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

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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; Tuschi 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 Schizosaccharomyces 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 Birchier, 2005; Wassenegger, 2005).

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 (Liave 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
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 Polycistronic 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 Polycistronic 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 (Ler) background (Kidner and Martienssen, 2004), whereas dcl1-9 was isolated in a mixed Ler-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 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 entries 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 SPLs 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 SBP box. The smaller class of SPLs 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 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 (Liave 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 mRNA 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 SAPL 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
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\#9 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 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.
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 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 Ler (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

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).

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 (Ler), ago1-11, and ago1-9 (left three lanes, each panel) and the wild type (Ler) and dcl1-9 (right two lanes, each panel) at 21 d.

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 (Ler), 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 (Ler) and ago1-9 plants were also used. Vertical bars (at right) indicate the positions of miRNA-homologous regions of targets.
sizes; this was most prominent with At1g62670, which had at least five visible bands within this range in Ler 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 (Kidner and Martienssen, 2004), or attributable to targeting of the miR166b precursor by TAS3 (Garcia et al., 2006).

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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
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 SPLs 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 \textit{ago1} and \textit{dcl1}.

RNAs from the wild type (Ler), \textit{ago1-9}, and \textit{ago1-11} at 9 and 21 d and from \textit{dcl1-9} at 21 d were reverse-transcribed with strand-specific primers corresponding to At1g62670 (A), \textit{HAP2C} (B), \textit{SPL10} (C), and \textit{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.
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 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|42530656|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 hy1 (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 Ler 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), whereas we used plants from the Ler 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; and increased small antisense RNA (asiRNA) levels. In addition to the 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 products.
siRNA signatures from products detected by primer extension. We examined MPSS most cases (M.W. Vaughn and R.A. Martienssen, unpublished data). not restricted to miRNA target genes and requires other polymerases in the action of RDR6 on cleaved (uncapped) mRNA, but antisense RNA is RNA downstream of the miRNA-complementary sites can arise through 21- to 22-nucleotide siRNAs by DCL1 (Borsani et al., 2005). Antisense sized class of siRNA that serves as a signal to initiate the production of target additional sense or antisense RNA molecules, through AGO1, register siRNA, or the action of a Dicer variant. The siRNA may then 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).

Figure 7. DCL1 and AGO1 in miRNA Action.

DCL1 is required for the processing of miRNA precursors. Processed miRNAs (in black) then target homologous miRNAs (dark gray), which are cleaved within the miRNA-homologous exons (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).

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 used for 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. Pool 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 ≤ 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 Russell, 2001) with exceptions as noted. RNA gel blots and hybridizations were performed using the NorthernMax-Gly kit (Ambion). Semi-quantitative RT-PCR was performed using the Sensiscript RT kit (Invitrogen). Genomic DNA was precipitated with 5% polyethylene glycol (Sigma-Aldrich) to concentrate the small RNAs (<200 nucleotides). All hybridizations were performed using [α-32P]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.

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