Supplementary Online Material for "Arabidopsis *ARGONAUTE4* controls locus specific siRNA accumulation, DNA and histone methylation", Daniel Zilberman, Xiaofeng Cao, Steven E. Jacobsen.

Materials and Methods

Mutant screen, mapping and complementation testing. The *clk-st* line and the EMS mutagenesis conditions have been described (*1-3*). The *ago4-1* mutant was backcrossed three times to line *clk-st* to eliminate extraneous mutations and to confirm recessive inheritance. To map *ago4-1*, we first introgressed the *clk-st* transgene into the Columbia wild type background by four successive backcrosses, and then isolated a line homozygous for the *clk-st SUP* transgene. This line was then selfed two times at which time it developed a strong *clark kent* phenotype due to trans silencing of the endogenous *SUP* gene by the *clk-st* transgene, as has been described (*3*). Then this Columbia *clk-st* line was crossed with a plant heterozygous for *ago4-1* in the original Ler *clk-st* background, and several F1 plants were allowed to self. Several F2 families were screened for segregation of the *ago4-1* suppressor phenotype. DNA was isolated from 116 F2 plants with the *ago4-1* suppressor phenotype (wild type *SUP* flowers) and analyzed by polymerase chain reaction with a series of molecular markers covering the five Arabidopsis chromosomes. These markers were derived from the database of polymorphisms between the Landsberg *erecta* and Colombia ecotypes generated by Cereon Genomics (http://www.arabidopsis.org/Cereon/index.html). *ago4-1* mapped to the middle of the long arm of chromosome II, between markers CER461423 and CER451165. *AGO4* lies in this genomic interval.

A transgene containing only the *AGO4* gene was constructed by cloning a SacI - XbaI fragment corresponding to positions 33650 to 45561 of BAC clone T20P8 (accession AC005623) into the pCAMBIA1300 vector. This plasmid was electrotransformed into *Agrobacterium* strain ASE and *ago4-1* plants were transformed using the floral dip method (*4*). Complementation was scored as the reappearance of a *clk* floral phenotype.

Bisulfite sequencing. Bisulfite sequencing was performed as previously described (*5*). PCR products were cloned using the TOPO TA cloning kit (Invitrogen), and individual clones were sequenced. Primer sequences and cycling conditions for each experiment are available upon request. The regions analyzed in *SUP* and *MEA-ISR* were previously described (*1, 6*). The region analyzed in *AtMu1* corresponds to positions 23421 to 23840 of the bottom strand of BAC clone T3F12 (accession AC002983). The region of *AtSN1* encompasses 156 nucleotides of the bottom strand of the element corresponding to positions 84459 to 84304 of BAC clone T15B3 (Accession AL163975). Data presented in Fig. 1A that is not shown in detail in Table S1 was derived from previously published sources and provided for comparison. (*1, 2, 6*).

Chromatin immunoprecipitation assays. ChIP assays were performed exactly as described (*7*). Several independent immunoprecipitations were performed on *clk-st* and *ago4-1 clk-st* tissues using H3K9 dimethyl specific antibodies (either immunopurified antibodies from Upstate Biotechnology, #07-212, or unpurified serum kindly provided by C. David Allis), with similar results. Multiplex PCR was performed

with primers for *SUP, AtSN1* or *Ta3* together with *ACTIN*. Images were captured using Kodak Digital Science System and quantitation was performed using ImageQuant software (Amersham). The enrichment of H3K9 methylation at either *SUP, AtSN1* or *Ta3* was calculated relative to that present at *ACTIN* by dividing the ratio of *SUP/ACTIN* in the sample, by the ratio of *SUP/ACTIN* in the input control lane, as previously described (*7, 8*).

PCR based molecular markers used in this study. A molecular marker used to genotype the *ago4-1* mutation was composed of the following combinations of oligonucleotide primers and restriction enzymes: (5'- TGACTGACAGCTGAAAATGGGATGTGGAT-3' and 5'-GCCACTCCCTAGAACTCACCACCTAAGTT -3' with *AvaII*). For the RT-PCR result shown in Fig. S1B, we performed first strand cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen) with the gene specific primer 5'- TTGAGATTCACTCACACCATCCCTGAA-3', followed by PCR with the primers 5'- CTGGAACTTCAACAACAAGGAATTTGTT-3' and 5'-TGTAGAAATCTACCAGCAACTCCTTGAT-3'.

Small RNA analysis. Total RNA was extracted from Arabidopsis inflorescences and enriched for small RNAs using polyethylene glycol precipitation (*9*). 50 micrograms of RNA per lane was run on 15% polyacrylamide-7M urea gels and electroblotted onto Zeta-Probe GT nylon membranes. Membranes were prehybridized and hybridized as described (*9*). Riboprobes were generated by cloning fragments of DNA into the pCR4-TOPO vector (Invitrogen) and *in vitro* transcribed using T7 (Ambion) RNA polymerase in the presence of ^{32}P-UTP. The *AtSN1* probe sequence corresponded to the sense strand of the element, positions 84459 to 84299 of BAC clone T15B3 (Accession AL163975). RNA probes were hydrolyzed to a size of 50-70nt. End labeled Ambion (Austin, TX) Decade RNA markers were used as size standards, and the positions of the 20 and 30 nucleotide markers are shown on Fig. 3A.

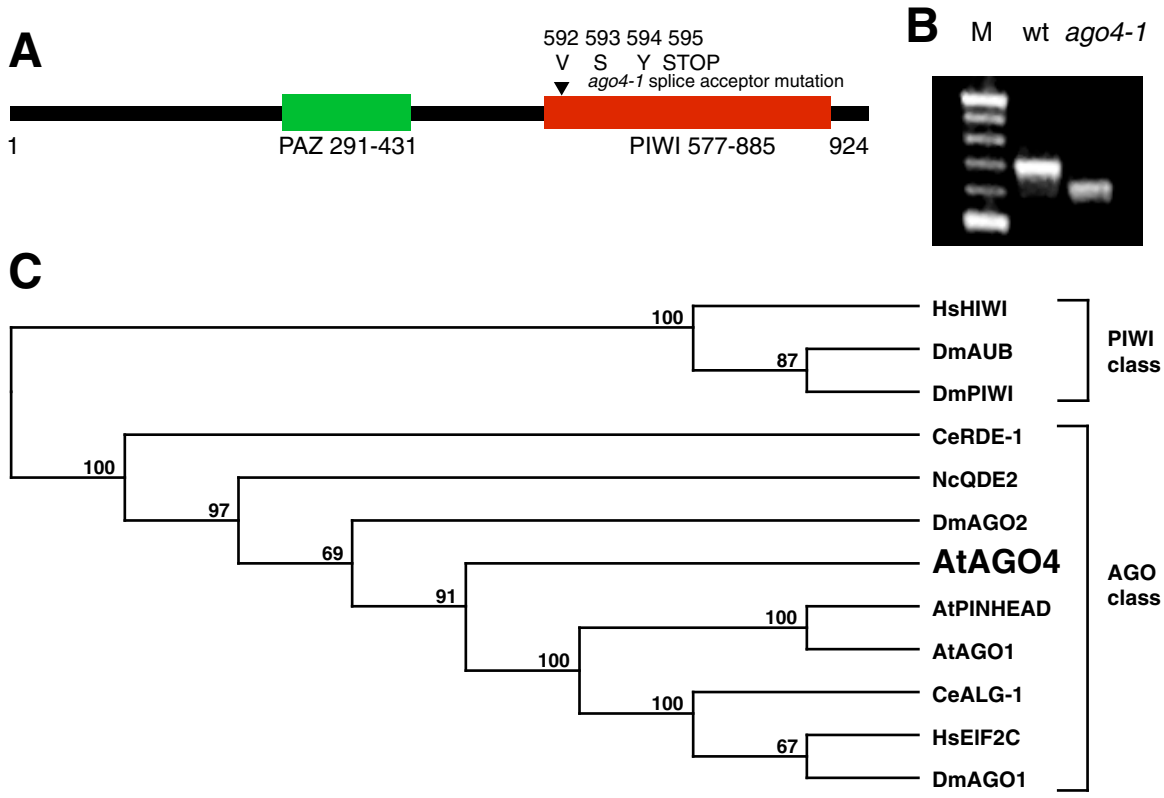

Fig. S1 *AGO4* mutant analysis and phylogeny. **A)** Schematic diagram of the AGO4 protein showing the PAZ and PIWI domains and the *ago4-1* mutation. **B)** RT-PCR showing that the *ago4-1* mutation causes incorrect splicing of the mRNA. Left lane (M) is a 100 base pair ladder of DNA fragments (NEB), shown from 1.0 to 0.5 kilobases. **C)** A bootstrap tree of an alignment of the PIWI domains of various AGO family proteins constructed using the UPGMA method in MacVector 7.0, using 1000 repetitions. AGO4 groups more closely with proteins of the AGO class than with proteins of the PIWI class (*10*).

Table S1. Number of cytosines methylated in different sequence contexts within cloned PCR products of bisulfite treated DNA.

SUPERMAN 1028 nucleotides (15 clones/genotype).

	CpG	CpNpG*	Asym†
Total number of sites	135	405	3060
Number methylated			
clk-st	22 (16%)	221 (55%)	497 (16%)#
ago4-1 clk-st	21 (16%)	80 (20%)	111 (3.6%)

MEA-ISR 189 nucleotides (18 clones/genotype).

	CpG	CpNpG	Asym
Total number of sites	144	36	270
Number methylated			
Wild type	137 (95%)	21 (58%)	69 (26%)
ago4-1	116 (81%)	0 (0%)	2 (0.7%)
kyp-1	133 (92%)	9 (25%)	45 (17%)

AtMu1 297 nucleotides (15 clones/genotype).

	CpG	CpNpG	Asym
Total number of sites	60	75	930
Number methylated			
Wild type	35 (58%)	26 (35%)	105 (11%)
ago4-1	28 (47%)	14 (19%)	45 (4.8%)

AtSN1 156 nucleotides (18 clones/genotype).

	CpG	CpNpG	Asym
Total number of sites	72	126	612
Number methylated			
Wild type	54 (75%)	88 (70%)	148 (24%)
ago4-1	30 (42%)	18 (14%)	5 (0.8%)
cmt3-7	35 (49%)	11 (8.7%)	53 (8.7%)
drm1 drm2	38 (53%)	38 (30%)	20 (3.3%)
drm1 drm2 cmt3-7	20 (28%)	0 (0%)	1 (0.2%)
kyp-2	59 (82%)	67 (53%)	179 (29%)

* CpGpG sites are counted as CpG sites and not included in the CpNpG category. †Asym (Asymmetric) is defined by cytosines within the context CpHpH, where H = A, T, or C. # Derived from previously published data (*1*).

Supporting references

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