Supplemental Data

The SRA Methyl-Cytosine-Binding Domain Links DNA and Histone Methylation

Lianna M. Johnson, Magnolia Bostick, Xiaoyu Zhang, Edward Kraft, Ian Henderson, Judy Callis, and Steven E. Jacobsen

Supplemental Experimental Procedures

Plant Material

The wild-type control in this study was the Columbia ecotype. *kyp-2* and *kyp-5* were isolated in the screen described in Jackson et al. [S1], and *kyp-6* is a T-DNA insertion line obtained from the SALK Institute Genomic Analysis Laboratory (SALK_041474) [S2]. The *met1-3* line was described in Saze et al. [S3]. The triple *drm1 drm2 cmt3* mutant was made by crossing the SALK T-DNA insertions lines (CMT3, SALK_148381; DRM1, SALK_095534; DRM2, SALK_150863). All plant material (DNA for bisulfite sequencing and tissue for ChIPs) was isolated from the first-generation homozygous plants for both the *met1-3* line and the *drm1 drm2 cmt3* triple mutant.

Bisulfite Sequencing

DNA was isolated and treated with bisulfite as previously described [S4]. In the *AtSN1* element, there are a total of 4 CG, 7 CNG, and 33 CNN residues. Ten to twelve independent clones were sequenced, and the average number of methylated CG, CNG, or CNN was determined (see Table S1 for all primer sequences). At *AtCOPIA4*, there are a total of 25 CG, 18 CNG, and 116 CNN residues. Seven to twelve independent clones were sequenced, and the average number of methylated CG, CNG, or CNN was determined.

Chromatin Immunoprecipitation

ChIPs were performed as described previously [S5] with the following modifications. 0.3 g of crosslinked tissue was ground by mortar and pestle and resuspended in 2 ml of lysis buffer plus inhibitors. Cells were disrupted for 12 min with a Dianode Bioruptor (30 s on, 30 s off; hi setting). H3K9me2-containing chromatin was immunoprecipitated with antibody from Upstate Biotechnology (#07-441). After reversal of crosslinking, the DNA was purified with a silica gel membrane (Qiagen) and analyzed by real-time PCR with Taqman probes (Primer 3 designed). The values represented are the average of two biological replicas done in duplicate with standard deviations shown.

Immunofluorescence

Interphase nuclei were isolated from leaves of 3-week-old plants, and immunolabeling was performed as previously described [S6]. H3K9me2 was detected with polyclonal antisera from Upstate Biotechnology (#07-441, diluted 1/150) followed by incubation with rhodamine red conjugated secondary antibodies from Jackson Immunoresearch (#111 295 144, goat anti-rabbit diluted 1/200). After staining, vectashield mounting medium with DAPI was added and nuclei were visualized with a Zeiss Axioskop 2 with the Zeiss Axiocam HRC color digital camera system with the Zeiss Axiovision software.

Purification of GST-Tagged SRA Proteins

GST fusion proteins were made either with the Gateway cloning system with pDEST15 as the final vector or with the vector pGEX2TK. Both of these vectors result in N-terminal GST fusion proteins. Protein expression in BL21-AI Chemically Competent Cells (Invitrogen) was induced by the addition of 2% arabinose and 1 mM IPTG (for pGEX2TK clones) and allowed to grow for 4 hr at room temperature or 15°C overnight. Cells were resuspended in 50 mM Tris (pH 7.5), 300 mM NaCl, 1% triton X-100, 10 mM EDTA, 10 mM DTT, 0.25 mg/ml lysozyme, 1 mM PMSF, 0.7 μg/ml pepstatin, and complete protease inhibitor cocktail (Roche) and disrupted for 15 min with a Dianode Bioruptor (30 s on, 30 s off; hi setting). After removal of cell debris by centrifugation, immobilized glutathione beads were added and allowed to rotate for 1 hr at 4°C. The beads were settled in a column and washed with 5 ml 50 mM Tris (pH 7.6), 10% glycerol, 0.1% triton, 1 M NaCl, 1 mM PMSF, and then 10 ml of the same buffer with 0.1 M NaCl. Protein was eluted with 50 mM Tris (pH 6.8), 10% glycerol, 0.1% triton, 0.3 M NaCl, 1 mM PMSF, 50 mM reduced glutathione, and 1 mM DTT. Protein was then dialyzed into the same buffer with 40% glycerol and no glutathione.

DNA Binding Assays

The oligonucleotides utilized in the binding assays are listed in Table S1 and were a gift from S. Pradhan. The complementary oligonucleotides were annealed, and the double-stranded product was purified by polyacrylamide gel electrophoresis. T4 polynucleotide kinase was used to label the oligonucleotides with ^{32}P. Binding reactions contained 15–73 pg probe (2500–5000 cpm), 25 mM Tris (pH 7.5), 5% glycerol, 60 mM NaCl, 0.4 mg/ml BSA (bovine serum albumin), 10 mM $MgCl_2$, 2 mM DTT, and either 0.5 mg/ml polyglutamate or 2.5 μg/ml nonspecific oligonucleotide as polyanion or 1.25 μg/ml

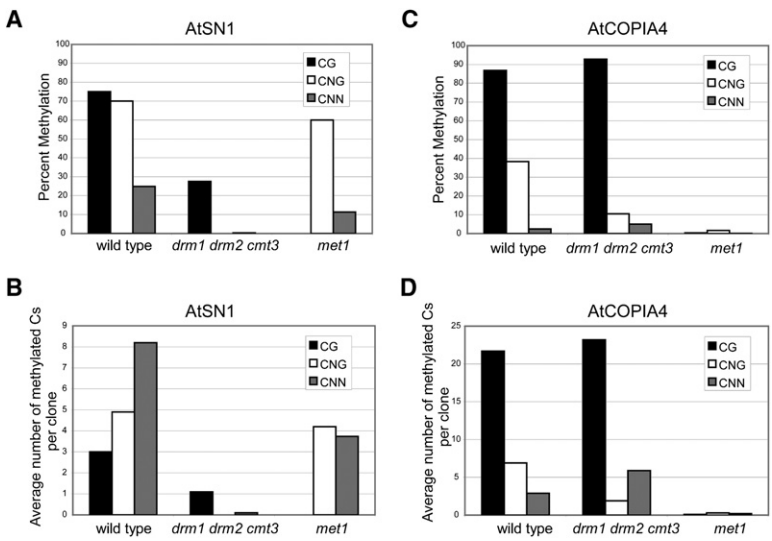

Figure S1. Bisulfite Sequence Data at *AtSN1* and *AtCOPIA4* Represented in Two Different Ways

(A and C) Data represented as the percent of CG, CNG, or CNN sites that are methylated.
(B and D) Data represented as the average number of methylated CG, CNG, or CNNs per clone.

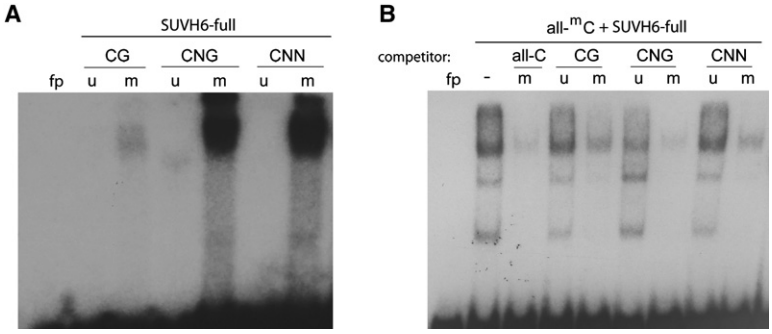

Figure S2. SUVH6 Binds Preferentially to Methylated DNA

(A) Mobility-shift assays where GST-SUVH6 full (amino acids 1–781) was added to a binding reaction with either an unmethylated (u) or methylated (m) CG, CNG, or CNN double-stranded oligonucleotide probe.

(B) GST-SUVH6 full was bound to oligonucleotides with cytosines methylated in all sequences contexts (all-^{m}C) and competed with 1000× excess double-stranded oligonucleotides that were unmethylated (u) or methylated (m) in CG, CNG, or CNN context. Free probe is indicated by fp and no competitor by minus sign. The lower bands are due to SUVH6 degradation products binding to DNA.

lambda DNA digested with HindIII as nonspecific competitor. Final protein concentration in the binding assay was between 25 and 350 nM, except for SUVH6-SRA where the concentration was 10 nM, and reactions were incubated at room temperature for 20 min. Binding was detected after electrophoresis in 6% acrylamide: bisacrylamide (40:1) gels containing 5% glycerol and Tris-borate-EDTA buffer by autoradiography.

Supplemental References

S1. Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature *416*, 556–560.

S2. Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana.* Science *301*, 653–657.

S3. Saze, H., Mittelsten Scheid, O., and Paszkowski, J. (2003). Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. Nat. Genet. *34*, 65–69.

S4. Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. (2001). Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science *292*, 2077–2080.

S5. Johnson, L., Cao, X., and Jacobsen, S. (2002). Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. *12*, 1360–1367.

S6. Jasencakova, Z., Soppe, W.J., Meister, A., Gernand, D., Turner, B.M., and Schubert, I. (2003). Histone modifications in *Arabidopsis*—high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin. Plant J. *33*, 471–480.

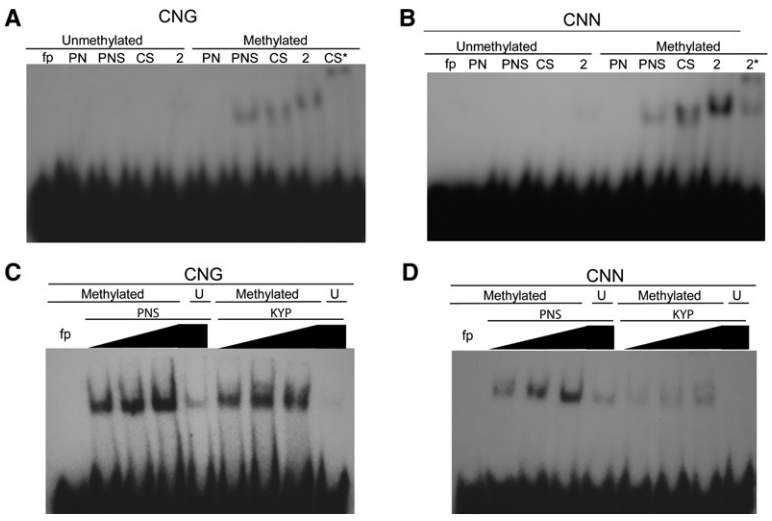

Figure S3. Specificity of DNA Binding by ORTH and KYP Proteins

(A and B) All ORTH1 and ORTH2 constructs containing the SRA domain can bind to CNG and CNN methylated substrates in addition to CG-methylated substrates (see Figure 4B). Mobility-shift assays with no protein (fp), ORTH1 with the amino-terminal PHD and RING domains (PN); PHD, amino-terminal RING, and SRA domains (PNS); carboxy-terminal RING and SRA domain (CS); and full-length ORTH2 (2) binding to unmethylated (lanes 1–5) or methylated (lanes 6–9), CNG (A) or CNN (B) oligonucleotides. Lane 10 contains binding of either CS (A) or 2 (B) to methylated CG supershifted by addition of an anti-GST antibody (*).

(C and D) Compare ORTH1 PNS and KYP in their ability to bind CNG and CNN methylated substrates (see also Figure 4D for CG-methylated substrates). Mobility-shift assays with increasing amounts (100, 200, and 400 ng) of PNS (lanes 2–5) or KYP (lanes 6–9) binding to CNG (C) or CNN (D), either methylated (lanes 2–4, 6–8) or unmethylated (lanes 5, 9).

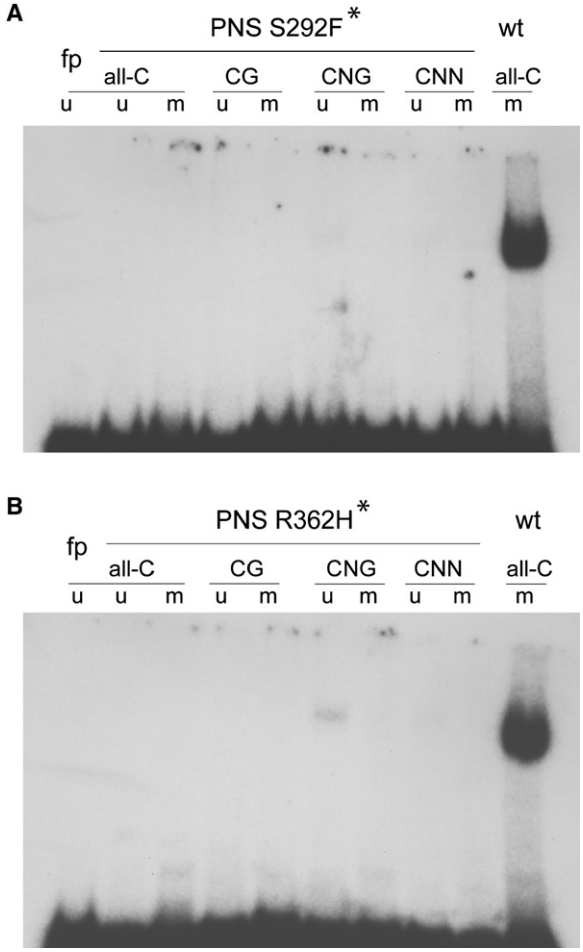

Figure S4. Point Mutations within the SRA Domain Disrupt Binding of ORTH1 to Methylated DNA

The binding of the N terminus of ORTH1 (PNS) containing a serine mutated to phenylalanine, S292F (A), or an arginine mutated to histidine, R362H (B). See Figure 2 for positions of mutations. Last lane in each panel shows wild-type ORTH1 PNS as a positive control. Oligonucleotides utilized contained cytosines in all sequence contexts (all-C), or a CG context (CG), CNG context (CNG), or CNN context (CNN), which were either methylated (m) or unmethylated (u). The first lane in each panel represents oligonucleotide with no added protein, or free probe (fp). *In ORTH1, the serine mutated corresponding to S200F in KYP is at position 292 and the arginine mutated corresponding to R260H in KYP is at position 362.

Table S1. Primer and Probe Sequences

Primer Number	Primer Name	Primer Sequence (5′-3′)
JP1595	*ACTIN*, real-time	CGTTTCGCTTTCCTTAGTGTTAGCT
JP1596	*ACTIN*, real-time	AGCGAACGGATCTAGAGACTCACCTTG
M-actin	*Actin*, probe	TCATCTTCTTCTTCTTCAAGGTGA
JP1821	*AtSN1*, bisulfite	CAATATACRATCCAAAAAACARTTAAAATAATATCTTAA
JP1822	*AtSN1*, bisulfite	GTTGTATAAGTTTAGTTTTAATTTTAYGGATYAGTATTAATTT
JP2669	*AtSN1*, real-time	GTTGGCCCAGTGGTAAATCTGTTGGCCCAGTGGTAAATCT
JP2683	*AtSN1*, real-time	TGGTGGTTGTACAAGCCTAGTT
M2	*AtSN1*, probe	ATCTCCCAGAGGCGGGACCC
JP3100	*AtCOPIA4*, bisulfite	GGTTGTYTGTGTTTTTTATGGTTYAGATTTTATA
JP3101	*AtCOPIA4*, bisulfite	ATAACTRAACCACARATTCARACCCATTTTCATTT
JP3067	*AtCOPIA4*, real-time	CTTGTTTGTCTTCCCCGTGT
JP3068	*AtCOPIA4*, real-time	TGACGAAGAGCGTACCTGTG
M1	*AtCOPIA4*, probe	CATTCATCACAGCCGACAAC
JP3010	all-C	aacgcagcatgcgctgctagcgcagctagcgctgcatg
JP3011	all-C	aacgcagcatgcgctgctagcgcagctagcgctgcatg
JP3018	all-^{m}C	aamgmagmatgmgmtgmtagmgmagmtagmgmtgmatg
JP3019	all-^{m}C	AAMATGMAGMGMTAGMTGMGMTAGMAGMGMATGMTGMG
JP3524	CG	CGCGaCGaCGCaCGaCGaCGCaCGaCGCGaaCGCGCGaa
JP3525	CG	TTCGCGCGTTCGCGTCGTGCGTCGTCGTGCGTCGTCGCG
JP3536	^{m}CG	MGMGaMGaMGCaMGaMGaMGCaMGaMGMGaaMGMGMGaa
JP3537	^{m}CG	TTMGMGMGTTMGMGTMGTGMGTMGTMGTGMGTMGTMGMG
JP3441	CNG	CAGCAGaCAGtCAGCAGttCAGCAGaCAGCAGcCAGCAG
JP3442	CNG	CTGCTGGCTGCTGTCTGCTGAACTGCTGACTGTCTGCTG
JP3443	^{m}CNG	MAGMAGaMAGtMAGMAGttMAGMAGaMAGMAGcMAGMAG
JP3444	^{m}CNG	MTGMTGGMTGMTGTMTGMTGAAMTGMTGAMTGTMTGMTG
JP3445	CNN	CACTCCCCCACTCTCCCACCCACTCACTCCCTCCCACTT
JP3446	CNN	AAGTGGGAGGGAGTGAGTGGGTGGGAGAGTGGGGGAGTG
JP3447	^{m}CNN	MAMTMCMCMAMTMTMCMAMCMAMTMAMTMCMTMCMAMTT

R represents a mix of G and A, Y represents a mix of C and T, and M represents 5-methyl cytosine.