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REVIEW

Form, Function, and Regulation of ARGONAUTE Proteins

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Both transcriptional (TGS) and posttranscriptional gene silencing (PTGS) are conserved eukaryotic gene regulatory mechanisms, integral for taming exogenous (viruses and bacteria) or endogenous (repetitive elements and transposons) invasive nucleic acids to minimize their impact on genome integrity and function. TGS and PTGS also are essential for controlling the expression of protein coding genes throughout development or in response to environmental stimuli. In plants and animals, at least one member of the conserved ARGONAUTE (AGO) protein family comprises the catalytic engine of the silencing complex, which is guided by sequence-specific small RNA to cognate RNA. In this review, we present general features of plant and animal AGO proteins and detail our knowledge on the 10 *Arabidopsis thaliana* AGOs.

FORMS OF ARGONAUTE PROTEINS

Based on their functional domains, eukaryotic ARGONAUTE (AGO) proteins can be divided into two major groups: the AGO and the PIWI subfamilies, although there are some outliers that share similarity to the PIWI subfamily (Tolia and Joshua-Tor, 2007; Hutvagner and Simard, 2008; Vaucheret, 2008). The founding member of AGO proteins is *Arabidopsis thaliana* AGO1, which was discovered in a forward genetic screen for genes involved in development (Bohmert et al., 1998). Null *ago1* mutants have pleiotropic phenotypes, including tubular shaped leaves that resemble the tentacles of an argonaute squid. Shortly after the developmental characterization of *ago1* mutants, the critical role of AGO proteins in small RNA-directed silencing was discovered in numerous organisms (Tabara et al., 1999; Catalanotto et al., 2000; Fagard et al., 2000). AGO proteins are deeply conserved and are expressed in a wide range of tissues at high levels in many organisms (Carmell et al., 2002). However, the number of AGO proteins encoded among organisms varies greatly (Tolia and Joshua-Tor, 2007; Hutvagner and Simard, 2008; Vaucheret, 2008). Among the common model systems, *Saccharomyces cerevisiae* does not express AGO proteins and does not appear to encode other small RNA pathway factors, whereas *Schizosaccharomyces pombe* expresses one AGO subfamily protein. On the other hand, *Caenorhabditis elegans* expresses 27 AGO proteins that fall into both subfamilies, while *Drosophila melanogaster* expresses five and humans express eight AGO proteins that fall into both subfamilies. Plant AGO proteins are all in the AGO subfamily. A phylogenetic comparison of the 10 *Arabidopsis* AGO proteins placed them in three major clades: the AGO1, AGO5, and AGO10 clade; the AGO2, AGO3, and AGO7 clade; and the AGO4, AGO6, AGO8, and AGO9 clade (Morel et al., 2002; Vaucheret, 2008). However, this classification is based purely on protein similarity and does not necessarily

indicate functional similarity. Indeed, in rice (*Oryza sativa*), the AGO1/AGO5/AGO10 clade was subdivided in two separate clades; thus, the 18 AGO proteins encoded by the rice genome were classified in four clades. The rice AGO2/AGO3/AGO7 and AGO4/AGO6/AGO8/AGO9 clades are almost identical to those in *Arabidopsis* and are each comprised of three proteins. By contrast, six proteins define the expanded AGO1/AGO10 rice clade, while six proteins define the distinct AGO5 rice clade (Nonomura et al., 2007).

FUNCTIONS OF AGO PROTEINS

Two factors define the working body of RNA silencing complexes: an AGO protein and a small RNA, which bind to AGO proteins and regulate gene expression by acting as sequence-specific guides, leading AGO proteins to perfectly or partially complementary RNAs. Because the first characterized AGO protein catalyzed targeted RNA cleavage, AGO proteins have been referred to as Slicer proteins (Liu et al., 2004; Song et al., 2004). However, this term is not accurate to describe all AGO proteins because not all are capable of slicing and, instead, may repress gene expression by other mechanisms. In particular, in animals, most microRNAs (miRNAs) regulate their target RNA by preventing productive translation, leading to RNA destabilization or sequestration in specialized cytoplasmic bodies, a process that does not involve cleavage and permits the RNA to reenter the translation pathway (Bhattacharyya et al., 2006; Eulalio et al., 2008; Bartel, 2009). Because plant and animal cleavage targets generally exhibit extensive complementarity to the corresponding small RNAs (Rhoades et al., 2002; Yekta et al., 2004), whereas animal targets that are regulated at the translational level have only limited complementarity outside of the seed region (nucleotides 2 to 7) of the miRNA (Lewis et al., 2003, 2005), it has been proposed that the degree of complementarity between a small RNA and its target determines the type of regulation that occurs.

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However, a growing number of exceptions have diminished the value of this hypothesis.

In addition, it is worth mentioning that AGO-mediated target cleavage can instigate additional processes required to achieve the proper threshold or level of regulation. For example, RNA cleavage can be followed by either DNA methylation and heterochromatin restructuring in plants (Qi et al., 2006; Law and Jacobsen, 2010) and fungi (Wassenegger, 2005) or DNA elimination during the formation of the macronucleus in protists (Duharcourt et al., 2009).

There is wide diversity of small RNA-directed AGO function in plants. In *Arabidopsis*, the plant model species in which RNA interference pathways have been most thoroughly deciphered, transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) result from the action of specialized AGO proteins and specialized small RNAs (summarized in Figures 1 and 2). The major endogenous classes of small RNAs in *Arabidopsis* are 21- and 22-nucleotide small RNAs (including miRNAs, *trans*-acting small interfering [ta-siRNAs] and natural *cis*-acting siRNAs

[nat-siRNAs]), which primarily direct posttranscriptional gene silencing (PTGS), and 24-nucleotide small RNAs (including p4- and p5-siRNAs that require PolIV or both PolIV and PolV, respectively, for their production), which direct DNA methylation and transcriptional gene silencing (TGS) (Vaucheret, 2006; Chapman and Carrington, 2007). In addition, endogenous inverted repeats were recently shown to give rise to 21-, 22- and 24-nucleotide small RNAs that trigger TGS or PTGS depending on their length (Dunoyer et al., 2010a). All small RNAs are processed from their long RNA precursors by RNaseIII enzymes. There are four DICER-LIKE (DCL) RNaseIII proteins in *Arabidopsis*. DCL1 mainly processes miRNAs and some nat-siRNAs, although DCL2 also participates in nat-siRNA biogenesis, DCL3 processes 24-nucleotide p4- and p5-siRNAs, endo-inverted repeat siRNAs, and long miRNAs, and DCL4 processes ta-siRNAs, endo-inverted repeat siRNAs, and some young miRNAs. DCL2 and DCL4 also produce siRNAs from exogenous double-stranded RNAs (dsRNAs) produced by RNA viruses and transgenes (Xie and Qi, 2008; Liu et al., 2009). To accommodate these

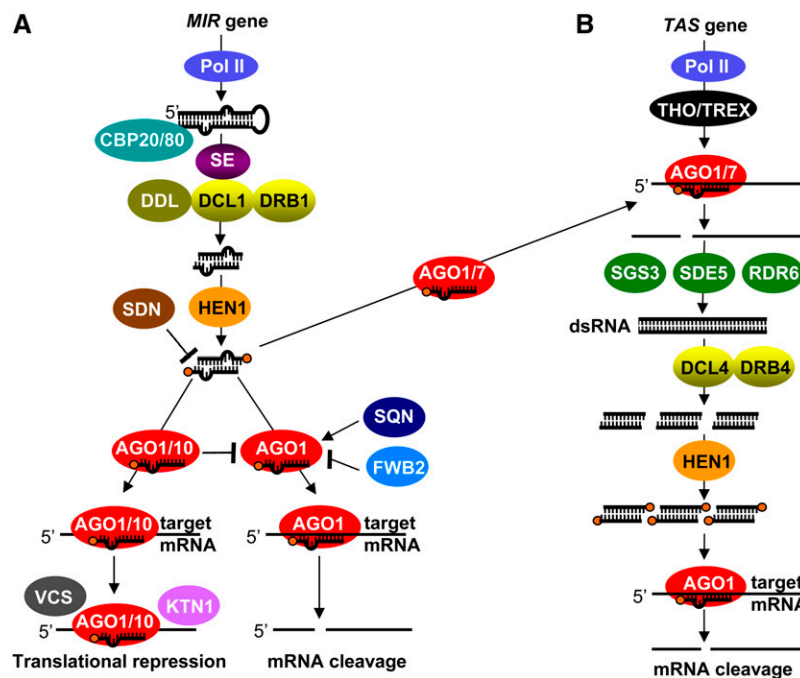


Figure 1. *trans*-Silencing: the miRNA and ta-siRNA Pathways.

(A) The miRNA pathway: *MIRNA* genes are transcribed by PolII into precursor RNAs that form hairpin stem-loops due to their self-complementary, a classical trait of miRNA precursors. Processing of miRNA precursors into a 21- to 22-nucleotide miRNA duplex with 2-nucleotide 3' overhangs requires Cap binding proteins CBP20 and CBP80, DDL (a forkhead protein), SE (a zinc finger protein), DRB1 (a dsRNA binding protein), and DCL1 (a RNaseIII). miRNA duplexes are methylated by HEN1 to protect against SDN exonuclease degradation. One strand of the miRNA duplex binds to either AGO1 or AGO10. The 21-nucleotide miRNAs guide AGO1 to partially complementary mRNA targets, and the PIWI (RNaseH-like) domain of AGO1 cleaves the target mRNAs. AGO1 is positively and negatively regulated by the cyclophilin protein SQN and the F-box protein FBW2, respectively, and negatively regulated by both AGO1 and AGO10. AGO1, AGO10, VCS, and KTN1 direct translational repression of RNA targets partially complementary to miRNAs. Although it is assumed that AGO10 binds to small RNAs, it has not been directly demonstrated. The role of VCS and KTN1 in this process is unclear. **(B)** The ta-siRNA pathway: Some miRNA-directed cleavage targets give rise to secondary ta-siRNAs. In these cases, 22-nucleotide miRNAs bound to AGO1 or miR390 bound to AGO7 (miR390 is the unique miRNA that binds to AGO7) guide cleavage of ta-siRNA precursors that are transcribed by PolII and rely on the THO/TREX RNA trafficking complex for their stability. Once cleaved, one of the two ta-siRNA precursor fragments is copied into dsRNA by RDR6, SGS3, and SDE5, and the dsRNA is cleaved into 21- to 22-nucleotide ta-siRNAs by the DRB4/DCL4 complex. The resulting ta-siRNAs are methylated by HEN1, and one strand of the duplex binds to AGO1 and directs cleavage of complementary mRNA targets.

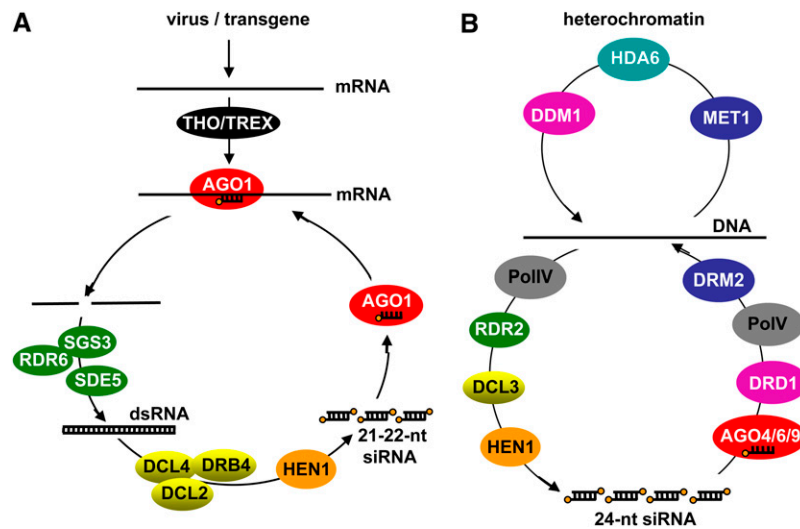


Figure 2. *cis*-Silencing: The Virus/Transgene and Heterochromatin siRNA Pathways.

(A) Virus and sense transgene PTGS: Viruses and transgenes can give rise to primary siRNAs by direct production of dsRNA (during RNA virus replication) or aberrant transcripts. Once a primary pool of siRNAs is produced, the PTGS process is initiated and an amplification loop is established whereby siRNA-guided AGO1 cleavage of viral or transgene transcripts leads to the RDR6/SGS3/SDE5-dependent production of dsRNA, which is processed by DCL4 or DCL2 into 21- to 22-nucleotide (nt) siRNAs that bind to AGO1 and guide cleavage of additional viral and transgene transcripts. Therefore, unlike *MIR* and *TAS* genes, viruses and transgenes both produce siRNAs and are subsequently targeted by these siRNAs.

(B) The 24-nucleotide siRNA heterochromatin pathway: Repeat DNA and transposons are transcriptionally silenced through DNA methylation and heterochromatin formation. Transcripts produced by PolIV are thought to be substrates for RDR2-mediated synthesis of dsRNA, which is processed by DCL3 into 24-nucleotide siRNAs. These siRNAs are methylated by HEN1 and loaded onto AGO4, AGO6, and AGO9, which direct DNA methylation through the DNA methyltransferase DRM2. DNA methylation of some loci also requires PolIV and the SWI2/SNF2-like chromatin remodeling factor DRD1. In addition to siRNA-dependent TGS, silencing of some loci is maintained in an siRNA-independent manner through the methyltransferase MET1, the histone deacetylase HDA6, and the SWI2/SNF2-like chromatin remodeling factor DDM1.

various classes of small RNAs, *Arabidopsis* encodes 10 AGO proteins. Transcriptional RNA silencing complexes involve AGO4, AGO6, and AGO9 and their associated 24-nucleotide small RNAs (Zilberman et al., 2003; Zheng et al., 2007; Havecker et al., 2010). By contrast, posttranscriptional RNA silencing complexes involve the cleavage-competent AGO1 and AGO7 proteins and their associated 21- to 22-nucleotide small RNA (either miRNA, ta-siRNA, or exogenously derived siRNA, such as those from viruses and transgenes) (Baumberger and Baulcombe, 2005; Qi et al., 2005; Montgomery et al., 2008a). AGO10 is required for translational control of several miRNA targets, including *AGO1* (Brodersen et al., 2008; Mallory et al., 2009). However, because an association of AGO10 with small RNA has not been shown, the mode of action of AGO10 remains elusive. Interestingly, AGO1 has been shown to perform translational repression of a miRNA-targeted transgene sensor (Brodersen et al., 2008), but it is not clear whether this mode of action is commonly used by AGO1 to regulate endogenous RNA targets. Moreover, the factors that determine whether AGO1 or AGO10 perform translational repression are not known.

DOMAINS OF AGO PROTEINS

AGO proteins are defined by three major functional domains: the PAZ, MID, and PIWI domains. X-ray crystallography of the

prokaryote *Thermus thermophilus* AGO protein revealed a bilobal structure. The N-terminal domain is composed of a variable N-terminal region, which may facilitate the separation of the small RNA:target duplex after slicing by interrupting the duplex structure, and the PAZ domain, which has been shown to anchor the 3' end of the bound small RNA. The C-terminal domain is composed of the MID and PIWI domains, which, due to a binding pocket at the junction of these domains, anchors the 5' end of the bound small RNA (Wang et al., 2008, 2009; Parker, 2010). Together, all three domains participate to correctly position the small RNA sequence relative to the RNA target.

The structure and catalytic site of the PIWI domain closely resembles that of an RNase H domain-containing protein from *Bacillus holodurans* (Song et al., 2004), and, through mutational analyses that deactivate the catalytic DDH or DDD amino acid core, the PIWI domain of AGO has been shown to execute the slicing of RNA targets. For example, in line with mutational studies of human Ago2 (Liu et al., 2004; Rivas et al., 2005), mutating Asp at position 760 of *Arabidopsis* AGO1, which is equivalent to the Asp residue at position 597 of human Ago2, to Ala rendered the AGO1D760A protein cleavage deficient, although the mutant protein was still able to bind small RNAs (Baumberger and Baulcombe, 2005). Likewise, mutating His-798, which is highly conserved among AGOs, to Pro produced a similar result. However, other properties beyond the catalytic amino acid core may

contribute to the cleaving efficiency of the PIWI domain. One such property was revealed by the crystal structure of the *T. thermophilus* AGO protein. It appears that two Arg residues (Arg-172 and Arg-548) contribute to the stabilization of the small RNA guide strand between nucleotides 10 and 11 (Wang et al., 2009), positions previously shown to be important for AGO cleavage activity in plants and animals (Elbashir et al., 2001; Mallory et al., 2004). Although these amino acids clearly appear to influence the small RNA:target duplex structure, they are not conserved among AGO proteins, putting into question their widespread importance to cleavage. In line with this, human Ago3, which has a DDH domain, does not appear to be cleavage competent, suggesting that a DDH domain per se is not sufficient for cleavage activity (Liu et al., 2004; Meister et al., 2004). Similarly, replacing the *Arabidopsis* AGO1 MID-PIWI domain with the MID-PIWI domain of AGO10, both of which possess the DDH catalytic core, does not restore silencing activity in an *ago1* hypomorphic mutant (Mallory et al., 2009), again implying that the DDH amino acid core is not sufficient to impart cleavage activity and that additional properties of the AGO1 MID-PIWI domain contribute to silencing activity.

Interestingly, it has been shown in animals that Ago2 can cleave pre-miRNA to generate the Ago2-cleaved precursor miRNA, suggesting that, in addition to carrying out the post-transcriptional regulatory activity of miRNAs, Ago proteins have a direct role in the maturation of miRNAs (Diederichs and Haber, 2007). Indeed, mouse miR-451 production is Drosha dependent but Dicer independent. Instead of being cleaved by Dicer, the pre-miR451 is cleaved by Ago2 and then further trimmed to generate the mature miR-451 (Cheloufi et al., 2010). The observation that Ago2 cleavage activity is essential for the production of some miRNAs helps to explain why catalytically inactive Ago2 transgenic mice are not viable and may help to explain why the cleavage activity of Ago2 is retained even though nearly all miRNAs direct translational repression rather than RNA cleavage. Whether AGO proteins play a role in *MIRNA* processing in plants is not known, although the accumulation of a subset of miRNAs is reduced in null *ago1* mutants (Vaucheret et al., 2004).

FACTOR INFLUENCING THE PARTITIONING OF SMALL RNAs INTO AGO COMPLEXES

In both animals and plants, immunoprecipitation of AGO proteins followed by pyrosequencing of their associated RNAs has revealed that numerous AGO proteins interact with abundant small RNA populations that vary greatly in sequence. For example, in *Drosophila*, AGO1 is the AGO protein involved in the miRNA pathway, whereas AGO2 functions in siRNA pathways (Okamura et al., 2004). Sorting of small RNAs into these AGOs is influenced by the small RNA duplex structure. Indeed, miRNA duplexes contain mismatches that are necessary for their incorporation into AGO1, whereas siRNAs are perfectly matched and bind to AGO2. Altering siRNA duplexes to include mismatches facilitates their association with AGO1 (Förstemann et al., 2007).

By contrast, immunoprecipitation of *Arabidopsis* AGO1, AGO2, AGO4, AGO5, AGO6, AGO7, and AGO9 has revealed that 5' nucleotide identity, together with small RNA length, contribute to

the sorting of small RNAs into these AGO proteins (Kim, 2008). For example, the majority of small RNAs associated with AGO1 have a 5' U and are 21 or 22 nucleotides long (Mi et al., 2008), whereas most small RNAs associated with AGO2 have a 5' A and are 21 nucleotides long (Mi et al., 2008; Montgomery et al., 2008a; Takeda et al., 2008). AGO4, AGO6, and AGO9 preferentially associate with small RNAs that are 24 nucleotides long and begin with a 5' A (Mi et al., 2008; Havecker et al., 2010), whereas AGO5 selectively associates with small RNAs that are 24 nucleotides long and begin with a 5' C (Takeda et al., 2008). Supporting the importance of small RNA length in determining their association with AGO proteins, certain loci give rise to small RNAs that vary in length and, as a function of this length, can repress gene expression either through PTGS or targeted chromatin remodeling through TGS depending on the AGO protein with which they associate. For example, rice pri-miR1850 produces both a DCL1-dependent 21-nucleotide miR1850 (termed canonical miRNA) and a DCL3-dependent 24-nucleotide miRNA sequence (termed long miRNA), which are arranged in tandem in the precursor sequence (Chellappan et al., 2010; Wu et al., 2010). Analysis of the distribution of canonical and long miRNAs associated with different AGO complexes revealed a preferential sorting of canonical miRNAs into AGO1 complexes (complexes that typically direct PTGS) and long miRNAs into AGO4 complexes (complexes that typically direct TGS). Indeed, some long miRNAs can direct sequence-specific DNA methylation at loci from which they are produced (Chellappan et al., 2010; Wu et al., 2010).

Of course, size and 5' nucleotide identity rules are not hard and fast. There are exceptions. For example, AGO7 exhibits a unique behavior by associating exclusively to miR390, a 5' A 21-nucleotide long miRNA that was expected to associate with AGO2 (Montgomery et al., 2008a). AGO7 also is unique because it forms a complex with the *TAS3* ta-siRNA precursor at a miR390 complementary site that cannot be cleaved due to mismatches at the center of the miR390:*TAS3* target RNA duplex (Axtell et al., 2006). Although the exact action of AGO7 on this sequence is not known, the miR390-AGO7-*TAS3* association at this site is absolutely required for *TAS3* ta-siRNA production. Indeed, replacing the noncleavable miR390-AGO7-*TAS3* module by various noncleavable miRNA-AGO1-*TAS3* modules prevents ta-siRNA production (Montgomery et al., 2008a).

AGO1 BINDS 22-NUCLEOTIDE miRNAs AND TRIGGERS SECONDARY siRNA PRODUCTION

The majority of canonical miRNAs are 21 nucleotides long and primarily associate with AGO1 to guide cleavage of partially complementary target RNAs. Once the target RNAs are cleaved, the 5' and 3' cleavage products are degraded by the 3'-5' exosome and 5'-3' exoribonucleases, respectively (Souret et al., 2004; Chekanova et al., 2007). However, some miRNAs are processed as 22-nucleotide small RNAs (in many cases this is due to asymmetric bulges in the stem-loop precursor) (Figure 3). Indeed, removing asymmetric bulges through mutagenesis lead to the production of canonical 21-nucleotide miRNAs (Chen et al., 2010; Cuperus et al., 2010). Like 21-nucleotide miRNAs, 22-nucleotide miRNAs associate with AGO1 and direct RNA target

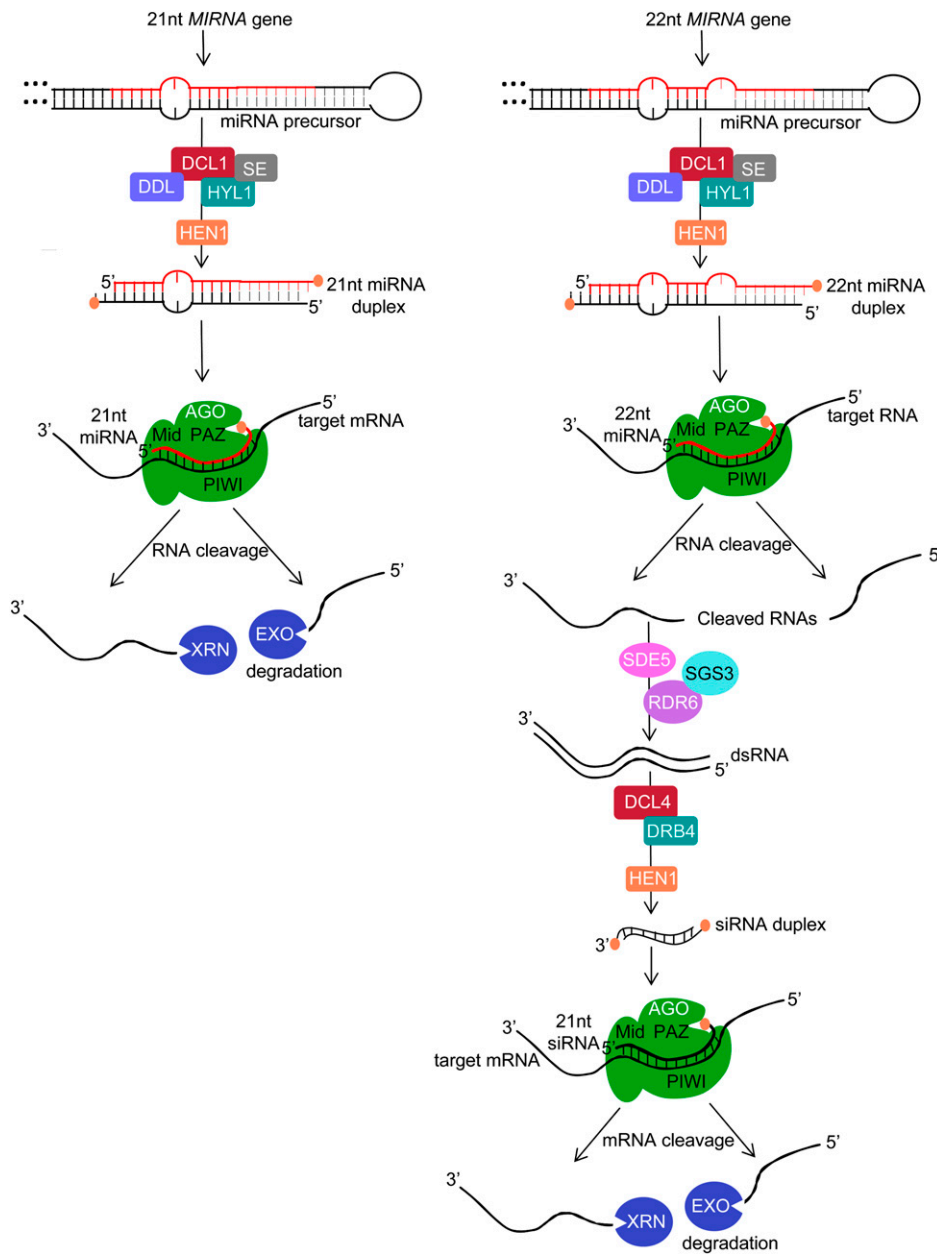


Figure 3. 21-Nucleotide miRNA versus 22-Nucleotide miRNA-Guided AGO1 Regulation in Plants.

Processing of miRNA precursors can give rise to either 21- or 22-nucleotide (nt) long miRNAs, depending on the structure of the miRNA precursor. Unlike 21-nucleotide miRNA precursors, 22-nucleotide miRNA precursors often have an asymmetric bulge in the miRNA duplex. In both cases, miRNA processing depends on the RNaseIII DCL1, the dsRNA binding protein HYL1, the zinc finger domain protein SERRATE, and the forkhead-associated domain protein DAWDLE. To protect against small RNA degrading enzymes, the miRNA duplex is methylated on each 3' end by the methylase HEN1. The mature miRNA strand then binds to AGO1 and guides RNA cleavage of partially complementary target RNAs (the main mode of action of the 21-nucleotide miRNA:AGO1 complex appears to be RNA cleavage, but both AGO1 and AGO10 have been shown to repress translation in a handful of cases). The majority of 21-nucleotide miRNA targets are mRNAs that code for transcription factors and developmental regulators. By contrast, 22-nucleotide miRNA generally target non-protein coding RNAs, such as the *TAS* RNAs. The target cleavage products generated from 21-nucleotide miRNA-directed cleavage are degraded by the 5'- to 3'-exoribonuclease (XRN4) and the exosome (EXO), whereas 22-nucleotide miRNA-directed cleavage leads to the recruitment of the putative RNA trafficking protein SDE5, the RNA binding protein SGS3, and the RNA-dependent RNA polymerase RDR6, which together produce dsRNA that is processed to 21-nucleotide siRNA duplexes (or occasionally 22-nucleotide duplexes) by the RNaseIII DCL4 and its dsRNA binding partner DRB4. *ta*-siRNA production from the 3' cleavage product generated by miR173-directed cleavage of the *TAS1* and *TAS2* precursors is shown as an example. The *ta*-siRNA duplexes are methylated on each 3' end by the methylase HEN1, and then one strand binds to AGO1 and guides cleavage of complementary target mRNAs, which, in all known cases, are protein-coding transcripts.

cleavage, but, rather than being degraded, it was shown that AGO1:22-nucleotide miRNA complexes are uniquely competent to instigate the production of RDR6-dependent siRNAs, from the RNA target 3' cleavage product (Figure 3). Indeed, AGO1 complexed with the natural 22-nucleotide form of miR173 leads to cleavage of the *TAS1c* precursor and subsequent RDR6-dependent phased 21-nucleotide ta-siRNA production. By contrast, when the *MIR173* precursor was modified to produce a 21-nucleotide miR173 rather than the 22-nucleotide form, ta-siRNA production was greatly reduced, even though the 21-nucleotide miR173 form was still able to bind to AGO1 and direct cleavage of the *TAS1c* precursor at the canonical site (Chen et al., 2010; Cuperus et al., 2010). Similar results were obtained for two additional 22-nucleotide miRNAs, miR472 and miR828. In addition, the *MIR168a* and *MIR168b* loci encode different ratios of 21- and 22-nucleotide miR168 species in partially overlapping tissues (Vaucheret, 2009), which may contribute in a tissue-specific manner to the feedback regulation of AGO1 by secondary siRNA arising from miR168-guided AGO1 mRNA cleavage products (Mallory and Vaucheret, 2009). Thus, miRNA length can affect the overall outcome of AGO1-mediated cleavage, although the exact mechanism by which this occurs is not known.

Curiously, although miR390 is 21 nucleotides long, not 22 nucleotides, it efficiently directs ta-siRNA production from the *TAS3* RNA target (Montgomery et al., 2008a). Moreover, miR390-induced *TAS3* ta-siRNA derive from 5' cleavage products, whereas miR173-induced ta-siRNA derive from 3' cleavage products (Montgomery et al., 2008b; Felippes and Weigel, 2009). One reason for the capacity of the 21-nucleotide miR390 to direct ta-siRNA production could be that it associates with AGO7 rather than AGO1, suggesting that both 21-nucleotide miRNA:AGO7

and 22-nucleotide miRNA:AGO1 complexes have the capacity to attract RDR6. However, the unique properties that allow these complexes to efficiently trigger ta-siRNA production are not understood.

TRANSCRIPTIONAL REGULATION OF AGO GENES

Analysis of the expression profiles of the 10 *Arabidopsis* AGO genes using the ATH1 array revealed varying levels and patterns of expression (Schmid et al., 2005) (Figure 4). Consistent with their respective essential roles in PTGS and TGS, AGO1 and AGO4 are widely expressed. By contrast, the highly specific expression profiles of AGO5 and AGO9 are consistent with the gamete-specific defects of rice *ago5* and *Arabidopsis ago9* mutants (Nonomura et al., 2007; Olmedo-Monfil et al., 2010). These data suggest that the specific actions of the different AGO is not only based on small RNA length and 5' nucleotide identity but also may be restricted by the limited expression domain of certain AGOs.

Reporter-based analysis has been used to compare the expression patterns of AGO proteins within two different clades in *Arabidopsis*: the AGO1/AGO5/AGO10 clade and the AGO4/AGO6/AGO9 clade. *pAGO10:YFP-AGO10* and *pAGO1:CFP-AGO1* reporter constructs, which rescued the corresponding mutant developmental defects and thus encoded functional proteins, indicated that AGO10 is initially expressed throughout the embryo but becomes limited to provascular strands and the adaxial sides of the cotyledons at about the globular stage. By contrast, AGO1 is expressed in the whole embryo with the strongest signal in the provascular cells from globular stage to

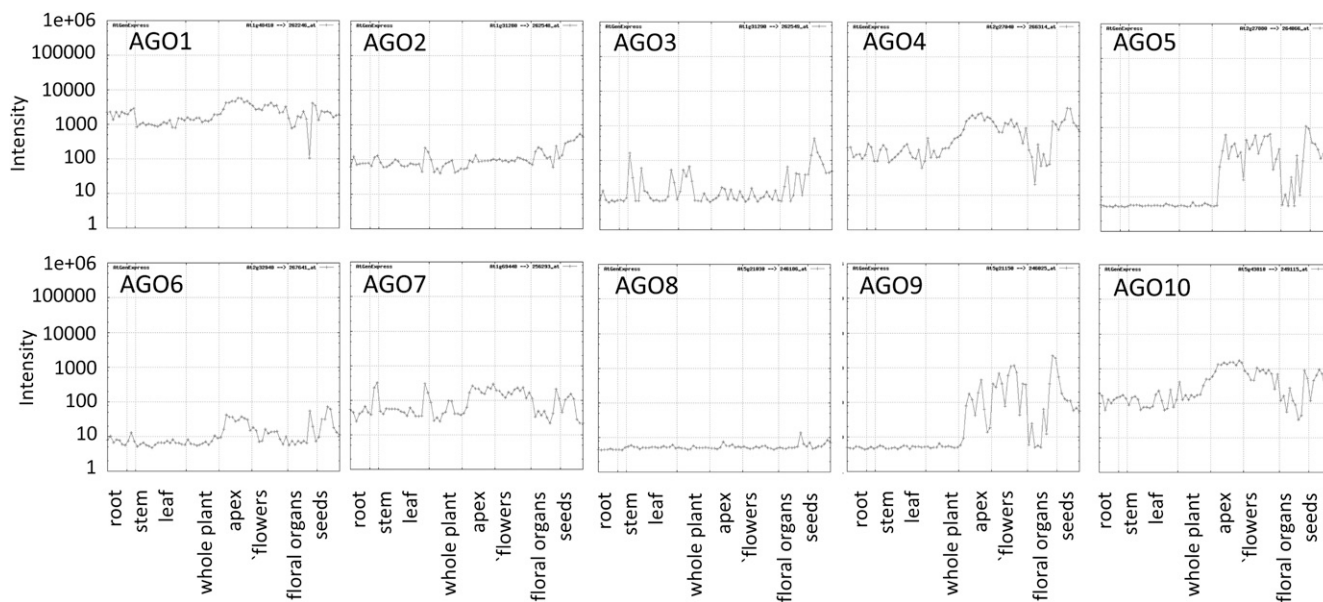


Figure 4. Expression Intensities of the 10 *Arabidopsis* AGO Genes.

ATH1 array expression profiles of the 10 *Arabidopsis* AGO genes. Expression data were retrieved using the AtGenExpress Visualization Tool. The expression of each AGO gene is shown at various developmental stages and in different tissues. Normalization methods, the tissue, and the developmental stages of each sample as well as additional information can be found at <http://jsp.weigelworld.org/expviz/expviz.jsp>.

early torpedo stage. Thus, AGO1 and AGO10 expression patterns overlap partially, with the AGO1 expression pattern being broader than that of AGO10 (Mallory et al., 2009). Furthermore, analysis of *pAGO1:GUS* expression in adult transgenic plants indicated that AGO1 is expressed throughout development, although expression is higher in meristematic and vascular tissues than in the rest of the plant (Vaucheret et al., 2006). Similar to AGO1, analysis of *pAGO4:GUS* transgenic plants revealed widespread expression in embryos, leaves, and flowers. By contrast, *pAGO6:GUS* expression is restricted to shoot and root growth points and the vascular tissue connecting these domains, and *pAGO9:GUS* expression is restricted to the embryonic shoot apex region and developing ovules (Havecker et al., 2010).

Expression profiling of the small RNAs bound to AGO4, AGO6, and AGO9 revealed that each AGO binds 24-nucleotide siRNAs that have a 5' adenosine and that derive from repeat and heterochromatic loci. However, each of these three AGOs showed a locus preference, likely due to their different expression patterns. To test this hypothesis, *ago4* mutants were transformed with *AGO4*, *AGO6*, and *AGO9* expressed from the *AGO4* promoter, and the profile of small RNA bound to these proteins was determined. When expressed from the *AGO4* promoter, both AGO6 and AGO9 had shifted siRNA binding profiles that closely resembled, but were not identical to, that of AGO4 and each other, suggesting that the spatial expression of these AGO proteins has an important contribution to determining siRNA binding but that other determinants influence small RNA associations (Havecker et al., 2010). As the small RNA profiles and expression patterns of additional AGO proteins become available, it will be interesting to compare them to the expression patterns of their target RNAs to understand more globally the limit of small RNA action and whether small RNA mobility plays a widespread role in the control of endogenous gene expression. Indeed, recent experiments have demonstrated that small RNAs are mobile through graft junctions (Dunoyer et al., 2010b; Molnar et al., 2010), raising the interesting possibility that their bound AGO proteins could also be trafficked from cell to cell or over long distances.

POSTTRANSCRIPTIONAL REGULATION OF AGO mRNA

Two *Arabidopsis* AGO genes encode mRNAs that are targeted by the miRNA pathway. *AGO1* mRNA is targeted by miR168 (Rhoades et al., 2002; Vaucheret et al., 2004), whereas *AGO2* mRNA is targeted by miR403 (Allen et al., 2005). Because the role of AGO2 has not been identified, the importance of miR403-mediated AGO2 regulation is not known. By contrast, miRNA regulation of AGO1 regulation has been more thoroughly investigated. AGO1 homeostasis is achieved by several regulatory loops, which permit the miRNA and siRNA pathways to function correctly. These loops include miR168-guided AGO1-mediated cleavage of *AGO1* mRNA (Vaucheret et al., 2004), increased accumulation of miR168 when AGO1 levels are high (Vaucheret et al., 2006), and translational repression of *AGO1* by AGO10 (Mallory et al., 2009). Moreover, homeostatic mechanisms for the correct functioning of *AGO1* in the siRNA pathway also exist and hinge on the production of a 21- or 22-nucleotide miR168 species (Vaucheret, 2009), which, after cleavage of *AGO1* mRNA, allows

the production of *AGO1* siRNAs that contribute to the regulation of *AGO1* mRNA level (Mallory and Vaucheret, 2009). Supporting the importance of miR168 in the regulation of AGO1, expression of a miR168-resistant version of *AGO1* causes developmental defects leading to the eventual death of the plant (Vaucheret et al., 2004). These defects can be suppressed by an artificial miRNA complementary to the miR168-resistant *AGO1* mRNA (Vaucheret et al., 2004), and the developmental defects of a null *ago1* mutant can be complemented by expressing the *AGO1* mRNA under the control of the *MIR168* promoter (Vaucheret et al., 2006), exemplifying the importance of the AGO1-miR168 regulatory module.

POSTTRANSLATIONAL REGULATION OF AGO PROTEINS

Although purified *Arabidopsis* AGO1 can cleave target RNA (Baumberger and Baulcombe, 2005), it does not exclude the possibility that additional proteins could modulate AGO1 cleavage activity in vivo. Supporting this hypothesis, mutants impaired in the SQN cyclophilin protein resemble hypomorphic *ago1* mutants and, as such, exhibit reduced miRNA accumulation, suggesting that SQN acts as a positive regulator of AGO1 (Schmid et al., 2005). Moreover, recent work showed that mutations in the F-box protein FBW2 suppress *sqn* mutations as well as hypomorphic *ago1* mutations. In addition, overexpression of FBW2 reduces AGO1 protein levels but not *AGO1* mRNA levels, suggesting that FBW2 acts as a negative regulator of AGO1 (Earley et al., 2010).

GW motif-containing proteins, some of which play a role in small RNA production, appear to be important for small RNA-mediated regulation through their interaction with AGO proteins. For example, *Arabidopsis* AGO4 interacts with the Pol V subunit NRPE1 through GW motifs located at the C-terminal domain of NRPE1 (El-Shami et al., 2007). Supporting this interaction, both proteins localize to nucleolus-associated bodies. In addition, AGO4 protein stability depends on upstream factors, such as the PolIV subunit NRPD1, RDR2, and DCL3, all of which are important for siRNA production, suggesting that AGO4 is stabilized through its association with siRNAs (Li et al., 2006).

The GW182 protein of *Caenorhabditis elegans*, *Drosophila*, and humans interacts with Ago proteins through its N-terminal GW repeats and is required for miRNA-mediated gene silencing (Eulalio et al., 2009; Tritschler et al., 2010). In animals, Ago proteins serve as adapters between small RNA, target mRNA, and the actual effectors of translational repression. Indeed, the PIWI domain of Ago proteins associates with GW proteins, which also associate with poly(A) binding proteins (PABPs). Association of GW proteins to PABPs counteracts the association of PABPs to eIF4G, which is required for translation initiation. The association of GW proteins to PABPs also counteracts the association between PABPs and the target RNA, allowing RNA deadenylation by the CAF1-CCR4-NOT complex, which, together with eIF4G displacement, promotes RNA decapping, resulting in the inhibition of translation (Tritschler et al., 2010). Supporting the hypothesis that small RNA:Ago complexes play the role of selective adapters, Ago function can be bypassed by tethering GW proteins directly to the cognate small RNA target (Pillai et al., 2004). In line with this, both the cleavage and translational repression

activities of *Arabidopsis* AGO1 (Brodersen et al., 2008) could be influenced by AGO1 interacting partners. Because AGO1 alone can cleave target RNA in vitro (Baumberger and Baulcombe, 2005), cleavage activity could be considered the default activity of AGO1. Therefore, AGO1 might exhibit translational repression activity only when associated with proteins that inhibit cleavage activity, forcing it to function in a manner similar to its closest paralog, AGO10, which appears to be devoid of cleavage activity despite the existence of the canonical DDH cleavage motif (Mallory et al., 2009).

Hsp90, a chaperone protein necessary for the proper folding and function of many proteins, interacts with human Ago2 and plant AGO1 in vitro and is important for stabilizing and recruiting human AGO2 to P-bodies (Pare et al., 2009; Iki et al., 2010; Johnston et al., 2010). Recently, it was shown that Hsp90 is required for siRNA duplexes to be loaded onto human Ago2 and plant AGO1 in vitro, suggesting that a general hallmark of RNA-induced silencing complex assembly involves Hsp90-directed AGO conformational changes that permit AGO proteins to associate with siRNAs to form active RNA-induced silencing complexes (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010).

Finally, in animals, hydroxylation and phosphorylation of Ago2 enhance its localization to cytoplasmic foci called P-bodies where miRNA- and siRNA-mediated silencing is supposed to occur (Heo and Kim, 2009). Whether P-bodies also support RNA silencing in plants is unknown, and whether hydroxylation or phosphorylation can affect the stability and localization of plant AGO proteins, in particular AGO1, the ortholog of animal Ago2, has not been reported.

AGO-VIRUS INTERACTIONS

In addition to regulating endogenous gene expression through miRNAs, AGO1 also plays a critical role in viral defense by binding viral siRNA to form the major antiviral RNA silencing complex (Azevedo et al., 2010). Accordingly, *ago1* mutants exhibit hypersusceptibility to virus infection (Morel et al., 2002). Induction of AGO1 mRNA accumulation is a general response to virus infection (Zhang et al., 2006; Csorba et al., 2007; Havelda et al., 2008), which should be expected to enhance virus resistance. However, most viruses counteract plant PTGS defenses through various evolutionarily refined strategies. Several viruses, including *Rose mosaic virus*, *Cucumber mosaic virus*, *Turnip crinkle virus*, *Crucifer-infecting tobacco mosaic virus*, and *Cymbidium ringspot virus*, achieve PTGS suppression by inducing miR168 expression, which in turn triggers translational repression of AGO1 through AGO10 (Várallyay et al., 2010). In the case of CymRSV, it is the viral suppressor protein (VSR) P19 that induces miR168 accumulation. Indeed, infection by CymRSV induces AGO1 mRNA and miR168 accumulation, resulting in AGO1 protein downregulation. By contrast, infection by Cym19stop, which is defective for P19, induces AGO1 mRNA but not miR168 accumulation, resulting in increased AGO1 protein levels and recovery from virus infection (Várallyay et al., 2010).

VSRs can inhibit AGO1 in several other ways. For example, *Cucumber mosaic virus 2b* binds AGO1 and inhibits its cleavage

activity (Zhang et al., 2006), whereas *Turnip crinkle virus* P38 and SPMMV P1 have GW motifs that allow competitive binding to AGO1 and inhibition of AGO1 activity (Azevedo et al., 2010; Giner et al., 2010). Furthermore, both BWYV P0 and PVX P25 target various AGO, including AGO1, for degradation. Whereas P25-mediated degradation acts through the proteasome pathway (Chiu et al., 2010), P0-mediated degradation is insensitive to inhibition of the proteasome pathway (Baumberger et al., 2007; Bortolamiol et al., 2007). Although some VSRs directly target AGO1, some viruses encode VSRs that do not directly target AGO1 but inhibit RNA silencing steps related to AGO1. For example, CIRV P19, BYV P21, and TEV HC-Pro bind siRNA duplex and prevent them from being loaded onto AGO1 (Chapman et al., 2004; Lakatos et al., 2006).

CONCLUSIONS

Although several recent discoveries have added to our knowledge of the roles of AGO proteins during gene regulation, there are some important questions that still remain unanswered. Do AGO proteins play a role in the processing of plant miRNA precursors similar to what has been shown in animals? What are the factors that determine whether AGO proteins will ultimately cleave or direct translational repression of their targets in plants? Most known plant small RNA targets have been identified based on high complementarity to the small RNA. Are there other types of target sites that can be efficiently regulated by small RNAs? Are these targets conserved among plant species? Do AGO proteins participate in the cell-to-cell or systemic trafficking of small RNAs in plants? Do *Arabidopsis* AGO2, AGO3, AGO5, and AGO8 participate to small RNA-directed regulation? Certainly these questions as well as others will be answered in the coming months as researchers continue to “unsilence” the mysteries of RNA silencing.

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