Genetic evidence that DNA methyltransferase DRM2 has a direct catalytic role in RNA-Directed DNA Methylation in *Arabidopsis thaliana*

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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 (defective in meristem silencing) 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 F2 mapping population by crossing homozygous dms8 plants with ecotype Landsberg erecta followed by
selfing of the resulting F1 hybrids to produce F2 progeny. F2 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 supporting information, Table S1). Subsequent Illumina whole genome sequencing revealed a mutation in this region in the gene encoding the DRM2 (At5g14620). The mutation (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 4,617,083) 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 hours light, 8 hours dark, 23°C).

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 (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 non-catalytic 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. 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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LITERATURE CITED


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FIGURE LEGENDS

Figure 1. Structure of 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. Open 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 four and results in premature stop codon owing 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 four are in bold 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, E449K). Conserved DNA methyltransferase catalytic domain motifs nine, three, and four (roman numerals above) are in boxes with catalytically important residues highlighted in red.

Figure 2. DNA methylation and small RNA accumulation in the drm2-3 mutant.
(A) Methylation of the target enhancer (black bar) and downstream region (shaded 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: non-transgenic wild type plants (Col-0); wild type plants containing only the T locus; a transgenic line in which most of the S locus has been deleted (1.6X). 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 was performed as described by KANNO et al. 2008 and DAXINGER et al. 2009.
Figure 1
Naumann et al.
Figure 2
Naumann et al.