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Biochemical Specialization within *Arabidopsis* RNA Silencing Pathways

Technique

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Summary

In plants, the RNA silencing machinery responds to numerous inputs, including viral infection, microRNAs, and endogenous siRNAs that may act both in *trans* and in *cis*. Additionally, the full spectrum of silencing outcomes has been demonstrated in plants, ranging from mRNA degradation to repression at the level of protein synthesis to chromatin remodeling. Genetic studies in *Arabidopsis* have indicated that individual response pathways are functionally compartmentalized. However, to date, no biochemical systems have been available to investigate the roles of specific proteins within silencing pathways or the effects of selected mutations on the biochemical activity of those components. Here, we describe the generation of *Arabidopsis* extracts that reproduce many aspects of RNA silencing reactions in vitro. We find that specific members of the Dicer and Argonaute families have distinct biochemical activities, which provides insight into their roles within RNA silencing pathways in *Arabidopsis*.

Introduction

RNA silencing in eukaryotes plays important roles in diverse biological processes, including spatial and temporal regulation of development, heterochromatin formation, and antiviral defense (Bartel, 2004; Baulcombe, 2004; Lippman and Martienssen, 2004). To achieve these outcomes, distinct double-stranded RNA (dsRNA) triggers are funneled into the RNA interference pathways, major components of which are Dicer and Argonaute proteins. Dicer enzymes initiate silencing by processing dsRNA substrates into 21–24 nucleotide (nt) small RNAs. These small RNAs join Argonaute-containing RNA-induced silencing complexes (RISC) that carry out effector functions leading to gene silencing at the transcriptional or posttranscriptional level (Hannon, 2002; Meister and Tuschl, 2004).

The number of Dicer and Argonaute family members varies greatly among organisms. In human and *C. elegans,* there is only one Dicer. In *Drosophila,* Dicer-1 and Dicer-2 are both required for small interfering RNA (siRNA)-directed mRNA cleavage, whereas Dicer-1 but not Dicer-2 is essential for microRNA (miRNA)-directed repression (Lee et al., 2004; Pham et al., 2004). *Arabidopsis* has four Dicer-like (DCL) proteins (Schauer et al., 2002), and genetic studies have suggested that these DCLs are functionally specialized. DCL1 is required for the accumulation of ~21 nt miRNAs and *trans*-acting siRNAs (ta-siRNAs) (Park et al., 2002; Peragine et al., 2004; Vazquez et al., 2004b), whereas DCL3 is involved in the accumulation of ~24 nt siRNAs implicated in DNA and histone methylation (Xie et al., 2004).

Animals and plants also contain multiple Argonaute proteins that seem to have specialized functions. In *C. elegans*, Rde-1 is involved in dsRNA-mediated silencing, whereas Alg-1 and Alg-2 are required for miRNAs to repress gene expression (Grishok et al., 2001; Tabara et al., 1999). In *Drosophila*, Ago2 is an essential component of the siRNA-directed RNAi response, while Ago1 is involved in miRNA function (Hammond et al., 2001; Okamura et al., 2004). In *Arabidopsis*, there are ten Argonaute proteins, some of which have been studied through the use of mutant alleles (Bohmert et al., 1998; Fagard et al., 2000; Morel et al., 2002; Moussian et al., 1998; Zilberman et al., 2003). AGO1 null plants have severe developmental defects and are impaired in PTGS (Bohmert et al., 1998; Fagard et al., 2000). AGO4 has been implicated in maintenance of DNA and histone methylation at specific loci. AGO4 is required for the accumulation of siRNAs corresponding to several loci, suggesting a role for this protein in epigenetic regulation (Zilberman et al., 2003, 2004).

Although *Arabidopsis* Dicer-like and Argonaute proteins have been linked with different aspects of silencing, insight into biochemical mechanisms underlying such specialization is lacking. Here, we report the development of biochemical tools that can be used to dissect the functions of individual *Arabidopsis* proteins within RNA silencing pathways. We show that two different Dicer-like proteins specifically produce 21 nt and 24 nt small RNAs, respectively. We find that 21 nucleotide small RNAs join AGO1 in vivo to form a cleavage-competent RISC but that AGO1 is less discriminating when primed with a small RNA in vitro.

Results

Dicer Activities in *Arabidopsis* Extracts

In plants, silencing pathways rely on distinct species of small RNAs ranging in size from 21–25 nucleotides with the most abundant size classes having discrete lengths of 21 or 24 nucleotides. We began by asking whether we could produce extracts from *Arabidopsis* that would generate known size classes of small RNAs. Extracts were prepared in several different ways, including protoplast formation followed by dounce homogenization and by grinding of snap-frozen material in liquid nitrogen. Using lysates prepared in the latter manner, we found that *Arabidopsis* extracts from either cultured cells or plant inflorescence tissues efficiently processed a long, radiolabeled dsRNA substrate into small RNAs (Figures 1A and 1B). As had previously been observed in wheat germ (Tang et al., 2003) and tobacco (A.M.D. and G.J.H., unpublished data), both ~21 nt and ~24 nt size classes of small RNAs were produced (Figures 1A and 1B).

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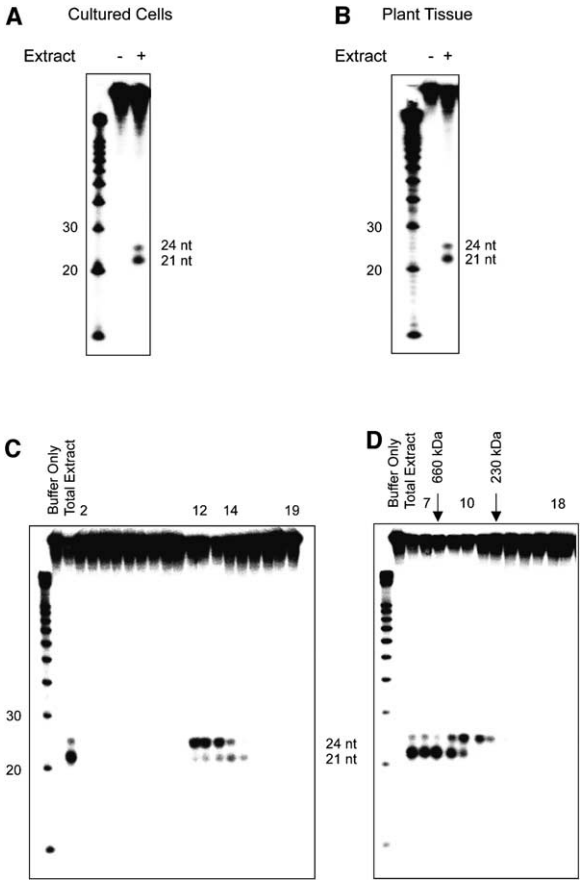

Figure 1. *Arabidopsis* Extracts Contain Multiple Dicer Activities

(A and B) A 400 bp, uniformly labeled, double-stranded RNA was incubated in the absence (–) or in the presence (+) of extract from an *Arabidopsis* cultured cell line (A) or in the absence (–) or in the presence (+) of extract from *Arabidopsis* inflorescence tissue from plants of the Columbia ecotype (B).

(C) Extracts from cultured cells were fractionated by ion exchange (Mono-Q) chromatography. Dicer activity was assayed for each fraction as in (A). Fraction numbers corresponding to the peak of activity are indicated.

(D) Extracts from cultured cells were fractionated by size-exclusion chromatography. Each fraction was assayed for Dicer cleavage activity, with fraction numbers corresponding to the peak of activity indicated. The fractions representing 230 and 660 kDa size markers, chromatographed separately, are shown.

In (A)–(D), the processed products were resolved in 15% denaturing gels; positions of the 21 and 24 nucleotide products are indicated. Decade RNA markers (Ambion) are shown for reference.

To determine whether these small RNAs might be produced by distinct DCL complexes, we examined the properties of the small RNA-generating activities using biochemical fractionation. Fractionation of cell extracts on an ion exchange (Mono-Q) column partially separated the two activities, with the 21 nt siRNA-producing activity peaking at fraction 12 and the 24 nt siRNA-producing activity peaking at fraction 14 (Figure 1C). In addition, size-exclusion chromatography revealed that the two activities reside in complexes with distinct sizes. The 21 nt siRNA-generating activity chromatographed at >660 kDa, whereas the 24 nt siRNA-generating activity sized at ~400 kDa (Figure 1D).

Considered together, our results are consistent with previous studies from wheat germ (Tang et al., 2003) and could indicate that either distinct DCLs or a single DCL in two distinct complexes generates different size classes of small RNAs. The *Arabidopsis* genome is predicted to encode four Dicer-like proteins, with DCL1 and DCL3 being required for the accumulation of ~21 nt and ~24 nt small RNAs, respectively (Park et al., 2002; Peragine et al., 2004; Vazquez et al., 2004b; Xie et al., 2004). We therefore set out to ask whether these in vivo observations reflected distinct intrinsic biochemical properties of these proteins.

DCL1 and DCL3 Produce 21 and 24 nt siRNAs, Respectively

To investigate whether DCL1 and DCL3 are directly responsible for the generation of 21 and 24 nt small RNAs, we examined DCL1 and DCL3 immunoprecipitates for their ability to cleave dsRNA. Consistent with the implications of previous genetic studies, immunoaffinity-purified DCL1 complexes processed dsRNAs into 21 nt siRNAs, whereas DCL3 complexes produced 24 nt siRNAs (Figure 2A). The specificity of each immunoprecipitation was confirmed by peptide-competition assays (Figure 2B). Western blots of fractions from the size-exclusion column with anti-DCL1 and anti-DCL3 antibodies indicated that DCL1 and DCL3 reside in >660 kDa and ~400 kDa complexes (Figure 1D and data not shown). Interestingly, maximal production of small RNAs by DCL1 immunoprecipitates requires ATP, whereas the activity of DCL3 appears to be ATP-independent (Figure S1 in the Supplemental Data available with this article online).

A real strength of *Arabidopsis* as a model system for studying mechanisms of RNA silencing is the availability of a large number of mutants that have been generated over the past several years. *dcl1-7* contains a mutation that changes proline 415 to serine, altering the RNA helicase domain of DCL1 (Golden et al., 2002). The mutations in *dcl2-1* and *dcl3-1* are T-DNA insertions that are predicted to produce truncated forms of DCL2 and DCL3 (Xie et al., 2004). Extracts from wild-type (wt) plants, Landsberg *erecta* (La-*er*, wt for *dcl1-7*) and Columbia (Col, wt for *dcl2-1* and *dcl3-1*), contained both 21 and 24 nt siRNA-generating activities (Figure 2C). In extracts from the *dcl2-1* mutant, both activities were still present at levels comparable to Col, suggesting that DCL2 is not the major enzyme that produces either 21 or 24 nt siRNAs (Figure 2C). In extracts from *dcl3-1* mutants, the 21 nt siRNA-generating activity was unaffected, but 24 nt siRNA-generating activity was absent. This indicates that DCL3 is either the sole or the predominant enzyme that produces 24 nt siRNAs (Figure 2C). This conclusion is also supported by the fact that accumulation of ~24 nt siRNAs in vivo was significantly decreased in *dcl3-1* but not in *dcl1-7* and *dcl2-1* mutants (Xie et al., 2004) (Figure S2). In the *dcl1-7* mutant, the accumulation of 21 nt miRNAs and *trans*-acting siRNAs [e.g., siR480(+)/sRNA255] was dramatically decreased (Peragine et al., 2004; Vazquez et al., 2004b; Xie et al., 2004) (Figure S2). Surprisingly, the 21 nt siRNA-generating activity was unaffected in *dcl1-7* extracts or immunoprecipitates (Figure 2C). These results suggest that

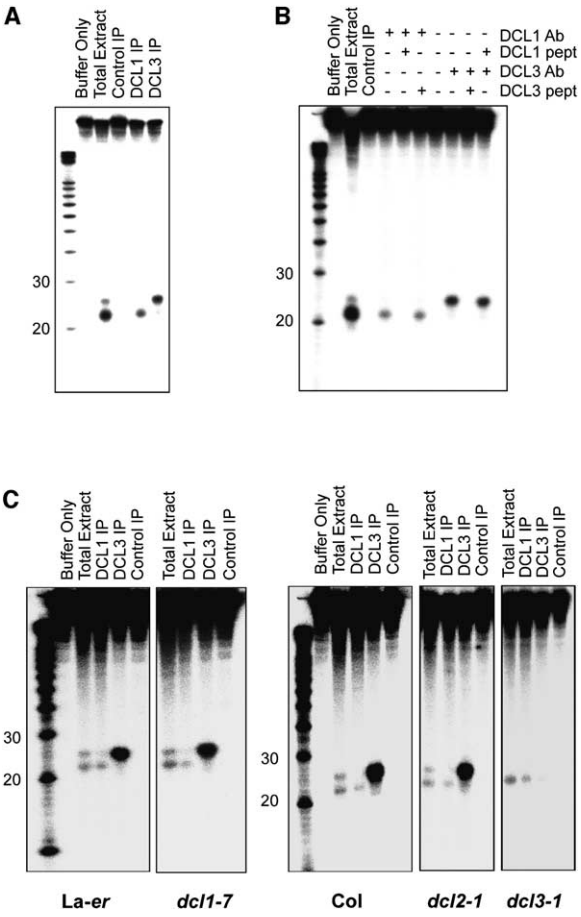

Figure 2. Biochemical Activities of *Arabidopsis* DCL1 and DCL3

(A) DCL1 and DCL3 complexes were immunoaffinity purified from cultured *Arabidopsis* cell extracts and incubated with a 400 bp dsRNA substrate. For comparison, incubations with total extract and a control immunoprecipitate (IP) prepared using an irrelevant *S. pombe* RdR1 antibody are shown.

(B) The immunoprecipitations used in (A) were tested for specificity by preincubating each antibody with either a control peptide or its respective antigenic peptide prior to use in immunoprecipitation, as indicated.

(C) DCL1 and DCL3 complexes were immunoaffinity purified from inflorescence extracts of Landsberg erecta (La-*er*), *dcl1-7* mutants, Columbia (Col), *dcl2-1* mutants, and *dcl3-1* mutants, as indicated. The activity of each complex was assayed as described in (A). Incubations with total extract and a control immunoprecipitate (IP) using an irrelevant *S. pombe* RdR1 antibody are shown for comparison.

In (A)–(C), positions of the 21 and 24 nucleotide products are indicated. Decade RNA markers are shown for reference.

the P415S substitution does not affect the catalytic activity of DCL1. We cannot rule out the possibility that DCL4 may also contribute to the activity generating the 21 nt siRNAs in *dcl1-7* extracts, although not in the immunoprecipitates. Intriguingly, both 21 nt and 24 nt siR480(+)/sRNA255 were detected in La-*er* but not in *dcl1-7* (Figure S2). This could indicate a requirement for cleavage of ta-siRNA-generating transcripts as guided by miRNAs whose biogenesis requires DCL1 (Allen et al., 2005).

AGO1 Forms a Cleavage-Competent RISC

To examine whether we could detect RISC activity in *Arabidopsis* lysates, we incubated in vitro-synthesized transcripts corresponding to At4g29770, a target of siR480(+)/sRNA255, with total extracts prepared from inflorescence tissues. In concentrated extracts (5 mg/ml total proteins), RNAs were nonspecifically degraded, and no specific RISC-directed cleavage was observed. However, specific cleavage products were readily detectable when extracts were diluted prior to use (Figure 3A).

In numerous systems, it has been shown that siRNAs and miRNAs are incorporated into Argonaute-containing RISCs that execute silencing effector functions. Indeed, in mammals, different RISCs have been shown to have biochemically distinct capabilities, with only Ago2-containing RISC being cleavage competent (Liu et al., 2004; Meister et al., 2004). We have recently shown that Argonaute uses an unusual DDH catalytic triad to coordinate a Mg^{2+} ion at the active site (Rivas et al., 2005). There are ten Argonaute proteins encoded within the *Arabidopsis* genome, and RT-PCR analysis indicates that they are all expressed in inflorescence tissues (data not shown). Alignment of *Arabidopsis* Argonaute proteins with human Ago2 revealed that eight *Arabidopsis* homologs (AGO1, AGO4, AGO5, AGO6, AGO7/ZIP, AGO8, AGO9, and ZLL/PNH) have all three conserved catalytic site residues (Figure S3). Thus, these Argonautes are potential *Arabidopsis* "Slicers". *ago1* mutants have severe developmental defects and are impaired in PTGS (Bohmert et al., 1998; Fagard et al., 2000; Morel et al., 2002). Accompanying developmental defects are decreased accumulation of miRNAs and ta-siRNAs and increased accumulation of mRNAs known to be miRNA or ta-siRNA targets (Vaucheret et al., 2004; Vazquez et al., 2004b). These genetic studies suggest AGO1 as a good "Slicer" candidate.

An examination of AGO1-associated RNAs showed that siR480(+)/sRNA255, miR159, miR171 and miR172 coimmunoprecipitated with AGO1, while ~24 nt siRNA02, siRNA1003 and *AtSN1*-derived siRNAs did not (Figure S4). To test whether the AGO1-bound siRNAs and miRNAs could direct cleavage, we incubated AGO1 immunoprecipitates with complementary targets. We found that AGO1 immunoprecipitates cleaved mRNA targets of siR480(+)/sRNA255 (At4g29770), miR159 (TCP4) and miR171 (SCL6) (Figure 3B). Deletion of the region complementary to the small RNA in each mRNA abolished this cleavage. These data indicated that AGO1 binds to miRNAs and siRNAs in vivo and forms cleavage-competent RISC. Intriguingly, the target of miR172, AP2 was not efficiently cleaved (Figure 3B), although miR172 was associated with AGO1 (Figure S4). This outcome is in agreement with previous studies, which indicated that miR172 functions predominantly by repressing protein synthesis (Aukerman and Sakai, 2003; Chen, 2004). Considered together, these results implicate AGO1 as a constituent of cleavage-competent *Arabidopsis* RISC.

Genetic Requirements for RISC Activity

The aforementioned RISC activity assay offered an opportunity to examine the genetic requirements for for-

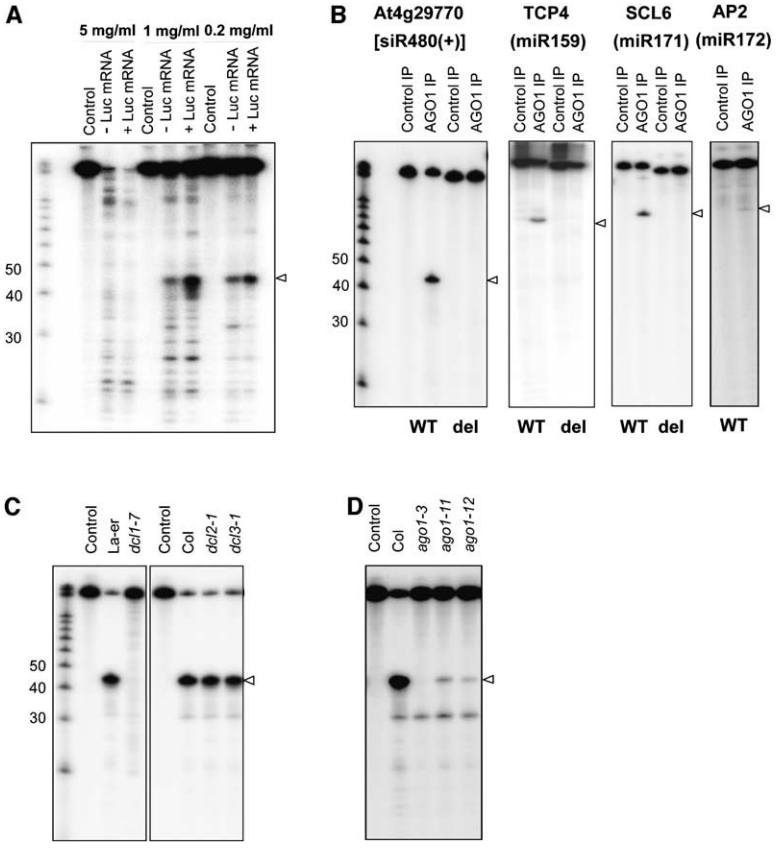

Figure 3. *Arabidopsis* Total Extracts and AGO1 Immunoprecipitates Are Competent for mRNA Cleavage

(A) ^{32}P-cap-labeled synthetic transcripts of siR480(+)/sRNA255 target gene, At4g29770, were incubated with inflorescence extracts of different total protein concentrations in the presence or absence of excess amount of cold luciferase (Luc) mRNAs as indicated. Incubation with buffer only was used as negative control.

(B) ^{32}P-cap-labeled synthetic siRNA or miRNA target mRNAs (wt, first two lanes of each panel) were incubated with control IPs and AGO1 IPs. RNAs with complete or partial deletion of the predicted target sites (del, final two lanes of each panel) were used to confirm the sequence specificity of cleavage.

(C) AGO1 immunoprecipitates were prepared from wild-type plants (La-*er* for *dcl1-7* and Col for *dcl2-1* and *dcl3-1*) and indicated *dcl* mutants. An *S. pombe* RdR1 antibody was used for control immunoprecipitations. RISC cleavage activity in the immunoprecipitates was assayed by incubation with ^{32}P-cap-labeled At4g29770 mRNA.

(D) AGO1 immunoprecipitates were prepared from wild-type (Col) and indicated *ago1* mutant plant extracts. RISC cleavage activity in the immunoprecipitates was assayed as described in (C).

In (A)–(D), cleavage products were resolved in 15% denaturing gels. Positions of 5′ cleavage products are indicated by the arrows. Decade RNA markers are shown for reference.

mation of active RISC. We prepared AGO1 immunoprecipitates from *dcl1-7*, *dcl2-1*, and *dcl3-1* mutants and incubated them with cap-labeled At4g29770 mRNA. In RISC prepared from *dcl1-7* mutants, we observed no target cleavage (Figure 3C). This is consistent with previous studies indicating a lack of ~21 nt small RNA accumulation in *dcl1-7* mutants in vivo. Using RISC prepared from *dcl2-1* and *dcl3-1* mutants, At4g29770 was cleaved at levels comparable to those observed in immunoprecipitates from wild-type (Figure 3C). We also prepared AGO1 immunoprecipitates from *ago1* mutants and tested the RISC activity toward At4g29770. In immunoprecipitates from an *ago1* null allele, *ago1-3*, there was no detectable cleavage, whereas in RISC prepared from plants bearing either of two hypomorphic alleles, *ago1-11* and *ago1-12*, a weak cleavage signal could be detected (Figure 3D).

In Vitro Reconstitution of RISC

We next sought to test whether exogenous siRNAs could be loaded into immunoaffinity-purified AGO1 complexes by adapting a protocol we have developed for human Ago2 (Rivas et al., 2005). Single-stranded siRNAs containing photoreactive residues at either their 3′ or 5′ ends could be specifically photocross-linked to AGO1, suggesting that the purified complex could bind to exogenously supplied small RNAs (Figure 4A). We therefore tested whether in vitro loaded siRNAs could direct target RNA cleavage by loading AGO1 immunoprecipitates with a 21 nt single-stranded siRNA targeting firefly luciferase. Preformed complexes incubated with cap-labeled luciferase mRNA were able to catalyze mRNA cleavage at the expected positions (Figure 4B). In these reactions, ATP was not essential but could enhance RISC activity (Figure 4C), reminiscent of the ATP requirement for product release in *Drosophila* (Haley and Zamore, 2004). As shown for human and fly RISC (Martinez and Tuschl, 2004; Rivas et al., 2005; Schwarz et al., 2004). Mg^{2+} was required for activity (Figure 4D). The 5′-phosphate of the siRNA was not essential (Figure 4E). This is similar to what has recently been observed for reconstitution of a minimal mammalian RISC using recombinant Argonaute protein and a single-stranded siRNA (Rivas et al., 2005). Interestingly, 24 nt siRNAs could also be loaded to AGO1 immunoprecipitates (Figure 4F) to form cleavage-competent RISC (Figure 4G). This suggests that distinctions within pathways in *Arabidopsis* RNA silencing are probably determined via programmed interactions between complexes that produce and load different siRNA classes rather than by limitations on the sizes of small RNAs that can bind to specific Argonaute proteins.

2′- or 3′-*O*-methyl Modification of an siRNA Does Not Affect RISC Activity In Vitro

It was recently shown that *Arabidopsis* miRNAs are methylated at either the 2′- or the 3′-*O*-ribose position on their 3′ terminal nucleotide (Yu et al., 2005). Thus, it was hypothesized that this methylation event places a critical tag on plant small RNAs that may be essential

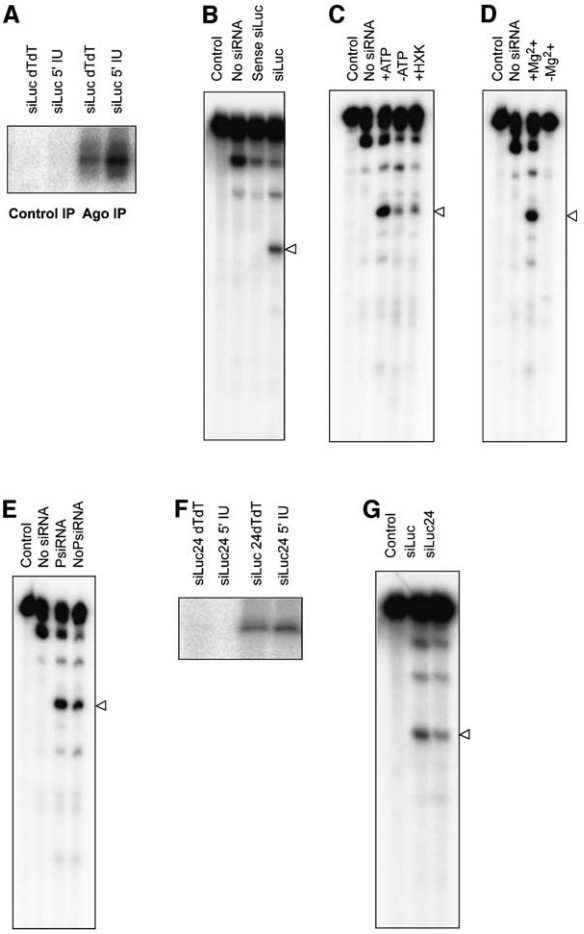

Figure 4. In Vitro Reconstitution of RISC

(A) AGO1 and control immunoprecipitates, as indicated, were incubated with single-stranded ^{32}P-labeled 21 nt siRNAs bearing photoreactive dT residues at the two 3′ positions (lucdTdT) or iodo-U in the 5′ position (luc5′-IU). Mixtures were irradiated with UV light as described in the Methods. Crosslinked products were resolved by 10% SDS-PAGE.

(B) AGO1 immunoprecipitates were mixed with single-stranded siRNAs that corresponded either to the sense strand (sense siLuc) or antisense strand (siLuc) of the target RNA. Reconstituted RISC was tested for cleavage activity by incubation with a ^{32}P-cap-labeled 300 nt luciferase mRNA. As a control, siLuc was mixed with *S. pombe* RdR1 immunoprecipitate. The reactions were resolved on a 5% denaturing gel.

(C) RISC reconstitution and activity tests were performed with siLuc as described in (B). +ATP: reactions contained ATP, GTP, and an ATP-regenerating system. –ATP: lanes lacked ATP, GTP, and the regenerating system. +HXK: AGO1 immunoprecipitates were pretreated with hexokinase and glucose and reacted in the absence of added ATP.

(D) RISC reconstitution and activity tests were performed by incubation with siLuc as described in (B) in the presence or absence of added Mg^{2+}, as indicated.

(E) RISC was charged with an siRNA either containing or lacking a 5′ phosphate (as indicated) under conditions of siRNA excess.

(F) UV-crosslinking assays were performed as described in (A) except that 24 nt siRNAs (siLuc24), extended at the 3′ and 5′ ends by two and one nucleotides respectively, were used.

(G) RISC reconstitution and activity tests were performed as described in (B) except that a 21 (siLuc) or 24 nt (siLuc24) siRNA was used as indicated.

In appropriate panels, the open arrow indicates the position of the 5′ cleavage product.

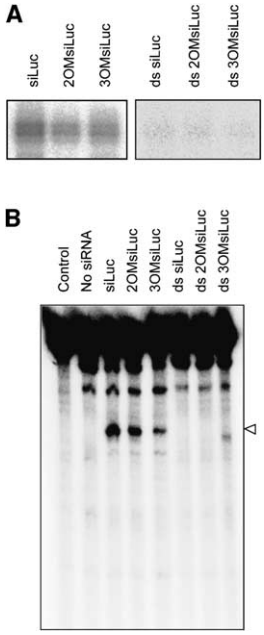

Figure 5. Impact of siRNA Methylation on RISC Assembly

(A) UV photocrosslinking assays were performed with 2′- or 3′-*O*-methyl modified siRNAs and siRNA duplexes.

(B) RISC reconstitution and activity tests were performed with 2′- or 3′-*O*-methyl modified siRNAs and siRNA duplexes.

for their function in the RNA silencing pathway (Yu et al., 2005). The aforementioned results suggested that methylation of the siRNA is not essential for RISC assembly or activity in our in vitro system. However, it remained possible that the presence of the modification could enhance function. We prepared siRNAs containing either 2′- or 3′-*O*-methyl groups and assessed their competency to form RISC. We found that incorporation of a methyl group either at 2′- or 3′-*O*-ribose position of the last nucleotide did not enhance the ability of the siRNA crosslink to immunoaffinity-purified AGO1 (Figure 5A). If anything, the presence of a 2′- or 3′-*O*-methyl attenuated cleavage activity rather than enhanced it (Figure 5B). As was seen for the unmethylated siRNA duplex, methylated siRNA duplexes could not bind to AGO1 immunoprecipitates (Figure 5A) and form functional RISC (Figure 5B). Neither have we succeeded in loading these duplexes into AGO1-containing RISC by incubation in *Arabidopsis* whole-cell lysates (data not shown). Considered together, our results suggest that methylation of small RNAs does not have a positive effect within RISC itself. However, such modifications could impact the pathway at numerous other points, including a possible function during RISC loading.

Discussion

In plants, two size-classes (~21 nt and ~24 nt) of small RNAs have been correlated with different functions in RNA silencing pathway based upon genetic studies. In general, 21 nt small RNAs target mRNAs for degrada-

tion and also are implicated in short-range cell-cell movement of RNA silencing (Baulcombe, 2004). In contrast, 24 nt siRNAs are correlated with DNA and histone methylation and long-distance trafficking of silencing signals (Baulcombe, 2004; Lippman and Martienssen, 2004). This study is consistent with those previous genetic data. Here, we provide direct biochemical evidence that DCL1 and DCL3 generate 21 nt and 24 nt siRNAs respectively. Specifically how Dicer measures and processes dsRNA into ~21–24 nt products remains a mystery; however, several studies indicate that the PAZ domain aids in end recognition to determine a cleavage site at a precise distance from the end (Song et al., 2003; Zhang et al., 2004). Thus, the difference in the sizes of DCL1 and DCL3 products could be attributed to intrinsic structural differences between DCL1 and DCL3 proteins. Alternatively, the size of their small RNA products may be dictated by different factors that associate with DCL1 and DCL3. Size-exclusion chromatography has indicated that DCL1 and DCL3 reside in >660 kDa and ~440 kDa complexes, respectively. This suggests that these two DCLs exist in complexes with other proteins. In animals, dsRBD containing proteins have been shown to stably associate with Dicers and to be critical for their function (Liu et al., 2003; Tabara et al., 2002; Tomari et al., 2004). In *Arabidopsis*, two dsRBD proteins, HEN1 and HYL1, have demonstrated roles in RNA silencing pathways (Han et al., 2004; Park et al., 2002; Vazquez et al., 2004a). Recently, it has been shown that HYL1 interacts with DCL1 in vitro (Hiraguri et al., 2005).

In *Arabidopsis*, miRNAs and ta-siRNAs suppress gene expression predominantly through cleavage of target mRNAs. We demonstrated that miRNAs and ta-siRNAs are present in complexes with AGO1 and that such complexes can cleave miRNA and ta-siRNA targets. Furthermore, we showed that exogenous siRNAs could be loaded into AGO1 immunoprecipitates to form a cleavage-competent RISC. A combination of preexisting genetic data with these biochemical studies strongly support that AGO1 is a core component of cleaving RISC. In vivo studies have cataloged differential effects of specific *ago1* mutations on the accumulation of miRNAs and the cleavage of known miRNA target genes (Vaucheret et al., 2004). The approaches described in this study provide a direct means to correlate sequence and structure alterations with biochemical properties and biological functions of the *Arabidopsis* AGO1 protein.

We have shown that DCL1 and AGO1, but not DCL2 and DCL3, are needed for AGO1-containing RISC activity toward At4g29770, a target of ta-siRNA480. It will be interesting to test whether other RNAi components, e.g., DCL4, HEN1, RDR6, and HYL1, are also required for the RISC activity. It will be also interesting to examine whether miRNA- and ta-siRNA-containing RISC activities require different components.

Besides AGO1, there are seven other *Arabidopsis* Argonaute proteins (AGO4, AGO5, AGO6, AGO7/ZIP, AGO8, AGO9, and ZLL/PNH) that possess the key catalytic site residues of Slicer (Liu et al., 2004; Rivas et al., 2005; Song et al., 2004)(Figure S4). These are also potentially capable of forming catalytically competent *Arabidopsis* RISCs. Among them, AGO7/ZIP and ZLL/PNH are of special interest. AGO7/ZIP plays a role in the regulation of vegetative developmental timing (Hunter et al., 2003). ZLL/PNH is involved in maintaining undifferentiated stem cells in the shoot apical meristem and has functions overlapping with those of AGO1 (Lynn et al., 1999; Moussian et al., 1998).

Many loci have been mapped in *Arabidopsis* that have an impact on the integrity of silencing pathways in vivo. The approaches presented here may allow the proteins encoded by those genes to be placed within a biochemical framework, initially assaying their impact on Dicer and RISC activities, but in the longer term creating a more fully elaborated biochemical model of RNA silencing in plants.

Experimental Procedures

Plant Materials and Growth Conditions

Arabidopsis suspension cells were cultured in Gamborg's B-5 medium (Sigma) supplemented with 20 g/l of sucrose, 0.5 g/l MES, and 0.1 mg/l of 2,4-Dichlorophenoxyacetic Acid (2,4-D) adjusted to pH 5.7 with KOH. Cells were maintained by shaking at 200 rpm in constant light at 22°C and sub-cultured weekly by 1:25 dilution. *Arabidopsis* plants were grown in a growth chamber under long day conditions. *Arabidopsis* ecotypes Columbia (Col) and Landsberg *erecta* (La-*er)* were ordered from the *Arabidopsis* Biological Research Center (ABRC). The *dcl1-7*, *dcl2-1*, and *dcl3-1* mutants lines were previously described (Golden et al., 2002; Xie et al., 2004) and kindly provided by J. Carrington (Oregon State University). The *ago1-3*, *ago1-11*, and *ago1-12* mutant materials were gifts from M. Ronemus and R. Martienssen (Cold Spring Harbor Laboratory). *ago1-3* is a null allele (Bohmert et al., 1998), *ago1-11* has a deletion of aa 826–845 and *ago1–12* has a H763L substitution in the PIWI domain.

Arabidopsis Extract Preparation

Arabidopsis cultured cells or inflorescence tissues were collected, ground into fine powder under liquid nitrogen, and then homogenized in 1 ml/g of extraction buffer (20 mM Tris. HCl, 4 mM MgCl2, [pH 7.5]) containing 5 mM DTT and 1 tablet/10mL (Roche) protease inhibitor cocktail. Cell debris was removed by centrifugation at 22,000g at 4°C for 20 min. The supernatant was collected and protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad). For Dicer activity assays and immunoprecipitations, protein concentrations were adjusted to 0.5 mg/ml and to 5–10 mg/ml with extraction buffer, respectively.

Chromatography

All columns and media were purchased from Amersham Biosciences. Buffers used for Mono-Q were buffer A (20 mM Tris.HCl [pH 7.5], 4 mM MgCl2, 2 mM DTT) and buffer B (20 mM Tris.HCl [pH 7.5], 4 mM MgCl2, 1 M NaCl, 2 mM DTT). For size chromatography, Sephacryl S-300 HR column was used with buffer At-size (20 mM Tris.HCl [pH 7.5], 150 mM NaCl, 4 mM MgCl2, 4% glycerol, 2 mM DTT). Five and 10 mg total proteins were loaded onto Mono-Q and size columns, respectively.

Peptides, Antibodies, and Immunoprecipitation

Peptides were designed based on amino acid sequences deposited in GenBank (DCL1, NM_099986; DCL3, NM_114260; AGO1, NM_179453). The peptides used were DCL1C (N-CEPMPSVKKAKD-C), DCL3N (N-MHSSLEPEKMEC-C), and AGO1N (N-MVR KRRTDAPSC-C). The peptides were conjugated to mcKLH using the Imject Maleimide Activated mcKLH Kit (Pierce) and used to raise rabbit polyclonal antibodies (Covance). Antibodies were affinity purified before use. In a standard immunoprecipitation reaction, 0.5 ml of *Arabidopsis* extract was precleared by incubation with 5 μl of Protein A/G-agarose (Roche) at 4°C for 30 min. Precleared extracts were then incubated with 2.5–5 μl of antibodies and 10 μl of Protein A/G agarose at 4°C for 2 hr. Immunoprecipitates were washed three times (20 min each) in extraction buffer.

Dicer Activity Assays

Dicer activity of *Arabidopsis* extracts and DCL1/DCL3 immunoprecipitates was assayed by incubation with uniformly ^{32}P-labeled luciferase dsRNAs in a 20 μl reaction containing 1 μl dsRNAs (50,000 cpm), 15 μl extract or immunoprecipitate, and 4 μl 5× Dicer reaction buffer (0.5 M NaCl, 5 mM ATP, 1 mM GTP, 6 mM MgCl2, 125 mM creatine phosphate, 150 μg/ml creatine kinase, and 2 U RNAsin Rnase Inhibitor) at 25°C for 2 hr. The reaction was stopped by adding 250 μl Trizol plus glycogen. Products were resolved on a 15% denaturing PAGE gels. When performed under no ATP conditions, the extracts or immunoprecipitates were pretreated with hexokinase in the presence of 10 mM glucose, and ATP, GTP, and the regeneration system were omitted from the reaction. In peptide-competition assays, antibodies were preincubated with excess peptide at 25°C for 30 min prior to use for immunoprecipitation.

mRNA Cleavage Assay and In Vitro RISC Reconstitution

mRNA cleavage assays were done as described (Liu et al., 2004; Rivas et al., 2005) with some modifications. *Arabidopsis* total extracts or AGO1 immunoprecipitates were prepared in 15 μl extraction buffer, and then mixed with 4 μl 5x cleavage buffer (0.25 M NaCl, 5 mM ATP, 1 mM GTP, 6 mM $MgCl_2$, 125 mM creatine phosphate, 150 μg/ml creatine kinase, and 2 unit/μl RNAsin RNase inhibitor) and 1 μl ^{32}P-cap-labeled target mRNAs. After incubation at 25°C for 2 hr, reactions were stopped by adding 250 μl Trizol with glycogen. Reactions were purified and resolved on a 15% denaturing PAGE gel.

For reconstitution of RISC activity, phosphorylated siRNAs (unphosphorylated when indicated) were added to a final concentration of 200 nM. After incubation at 25°C for 30 min, end-labeled target RNAs were added and the reactions were then incubated at 25°C for 2 hr. Cleavage products were resolved on a 5% denaturing PAGE gel.

Supplemental Data

Supplemental Data include four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/19/3/421/DC1/.

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