Supplemental Data

Role of *Arabidopsis ARGONAUTE4* in RNA-Directed DNA Methylation Triggered by Inverted Repeats

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Supplemental Experimental Procedures

Plant Materials and Agrobacterium-Mediated Transformation

The *ago4-1* mutant line, AP1RNAi line, H, and K transgene lines used in this study were previously described [S1–S3]. The AP1RNAi binary vector has been described [S2]. This plasmid was transformed into *Agrobacterium* strain ASE and wild-type L*er* and *ago4-1* plants were transformed by vacuum infiltration [S4].

Genotyping

PCR-based molecular markers used to genotype the *ago4-1* mutation and the H and K transgenes have been previously described [S1, S5]. Primers JP 2048 (5′-GTTAGATGTTCTTACCCATTGCATATGTT-3′) and JP2049 (5′-GGAATCACAGTTGTACTTGTGCAGATCTT-3′) were used to detect a simple sequence-length polymorphism between the Columbia and L*er* ecotypes located between positions 53332 and 53363 of AGI BAC F23O10 that was derived from the database of polymorphisms generated by Cereon Genomics (http://www.*Arabidopsis*.org/Cereon/index.html). This polymorphism is closely linked to the *AP1* gene and was used to distinguish between Columbia and L*er AP1*.

Bisulfite Sequencing

Genomic DNA was extracted from leaf tissue pooled from multiple plants, and bisulfite sequencing was performed as previously described [S6]. In the case of the AP1 T1 experiment (Figure 2F), tissues from multiple, independent transgenic lines were pooled. The 35S promoter and promoter-proximal repeat of the AP1RNAi and H transgenes were analyzed by using a single PCR product, and the genomic DNA was digested with a restriction enzyme (RsaI for AP1RNAi and HaeIII for H) that cuts in the spacer between the repeats to avoid snapback. PCR products were cloned by using the TOPO TA cloning kit (Invitrogen), and 18 individual clones were sequenced in each experiment.

Primer sequences are as follows: JP1936 (5′-TTGAAAATTGAT GATGATAATATGGTTTGAAATAAATTTG-3′) and JP1937 (5′-TAACT TCAATAAAATTACAACACCTAACRTAACTAACTTA-3′) for the endogenous *AP1* gene, JP2097 (5′-ATYGTGGAAAAAGAAGAYGTTT TAATTAYGTTTTTAAAGT-3′) and JP2099 (5′-CTTAAAACTCAACAR AAACARTAAAATCARCATAACCAAAA-3′) for the AP1RNAi transgene, JP1868 (5′-TATTGATAGTTTAAATTGAAGGTGGGAAATGATA ATT-3′) and JP1869 (5′-CCAAATAACCTCTCCACCCAAACAACCA AAA-3′) for the K transgene, and JP2099 and JP2115 (5′-CATCAT CAAAAACCRRACAAAAAATCCRRACARATTATTTAA-3′) for the H transgene.

RT-PCR

5 μg of total RNA was used for priming cDNA synthesis with oligo dT by using the SuperScript III First Strand Synthesis System (Invitrogen). Sequence corresponding to the *AP1* cDNA was amplified by using PCR with primers 5′-CGAATACTCCACTGATTCTTGTATG GAGA-3′ and 5′-ATTTTTTCCCTCTCCTTGATCTGTTTAGA-3′. These primers do not anneal to the AP1RNAi transgene. Sequence corresponding to the *UBQ10* cDNA was amplified by using PCR with primers 5′-CAAAGAGCTCTTCTTCTTCACAATTCAGA-3′ and 5′- CAA GATGAAGGGTGGACTCCTTCTGGATA-3′.

Small RNA Analysis

Total RNA was extracted from *Arabidopsis* inflorescences and enriched for small RNAs by using polyethylene glycol precipitation [S3]. RNA blots were prepared as previously described [S1]. RNA probes were used to detect *AP1*, *NOS* promoter, and cluster 2 small RNA. The coordinates of the *AP1* probe correspond to nucleotides 454–850 of mRNA accession Z16421; *NOS* and cluster 2 probes have been described [S5, S7]. Hybridization was performed as previously described [S1]. End-labeled DNA oligonucleotide probes were used to detect AtSN1 siRNA, siRNA 1003, siRNA02, and microRNA 173. The JP2107 oligo (5′-ACCAACGTGTTGTTGGCCCAGTGGTAAATC TCTCAGATAGAGG-3′) was used as the AtSN1 probe; 5S rDNA, siRNA02, and microRNA 173 probes have been described [S7, S8]. Hybridization was performed as previously described [S8].

Supplemental References

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S8. Park, W., Li, J.J., Song, R.T., Messing, J., and Chen, X.M. (2002). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Curr. Biol. *12*, 1484–1495.

Table S1. Number of Cytosines Methylated within 18 Cloned PCR Products of Bisulfite-Treated DNA

AP1 Data from Figure 1C	CG	CNG[a]	Asym[b]
Total number of sites	18	234	774
Sites per clone	1	13	43
	Number Methylated		
AP1RNAi	17 (94%)	161 (69%)	225 (29%)
AP1RNAi *ago4-1*	12 (67%)	70 (30%)	31 (4.0%)
AP1	10 (56%)	10 (4.3%)	6 (0.8%)
AP1 *ago4-1*	9 (50%)	15 (6.4%)	3 (0.4%)

AP1 Data from Figure 2B	CG	CNG[a]	Asym[b]
Total number of sites	18	234	774
Sites per clone	1	13	43
	Number Methylated		
AP1RNAi	4 (22%)	38 (16%)	86 (11%)
AP1RNAi *ago-4*	5 (28%)	44 (19%)	29 (3.7%)

AP1 Data from Figure 2C	CG	CNG[a]	Asym[b]
Total number of sites	18	234	774
Sites per clone	1	13	43
	Number Methylated		
AP1RNAi	10 (56%)	151 (65%)	233 (30%)
AP1RNAi *ago-4*	13 (72%)	70 (30%)	35 (4.5%)

AP1 Data from Figure 2F	CG	CNG[a]	Asym[b]
Total number of sites	18	234	774
Sites per clone	1	13	43
	Number Methylated		
AP1RNAi	14 (78%)	143 (61%)	268 (35%)
AP1RNAi *ago-4*	11 (61%)	72 (30%)	44 (5.7%)

*NOS*pro Data from Figure 1I	CG	CNG[a]	Asym[b]
Total number of sites	378	270	792
Sites per clone	21	15	44
	Number Methylated		
HxK	267 (71%)	121 (45%)	302 (38%)
HxK *ago4-1*	290 (77%)	56 (21%)	83 (10%)
K	202 (53%)	28 (10%)	12 (1.5%)
K *ago4-1*	223 (59%)	36 (13%)	1 (0.1%)

*NOS*pro Data from Figure 2E	CG	CNG[a]	Asym[b]
Total number of sites	378	270	792
Sites per clone	21	15	44
	Number Methylated		
HxK	135 (36%)	102 (38%)	240 (30%)
HxK *ago4-1*	185 (49%)	39 (11%)	42 (5.3%)

(*continued*)

Table S1. Continued

35S-AP1RNAi Data from Figure 1K	CG	CNG[a]	Asym[b]
Total number of sites	72	18	378
Sites per clone	4	1	21
	Number Methylated		
AP1RNAi	42 (58%)	14 (78%)	71 (19%)
AP1RNAi *ago-4*	1 (1.4%)	0 (0%)	1 (0.3%)

AP1-IR Data from Figure 1J	CG	CNG[a]	Asym[b]
Total number of sites	126	198	540
Sites per clone	7	11	30
	Number Methylated		
AP1RNAi	102 (81%)	144 (73%)	90 (17%)
AP1RNAi *ago-4*	2 (1.6%)	0 (0%)	2 (0.4%)

35S-H Data Not Shown In Figures	CG	CNG[a]	Asym[b]
Total number of sites	54	36	360
Sites per clone	3	2	20
	Number Methylated		
HxK	0 (0%)	2 (5.6%)	0 (0%)
HxK *ago-4*	1 (1.9%)	0 (0%)	1 (0.3%)

SPACER Data Not Shown in Figures	CG	CNG[a]	Asym[b]
Total number of sites	90	108	144
Sites per clone	5	6	8
	Number Methylated		
HxK	8 (8.9%)	8 (7.4%)	0 (0%)
HxK *ago4-1*	2 (2.2%)	1 (0.9%)	0 (0%)

NOS-IR Data from Figure 3L	CG	CNG[a]	Asym[b]
Total number of sites	324	216	612
Sites per clone	18	12	34
	Number Methylated		
HxK	272 (84%)	136 (63%)	334 (55%)
HxK *ago4-1*	96 (30%)	10 (4.6%)	49 (8%)

[a] CGG sites are counted as CG sites and are not included in the CNG category.

[b] Asym (asymmetric) is defined by cytosines within the context CHH, where H = A, T, or C.

The following regions were sequenced: AP1, 200 nucleotides of the endogenous *APETELA1* (*AP1*) gene; *NOS*pro, 300 nucleotides of the *NOS* promoter of the K transgene (K); 35S-AP1RNAi, 115 nucleotides of the 35S promoter of the AP1RNAi transgene; AP1-IR, 266 nucleotides of the AP1 inverted repeat region of the AP1RNAi transgene; 35S-H, 114 nucleotides of the 35S promoter of the H transgene; SPACER, 93 nucleotides of the spacer between the 35S promoter and the *NOS* promoter inverted repeat region of the H transgene; and *NOS*-IR, 257 nucleotides of the *NOS* promoter inverted repeat region of the H transgene.