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Note

Genetic Evidence That DNA Methyltransferase DRM2 Has a Direct Catalytic Role in RNA-Directed DNA Methylation in *Arabidopsis thaliana*

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ABSTRACT

RNA-directed DNA methylation (RdDM) is a small RNA-mediated epigenetic modification in plants. We report here the identification of DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) in a forward screen for mutants defective in RdDM in *Arabidopsis thaliana*. The finding of a mutation in the presumptive active site argues in favor of direct catalytic activity for DRM2.

RNA-directed DNA methylation (RdDM) is a small RNA-mediated epigenetic modification that is highly developed in flowering plants (Matzke *et al.* 2009; Law and Jacobsen 2010). Forward genetic screens in *Arabidopsis thaliana* have revealed that RdDM requires a complex transcriptional machinery that comprises two plant-specific, RNA polymerase II-related RNA polymerases, called Pol IV and Pol V. Additional requirements include specialized transcription factors, chromatin remodelling proteins, and other novel, plant-specific proteins whose functions in the RdDM mechanism are not well understood (Matzke *et al.* 2009; Law and Jacobsen 2010). A previous study based on a reverse genetics approach implicated the *de novo* DNA cytosine methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) in RdDM (Cao *et al.* 2003). So far, however, no publications have appeared reporting the identification of this protein in a forward genetic screen. In this communication, we report the identification of DRM2 in a forward screen for mutants defective in RdDM in *A. thaliana* ecotype Col-0. These findings substantiate the view that DRM2 is the major *de novo* DNA methyltransferase in the RdDM pathway and support a direct catalytic role for this enzyme in RdDM.

Our forward genetic screen is based on a two-component transgene silencing system (target and silencer) in which a target enhancer that is active in shoot and root meristem regions drives expression of a downstream gene encoding green fluorescent protein (GFP). Silencer-encoded 24-nt small RNAs trigger methylation of the target enhancer, leading to transcriptional silencing of the *GFP* gene. Following mutagenesis by ethyl methanesulfonate (EMS), mutants are identified by screening for reactivation of *GFP* expression in root meristems of seedlings (Kanno *et al.* 2008; Daxinger *et al.* 2009). To date, seven *dms* (*d*efective in *m*eristem *s*ilencing) mutants have been retrieved in this screen (see supporting information, Table S1).

To identify the mutated gene in a new mutant, *dms8*, we generated an F_2 mapping population by crossing homozygous *dms8* plants with ecotype Landsberg erecta followed by selfing of the resulting F_1 hybrids to produce F_2 progeny. F_2 seedlings that were GFP positive and hygromycin resistant (indicative of *GFP* reactivation in the presence of the silencer locus, which encodes resistance to hygromycin, Kanno *et al.* 2008) were used for mapping, using ATH1 microarrays (Hazen *et al.* 2005; Kanno *et al.* 2008) and codominant markers (Koneiczny and Ausubel 1993). Using these techniques, the mutation in *dms8* was localized to an interval on the top arm of chromosome 5, which does not contain any of the seven *DMS* genes identified so far (see Table S1). Subsequent Illumina whole genome sequencing revealed a mutation in this region in the gene encoding the DRM2 (At5g14620). The mutation

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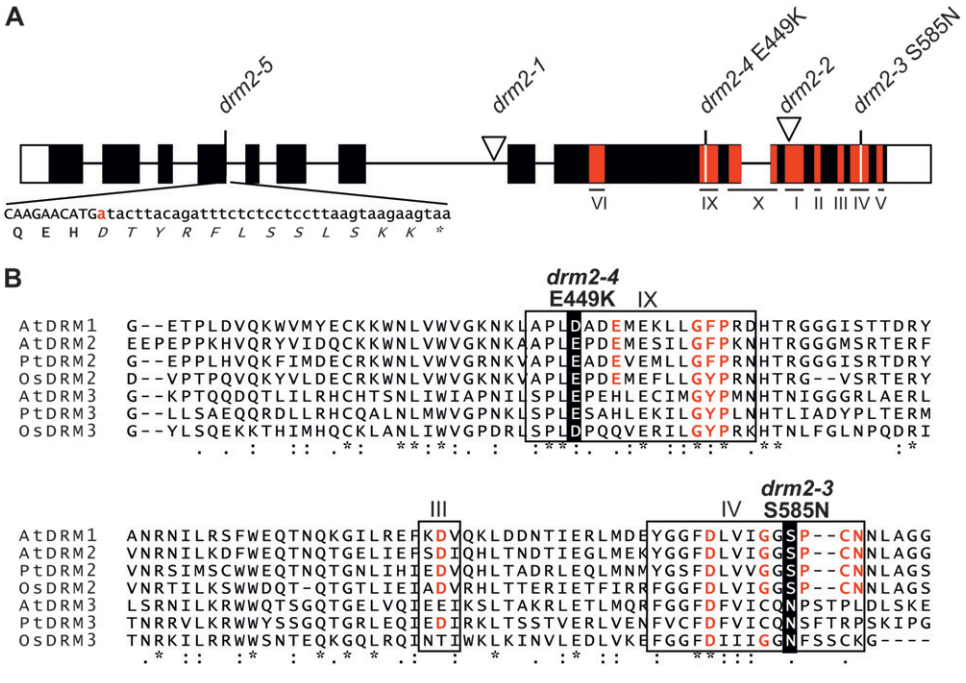

FIGURE 1.—Structure of the *DRM2* gene and positions of new point mutations. (A) Schematic presentation of the *DRM2* gene with indicated positions of new *drm2* mutations and previously identified T-DNA insertion mutants (triangles). Exons and introns are depicted by black boxes and lines, respectively. White boxes represent 5′ and 3′ untranslated regions. Red boxes and roman numerals with dashes above denote the eight conserved motifs of the DNA methyltransferase catalytic domain according to published nomenclature (CHANG 1995; CAO *et al.* 2000). The *drm2-5* mutation (G to A) is located in the 5′ splice site of intron 4 and results in premature stop codon due to intron retention. DNA sequence with A at the 5′ splice site in red and translation of this region are shown below. Exonic and intronic sequences are in upper and lower case, respectively. Last three amino acids encoded by exon 4 are in boldface type and those encoded by intron are in italics. Asterisk denotes the stop codon. (B) Sequence alignment of DRM1, DRM2, and DRM3 proteins from *Arabidopsis thaliana* (At), *Oryzae sativa* (Os), and *Populus trichocarpa* (Pt). For simplicity only regions containing newly identified point mutations resulting in amino acids changes are shown. Positions of mutations are highlighted on black background (*drm2-3*, S585N, *drm2-4*, and E449K). Conserved DNA methyltransferase catalytic domain motifs 9, 3, and 4 (roman numerals above) are in boxes with catalytically important residues highlighted in red.

(C/G to T/A, chromosome 5, nucleotide 4,715,556), resulted in the substitution of a conserved serine (S) in motif IV of the catalytic domain by asparagine (N) at amino acid 585, which is adjacent to invariant proline (P) and cysteine (C) residues that are essential for DRM2 catalytic activity (CAO *et al.* 2000; HENDERSON *et al.* 2010) (Figure 1). We designated our allele *drm2-3* in view of two existing *drm* mutants, both of which result from T-DNA insertions: *drm2-1* in ecotype WS (CAO and JACOBSEN 2002) and *drm2-2* in ecotype Col-0 (SALK_150863) (CHAN *et al.* 2006) (Figure 1).

Two additional *drm2* alleles, *drm2-4* and *drm2-5*, were identified by whole genome Illumina sequencing of two further uncharacterized *dms* mutants. The *drm2-4* allele (C/G to T/A, chromosome 5, nucleotide 4,716,083) has a glutamic acid-to-lysine substitution at amino acid 449 just before motif IX of the catalytic domain (Figure 1). The *drm2-5* allele (C/G to T/A, chromosome 5, nucleotide 4,717,922) has a splice site mutation that alters the open reading frame and produces a truncated protein lacking the entire catalytic domain (Figure 1). The *drm2* mutants do not display any obvious phenotypic abnormalities when grown under standard conditions (16 hr light, 8 hr dark, 23°).

Consistent with an essential role of DRM2 in RdDM, methylation of cytosines in all sequence contexts was largely eliminated at the target enhancer in the *drm2-3* mutant (Figure 2A) despite the presence of silencer-encoded 24-nt small RNAs (Figure 2B). In addition, methylation of cytosines in an asymmetric context (CHH, where H is A, T, or C) was reduced in 5S rDNA repeats, which are endogenous targets of RdDM (see lane "n.d." adjacent to "ddm1" in figure S7-d in GAO *et al.* 2010). These results substantiate the conclusion that DRM2 is the major DNA methyltransferase in the RdDM pathway and that it is essential for RNA-directed *de novo* methylation of cytosines in all sequence contexts.

The *drm2-3* allele (S585N) represents the first amino acid substitution to be identified in a highly conserved residue of the active site of DRM2 (CHANG 1995; CAO *et al.* 2000). The finding of this allele indicates that DRM2 actually catalyzes *de novo* methylation as opposed to the alternative hypothesis that the DRM2 protein recruits another DNA methyltransferase to carry out this function (DAMELIN and BESTOR 2005). Interestingly, the S585N substitution in *drm2-3* is also found in DRM3, a noncatalytic paralog of DRM2, in Arabidopsis, rice, and poplar (HENDERSON *et al.* 2010) (Figure 1). Thus, in addition to the absence of the invariant and essential P and/or C residues in DRM3 orthologs, the S-to-N change in these proteins may also contribute to the loss of catalytic activity. Despite its inability to catalyze DNA methylation, DRM3 is nevertheless needed for full methylation of repeats by DRM2 (HENDERSON *et al.* 2010).

In summary, we have identified three new alleles of *drm2* in a screen for mutants defective in RdDM in *A.*

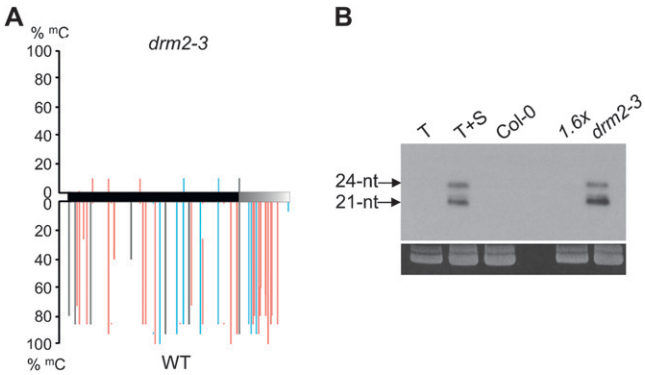

Figure 2.—DNA methylation and small RNA accumulation in the *drm2-3* mutant. (A) Methylation of the target enhancer (black bar) and downstream region (gray bar) in the *drm2-3* mutant (top) and wild-type (WT; bottom) plants containing the target and silencer loci was analyzed using bisulfite sequencing (carried out as described by Kanno *et al.* (2008) and Daxinger *et al.* (2009). CG, CHG, and CHH methylation are indicated by black, blue, and red lines, respectively. (B) Northern blot analysis of silencer-encoded small RNAs. Three size classes of small RNA, 21, 22, and 24 nt, are visible in wild-type plants containing the target (T) and silencer (S) loci (Daxinger *et al.* 2009) and, as shown here, in the *drm2-3* mutant. The small RNAs are not present in nontransgenic wild-type plants (Col-0), wild-type plants containing only the T locus, or in a transgenic line in which most of the S locus has been deleted (1.6×). The bottom panel shows ethidium bromide staining of the major RNA species on the gel as a loading control. Isolation of small RNAs and Northern blotting were performed as described by Kanno *et al.* (2008) and Daxinger *et al.* (2009).

thaliana. These new mutants, which result from EMS-induced point mutations in the Col-0 ecotype, will provide useful tools for further studies of DRM2 function and catalytic activity.

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Note added in proof: DRM2 has recently been identified in an independent forward genetic screen in M. V. Greenberg, I. Ausin, S. W. Chan, S. J. Cokus, J. J. Cuperus *et. al.*, 2011, Identification of genes required for de novo methylation in Arabidopsis. Epigenetics. **6**(3).

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TABLE S1

***dms* mutants**

Mutant Name	Common Name and AGI number	Function
DMS1	DRD1 (At2g16390)	SNF2-like chromatin remodeling protein (KANNO *et al.* 2004)
DMS2	NRPD2a (At3g23780)	common second largest subunit of Pol IV and Pol V (KANNO *et al.* 2005)
DMS3	DMS3 (At3g49250)	structural maintenance of chromosomes hinge domain-containing protein (KANNO *et al.* 2008)
DMS4	DMS4 (At2g30280)	IWR1 putative transcription factor (KANNO *et al.* 2010)
DMS5	NRPE1 (At2g40030)	unique largest subunit of Pol V (KANNO *et al.* 2005)
DMS6	DCL3 (At3g43920)	Dicer-like3 (DAXINGER *et al.* 2009)
DMS7	RDM1 (At3g22680)	small protein with novel fold (GAO *et al.* 2010)
DMS8	DRM2 (At5g14620)	*de novo* DNA methyltransferase (this study)

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