The Plant Cell, Vol. 18, 1559–1574, July 2006, www.plantcell.org

RESEARCH ARTICLES

MicroRNA-Targeted and Small Interfering RNA–Mediated mRNA Degradation Is Regulated by Argonaute, Dicer, and RNA-Dependent RNA Polymerase in *Arabidopsis* [W] [OA]

Michael Ronemus, Matthew W. Vaughn, and Robert A. Martienssen[1]

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

***ARGONAUTE1* (*AGO1*) of *Arabidopsis thaliana* mediates the cleavage of microRNA (miRNA)-targeted mRNAs, and it has also been implicated in the posttranscriptional silencing of transgenes and the maintenance of chromatin structure. Mutations in *AGO1* severely disrupt plant development, indicating that miRNA function and possibly other aspects of RNA interference are essential for maintaining normal patterns of gene expression. Using microarrays, we found that 1 to 6% of genes display significant expression changes in several alleles of *ago1* at multiple developmental stages, with the majority showing higher levels. Several classes of known miRNA targets increased markedly in *ago1*, whereas others showed little or no change. Cleavage of mRNAs within miRNA-homologous sites was reduced but not abolished in an *ago1* -null background, indicating that redundant slicer activity exists in *Arabidopsis*. Small interfering RNAs and larger 30- to 60-nucleotide RNA fragments corresponding to highly upregulated miRNA target genes accumulated in wild-type plants but not in *ago1*, the RNA-dependent RNA polymerase mutants *rdr2* and *rdr6*, or the Dicer-like mutants *dcl1* and *dcl3*. Both sense and antisense RNAs corresponding to these miRNA targets accumulated in the *ago1* and *dcl1* backgrounds. These results indicate that a subset of endogenous mRNA targets of RNA interference may be regulated through a mechanism of second-strand RNA synthesis and degradation initiated by or in addition to miRNA-mediated cleavage.**

INTRODUCTION

Double-stranded RNA (dsRNA) induces the posttranscriptional silencing (PTGS) of the corresponding gene via the degradation of homologous RNA (Fire et al., 1998; Waterhouse et al., 1998; Tuschl et al., 1999; reviewed in Hannon, 2002; Tijsterman et al., 2002). This process of RNA interference (RNAi) is thought to have an ancestral function in the defense against viruses and transposable elements (TEs), because mutants deficient in PTGS have increased susceptibility to viral infection (Voinnet et al., 1999; Mourrain et al., 2000) and some RNAi-deficient mutants of *Caenorhabditis elegans* also show increased transposon activation (Tijsterman et al., 2002). RNAi-mediated silencing extends to heterochromatic regions, such as the centromere repeats of *Schizoaccharomyces pombe*, in which RNAi participates in heterochromatin modification (Volpe et al., 2002). In plants, transgenes that undergo RNAi can also be silenced at the transcriptional level and undergo DNA methylation de novo (Baulcombe, 2005; Matzke and Birchler, 2005; Wassenegger, 2005).

[1] To whom correspondence should be addressed. E-mail martiens@cshl.edu; fax 516-367-8369.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Robert A. Martienssen (martiens@cshl.edu).

[W] Online version contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.106.042127.

A hallmark of RNAi is the presence of small interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999). The siRNAs are cleaved from dsRNA by a class of RNase III enzymes known as Dicers (Bernstein et al., 2001). After cleavage, siRNAs from both strands can then target additional RNA molecules for degradation. The siRNA involved in later rounds of RNAi can be derived from sequences not present in the initial triggering siRNA, a property termed transitive RNAi (Lipardi et al., 2001; Sijen et al., 2001) that is facilitated by the activity of RNA-dependent RNA polymerases (RdRPs) such as RDR2 and RDR6 of *Arabidopsis thaliana* (Dalmay et al., 2000; Mourrain et al., 2000; Xie et al., 2004).

Analogous to siRNAs are microRNAs (miRNAs) (Carrington and Ambros, 2003), a class of small RNAs differentiated from siRNAs by several features: nearly all miRNAs are complementary to sites within target mRNAs but generally contain one or more mismatches; miRNAs are processed from larger noncoding RNA precursors that contain stem-loop structures processed by a Dicer; and miRNAs are highly conserved in sequence, expression, and function. In plants, miRNAs act through several possible mechanisms: posttranscriptional cleavage of mRNA (Llave et al., 2002; Kasschau et al., 2003; Palatnik et al., 2003); inhibition of translation (Aukerman and Sakai, 2003; Chen, 2004); and RdRP-mediated second-strand synthesis and *trans*-acting siRNA (ta-siRNA) production initiated by miRNA action (Volpe et al., 2002; Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). Cleavage of miRNA target genes has been documented regardless of mode of action. The prevalence of the putative translational block has not been assessed systematically (Jones-Rhoades and

Bartel, 2004), and some evidence indicates that a transcriptional feedback mechanism may also be active (Schwab et al., 2005).

ARGONAUTE1 (*AGO1*) was originally characterized as a novel factor required for normal leaf development in *Arabidopsis* (Bohmert et al., 1998). Subsequently, *AGO1* and its homologs from animals, fungi, and plants were demonstrated to mediate RNA silencing, and many play roles in development (reviewed in Carmell et al., 2002; Tijsterman et al., 2002). The AGO family is defined by the presence of two conserved regions, the PAZ and PIWI domains; the PAZ domain interacts with the 2-bp 3′ overhangs of siRNA or miRNA duplexes (Song et al., 2003), whereas the PIWI domain mediates slicing of the target mRNA substrate through a cryptic RNase H–like activity(Liu et al., 2004; Song et al., 2004). AGO1-like proteins are the sole conserved components of the RNA-induced silencing complex (RISC), a nuclease complex that carries out small RNA–mediated target degradation (Hammond et al., 2001) and is present in various forms in organisms as diverse as *Drosophila* and *S. pombe* (Verdel et al., 2004; Pham and Sontheimer, 2005).

In *Arabidopsis*, *ago1* mutants have pleiotropic abnormalities in plant architecture, including small, unexpanded cotyledons and narrow, bladeless leaves with altered polarity. Axillary meristems are absent, and in the inflorescence, only a short shoot initiates and flowers have altered organ morphology. In more severe cases, the flowers are completely radialized (Bohmert et al., 1998; Kidner and Martienssen, 2004). Mutants are generally sterile, although weak alleles can be fertile in some backgrounds (Morel et al., 2002). Strong alleles can lack the shoot apical meristem, indicating that *AGO1* is required for stem cell maintenance, although with incomplete penetrance. Mutations in *PNH* enhance the meristematic defects in *ago1*, resulting in embryonic arrest (Lynn et al., 1999), and mutations in animal homologs, such as *piwi* and *Ago1* of *Drosophila*, also have stem cell defects, indicating that stem cell maintenance may be a basic function of AGO1-like factors (Kidner and Martienssen, 2004). Phenotypic and double mutant analyses indicate that AGO1 may regulate stem cell function via SHOOT MERISTEMLESS. AGO1 is also required for normal expression of the determinacy regulators LEAFY, APETALA1, and AGAMOUS. The Polycomb group gene *CURLY LEAF* (*CLF*) is overexpressed in the *ago1* background, and mutations in *AGO1* can partially suppress *clf*-induced defects in floral meristem identity, indicating that RNAi-based mechanisms may regulate the function of Polycomb group factors (Kidner and Martienssen, 2005).

Arabidopsis AGO1 is required for PTGS of sense transgenes (Morel et al., 2002) but not PTGS of inverted-repeat transgenes (Boutet et al., 2003). Initial reports using weak *ago1* alleles suggested that AGO1 did not function in the miRNA pathway (Boutet et al., 2003), but more recent evidence has shown that AGO1 possesses the slicer activity responsible for cleaving mRNA targets of miRNA (Baumberger and Baulcombe, 2005; Qi et al., 2005). It is unclear whether all miRNA target mRNAs are sliced by AGO1 in vivo or whether other AGO family members possess slicer activities. Processing of all examined miRNAs is strongly reduced in *dcl1* (Park et al., 2002; Reinhart et al., 2002; Kasschau et al., 2003; Papp et al., 2003), but silencing by an RNA hairpin is unaffected (Finnegan et al., 2003). Conversely, small RNAs from silenced sense transgenes fail to accumulate in a weak *ago1* background (Boutet et al., 2003) but do accumulate in *dcl1* (Papp et al., 2003), despite the siRNA-producing activity of DCL1 in vitro (Qi et al., 2005). These findings indicate that AGO1 and DCL1 have distinct roles in miRNA processing, siRNA production, and PTGS pathways.

We have examined gene expression profiles in *ago1* and compared them with those of wild-type and *dcl1* plants grown under identical conditions. Given the phenotypic similarity of *ago1* and *dcl1*, genes upregulated in both mutant backgrounds are likely to be targets of a common miRNA-mediated regulatory mechanism. In addition to miRNA-guided cleavage of target messages, we have found that this common mechanism implicates the production of secondary siRNAs, indicating the existence of a mechanism for the RNAi-mediated silencing of endogenous genes similar to that responsible for generating ta-siRNAs (Allen et al., 2005).

RESULTS

Global Expression Changes in *ago1* and *dcl1*

We profiled gene expression in *ago1* and *dcl1* using the Affymetrix AtGenome1 microarrays, which contain 8297 features; of these, ~7805 represent annotated coding sequences from *Arabidopsis* (Ghassemian et al., 2001). To distinguish between possible direct and downstream consequences of mutations in *AGO1*, we used multiple *ago1* alleles and compared these results with those obtained from *dcl1-9* (Jacobsen et al., 1999) grown under identical conditions. Both *ago1-9* (a null allele) and *ago1-11* (a moderate allele) were isolated in the Landsberg *erecta* (L*er*) background (Kidner and Martienssen, 2004), whereas *dcl1-9* was isolated in a mixed L*er*-Wassilewskija background (Jacobsen et al., 1999). Target RNA populations were collected from *ago1-9* and *ago1-11*, as well as matched sibling controls, at 9 and 21 d after seed germination. Because *dcl1-9*/*dcl1-9* cannot be readily distinguished from phenotypically wild-type siblings at 9 d, 21-d seedlings were collected. A core set of 4214 genes representing >80% of the genes expressed in wild-type plants at both 9 and 21 d were detected in all wild-type and mutant data sets (see Supplemental Table 1 online).

Genes were classified as misexpressed if their expression increased or decreased by at least 1.5-fold and the changes were statistically significant between replicates (see Supplemental Table 1 online). Based on these criteria, *ago1-9* had the highest number of misexpressed genes (7.5% at 9 d and 4.4% at 21 d), of which the majority increased rather than decreased. In *ago1-11*, there were fewer changes in expression; only 1.7% (9 d) and 1.2% (21 d) of genes changed significantly, with >50% of genes downregulated. Relative to *ago1-9* and *ago1-11*, an intermediate number of genes changed in *dcl1-9* (2.3%), but these were skewed heavily (>80%) toward higher mRNA levels.

We manually classified genes that changed in *ago1* and *dcl1* according to Gene Ontology Consortium annotations (http://www.geneontology.org/). In general, the categories of genes that changed in expression reflected approximately the same distribution as those expressed in the wild type, except that transcription factors were moderately overrepresented among genes upregulated in *ago1* and *dcl1* (see Supplemental Table 2 online).

This finding indicates that many of the observed changes in gene expression may occur well downstream of direct consequences of mutations in *AGO1* and *DCL1*. There was substantial overlap among upregulated genes in both *ago1* alleles and *dcl1-9* but not among downregulated genes. More than 50% of genes that were upregulated in *ago1-11* at 9 d also increased in *ago1-9*, and more than half of the genes that increased in *dcl1-9* also did so in at least one *ago1* allele. By contrast, only ~20% of genes that decreased in *dcl1-9* also did so in *ago1* (see Supplemental Table 1 online).

To ascertain the contributions of AGO1 and DCL1 to the regulation of TEs, we identified features on the AtGenome1 microarray that were homologous with entities in Repbase, a compendium of TE and repeat sequences from a number of organisms, including *Arabidopsis* (Jurka et al., 2005). Approximately 230 of the features on the AtGenome1 microarray represent TEs. Of these, nearly one-third were not expressed in wild-type or mutant plants under any conditions, and very few displayed significant upregulation in *ago1* or *dcl1* (see Supplemental Table 1 online). This finding indicates that AGO1 and DCL1 may function in the silencing of specific TEs, but neither is required for general silencing.

Some miRNA Target Gene mRNAs Accumulate in *ago1* and *dcl1*

The most prominent changes in gene expression in the *ago1* and *dcl1* backgrounds were observed for known miRNA target genes (Figure 1). Probe sets homologous with 22 known miRNAs and ta-siRNAs were present on the microarray (see Supplemental Table 3 online), but of these, only genes homologous with specific classes of miRNAs showed consistent upregulation in *ago1* and/or *dcl1* under our experimental conditions. The most prominent of these were the *SPL* genes, defined by the presence of the SBP box, an 80–amino acid DNA binding motif (Cardon et al., 1999). The upregulated *SPL*s fall into two classes: one with coding regions of 1.1 to 1.2 kb and microRNA complementarities at the 3′ end of the coding region (*SPL2*, *SPL6*, *SPL9*, *SPL10*, and *SPL11*); and a second with short coding regions of 0.4 to 0.6 kb and miR complementarities within the 3′ untranslated region (UTR) (*SPL3*, *SPL4*, and *SPL5*). *SPL10* and *SPL11* are 85% identical and are located 1.3 kb apart on chromosome 1, forming a natural inverted repeat. *SPL2*, *SPL6*, and *SPL9* also contain considerable sequence identity, which is highest within the sequence encoding the SPB box. The smaller class of *SPL*s are also similar to each other, but mainly within their SBP box–encoding regions and within the microRNA-homologous regions of the 3′ UTR.

HAP2C and At1g54160 are two closely related members of a class of CCAAT box binding B subunits homologous with miR169. Both are located on chromosome 1 and contain a 54–amino acid CCAAT box binding motif in the center of the protein and miRNA complementarity within the 3′ UTR. Putative targets of miR160 and miR161 also showed marked upregulation in *ago1* and *dcl1*. *ARF16* is one of three related auxin response factors homologous with miR160; it contains a conserved DNA binding domain in the N terminus, which binds auxin response elements. At1g62670, which matches miR161, is a pentatricopeptide (PPR) repeat gene. The PPR motif is a degenerate 35–amino acid tandem repeat; it is thought that the PPR repeat may bind RNA, and many of the PPR proteins in *Arabidopsis* are predicted to be targeted to organelles (Schmitz-Linneweber et al., 2005). At1g62670 has at least four homologs with >70% nucleotide sequence identity throughout the coding region that are also homologous with miR161. *CSD1* (encoding a superoxide dismutase), a gene homologous with miR398 (Jones-Rhoades and Bartel, 2004), also showed a moderate trend of upregulation, particularly at 21 d. The class III HD-ZIP genes homologous with miR165/166, namely *PHB*, *PHV*, *ATHB8*, and *ATHB15*, increased approximately twofold in *ago1-9* and *dcl1-9* at 21 d. The action of miRNA on this class of genes is well documented in vitro (Tang et al., 2003) and in vivo (McConnell et al., 2001; Emery et al., 2003; Kidner and Martienssen, 2004), but the spatially restricted expression of both target and miRNA obscures large changes in mRNA accumulation after loss of the miRNA (Kidner and Martienssen, 2004).

Several miRNA targets were not upregulated in *ago1* and *dcl1* (see Supplemental Table 3 online). Both the miR170/171-targeted *SCL6* genes and the miR319-targeted *TCP2* and *TCP10* genes have been shown to undergo miRNA-mediated cleavage that is abolished by mutations within the miR-homologous site (Llave et al., 2002; Palatnik et al., 2003), but levels of these mRNAs were not greatly affected in the *ago1* and *dcl1* backgrounds. Similarly, targets of miR172 such as *AP2*, which undergoes mRNA cleavage but has an apparent miRNA-induced translational block, showed little change in mRNA levels in either the *ago1* background or *dcl1-9*. Among other miRNA target genes that did not show marked upregulation were those homologous with miR159, miR394, miR395, and miR397.

Validation of Targets Identified by Microarray Analysis

An important consideration in determining the expression levels of miRNA targets by microarray analysis is the position of probes with respect to miRNA cleavage sites. In many cases, polyadenylated 3′ cleavage products are stable and will not change in abundance if cleavage is blocked. Probes corresponding to the *SPL* and *AP2* genes, *HAP2C*, and miR159 targets span the miRNA site, but probes corresponding to At1g62670, *SCL6*, other targets of miR169 and most other targets on the array do not.

Therefore, RNA gel blots were used to validate mRNA accumulation in *ago1* and *dcl1*, using *TUB4* (β-tubulin) as a control (Figure 2). For putative miRNA target genes, hybridization probes flanked the miR-homologous sites by at least 100 nucleotides. Full-length messages from *SPL2*, *SPL3*, and *SPL10* were upregulated in both *ago1* alleles and *dcl1* (Figure 2), as were *SPL5*, *SPL6*, *SPL9*, and *SPL11* (data not shown). *HAP2C* and At1g54160 (Figure 2) and At1g17590 (data not shown) also increased significantly in expression in the mutants; both At1g54160 and At1g17590 were nearly undetectable in the wild type. Similarly, the PPR repeat gene At1g62670 showed little expression in the wild type but was expressed at high levels in *ago1* and *dcl1* (Figure 2).

Smaller transcripts were detected corresponding to At1g62670, *HAP2C*, *SPL2*, *SPL3*, and *SPL10*; these RNAs did not

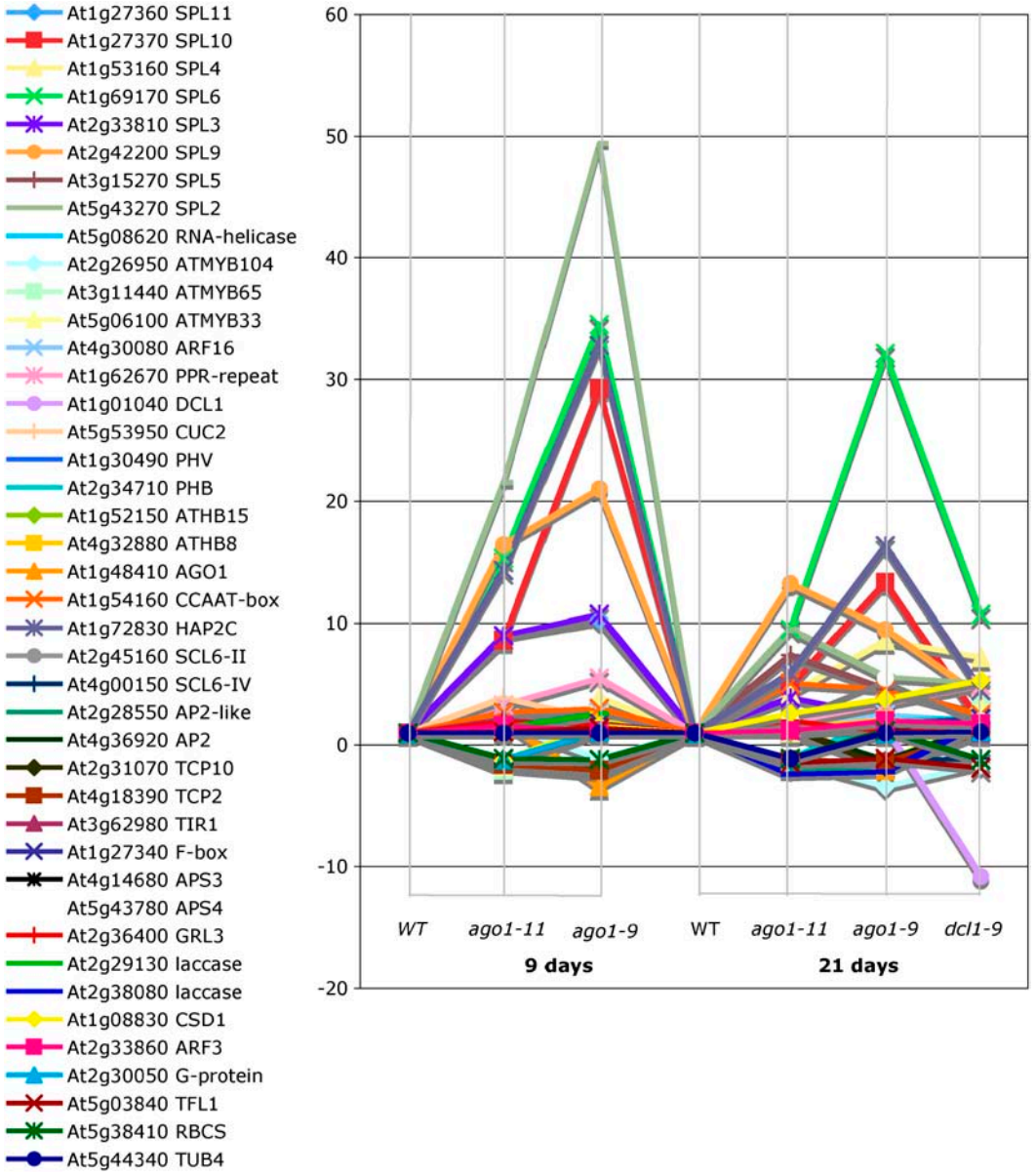

Figure 1. Graphic Display of Transcript Levels from miRNA Targets in *ago1* and *dcl1*.

Fold change values for known miRNA target transcripts represented on the AtGenome1 array are shown for wild-type, *ago1-11*, and *ago1-9* 9-d-old seedlings and for wild-type, *ago1-11*, *ago1-9*, and *dcl1-9* 21-d-old plants. Changes are most severe in *ago1-9*.

accumulate preferentially in wild-type or mutant plants and generally did not appear to change in quantity (Figure 2). Similar RNAs from two of the miR171-targeted *SCL6* genes represent miRNA-mediated cleavage products (Llave et al., 2002). 5′ rapid amplification of cDNA ends analyses of a number of additional miRNA target genes, including *SPL2*, have shown that stable cleavage products also accumulate (Llave et al., 2002; Kasschau et al., 2003; Palatnik et al., 2003). In some examples, levels of full-length mRNAs of miRNA-targeted genes increased in *dcl1* (Kasschau et al., 2003). The sizes of the RNAs we observed were also consistent with mRNA cleavage.

To determine the nature and extent of cleavage within target mRNAs, we used a primer extension assay. Primers were situated in unique regions 70 to 120 nucleotides downstream of the miRNA-homologous sites. Prominent cleavage products terminating within the miRNA-homologous sites were detected for *HAP2C*, *SPL2*, *SPL10*, and At1g62670 in total RNA from the wild type as well as *ago1-11* and *dcl1-9* (Figure 3). In *SPL10* and At1g62670, additional larger bands were observed, indicating an additional site ~10 nucleotides upstream, accounted for by an alternative isoform of miR161 in the case of At1g62670. Additional faint bands outside of the miRNA-homologous sites were

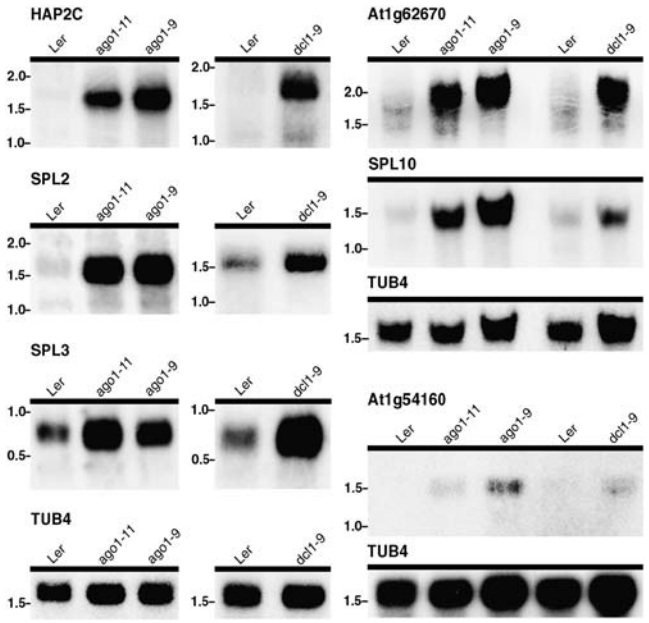

Figure 2. RNA Gel Blot Analysis of Genes Upregulated in *ago1* and *dcl1*.

Antisense riboprobes flanking the miRNA-homologous sites by 100 to 200 nucleotides were hybridized to 10 μg of total RNA from the wild type (L*er*), *ago1-11*, and *ago1-9* (left three lanes, each panel) and the wild type (L*er*) and *dcl1-9* (right two lanes, each panel) at 21 d.

sometimes observed. Thus, 3′ cleavage products were present at similar levels in the wild type and in both *ago1* and *dcl1* backgrounds. However, as intact mRNA levels were increased significantly, slicing activity was substantially reduced in these mutants, as expected. An abundant *SCL6-IV* 3′ cleavage product was also present in the null mutant *ago1-9*, although mRNA from *SCL6-IV* did not appear to increase in abundance by microarray analysis. These cleavage products were not observed in mature leaves or shoots but were present in inflorescence and seedling RNAs, in agreement with previous studies (Llave et al., 2002). Microarray probes from this gene corresponded only to these stable 3′ cleavage products, so we used RT-PCR to confirm that exons from the 5′ cleavage product were not significantly upregulated in *ago1-9* (data not shown). In this case, cleavage seemed to be relatively unaffected in *ago1-9*, despite the depletion of miR171 (see below).

Novel siRNAs Corresponding to miRNA Target Genes Accumulate in the Wild Type but Not in *ago1* or *dcl1*

After mRNA cleavage, noncoding miRNA targets in *Arabidopsis* serve as templates for the production of ta-siRNAs through an RdRP-mediated pathway (Allen et al., 2005). We examined whether siRNA corresponding to upregulated miRNA target genes accumulated in the wild type, *ago1*, and *dcl1-9* as well as in *dcl3*, *rdr2*, and *rdr6*. *DCL3* encodes a homolog of Dicer required for the production of siRNA from endogenous targets of RNAi-mediated silencing, such as *Arabidopsis* SINE At *SN1*; *RDR2* encodes an RdRP that functions in the same pathway. Neither DCL3 nor RDR2 is required for miRNA processing (Xie et al., 2004). *RDR6* also encodes an RdRP, which is required for transgene RNAi (Dalmay et al., 2000; Mourrain et al., 2000), the systemic spread of the silencing signal (Himber et al., 2003), and the production of ta-siRNAs (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). Microarray analysis indicates that the levels of sense mRNA expression of *SPL10*, *HAP2C*, and At1g62670 do not change detectably in the *dcl3*, *rdr2*, and *rdr6* backgrounds (Allen et al., 2005).

Using sense strand probes 5′ of the miRNA-homologous sites of At1g62670, *HAP2C*, and *SPL10*, we were able to detect putative siRNA of 21 to 22 nucleotides as well as additional size classes in wild-type RNA from both Columbia (Col) and L*er* (Figures 4A to 4C). These siRNAs were virtually absent in *dcl1-9* and *ago1-9* and were significantly reduced in amount in *ago1-11*. The siRNAs were also absent in *dcl3*, *rdr2*, and *rdr6*, implicating at least one additional Dicer and two RdRPs in their production and indicating that other siRNA pathways may be linked to the action of miRNA in vivo. Additional fragments of higher molecular weight, typically 44 to 45 and 55 to 60 nucleotides, were also consistently present, and their abundance correlated with the presence of the 21- to 22-nucleotide species. Some heterogeneity was also apparent within the 20- to 30-nucleotide size class, both between strains and in the overall distribution of

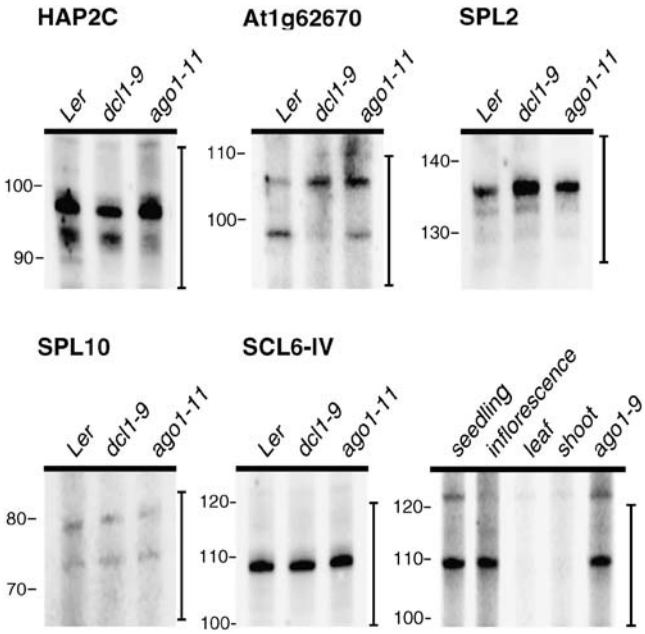

Figure 3. Primer Extension Analysis of miRNA Target Gene 3′ Cleavage Products.

Primers correspond to unique regions of genes 70 to 120 nucleotides downstream of the miRNA-homologous sites. RNAs were from 21-d wild type (L*er*), *ago1-11*, or *dcl1-9*; for *SCL6-IV* (bottom right panel), RNAs from 11-d seedlings as well as leaf, shoot, and inflorescence tissues from 25-d wild-type (L*er*) and *ago1-9* plants were also used. Vertical bars (at right) indicate the positions of miRNA-homologous regions of targets.

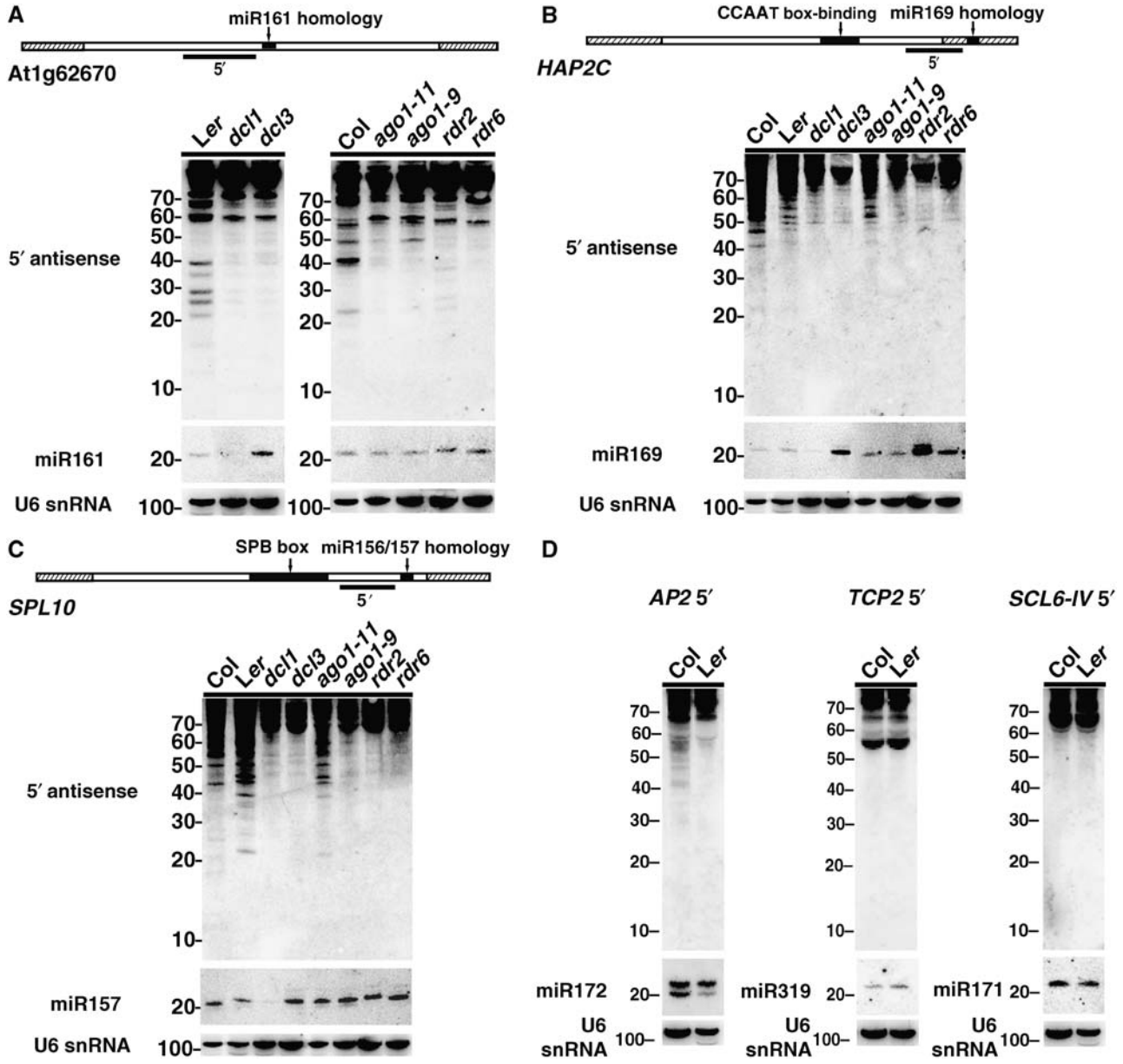

Figure 4. Small RNA Profiles of Genes Upregulated in *ago1* and *dcl1*.

Small antisense RNAs in the wild type (Col/L*er*), *dcl1*, *dcl3*, *ago1-11*, *ago1-9*, *rdr2*, and *rdr6* at 21 d were detected by 5′ sense probes for At1g62670 **(A)**, *HAP2C* **(B)**, and *SPL10* **(C)**. No small antisense RNAs in the 21- to 26-nucleotide size class corresponding to the miRNA target genes *AP2*, *TCP2*, or *SCL6-IV* (all in **[D]**) were detected by 5′ sense probes in either Col or L*er* wild-type RNA. Each lane is loaded with 20 to 25 μg of polyethylene glycol–precipitated total RNA; the positions of probes in **(A)** to **(C)** are indicated above each set of panels. Hatched boxes at the ends of each gene represent 5′ and 3′ UTRs. The top panels show antisense siRNA products upstream of the miRNA-homologous sites detected by 5′ sense probes; the middle panels show the same blots probed with sense strand oligonucleotides corresponding to the cognate miRNA for each gene. The bottom panels represent each blot probed with a U6 small nuclear (snRNA)-specific oligonucleotide as a loading control.

sizes; this was most prominent with At1g62670, which had at least five visible bands within this range in L*er* and only a single band of ~21 nucleotides in Col. Faint bands of 21 to 22 nucleotides were also visible with 5′ antisense strand probes corresponding to *HAP2C* and *SPL10* but not At1g62670; higher molecular weight bands of >40 nucleotides were observed for all three genes (data not shown).

The small RNA gel blots were also hybridized with probes corresponding to the miRNAs that target each class of genes: miR157, miR161, and miR169 (Figures 4A to 4C, middle panels)

and with a probe that detects the U6 snRNA as a loading control (Figures 4A to 4C, bottom panels). As expected, levels of all three miRNAs were significantly reduced in *dcl1-9*. Expression of miR157 was equivalent in all other genotypes. For miR161 and miR169, levels of expression were similar to wild-type levels in both *ago1* alleles but were increased in *dcl3*, *rdr2*, and *rdr6*. It is possible that these mutants are defective in a coupled miRNA–siRNA degradation mechanism leading to the overaccumulation of miRNA. Increases in miR165/166 have been reported previously in *rdr6*, which enhances the phenotype of *asymmetric leaves1* (Li et al., 2005). However, in this case, enhancement depends on the *TAS3* target gene *ETTIN*/*ARF3* and not on miR165 target genes (Garcia et al., 2006). Increases in miR165/166 may be an indirect consequence of the loss of leaf polarity, as they are in *ago1-9* (Kidner and Martienssen, 2004), or attributable to targeting of the miR166b precursor by *TAS3* (Garcia et al., 2006).

To determine whether the presence of putative upstream siRNAs was a general characteristic of miRNA-targeted genes, we also probed small RNA gel blots with sense strand probes 5′ to the cleavage sites of the miRNA target genes *APETALA2*, *SCL6-IV*, and *TCP2* (Figure 4D, top panels). None of these genes changed significantly in expression in *ago1* or *dcl1* (Table 1). All three genes are expressed at levels comparable to those seen for *SPL10*, *HAP2C*, and At1g62670 and are experimentally validated miRNA targets that undergo mRNA cleavage, which can be abolished by mutations within the miRNA-homologous site (Llave et al., 2002; Aukerman and Sakai, 2003; Palatnik et al., 2003; Chen, 2004). We did not detect any 21- to 26-nucleotide small RNA species in either the Col or L*er* wild type corresponding to any of these genes, although we did observe minor accumulation of a 40- to 45-nucleotide band in *AP2* in the Col background. The cognate miRNAs for all three genes were detected on the same blots at moderate to high levels (Figure 4D, middle panels), as was the U6 snRNA (Figure 4D, bottom panels).

We assessed the small RNA profiles of genes that showed increased expression in *ago1* and *dcl1* by comparing our expression data with the MPSS database (http://mpss.udel.edu/at/) of *Arabidopsis* small RNAs (see Supplemental Table 3 online), which contains >75,000 nonredundant sequences from 2-week-old seedlings and 5-week-old inflorescences (Lu et al., 2005). We found that many of the miRNA target genes with significant increases in expression in *ago1* had additional small RNA complementarities in seedling libraries (Table 1). The PPR repeat gene At1g62670 had multiple hits throughout its coding region both 5′ and 3′ of its miRNA complementarity, whereas *HAP2C* also had several matches centered on its miRNA complementarity that appear to be sequence variants of miR169 possessing perfect complementarity to the target mRNA. Other miRNA targets with small RNA matches (other than miRNA hits) included *TIR1* and *AGO1* itself, which were upregulated in *ago1-11* at 9 d. Of all significantly upregulated

Table 1. Genes Upregulated in *ago1* at 9 d with Matches in the MPSS Database

Feature	Locus Identifier	Fold Change	Abundance	Count	Context
ago1-9 (9 d)					
HAP2C	At1g72830	33.2	58	1	miRNA sequence variant
Plantacyanin	At2g02850	21.0	121	2	Predicted miRNA target
Hydroperoxide lyase (HPL1)	At4g15440	9.3	56	7	Intronic tandem repeats
PPR repeat	At1g62670	5.5	111	17	miRNA target; many dispersed small RNA hits
Gly hydroxymethyltransferase	At4g13930	2.7	6	2	
HARBINGER	At4g10890	2.7	2	1	Transposon
60S ribosomal protein L34	At1g26880	2.7	2	1	
Putative β-galactosidase	At4g36360	2.5	2	1	Intronic tandem repeats
60S ribosomal protein L11	At4g18730	2.4	3	1	
60S ribosomal protein L31	At4g26230	2.3	2	1	
6,7-Dimethyl-8-ribityllumazine synthase	At2g44050	2.1	2	1	
Expressed protein	At3g52500	2.0	4	2	3′ UTR/downstream tandem repeats (ATHPOGON13)
RNA binding protein (cp31)	At4g24770	1.9	4	2	VANDAL21 insertion in first exon
Lipid transfer/seed storage protein	At2g13820	1.8	91	19	Adjacent to GYPSY retroelement, intronic repeats
Kelch repeat protein	At1g54040	1.8	125	16	Upstream/intronic tandem repeats
Transferase family protein	At3g48720	1.7	31	7	Intronic AtMu10 homology
Acyltransferase-like protein	At1g11860	1.7	5	2	
40S ribosomal protein S3A	At4g34670	1.6	11	2	
Ketol-acid reductoisomerase	At3g58610	1.6	3	1	
Sugar transporter	At1g20840	1.5	12	1	Possible miRNA target
Putative glycosyltransferase	At4g02500	1.5	3	2	Possible miRNA target
ago1-11 (9 d)					
Transport inhibitor response 1 (*TIR1*)	At3g62980	2.1	93	4	miRNA target; several dispersed small RNA hits
AGO1	At1g48410	1.9	28	4	miRNA target; several dispersed small RNA hits
Plastocyanin	At1g76100	1.6	3	1	

miRNA targets in either *ago1* allele, only the *SPL* genes did not show any evidence of additional small RNAs in the MPSS collection.

Antisense RNAs Corresponding to Upregulated miRNA Targets

siRNA is derived from dsRNA by Dicer-mediated cleavage, so we performed semiquantitative strand-specific RT-PCR to determine whether antisense RNAs accumulated. Primers were designed from unique regions of At1g62670, *HAP2C*, and *SPL10* upstream (5′) and downstream (3′) of their miRNA-complementary sites. For upstream sequences, levels of sense-specific RT-PCR products reflected the microarray and RNA gel blot data; expression was lowest in the wild type and increased in both *ago1* and *dcl1* (Figure 5). Antisense RNAs within the same regions were also observed, and their accumulation mirrored that of sense RNAs at both 9 and 21 d. Antisense expression was highest in *ago1-9* and was much lower but detectable in the wild type. Sense and antisense RT-PCR products were also observed downstream of the miRNA-complementary sites of At1g62670, *HAP2C*, and *SPL10*. Downstream antisense products accumulated to a greater degree in *ago1* and *dcl1*, but at 21 d the levels of downstream sense products from At1g62670 and *SPL10* were unchanged in *ago1* and *dcl1*, indicating that 3′ cleavage products were stable in the wild type. We used α-tubulin (*TUA3*) as an amplification control (Figure 5D); no detectable antisense RNA was seen at comparable levels.

We considered the possibility that antisense RNA was derived by transcription of the noncoding DNA strand at the SPL10 and HAP2C loci. The 5′ RT-PCR primers spanned introns for *SPL10* and *HAP2C*; cDNA corresponding to spliced mRNA was amplified from both sense and antisense RNA, indicating that antisense RNA was derived from sense RNA rather than from genomic DNA templates (Figure 5). A second *SPL10* cDNA of higher molecular weight was observed with a size corresponding to that of unspliced message. In this case, transcription in the reverse direction could be accounted for by the presence of an inverted tail-to-tail duplication with *SPL11*.

We next investigated which RdRP might be responsible for antisense production. Levels of 5′ and 3′ antisense RNAs for At1g62670, *HAP2C*, and *SPL10* were much lower than for the sense strand, ranging from ~10-fold less in *HAP2C* to nearly 100-fold less in At1g62670 (Figure 5). Therefore, it was difficult to determine whether these low levels were further reduced in *rdr2* and *rdr6* (data not shown). Instead, we examined the accumulation of antisense RNA from the superoxide dismutase gene *CSD1*, which is highly expressed in wild-type cells and upregulated significantly (four- to fivefold) at 21 d in *ago1-9* and *dcl1-9* (Figure 2). The miR398 recognition site is close to the start of transcription, so that transcripts could only be examined downstream (Figure 6). Sense transcripts were upregulated in agreement with the microarray data. Antisense transcripts could be readily detected in wild-type plants but were absent from *rdr6* and *ago1-9*. Therefore, antisense transcripts in this case depended on cleavage by AGO1 and RdRP. Antisense transcripts corresponding to *SCL6-IV* were also detected, both upstream and downstream of the miR171 target site. Once again, downstream antisense transcripts were absent in *rdr6*, but upstream antisense transcripts were retained (data not shown).

Transposons and Repeats Regulated by Ago1

A number of target genes with no known miRNA complementarities were upregulated in *ago1* and also had multiple small RNA signatures in the MPSS database (see Supplemental Table 2 online). The increases in expression were much lower than those seen for upregulated miRNA targets. We further examined the mRNA and small RNA expression of several of these genes and their intronic repeats (*HPL1*/At4g15440 and At2g13820, a lipid transfer protein) as well as a TE (At1g08740/*VANDAL14*) that was upregulated in both alleles of *ago1*. In agreement with the microarray data, *HPL1* and At2g13820 increased in *ago1-9* relative to the wild type at 21 d, whereas *VANDAL14* was silent in wild-type plants and expressed in *ago1-9* (see Supplemental Figure 1 online). The MPSS signatures from *HPL1* and At2g13820 were localized almost exclusively to intronic repeats. We verified that 24- to 28-nucleotide small RNAs were produced from these repeats, as well as from *VANDAL14*, but levels were increased rather than reduced in *ago1-9* (see Supplemental Figure 1 online), along with several larger RNAs of 35 to 45 nucleotides. We also examined the DNA methylation of the intronic repeats and the *VANDAL14* element by methylation-specific PCR (Lippman et al., 2003). All three sequences were heavily methylated, but the overall level of DNA methylation did not change significantly in *ago1-9* (data not shown).

DISCUSSION

Mutants in *AGO1* and *DCL1* have increased gene expression, consistent with general roles in repressing gene expression. Many of the changes reflect secondary consequences of a loss of RNAi, but we have been able to identify candidates for direct targets by combining results from severe and moderate *ago1* alleles with the Dicer mutant *dcl1-9* as well as by searching for matches with small RNAs. There were fewer changes in *ago1-11* than in *ago1-9*, consistent with its less severe phenotype. These differences may reflect the loss of the PAZ domain, which is present in *ago1-11* but not in *ago1-9* (Kidner and Martienssen, 2004). More genes were upregulated in *dcl1-9* than in *ago1-11*, but with generally lower fold changes, despite their similar phenotypes. miRNAs are ideally suited for regulating development in the highly redundant polyploid genomes of plants because a single miRNA can regulate entire gene families. Nearly all of the genes that showed upregulation in *ago1* and *dcl1* are members of multigene families, and of these, highly similar members within each gene family generally showed coordinate changes in expression. Unfortunately, very little is known of the functions of the miRNA-targeted gene families, such as the *SPL* genes and the CCAAT box B transcription factors that are upregulated in *ago1* and *dcl1*, although some *SPL*s are upregulated at the vegetative-to-floral transition (Schmid et al., 2003) and may promote flowering (Cardon et al., 1999).

Although TEs were relatively unaffected in *ago1* and *dcl1*, a small number of TEs did show increased expression, consistent with previous observations that AGO1 is required to silence a

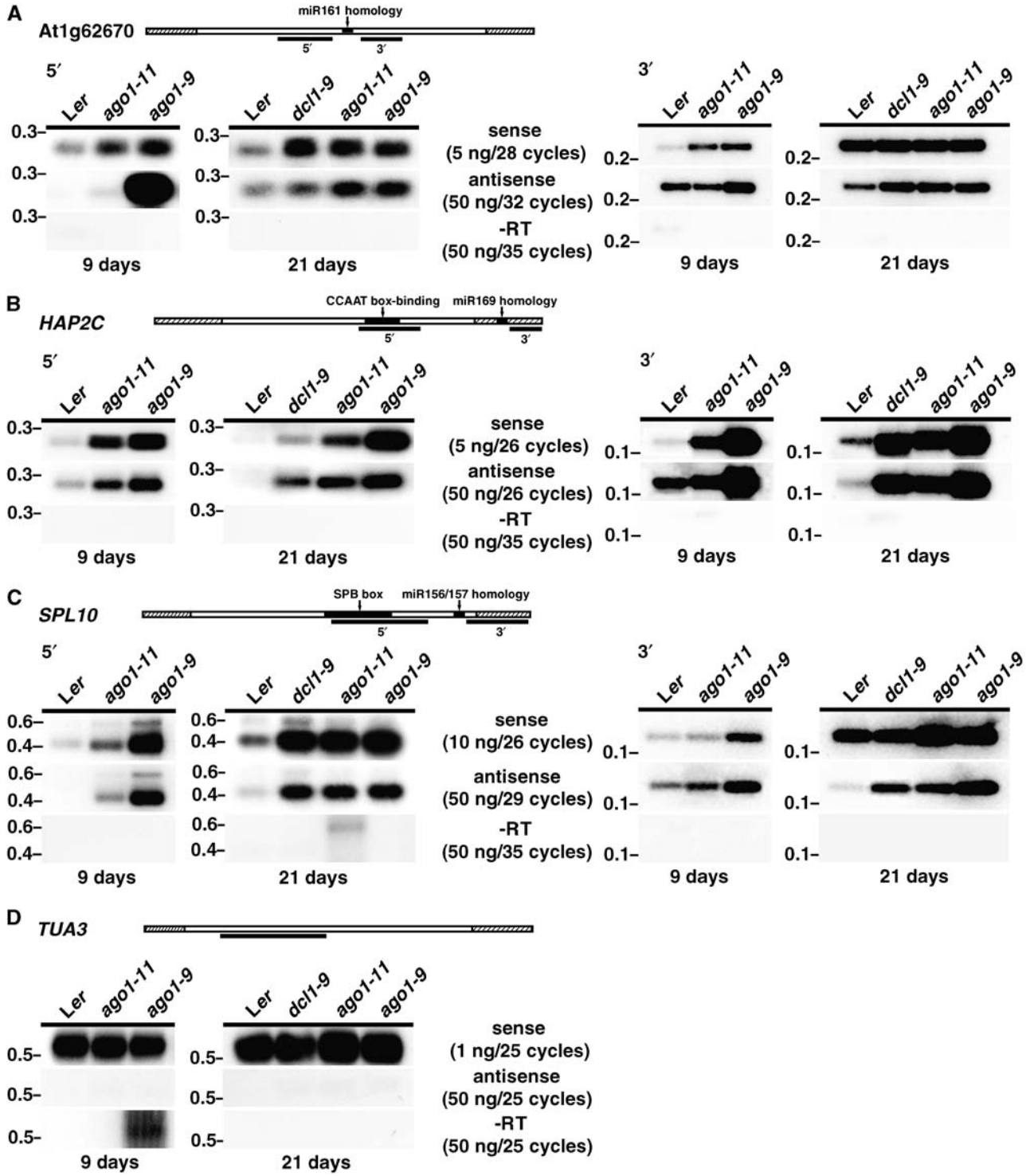

Figure 5. Sense and Antisense Expression of Genes Upregulated in *ago1* and *dcl1*.

RNAs from the wild type (L*er*), *ago1-9*, and *ago1-11* at 9 and 21 d and from *dcl1-9* at 21 d were reverse-transcribed with strand-specific primers corresponding to At1g62670 **(A)**, *HAP2C* **(B)**, *SPL10* **(C)**, and *TUA3* **(D)**, then amplified by PCR. RT-PCR conditions ranged from 1 to 50 ng of total RNA and 25 to 32 cycles; representative panels of approximately equal hybridization intensity are shown. The top panels correspond to sense products; the middle panels refer to antisense products; and the bottom panels are controls lacking reverse transcription. All sizes are indicated in kilobases.

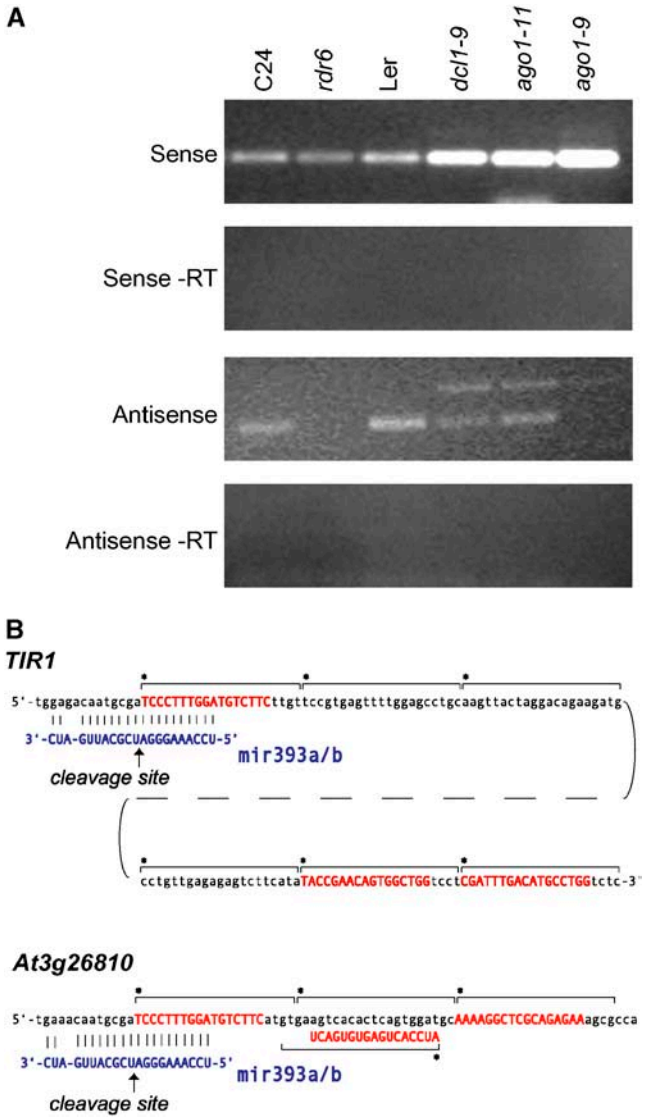

Figure 6. Antisense Production Downstream of miRNA Complementarity Leads to Secondary siRNAs.

(A) Strand-specific RT-PCR of *CSD1*, 3′ of the miRNA recognition site. The *CSD1* sense strand is upregulated in *dcl1-9*, *ago1-11*, and *ago1-9*. The *CSD1* antisense strand is lost in *rdr6* and *ago1-9* but not in *ago1-10* or *dcl1-9*.

(B) In-register phasing of siRNA from *TIR1* and one of its homologs, At3g26810. Predicted miRNA cleavage sites (Jones-Rhoades and Bartel, 2004) are in the 21-nucleotide register, with the 17-nucleotide MPSS signatures from siRNA corresponding to each gene (red). miR393 is shown in blue.

handful of transposons (Lippman et al., 2003). We also observed increased expression of a number of non-miRNA target genes containing intronic repeats in *ago1-9*. These *ago1*-induced changes in expression resemble the previously documented effects of mutations in *AGO1* on a transgene undergoing PTGS (Morel et al., 2002). However, the levels of putative siRNAs corresponding to these TEs and repeats increased rather than decreased in *ago1-9*. These small RNAs represent the 24- to 26-nucleotide class of heterochromatic siRNAs (Hamilton et al., 2002), which may not interact with AGO1 (Baumberger and Baulcombe, 2005). It is possible, therefore, that AGO1 regulates these genes posttranscriptionally, resulting in increased levels of transcript and 24-nucleotide siRNAs generated by other means. The presence of repeated sequences in unspliced ESTs derived from these genes (such as gi|42530564|gb|BX836481.1 from At2g13820) could provide a substrate for this PTGS as they match siRNAs. *VANDAL14* is a member of a small, young TE family with six members possessing intact open reading frames and >99% sequence identity dispersed throughout the *Arabidopsis* genome. AGO1 might silence young families of TEs posttranscriptionally through its slicer activity. The transcriptional silencing machinery could then act to maintain a more repressive chromatin state (Lippman et al., 2003).

AGO1 Regulates a Subset of miRNA Target Genes

The upregulation of miRNA targets that we observed in *ago1* is further evidence that AGO1 is involved in miRNA function (Boutet et al., 2003; Kidner and Martienssen, 2004; Vaucheret et al., 2004). But many miRNA target genes, including *SCL6-IV*, *TCP2*, and *AP2*, are not upregulated, in agreement with other microarray analyses involving *dcl1*, *hen1*, and *hyl1* (Peragine et al., 2004; Allen et al., 2005). These studies contrast somewhat with other reported findings of miRNA target gene expression in the *ago1* background (Vaucheret et al., 2004), in which large increases in the expression of 10 different miRNA target genes were detected in the null allele *ago1-3*, as well as decreased abundance of some but not all miRNAs. This discrepancy most likely results from the use of more mature plants from the Col background grown under short-day conditions (Vaucheret et al., 2004), whereas we used plants from the L*er* background at much earlier stages of development grown under long-day conditions. It is also possible that the differences reflect pleiotropic consequences of strong *ago1* alleles, in which later development is severely disrupted, especially in the inflorescence, which may lead to a general decrease in miRNA levels. However, both data sets indicate that miR157 and miR161 do not change very much even in null alleles of *ago1*, whereas their target genes show large increases in expression. Conversely, miR171 is much lower in *ago1* than in the wild type (see Supplemental Figure 1 online), yet we observed no change in expression of the homologous miRNA target genes *SCL6-II* and *SCL6-IV*. *SCL6-III* expression has been reported to increase under short-day conditions (Vaucheret et al., 2004), but expression of the *SCL6* genes (including *SCL6-III*) does not change greatly in other miRNA-deficient backgrounds (Allen et al., 2005).

AGO1 has mRNA slicer activity (Baumberger and Baulcombe, 2005; Qi et al., 2005), and although we did not detect changes in cleavage products from At1g62670, *HAP2C*, *SPL2*, and *SPL10* in *ago1-11* and *dcl1-9*, uncleaved mRNA levels were substantially increased on RNA gel blots. These data indicate that the rate of mRNA cleavage is greatly reduced in both *ago1* and *dcl1*, although some slicing activity clearly remains. Both *dcl1-9* and *ago1-11* are weak alleles; in *ago1-11*, a splice-site mutation is

predicted to result in a mutant AGO1 protein that retains the PAZ domain but is missing 20 amino acids from the PIWI domain, including the second of two Asp residues considered essential for slicer activity (Liu et al., 2004; Song et al., 2004), although a small amount of normally spliced AGO1 mRNA can still be detected by RT-PCR (Kidner and Martienssen, 2004). But loss of slicing alone is unlikely to account for the larger changes in expression of some miRNA targets seen in strong *ago1-9*, in which abundant *SCL6-IV* 3′ cleavage products can still be detected (Figure 3) despite the severe reduction of miR171 (see Supplemental Figure 1 online). This finding indicates that additional slicing activity exists in *Arabidopsis*, and as the Argonaute homolog most similar to AGO1, PNH represents a likely candidate for such an activity (Kidner and Martienssen, 2004); the phenotype conferred by *ago1* is strongly enhanced in the *pnh* background (Lynn et al., 1999), although it is not known whether miRNA target genes are misregulated.

The presence of stable 3′ cleavage products (Llave et al., 2002; Kasschau et al., 2003; Palatnik et al., 2003) may account for the unchanged levels of total mRNA detected by microarray analysis; for example, the probe sets representing *SCL6-IV* are downstream of the cleavage site and would detect stable 3′ cleavage products. Mutations in At *XRN4*, an *Arabidopsis* homolog of the yeast mRNA-degrading exonuclease Xrn1p, result in stabilization of the 3′ cleavage products of specific miRNA target genes such as *SCL6-II* as well as targets of miR159 and miR160 (Souret et al., 2004). *SPL2*, *SPL9*, and *SPL10* had probe sets located both 5′ and 3′ of the miRNA-homologous sites and showed significant changes of similar magnitude in multiple probe sets. But the level of *SPL10* 3′ cleavage product is unaffected in the *xrn4* background, indicating that this specificity does not account for differential regulation (Souret et al., 2004). Another explanation for the differential regulation lies in the expression of miRNA itself. In genes such as *PHB*, miR165 is restricted to only a few cell types and cleavage may have little effect on total mRNA levels from mixed tissues (Kidner and Martienssen, 2004). If the *SPL* genes are normally expressed in the inflorescence but silenced in vegetative tissues, this could account for their more dramatic upregulation in *ago1* mutant seedlings. But this argument is not completely satisfactory either, as many floral target genes (such as *AP2*) are only weakly upregulated in mutant seedlings.

miRNAs Can Trigger the Production of Secondary siRNAs

In plants, all miRNA interactions with target mRNAs appear to result in cleavage within the miRNA-homologous site of the mRNA (Llave et al., 2002). At least three subsequent (and potentially overlapping) downstream consequences of the miRNA–mRNA interaction are thought to exist: posttranscriptional decrease in overall levels of mRNA; decreased protein levels as a result of translational inhibition (Aukerman and Sakai, 2003; Chen, 2004); and RdRP-directed second-strand synthesis that generates ta-siRNAs through multiple Dicers (DCL1 and DCL4), which in turn target other mRNAs for miRNA-like cleavage (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). Similarly, the silencing of reporter genes fused to a miRNA target site has also been shown to involve the production of secondary siRNAs (Parizotto et al., 2004). More recently, it has been shown that the coexpression of naturally occurring antisense RNAs can also lead to the production of siRNAs through a process that also involves multiple Dicers, in this case DCL1 and DCL2 (Borsani et al., 2005).

Our data most clearly support a model in which miRNA-mediated cleavage activity and secondary siRNA production are required to regulate the expression of some, but not all, miRNA target genes by AGO1 and DCL1. From a mechanistic standpoint, this resembles the production of ta-siRNAs: after miRNA-directed slicing of the target mRNA, RdRP activity synthesizes a complementary antisense strand (Figure 7). Synthesis upstream of the miRNA site could potentially use the miRNA as a primer, but synthesis downstream is thought to follow the loss of the 5′ cap by cleavage and requires RDR6 (Gazzani et al., 2004). Downstream antisense strand was detected in each of the genes examined, including *SCL6-IV*, and depended, at least in part, on RDR6. Upstream antisense strand did not depend on RDR6 but instead may depend on the miRNA itself. Unlike most miRNAs, miR157, miR161, and miR171 all match their targets perfectly at the 3′ end and could potentially be extended by polymerase activity. Only one isoform of miR169 can be so extended, and the levels of antisense transcript were much lower for *HAP2C*. Antisense RNA upstream of the miRNA target site does not depend on RDR2 or RDR6 and may use a different polymerase altogether. In fact, ~30% of *Arabidopsis* genes have existing antisense RNA, regardless of the presence of miRNA (Yamada et al., 2003). Many of these antisense transcripts correspond only to exons, and so presumably use sense transcripts as a template, but their synthesis is unaffected in *rdr2* and *rdr6* (M.W. Vaughn and R.A. Martienssen, unpublished data). These long antisense transcripts could also account for second-strand synthesis downstream of the miRNA site (Figure 7), as has been observed in transgene systems (Voinnet et al., 1998; Vaistij et al., 2002).

Dicer activity presumably processes this dsRNA into siRNAs, which can in turn target additional mRNA molecules, even those lacking homology with the initiating miRNA. Additional rounds of siRNA targeting, RdRP-mediated second-strand synthesis, and Dicer activity might then serve to amplify the initial signal. This would account for the loss of siRNA in *rdr2* and *rdr6*, even though antisense RNA was still present in these mutant strains. In this model, AGO1 could act in the miRISC and facilitate the formation of downstream siRISCs (using other AGO proteins), as AGO1 is associated with both miRNAs and siRNAs (Baumberger and Baulcombe, 2005). As with AGO1, DCL1 could act at multiple steps in this coupled miRNA–siRNA mechanism: first to process miRNA precursors, then to generate 21-nucleotide siRNAs from dsRNA produced by RdRP activity. dsRNA dicing activity has been described for DCL1 both in vitro (Qi et al., 2005) and in vivo (Borsani et al., 2005).

In addition to 21- to 22-nucleotide siRNAs, we detected multiple species of small RNAs, ranging from 30 to 65 nucleotides in length. Although their origin is unclear, they could be aborted products of RdRP. Alternatively, slicing guided by adjacent, in-register siRNAs might be expected to generate specific fragments in this size range. Cleavage by siRNAs adjacent to the miRNA site might also be responsible for longer cleavage

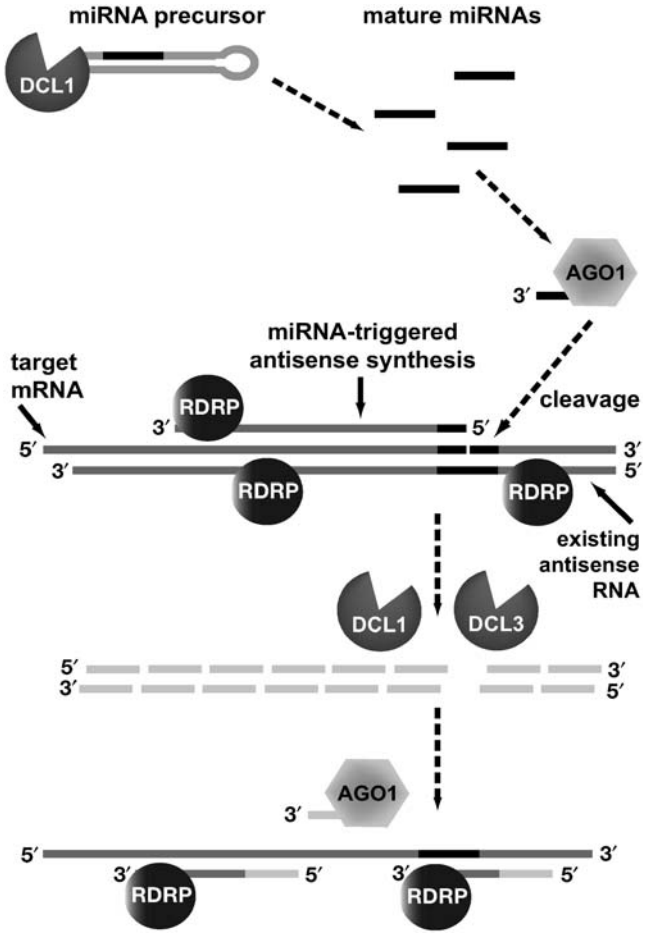

Figure 7. DCL1 and AGO1 in miRNA Action.

DCL1 is required for the processing of miRNA precursors. Processed miRNAs (in black) then target homologous mRNAs (dark gray), which are cleaved within the miRNA-homologous site (shown in black). RdRP activity is then recruited to the miRNA/mRNA duplex and/or cleavage site; the RdRP activity transcribes the mRNA upstream into antisense RNA. After production of dsRNA and the miRNA-mediated triggering of RNAi, AGO1 recruits Dicer activity to the duplex. Twenty-one- to 22-nucleotide siRNAs (light gray) processed from dsRNA by DCL1 accumulate in wild-type plants, as do larger products, which may result from incomplete processing, abortive RdRP activity, slicing guided by in-register siRNA, or the action of a Dicer variant. The siRNA may then target additional sense or antisense RNA molecules, through AGO1, RDR2, RDR6, and DCL3, which may be required to produce a specialized class of siRNA that serves as a signal to initiate the production of 21- to 22-nucleotide siRNAs by DCL1 (Borsani et al., 2005). Antisense RNA downstream of the miRNA-complementary sites can arise through the action of RDR6 on cleaved (uncapped) mRNA, but antisense RNA is not restricted to miRNA target genes and requires other polymerases in most cases (M.W. Vaughn and R.A. Martienssen, unpublished data).

products detected by primer extension. We examined MPSS siRNA signatures from *PPR* repeat genes, and from *AGO1* and *TIR1*, for evidence that they were generated in register with miRNA cleavage sites, by analogy with ta-siRNAs (Allen et al., 2005). Indeed, siRNAs from *TIR1* and its homologs were in the 21-nucleotide register with the upstream miRNA cleavage site predicted by the sequence of miR393 (Figure 6; see Supplemental Figure 1 online). There was no such bias for MPSS siRNAs corresponding to *PPR* repeat genes, possibly because they are members of gene families or because they are targeted by two or three different small RNAs (miR161, miR400, and *TAS2*). MPSS siRNA signatures for the ta-siRNA loci *TAS1*, *TAS2*, and *TAS3* were not perfectly in-register either, presumably because the MPSS collection includes multiple size classes of siRNAs and not just 21-nucleotide ta-siRNAs.

Digestion of dsRNAs into siRNAs may account for the low levels of antisense transcripts in wild-type cells and for their increase in *ago1* and *dcl1*. The siRNAs we observed corresponding to *SPL10*, *HAP2C*, and At1g62670 are lost in *ago1* and *dcl1*, as well as in *dcl3*, *rdr2*, and *rdr6*, indicating that at least two RdRPs and one additional Dicer are required for their production. But why would multiple Dicers and RdRPs be required? It may be that RDR2 and RDR6 carry out different steps in the production of siRNAs, such as the initial synthesis of antisense RNA, the siRNA-mediated amplification of dsRNA, and the systemic spread of the silencing signal (Himber et al., 2003). Both *dcl3* and *dcl4* enhance the phenotype conferred by *dcl1*, and DCL2, DCL3, and DCL4 are thought to have partial redundancy in the production of multiple classes of siRNAs (Gasciolli et al., 2005; Xie et al., 2005). DCL4 produces the 21-nucleotide siRNA signal responsible for cell-to-cell transmission of some instances of RNAi (Dunoyer et al., 2005), but it also cooperates with DCL1 in mediating the creation and activity of natural antisense transcript siRNAs (Gasciolli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005). DCL2, generally thought to be responsible for viral siRNA production, also acts coordinately with DCL1 in generating nat-siRNAs, possibly by the processing of a 24-nucleotide species that serves to trigger the downstream accumulation of 21-nucleotide siRNAs (Borsani et al., 2005).

In *dcl3*, *rdr2*, and *rdr6*, the loss of siRNA does not alter the expression of miRNA-targeted genes seen in *ago1* and *dcl1* (Allen et al., 2005), and their developmental phenotypes are much weaker (Peragine et al., 2004; Xie et al., 2004). This indicates that the siRNA pathway acts downstream of miRNA-mediated cleavage and does not alter gene expression significantly by itself. A coupled miRNA/siRNA mechanism might prove advantageous in regulating target genes in tissues in which the miRNA is not expressed, or it might allow miRNA action to be amplified under conditions in which a limiting amount of miRNA is present.

Specificity of Secondary siRNA Production

Approximately 30% of annotated *Arabidopsis* genes have significant levels of antisense RNA (Yamada et al., 2003), but the majority of these genes do not possess small RNA signatures in the MPSS database (Lu et al., 2005), indicating that some type of trigger is necessary to induce RNAi. Even *HAP2C* and *SPL10*, two genes that have homologous siRNAs on blot analyses, do not have matches in the MPSS collection. Fifteen known miRNAs were also missing from the MPSS collection (Lu et al., 2005), and this may reflect low abundance in the sampled tissues: secondary siRNAs corresponding to At1g62670 were easily detected on

blots and readily apparent in the MPSS collection, whereas those specific to *SPL10* and *HAP2C* were much more difficult to detect even with greater amounts of RNA.

Our findings are consistent with a scenario in which AGO1 and miRNAs trigger the entry of homologous mRNAs into the siRNA pathway, but it remains unclear what cues are required. One possibility is that modifications of miRNA, such as methylation of the 3′ end by HEN1, may favor a specific pathway. Alternatively, nearly all plant miRNAs contain highly conserved mismatches relative to their targets, the basis of which remains uncertain, but these could also influence entry into the siRNA pathway. Point mutations in the miRNA target sites of miR165/166 targets such as *PHV* and *PHB* lead to dominant phenotypes (McConnell et al., 2001; Kidner and Martienssen, 2004), as does the ectopic expression of miRNA-resistant transgenes (Palatnik et al., 2003). It is reasonable to expect that miRNA-resistant mutant alleles of genes or transgenes that are also siRNA-regulated might have more limited phenotypic effects because of several potential factors: an intact siRNA pathway; multiple homologs with intact miRNA sites; and, with transgenes, the presence of wild-type copies of the gene still subject to miRNA-based regulation. The prospect of miRNA-mediated epigenetic silencing also should not be ignored. We did not observe changes in DNA methylation corresponding to the target genes examined here (Bao et al., 2004; Ronemus and Martienssen, 2005), but secondary siRNAs promote their own production (Figure 7), and once they accumulate, they could lead to targeting in subsequent generations.

METHODS

Plant Growth and RNA Extraction

Arabidopsis thaliana seeds were sown on Murashige and Skoog medium (Invitrogen), stratified for 72 h at 4°C, and then placed at 21°C under long-day (16 h of light) conditions. Plants were harvested at 9 and 21 d as indicated; *dcl1/dcl1* and +/+ individuals were identified by PCR genotyping of the T-DNA insertion (Jacobsen et al., 1999). RNA was extracted with the Trizol reagent (Invitrogen). Pools of 8 to 12 individual seedlings were prepared separately for each replicate in the microarray analysis. Pooled RNA populations from remaining seedlings were used in all other experiments. We obtained *dcl3-1* and *rdr2-1* lines from James Carrington (Xie et al., 2004). *rdr6-16* is in the Landsberg background (Garcia et al., 2006).

Microarray Hybridization

Hybridizations to the *Arabidopsis* AtGenome1 GeneChip (Affymetrix) were done as described in the GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, we used 15 to 25 μg of total RNA per replicate to prepare cDNAs using the SuperScript cDNA synthesis kit (Invitrogen). The cDNAs served as templates for biotinylated copy RNA synthesis with the BioArray kit (Enzo Diagnostics). We hybridized three to four biological replicates from each strain at 9 and 21 d.

Microarray Data Analysis

Expression levels of individual features on the AtGenome1 microarray were assessed using MAS 5.0 (Affymetrix). Unscaled data files were then imported into GeneSpring 5.1 (Silicon Genetics) and normalized using raw signal values against banks of positive and negative controls (see Supplemental Table 5 online). The positive control set consisted of a panel of 42 expressed features that varied by ≤15% in expression between wild-type and mutant lines across a large number of wild-type and mutant data sets, normalized by scaling of median expression levels (on a per chip basis). The negative controls were a set of antisense features present on the AtGenome1 array. All expression values were divided by the median of the positive controls within that sample; background was subtracted from each raw value based on the median value of the negative controls within that sample. Normalized expression values of ≤0.0 were artificially set to a positive value representing 1% of the median expression level of the positive controls. Significance was established by one-way analysis of variance ($P \leq 0.01$). Fold change was calculated by the ratio of normalized and/or corrected mutant to wild-type expression values. A gene was considered expressed if its average expression met or exceeded a threshold of 10% of the median of the positive controls. Microarray data from these experiments are available via Gene Expression Omnibus (GEO) accession number GSE4684.

Gene Classification

Features on the microarray were classified based on their Gene Ontology Consortium (GO) database entries (http//:www.geneontology.org/). For genes with multiple GO entries, we cross-referenced with The Institute for Genomic Research *Arabidopsis* Genome Annotation Database (http://www.tigr.org/tdb/e2k1/ath1/ath1.shtml) as well as our own annotation. We classified a feature as unknown only if it returned a null query from the GO database.

RNA Expression Analyses

Analyses were performed using standard procedures (Sambrook and Rusell, 2001) with exceptions as noted. RNA gel blots and hybridizations were performed using the NorthernMax-Gly kit (Ambion). Semiquantitative RT-PCR was performed using the Sensiscript RT kit (Qiagen) with concentrations of total RNA ranging from 1 to 50 ng. First-strand synthesis was terminated by 15 min of incubation at 95°C, followed by 25 to 35 cycles of PCR; products were then blotted and probed. Small RNA gel blots were analyzed according to published methods (Hamilton and Baulcombe, 1999); total RNAs were precipitated with 5% polyethylene glycol (Sigma-Aldrich) to concentrate the small RNAs (<200 nucleotides). All hybridizations were performed using [α-^{32}P]UTP-labeled strand-specific riboprobes prepared with the MAXIscript T3/T7 kit (Ambion).

Primer Extension

We performed primer extension assays using standard procedures (Newman, 1987), modified as follows: annealing reactions were heated to 80°C for 5 min, then cooled to 48°C at –0.1°C/s. After a 1-h incubation at 48°C, reverse transcription was done at 42°C for 1 h and samples were analyzed by 8% PAGE.

Accession Number

Microarray data have been deposited at GEO under accession number GSE4684.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Overview of Transcript Levels in the Wild Type, *ago1*, and *dcl1*.

Supplemental Table 2. Gene Ontology Classification of Transcripts That Change Significantly in *ago1* and *dcl1*.

Supplemental Table 3. miRNA Targets and Genes Showing Consistently Altered mRNA Levels in *ago1* and *dcl1*.

Supplemental Table 4. Fold Changes of Features That Change Significantly in Expression in *ago1* and *dcl1*.

Supplemental Table 5. Positive and Negative Controls for the AtGenome1 Microarray.

Supplemental Table 6. Primer Sequences for Figures 1 to 5.

Supplemental Figure 1. Small RNA and mRNA Expression of Repeat-Containing Genes and Transposons in *ago1-9*.

ACKNOWLEDGMENTS

We thank C. Kidner for providing *ago1-9* and *ago1-11* and S. Jacobsen and E. Meyerowitz for providing *dcl1-9*. We are grateful to C. Kidner, A. Caudy, V. Colot, G. Hannon, Z. Lippman, T. Volpe, and K. Zito for helpful discussions and to B. Reinhart and D. Bartel for sharing unpublished data. We thank K. Gold and K. Slotkin for help with antisense analysis. M.R. was supported by postdoctoral fellowships from the Cold Spring Harbor Association and the National Institutes of Health (Grant GM-064269). M.W.V. was supported by a National Science Foundation bioinformatics postdoctoral fellowship (Grant DBI-0306035). This work was supported by the National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service (Grant 2005-35319-15280 to R.A.M.).

Received February 26, 2006; revised May 3, 2006; accepted May 16, 2006; published June 23, 2006.

REFERENCES

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell **121,** 207–221.

Aukerman, M.J., and Sakai, H. (2004). Correction: Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell **16,** 555.

Bao, N., Lye, K.W., and Barton, M.K. (2004). MicroRNA binding sites in Arabidopsis class III HD-ZIP mRNAs are required for methylation of the template chromosome. Dev. Cell **7,** 653–662.

Baulcombe, D. (2005). RNA silencing. Trends Biochem. Sci. **30,** 290–293.

Baumberger, N., and Baulcombe, D.C. (2005). Arabidopsis ARGONAUTE1 is an RNA slicer that selectively recruits microRNAs and short interfering RNAs. Proc. Natl. Acad. Sci. USA **102,** 11928–11933.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409,** 363–366.

Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. (1998). AGO1 defines a novel locus of Arabidopsis controlling leaf development. EMBO J. **17,** 170–180.

Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R., and Zhu, J.K. (2005). Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell **123,** 1279–1291.

Boutet, S., Vazquez, F., Liu, J., Beclin, C., Fagard, M., Gratias, A., Morel, J.B., Crete, P., Chen, X., and Vaucheret, H. (2003). Arabidopsis HEN1. A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Curr. Biol. **13,** 843–848.

Cardon, G., Hohmann, S., Klein, J., Nettesheim, K., Saedler, H., and Huijser, P. (1999). Molecular characterisation of the Arabidopsis SBP-box genes. Gene **237,** 91–104.

Carmell, M.A., Xuan, Z., Zhang, M.Q., and Hannon, G.J. (2002). The Argonaute family: Tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. **16,** 2733–2742.

Carrington, J.C., and Ambros, V. (2003). Role of microRNAs in plant and animal development. Science **301,** 336–338.

Chen, X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science **303,** 2022–2025.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D.C. (2000). An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell **101,** 543–553.

Dunoyer, P., Himber, C., and Voinnet, O. (2005). DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. Nat. Genet. **37,** 1356–1360.

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F., and Bowman, J.L. (2003). Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Curr. Biol. **13,** 1768–1774.

Finnegan, E.J., Margis, R., and Waterhouse, P.M. (2003). Posttranscriptional gene silencing is not compromised in the Arabidopsis CARPEL FACTORY (DICER-LIKE1) mutant, a homolog of Dicer-1 from Drosophila. Curr. Biol. **13,** 236–240.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans.* Nature **391,** 806–811.

Garcia, D., Collier, S., Byrne, M.E., and Martienssen, R.A. (2006). Specification of leaf polarity in Arabidopsis via the trans-acting siRNA pathway. Curr. Biol. **16,** 933–938.

Gasciolli, V., Mallory, A.C., Bartel, D.P., and Vaucheret, H. (2005). Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. Curr. Biol. **15,** 1494–1500.

Gazzani, S., Lawrenson, T., Woodward, C., Headon, D., and Sablowski, R. (2004). A link between mRNA turnover and RNA interference in Arabidopsis. Science **306,** 1046–1048.

Ghassemian, M., Waner, D., Tchieu, J., Gribskov, M., and Schroeder, J.I. (2001). An integrated Arabidopsis annotation database for Affymetrix Genechip data analysis, and tools for regulatory motif searches. Trends Plant Sci. **6,** 448–449.

Hamilton, A., Voinnet, O., Chappell, L., and Baulcombe, D. (2002). Two classes of short interfering RNA in RNA silencing. EMBO J. **21,** 4671–4679.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286**, 950–952.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., and Hannon, G.J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293,** 1146–1150.

Hannon, G.J. (2002). RNA interference. Nature **418,** 244–251.

Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C., and Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO J. **22,** 4523–4533.

Jacobsen, S.E., Running, M.P., and Meyerowitz, E.M. (1999). Disruption of an RNA helicase/RNAse III gene in Arabidopsis causes unregulated cell division in floral meristems. Development **126,** 5231–5243.

Jones-Rhoades, M.W., and Bartel, D.P. (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol. Cell **14,** 787–799.

Jurka, J., Kapitonov, V.V., Pavlicek, A., Klonowski, P., Kohany, O., and Walichiewicz, J. (2005). Repbase Update, a database of eukaryotic repetitive elements. Cytogenet. Genome Res. **110,** 462–467.

Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A., and Carrington, J.C. (2003). P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. Dev. Cell **4,** 205–217.

Kidner, C.A., and Martienssen, R.A. (2004). Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. Nature **428,** 81–84.

Kidner, C.A., and Martienssen, R.A. (2005). The role of ARGONAUTE1 (AGO1) in meristem formation and identity. Dev. Biol. **280,** 504–517.

Li, H., Xu, L., Wang, H., Yuan, Z., Cao, X., Yang, Z., Zhang, D., Xu, Y., and Huang, H. (2005). The putative RNA-dependent RNA polymerase RDR6 acts synergistically with ASYMMETRIC LEAVES1 and 2 to repress BREVIPEDICELLUS and MicroRNA165/166 in Arabidopsis leaf development. Plant Cell **17,** 2157–2171.

Lipardi, C., Wei, Q., and Paterson, B.M. (2001). RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell **107,** 297–307.

Lippman, Z., May, B., Yordan, C., Singer, T., and Martienssen, R. (2003). Distinct mechanisms determine transposon inheritance and methylation via small RNA and histone modification. PLoS Biol. **1,** 420–428.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science **305,** 1437–1441.

Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. (2002). Cleavage of *Scarecrow-like* mRNA targets directed by a class of *Arabidopsis* miRNA. Science **297,** 2053–2056.

Lu, C., Tej, S.S., Luo, S., Haudenschild, C.D., Meyers, B.C., and Green, P.J. (2005). Elucidation of the small RNA component of the transcriptome. Science **309,** 1567–1569.

Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P., and Barton, M.K. (1999). The PINHEAD/ZWILLE gene acts pleiotropically in Arabidopsis development and has overlapping functions with the ARGONAUTE1 gene. Development **126,** 469–481.

Matzke, M.A., and Birchler, J.A. (2005). RNAi-mediated pathways in the nucleus. Nat. Rev. Genet. **6,** 24–35.

McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. (2001). Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature **411,** 709–713.

Morel, J.B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H. (2002). Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell **14,** 629–639.

Mourrain, P., et al. (2000). Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101,** 533–542.

Newman, A. (1987). Specific accessory sequences in *Saccharomyces cerevisiae* introns control assembly of pre-mRNAs into spliceosomes. EMBO J. **6,** 3833–3839.

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature **425,** 257–263.

Papp, I., Mette, M.F., Aufsatz, W., Daxinger, L., Schauer, S.E., Ray, A., van der Winden, J., Matzke, M., and Matzke, A.J. (2003). Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. Plant Physiol. **132,** 1382–1390.

Parizotto, E.A., Dunoyer, P., Rahm, N., Himber, C., and Voinnet, O. (2004). In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. Genes Dev. **18,** 2237–2242.

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

Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H.L., and Poethig, R.S. (2004). SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. Genes Dev. **18,** 2368–2379.

Pham, J.W., and Sontheimer, E.J. (2005). Molecular requirements for RNA-induced silencing complex assembly in the Drosophila RNA interference pathway. J. Biol. Chem. **280,** 39278–39283.

Qi, Y., Denli, A.M., and Hannon, G.J. (2005). Biochemical specialization within Arabidopsis RNA silencing pathways. Mol. Cell **19,** 421–428.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. (2002). MicroRNAs in plants. Genes Dev. **16,** 1616–1626.

Ronemus, M., and Martienssen, R. (2005). RNA interference: Methylation mystery. Nature **433,** 472–473.

Sambrook, J., and Rusell, D.W. (2001). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schmid, M., Uhlenhaut, N.H., Godard, F., Demar, M., Bressan, R., Weigel, D., and Lohmann, J.U. (2003). Dissection of floral induction pathways using global expression analysis. Development **130,** 6001–6012.

Schmitz-Linneweber, C., Williams-Carrier, R., and Barkan, A. (2005). RNA immunoprecipitation and microarray analysis show a chloroplast pentatricopeptide repeat protein to be associated with the 5′ region of mRNAs whose translation it activates. Plant Cell **17,** 2791–2804.

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M., and Weigel, D. (2005). Specific effects of microRNAs on the plant transcriptome. Dev. Cell **8,** 517–527.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107,** 465–476.

Song, J.J., Liu, J., Tolia, N.H., Schneiderman, J., Smith, S.K., Martienssen, R.A., Hannon, G.J., and Joshua-Tor, L. (2003). The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nat. Struct. Biol. **10,** 1026–1032.

Song, J.J., Smith, S.K., Hannon, G.J., and Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. Science **305,** 1434–1437.

Souret, F.F., Kastenmayer, J.P., and Green, P.J. (2004). AtXRN4 degrades mRNA in Arabidopsis and its substrates include selected miRNA targets. Mol. Cell **15,** 173–183.

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.

Tijsterman, M., Ketting, R.F., and Plasterk, R.H. (2002). The genetics of RNA silencing. Annu. Rev. Genet. **36,** 489–519.

Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., and Sharp, P.A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. **13,** 3191–3197.

Vaistij, F.E., Jones, L., and Baulcombe, D.C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell **14,** 857–867.

Vaucheret, H., Vazquez, F., Crete, 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.

Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A.C., Hilbert, J.L., Bartel, D.P., and Crete, P. (2004).

Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Mol. Cell **16,** 69–79.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science **303,** 672–676.

Voinnet, O., Pinto, Y.M., and Baulcombe, D.C. (1999). Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. USA **96,** 14147–14152.

Voinnet, O., Vain, P., Angell, S., and Baulcombe, D.C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell **95,** 177–187.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science **297,** 1833–1837.

Wassenegger, M. (2005). The role of the RNAi machinery in heterochromatin formation. Cell **122,** 13–16.

Waterhouse, P.M., Graham, M.W., and Wang, M.B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA **95,** 13959–13964.

Xie, Z., Allen, E., Wilken, A., and Carrington, J.C. (2005). DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA **102,** 12984–12989.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., and Carrington, J.C. (2004). Genetic and functional diversification of small RNA pathways in plants. PLoS Biol. **2,** E104.

Yamada, K., et al. (2003). Empirical analysis of transcriptional activity in the Arabidopsis genome. Science **302,** 842–846.

Yoshikawa, M., Peragine, A., Park, M.Y., and Poethig, R.S. (2005). A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. Genes Dev. **19,** 2164–2175.