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 Ler 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, S8]. Primers 5'–CGAATTCAACTGGTTTCTGACTTCTGACGTTTT-3' and 5'–GAATACAACTGGTTTCTGACTTCTGACGTTTT-3' were used to detect a simple sequence-length polymorphism between the Columbia and Ler ecotypes located between positions S3332 and S3333 of AGI BAC F23010 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 Ler 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. S5. Cao, X., Aufsatz, W., Zilberman, D., Mette, M.F., Huang, M.S., and Jacobsen, S.E. (2000). Ectopic hypermethylation of flower-specific gene, act in microRNA metabolism in Arabidopsis thaliana. Curr. Biol 10, 179–186.

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'–GGATCTTCCACGATCTTGGATGAGA-3' and 5'–GTTCAGATCTTCTTCCACGATCTTGGATGAGA-3' for the H transgene, and 5'–CAAGAAGGTTGGATCCCTCTCAGATCTTGGATGAGA-3' for the K transgene, and 5'–CATGATCTTCTTCCACGATCTTGGATGAGA-3' for the AP1RNAi transgene.

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'–ACCAACGTTTTGAGCCAGTTGGCTAAATC TCTCGATAGAG-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


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

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<th>CG</th>
<th>CNG</th>
<th>Asym</th>
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<td>234</td>
<td>774</td>
</tr>
<tr>
<td>Sites per clone</td>
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<td>13</td>
<td>43</td>
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<tr>
<td><strong>AP1RNAi</strong></td>
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</tr>
<tr>
<td>Total number of sites</td>
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<td>161</td>
<td>225</td>
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<tr>
<td>Sites per clone</td>
<td>(94%)</td>
<td>(69%)</td>
<td>(29%)</td>
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<td><strong>AP1RNAI ago4-1</strong></td>
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<td>70</td>
<td>31</td>
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<tr>
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<td>Total number of sites</td>
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<td>234</td>
<td>774</td>
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<tr>
<td>Sites per clone</td>
<td>1</td>
<td>13</td>
<td>43</td>
</tr>
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<td>(16%)</td>
<td>(11%)</td>
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<td>Sites per clone</td>
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<td>(85%)</td>
<td>(30%)</td>
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<td>Sites per clone</td>
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<td>(81%)</td>
<td>(35%)</td>
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<tr>
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<tr>
<td><strong>HxK</strong></td>
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<td><strong>HxK ago4-1</strong></td>
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</tbody>
</table>

*CGG sites are counted as CG sites and are not included in the CNG category.

*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; NOSpro, 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.

(continued)