Supplemental Data

AGO1 Homeostasis Requires Coexpression of *MIR168* and *AGO1*, Posttranscriptional Stabilization of miR168 by AGO1, and miR168-Guided *AGO1* Cleavage

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Supplemental Results

Plants Expressing an AGO1 mRNA Perfectly Complementary to miR168 Exhibit Increased AGO1 mRNA Cleavage but Develop Normally Due To Reduced miR168 Levels

To test further the biological relevance of our posttranscriptional autoregulatory model, we analyzed miRNA and *AGO1* levels in plants that express an *AGO1* mRNA perfectly complementary to miR168 (*0m-AGO1*) (Supplemental Figure 3A). To compare the cleavage efficiency of wild-type (*WT-AGO1*) and mutant (*0m-AGO1*) RNAs, we used an *in vitro* assay that relies on the endogenous miRNA-programmed RISC activity of wheat germ extract (Tang et al., 2003), knowing that miR168 is present in these extracts (Axtell and Bartel, 2005). The cleavage rate of a *0m-AGO1* RNA, which was generated by mutagenesis of an *AGO1* cDNA, was 2.5-fold faster than that observed for *WT-AGO1* RNA (Supplemental Figure 3B), indicating that the three native mismatches negatively impact the efficiency of miRNA-directed mRNA cleavage *in vitro*.

To analyze the impact of increased *AGO1* mRNA cleavage on plant development and miRNA accumulation, the same mutagenesis was performed on an 8-kb *AGO1* genomic fragment that contains all the upstream and downstream regulatory elements required for wild-type AGO1 function (Vaucheret et al., 2004). We separately introduced the *WT-AGO1* and *0m-AGO1* constructs into the *ago1-1* null allele. This null allele contains a T-DNA that interrupts the endogenous *AGO1* gene, abolishing the production of *AGO1* mRNA, and that confers hygromycin resistance to the plant. Because homozygous *ago1-1* null alleles are sterile, we transformed fertile plants heterozygous for the *ago1-1* allele. Transformants that were kanamycin resistant, due to the presence of the *WT-AGO1* or *0m-AGO1* transgene, were first selected and the plants that did not exhibit an AGO1 cosuppression phenotype (Vaucheret et al., 2004) were allowed to self-fertilize. Seeds collected from these plants were selected on hygromycin and the plants that produced 100% hygromycin-resistant progeny were identified as homozygous for *ago1-1*. The two *ago1-1::WT-AGO1* and three *ago1-1::0m-AGO1* plants that were identified exhibited a wild-type phenotype (data not shown), indicating that neither the increased cleavage rate of *AGO1* mRNA (Supplemental Figure 3B) nor the conservative E to D amino acid substitution generated by the abolition of the three native mismatches (Supplemental Figure 3A) substantially impacted plant development. One *ago1-1::WT-AGO1* and one *ago1-1::0m-AGO1* plant that appeared to carry the construct inserted at a single locus (i.e. primary transformants that segregated 3:1 kanamycin-resistant:kanamycin-sensitive progeny) were kept for further analyses. *AGO1* mRNA accumulated at ~two-fold lower level in the *ago1-1::0m-AGO1* plant compared with the *ago1-1::WT-AGO1* plant (Supplemental Figure 3C), consistent with the increased cleavage rate of *0m-AGO1* RNA observed in vitro (Supplemental Figure 3B). miR156 and miR160, which are representative of miRNAs that are insensitive to perturbation in AGO1, and miR165/166 and miR159, which are representative of miRNAs that are weakly sensitive to perturbation in AGO1, accumulation was equivalent in the *ago1-1::WT-AGO1* and *ago1-1::0m-AGO1* plants (Supplemental Figure 3D). In contrast, the accumulation of miR168 was almost two-fold lower in the *ago1-1::0m-AGO1* plant compared to the *ago1-1::WT-AGO1* plant, and this decreased accumulation of miR168 was not due to decreased transcription of the MIR168 genes because the accumulation of the processed MIR168a loop was similar in the two plants (Supplemental Figure 3D). Rather, the decrease in the accumulation of miR168 likely was due to reduced *AGO1* mRNA (and AGO1 protein) levels because of the increased cleavage efficiency of *0m-AGO1* mRNA, which is perfectly complementary to miR168. In this scenario, the new equilibrium between miR168 and AGO1 levels maintained miRNA homeostasis and proper RISC functioning, consistent with the absence of developmental defects in *ago1-1::0m-AGO1* plants. These results support our model, in which a posttranscriptional regulatory loop involving AGO1 and miR168 is required to maintain a proper balance of the miRNA pool and AGO1 levels, which together control the mRNA levels of miRNA targets.

Supplemental References

Tang, G., Reinhart, B. J., Bartel, D. P., and Zamore, P. D. (2003). A biochemical framework for RNA silencing in plants. Genes Dev *17*, 49-63.

Vaucheret, H., Vazquez, F., Crété, P., and Bartel, D. P. (2004). The action of *ARGONAUTE1* in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes Dev *18*, 1187-1197.

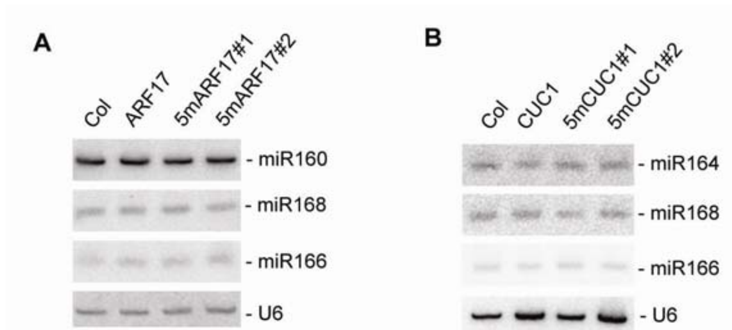

Figure S1. (A) miRNA accumulation in wild-type Col, a *ARF17* control and two individual *5m-ARF17* plants was determined by RNA gel blot analysis using 10 µg of RNA extracted from rosette leaves. Blots were successively hybridized to different probes complementary to miRNAs and a probe complementary to U6 as a loading control. (B) miRNA accumulation in wild-type Col, a *CUC1* control and two individual *5m-CUC1* plants was determined by RNA gel blot analysis using 10 µg of RNA extracted from rosette leaves. Blots were successively hybridized to different probes complementary to miRNAs and a probe complementary to U6 as a loading control.

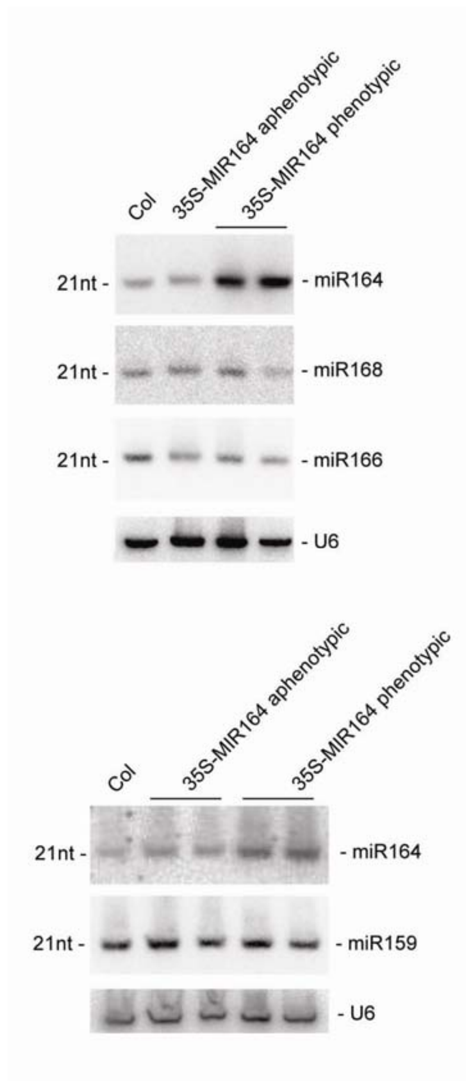

Figure S2. miRNA accumulation in wild-type Col, an aphenotypic 35S:MIR164b plant, and two individual 35S:MIR164b plants was determined by RNA gel blot analysis using 10 μg of RNA extracted from floral inflorescence. Blots were successively hybridized to different probes complementary to miRNAs and a probe complementary to U6 as a loading control.

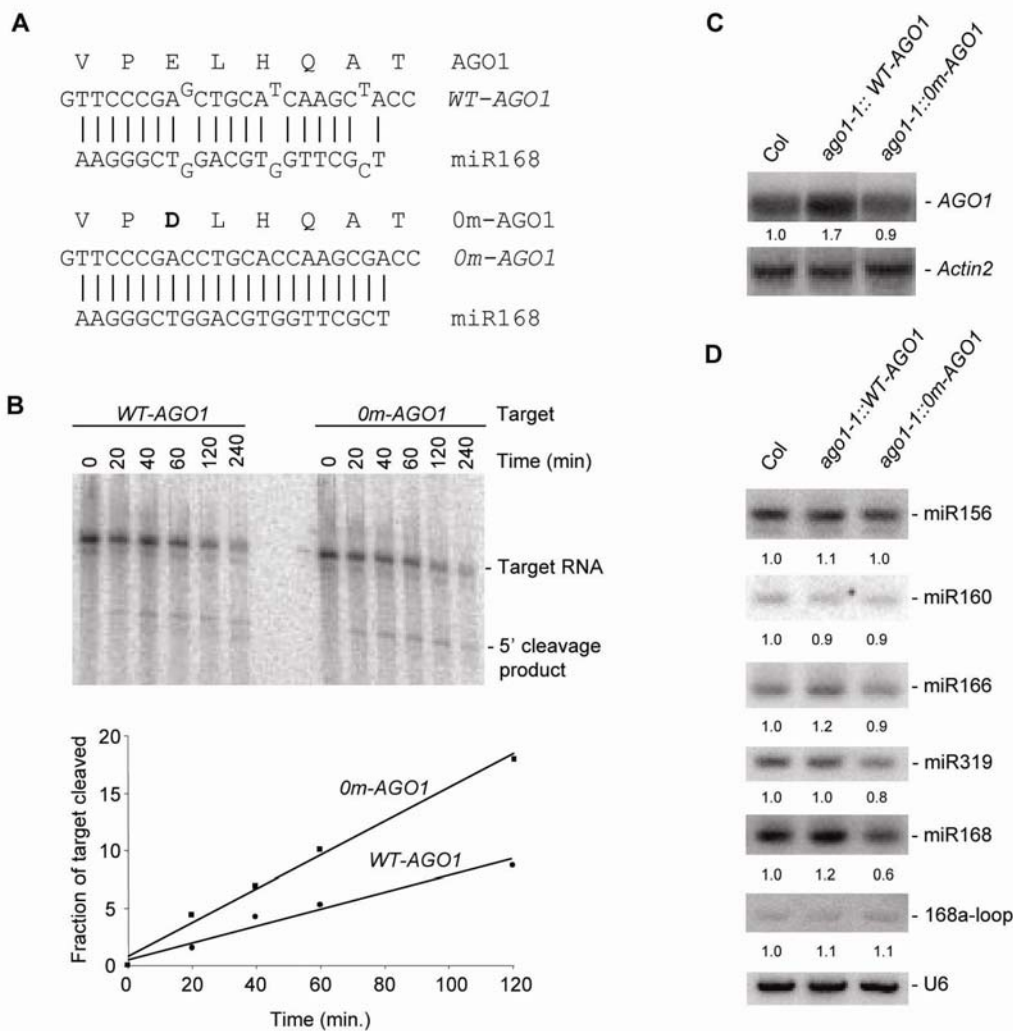

Figure S3. Plants expressing an *AGO1* mRNA perfectly complementary to miR168 exhibit increased *AGO1* mRNA cleavage but develop normally due to reduced miR168 level.

(A) The *WT-AGO1* mRNA naturally has three mismatches with miR168. Two of the three mutations that eliminate mismatches in the *0m-AGO1* mRNA are silent, whereas one mutation introduces a conservative E to D amino acid change.

(B) 5'-radiolabelled transcripts prepared from *WT-AGO1* and *0m-AGO1* cDNAs were introduced into wheat germ extracts, and the time course of cleavage was examined on a sequencing gel. Quantification of the data is shown below.

(C) *AGO1* mRNA accumulation in wild-type Col and *ago1-1::0m-AGO1* and *ago1-1::WT-AGO1* plants was determined by RNA gel blot analysis using 10 µg of RNA. The *Actin2* signal is shown as a loading control. Normalized values of *AGO1* mRNAs to *Actin2* mRNAs are indicated, with levels in Col control plants set at 1.0.

(D) RNA gel blot analysis of 10 µg of the same RNA as in (C) with miR156, miR166, miR168 and miR168a-loop probes. The blot was stripped and hybridized with a probe complementary to U6 as a loading control.

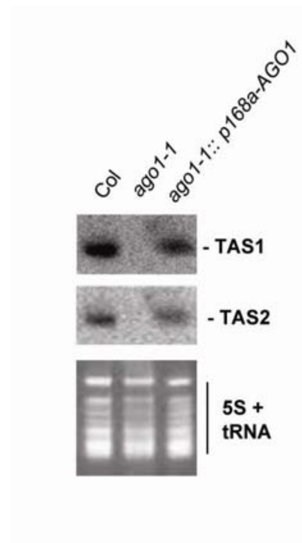

Figure S4. Expression of the *AGO1* cDNA under the control of the *MIR168a* promoter rescues the production of ta-siRNAs in null *ago1-1* alleles.

RNA gel blot analysis of 10 µg of RNA with TAS1 and TAS2 probes. Ethidium bromide-stained 5S and tRNA are shown as a loading control.