNA by Dicer, an RNaseIII–RNA helicase, and their synthesis often requires an RNA-dependent RNA polymerase. Argonaute proteins associate with RNA silencing effector complexes. *RDR2*, *DCL3*, *SDE4*, and *AGO4* are required to maintain siRNAs derived from several endogenous loci (5–7). Thus our results suggest that a canonical RNA silencing pathway mediates de novo methylation of transformed direct repeats.

Non-CG methylation at some loci is thought be maintained by persistent activity of de novo methyltransferases. For instance, *drm1 drm2* eliminates non-CG methylation at direct repeats from the endogenous *FWA* and *MEA-ISR* loci (8). We found that this methylation was also strongly reduced in *rdr2*, *dcl3*, *sde4*, and *ago4* (Fig. 1).

Our data suggest that RDR2, DCL3, SDE4, and AGO4 comprise an siRNA-metabolizing pathway that guides the DRM de novo methyltransferases, both to initiate DNA methylation at direct repeats and to perpetuate non-CG DNA methylation. Previous observations have associated RNA viruses and inverted repeat transgenes with DNA methylation of homologous genomic sequences (4). RNA thus appears to be a general means of targeting de novo DNA methylation, which may indicate how sequence-specific gene silencing is established in a variety of epigenetic phenomena.
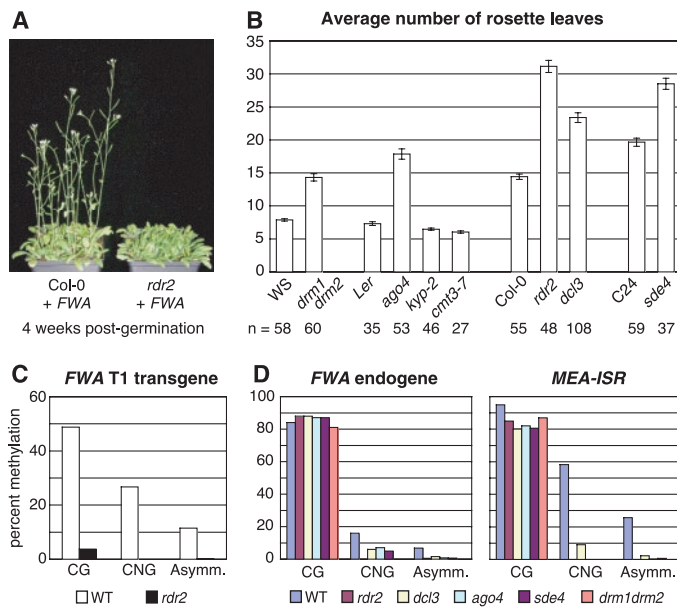

Fig. 1. (A) *FWA*-transformed Col and *rdr2*. (B) Flowering time in *FWA* transformants. Late flowering increases rosette leaf number. Mutants are grouped with their isogenic ecotype. Bars show standard error; n = number of independent transformants. (C) *FWA* transgene DNA methylation at CG, CNG, and asymmetric cytosines. (D) DNA methylation of endogenous *FWA* and *MEA-ISR*.

References and Notes

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