Evolution, Functions and Mysteries of Plant ARGONAUTE Proteins

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Abstract

ARGONAUTE (AGO) proteins bind small RNAs (sRNAs) to form RNA-induced silencing complexes for transcriptional and post-transcriptional gene silencing. Genomes of primitive plants encode only a few AGO proteins. The *Arabidopsis thaliana* genome encodes ten AGO proteins, designated AGO1 to AGO10. Most early studies focused on these ten proteins and their interacting sRNAs. AGOs in other flowering plant species have duplicated and diverged from this set, presumably corresponding to new, diverged or specific functions. Among these, the grass-specific AGO18 family has been discovered and implicated as playing important roles during plant reproduction and viral defense. This review covers our current knowledge about functions and features of AGO proteins in both eudicots and monocots and compares their similarities and differences. Based on these features, we propose a new nomenclature for some plant AGOs.

Introduction

In eukaryotes, sRNA-mediated gene silencing is critical for developmental regulation, responses to environmental stimuli, and epigenetic control of transposable elements (TEs) [1,2]. All types of described sRNAs are known to associate with AGO proteins to form RNA-induced silencing complexes (RISCs). Each RISC is guided by the bound sRNAs to achieve specific interactions with target transcripts based on sequence complementarity. These interactions can result in mRNA cleavage, translational repression, or chromatin modification [3,4]. Based on their functional domains, eukaryotic AGO proteins can be divided into two major groups: the AGO and the PIWI subfamilies. PIWIs and their interacting sRNAs (piRNAs) are both found predominantly in animal germ lines [5]. Plant genomes encode multiple AGO proteins, all in the AGO subfamily [3,4].

Taking advantage of sequenced plant genomes, we examined the AGO phylogenetic relationships using four selected angiosperms (two eudicots, two grasses) and utilized several basal non-flowering plant taxa to track the expansion of AGOs over a long period of plant evolution (Figure 1). The Arabidopsis genome encodes ten AGO proteins [3], but this number is increased in other flowering plants: 22 AGO proteins in soybean (*Glycine max*, a paleopolyploid) [6], 19 in rice (*Oryza sativa*) [7], and 17 in maize (*Zea mays*) [8,9] (Figure 1). All flowering plant AGOs group into three major clades: AGO1/5/10, AGO2/3/7, and AGO4/6/8/9 [3]. For simplicity, we designated these as clades I, II, and III (Figure 1). The grasses exhibit an expanded first clade and equivalent or similar size second and third clades. Additionally, grasses have an extra sub-clade – AGO18, a deep branch in the AGO1/5/10 clade (Figure 1). Unless specified, the term “AGO” in this review is referring to a class of AGOs in all flowering plants, while particular AGOs in a species are named with two letters representing the abbreviated species names, for example, AtAGO for an Arabidopsis AGO protein.

Features and functions of plant AGO proteins

The AGO family expanded during plant evolution with numerous duplications and losses [10]. In ancient unicellular or multicellular green algae (*Micromonas pusilla*, *Volvox carteri*, *Coccomyxa subellipsoidea*, *Chlamydomonas reinhardtii*), few (≤ 3) AGO genes are present [11]. The family expanded to seven members in mosses (*Physcomitrella patens*) and lycophytes (*Selaginella moellendorffii*), and further to ten or more members in flowering plants (Figure 1). The expansion of the plant AGO family suggests a functional diversification of AGO proteins presumably reflecting expanding sRNA-directed regulatory pathways.

Our understanding of AGO functions and their small RNA binding preferences is informed by sequencing of sRNAs associated with AGO proteins and by functional analysis of *ago* mutants in Arabidopsis and other plants. In Table 1, well-characterized plant AGO proteins are listed with their interacting sRNAs, binding preferences, and demonstrated functions. Because of the rapid rate of whole genome sequencing, AGO protein identification via genome annotation has outpaced functional characterization, the latter typically done via mutant analysis [6-9]. Because of the errors in genome

annotation and the naming history, AGO nomenclature in flowering plants is somewhat inconsistent and confusing. We propose a phylogeny-based nomenclature and list names assigned in previous literature, as a guide to published analyses (Table 2).

Clade I --- AGO1/5/10

AtAGO1 is the founding member of the *AGO* gene family, so named because Arabidopsis *ago1* mutants resemble the tentacles of an *Argonauta* squid [12]. AGO1 preferentially associates with sRNAs with a 5' uridine; it plays an essential role in miRNA-mediated regulation of development and stress responses [12-15]. Although only one *AGO1* exists in *A. thaliana*, four *AGO1* homologs have been identified and characterized in rice [15]. Deep sequencing of sRNAs bound in OsAGO1a, OsAGO1b, and OsAGO1c complexes in seedlings showed that most miRNAs are evenly distributed in the three AGO1 complexes tested, but a subset of miRNAs are specifically incorporated into or excluded from one of these three AGO1s [15]. These observations suggest that there are both redundant and specialized functions among the rice *AGO1* genes. The binding specificity of OsAGO1d has not yet been analyzed.

AtAGO10 is closest to *AtAGO1*, and it regulates shoot apical meristems by sequestering miR165/166 [16-18]. The miR165/166 family is produced from nine Arabidopsis loci and targets class III homeodomain-leucine zipper (HD-ZIP III) transcription factors. *AtAGO10* recognizes distinct structural features in miR165/166 duplexes that are absent from other miRNAs and it has a higher affinity for miR166 than does *AtAGO1*. As a result, *AtAGO10* specifically sequesters miR165/166, preventing their loading into *AtAGO1*, and thereby upregulating *HD-ZIP III* transcripts [16-18]. One and two AGO10 homologs were identified in rice and maize, respectively [7,8], while eight were found in soybean (Figure 1) [6]. The expansion of the GmAGO10 family likely co-evolved with the expansion of the miR165/166 family, as 21 copies of miR165/166 are annotated in the soybean genome [19,20].

AtAGO5 is specifically expressed in the somatic cells surrounding megaspore mother cells and in megaspores [21]. While Arabidopsis *ago5* null mutants lack obvious defects, a semi-dominant allele is impaired in initiation of megagametogenesis [21]. The grass *AGO5* family has expanded, with five homologs in rice and three in maize (Figure 1). Among these, only *MEL1* (*MEIOSIS ARRESTED AT LEPTOTENE1*, *OsAGO5c*) has been well studied. Rice *mell/ago5c* mutants display abnormal periclinal cell division in the somatic tissues of anther lobes and later exhibit defects in pollen mother cells (PMCs) [22]. It is still unclear whether *MEL1* affects rice sporogenesis directly by acting in PMCs and meiocytes, or whether the earlier somatic defects indirectly cause meiotic arrest. Recently it was shown that *MEL1* binds to 21-nt phased small RNAs (phasiRNAs), a highly abundant sRNA group in grass anthers [23-25]. Like *AtAGO5*, *MEL1* binds preferentially to sRNAs with a 5' cytosine [23]. Interestingly, this preference was not observed in the total 21-nt phasiRNA population of rice or maize [24-27], suggesting that *MEL1* may not be the only AGO protein that binds 21-nt phasiRNAs.

The *AGO18* sub-clade has been found only in grass genomes. In rice, AGO18 protein is not detectable in normal seedlings but is induced by viral infection [28]. Overexpression

of *OsAGO18* confers broad-spectrum virus resistance in rice. A Tos17 retrotransposon insertional mutant of *ago18* is more sensitive to viral infection, although no other plant phenotypes were observed [28]. Interestingly, viral siRNAs are highly enriched in OsAGO1 complexes but only account for a small percentage of sRNAs bound to OsAGO18 [28]. Therefore, OsAGO18 regulates or indirectly contributes to antiviral defense response by a strategy other than directly binding viral siRNAs.

In maize, two copies of *AGO18* exist, one of which (*ZmAGO18b*) is enriched in the tapetum and germinal cells during male meiosis [9]. This pattern of expression mimics the accumulation of the 24-nt phasiRNAs that are derived from unique intergenic regions and are processed by DICER-LIKE 5 (DCL5)/DCL3b (originally named DCL3b, renamed as DCL5 thereafter) [25,26]. DCL5, AGO18, as well as the 24-nt phasiRNAs are all absent from eudicot genomes analyzed to date [25,29]. Based on the co-expression of *ZmAGO18* and 24-nt phasiRNAs, and the coincident novelty of their appearance, *ZmAGO18b* is hypothesized to bind the 24-nt phasiRNAs [25]. How and by virtue of what selective pressures this apparently novel sRNA pathway evolved in grasses is still a mystery.

Clade II --- AGO2/3/7

In Arabidopsis, AGO2 primarily associates with miRNAs with a bias for a 5' adenosine [14,30] and functions in antibacterial immunity [31]. Besides miRNAs, it also binds virus-derived small interfering RNAs (vsiRNAs) [32] and small RNAs induced by double-strand DNA breaks (diRNAs) [33]. *AtAGO3* is very similar to *AtAGO2*, and they are 3 kb apart in a direct tandem repeat [3]. Neither rice nor maize encodes an *AGO3* ortholog, but both have a pair of similar *AGO2* genes, designated *AGO2a* and *AGO2b* [7,9].

The function of the third member of this clade is better known and highly conserved: AGO7 predominantly binds miR390 and triggers the generation of *TAS3*-derived *trans*-acting siRNAs (tasiRNAs). *TAS3*-tasiRNAs target several *AUXIN RESPONSE FACTORS* and regulate dorsiventral leaf patterning and lateral organ development in flowering plants [34-38].

Clade III --- AGO4/6/8/9

Arabidopsis AGO4 primarily binds 24-nt, repeat and heterochromatin-associated siRNAs and functions in RNA-directed DNA methylation (RdDM) [39-40]. Transcripts from RNA POLYMERASE IV (Pol IV) are copied into double-stranded RNAs (dsRNAs) by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2). The dsRNAs are processed by DCL3 into 24-nt siRNAs and incorporated into AtAGO4. These AtAGO4-bound siRNAs guide the targeting of nascent scaffold transcripts from RNA POLYMERASE V by sequence complementarity and recruit DNA methyltransferase activity to mediate *de novo* methylation [41].

Besides the RdDM pathway directed by 24-nt siRNAs, a recent report found that Arabidopsis 21-nt tasiRNAs can also direct RdDM [42]. Instead of being processed by DCL4 and loaded into AGO1 for target mRNA cleavage in *trans*, these tasiRNAs are

processed by DCL1 and loaded into AtAGO4 or AtAGO6; these RISCs direct methylation of TAS loci [42]. Pol II transcripts from TEs can be converted to dsRNAs by RDR6 to produce 21-22 nt endogenous siRNAs (endo-siRNAs), which can be loaded into AtAGO1 for post-transcriptional gene silencing [43,44] or loaded into AtAGO6 to promote methylation of transcriptionally active TEs [45]. DNA methylation directed by both tasiRNAs and endo-siRNAs is independent of Pol IV, DCL3 and RDR2, but does depend on RDR6 [41].

In Arabidopsis, AGO9 controls female gamete formation by repressing the specification of germ cell fate in the somatic companion cells [46]. *ago9* mutants of *A. thaliana* contain multiple megaspore mother cells. AtAGO9 preferentially interacts with 24-nt sRNAs derived from TEs and is necessary to silence TEs in female gametes [46]. *AtAGO8* and *AtAGO9* are very similar in sequence [3], but *AtAGO8* transcripts are barely detectable in all tested tissues; *AtAGO8* is proposed to be a pseudogene [4].

ZmAGO9 (initially named *ZmAGO104* in the literature, Table 2) is also specifically expressed in the somatic cells surrounding the precursors of the gametic cells [47]. But unlike AtAGO9 which represses germ cell fate in the somatic cells, ZmAGO9 represses somatic cell fate in the germ cells. The *ago104* mutant exhibits unreduced (diploid) gametes, abnormal chromatin condensation during meiosis, and hypomethylated centromeric and knob-repeat DNA at non-CG sites [47]. In rice, an AGO member (previously OsAGO4b) shows a close homology to ZmAGO9 (Figure 1), we therefore suggest renaming it as OsAGO9.

Why so many AGOs?

In ancient alga, all AGOs belong to a single group, which diversified into distinct clades in bryophytes (Figure 1). The presence of these AGO clades in primitive plants suggests that sRNA-guided processes were already well-established long before the seed plants. Ferns (present ~200 mya prior to flowering plants) are a major missing link in terms of genome sequences, required for connecting the lycophytes to the more recent gymnosperms and angiosperms. Within the angiosperms, the major groups of AGO proteins have all been retained; certain subgroups have expanded differently in monocots and eudicots (Figure 1).

Genome duplication is one explanation for the proliferation of the AGOs. For example, soybean is a paleopolyploid [48] and its genome encodes two copies of AGO1, AGO2, AGO4/9, AGO5, AGO6, and AGO7 (Figure 1). Gene duplication events and promoter subfunctionalization could generate cell type specificity or the ability to respond to specific conditions. *AtAGO2* and *AtAGO3*, *AtAGO8* and *AtAGO9*, *OsAGO2a* and *OsAGO2b*, *OsAGO5a* and *OsAGO5e*, as well as *OsAGO4a* and *OsAGO4b* have all been suggested to arise from gene duplication [3,7]. These now-diverged genes have non-redundant expression profiles [4,7] and hence are amenable to genetic disruption in single mutants. Functional specialization of AGOs could also occur to bind a certain type of sRNAs for a specific function, as exemplified by the specialization of AGO10-miR165/166 and of AGO7-miR390 [2].

Although *AGO* genes have been extensively studied in *Arabidopsis*, only a few members of the AGO family have been analyzed in depth in the monocots and other eudicots (Table 1). In those species, many fundamental questions regarding plant AGOs remain to be answered. Do they function exactly the same as or similarly to their homologs in *Arabidopsis*? If multiple homologs exist, do they have redundant or specific roles? Why are some genes, such as *OsAGO17*, found only in rice and are missing in other species tested? From where did the grass-specific AGO18 originate? Biochemical studies are also a high priority for future research: Are all sRNAs bound to an AGO? Can RISC functions be redirected by exchange of sRNAs?

In summary, the diversification of sRNA-mediated regulation in plants is a fascinating story. This regulation is mediated by AGO proteins bound to guide sRNAs. In this review we have attempted to summarize what is currently known about the phylogeny of AGO proteins in flowering plants, the diverse roles and preferences of the individual AGO types, and the directions for future research.

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Table 1 Characterized AGO proteins in plants *

Clade	AGO name	Function	5'-nucleotide preference	sRNA bound	Reference
Clade I: AGO1/5/10	AtAGO1	plant development regulation and stress responses	U	21-nt miRNAs	12, 14
	OsAGO1a	miRNA-directed target gene regulation	U	21-nt miRNAs	15
	OsAGO1b	miRNA-directed target gene regulation	U	21-nt miRNAs	15
	OsAGO1c	miRNA-directed target gene regulation	U	21-nt miRNAs	15
	AtAGO5	initiation of megagametogenesis	C	siRNAs	14,21
	OsAGO5c	regulation of cell division of premeiotic germ cells	C	21-nt siRNAs	22,23
	AtAGO10	regulation of shoot apical meristems	n/d	miR165/166	16-18
	OsAGO18	broad-spectrum virus resistance	n/d	n/d	28
	ZmAGO18b	tapetum and germ cell development	n/d	n/d	9,25
Clade II: AGO2/3/7	AtAGO2	antibacterial immunity, viral defense, and DSB-induced sRNAs activity	A	21-nt miRNAs, vsiRNAs, diRNAs	30-33
	AtAGO3	n/d	n/d	n/d	
	AtAGO7	TAS3-derived tasiRNA biogenesis	n/d	miR390	34
	OsAGO7	TAS3-derived tasiRNA biogenesis	n/d	miR390	35,38
	ZmAGO7	TAS3-derived tasiRNA biogenesis	n/d	miR390	36
Clade III: AGO4/6/8/9	AtAGO4	RdDM pathway methylation of tasiRNA-generating loci and transcriptionally active TEs	A	24-nt siRNAs	39,40,42
	AtAGO6	proposed to be a pseudogene	A	24-nt siRNAs and 21-22-nt endo-siRNAs	45
	AtAGO8	germ cell fate repression in the somatic companion cells surrounding MMC	n/d	n/d	
	AtAGO9	somatic cell fate repression in the germ cells	A	24-siRNAs	46
	ZmAGO9	repression in the germ cells	n/d	n/d	47

* Due to space limitations, only the main function and major associated sRNA type is listed.

n/d not determined.

Table 2. Proposed nomenclature of AGO proteins in the four selected flowering plants*

Gene identifier (eudicots)	Proposed name	Previous name	Gene identifier (monocots)	Proposed name	Previous name
AT1G48410		AtAGO1 [†]	LOC_Os02g45070		OsAGO1a [†]
AT1G31280		AtAGO2 [†]	LOC_Os04g47870		OsAGO1b [†]
AT1G31290		AtAGO3 [†]	LOC_Os02g58490		OsAGO1c [†]
AT2G27040		AtAGO4 [†]	LOC_Os06g51310		OsAGO1d [†]
AT2G27880		AtAGO5 [†]	LOC_Os04g52540	OsAGO2a	OsAGO2 ⁷
AT2G32940		AtAGO6 [†]	LOC_Os04g52550	OsAGO2b	OsAGO3 ⁷
AT1G69440		AtAGO7 [†]	LOC_Os01g16870		OsAGO4a [†]
AT5G21030		AtAGO8 [†]	LOC_Os01g16850	OsAGO4b	OsAGO15 ⁷
AT5G21150		AtAGO9 [†]	LOC_Os03g47830	OsAGO5a	OsAGO11 ⁷
AT5G43810		AtAGO10 [†]	LOC_Os07g09020	OsAGO5b	OsAGO14 ⁷
Glyma16g34300		GmAGO1a [†]	LOC_Os03g58600	OsAGO5c	OsMEL1 ⁷
Glyma09g29720		GmAGO1b [†]	LOC_Os03g57560	OsAGO5d	OsAGO13 ⁷
Glyma20g02820	GmAGO2a	GmAGO3a ⁶	LOC_Os03g47820	OsAGO5e	OsAGO12 ⁷
Glyma15g13260	GmAGO2b	GmAGO3b ⁶	LOC_Os07g16224	OsAGO6	OsAGO16 ⁷
Glyma14g04510		GmAGO4a [†]	LOC_Os03g33650	OsAGO7	SHL4 ⁷
Glyma02g44260		GmAGO4b [†]	LOC_Os04g06770	OsAGO9	OsAGO4b ⁷
Glyma20g12070		GmAGO4c [†]	LOC_Os06g39640	OsAGO10	OsPNH1 ⁷
Glyma12g08860		GmAGO5a [†]	LOC_Os02g07310		OsAGO17 [†]
Glyma11g19650		GmAGO5b [†]	LOC_Os07g28850		OsAGO18 [†]
Glyma13g26240		GmAGO6a [†]	GRMZM2G441583		ZmAGO1a [†]
Glyma15g37170		GmAGO6b [†]	AC209206.3_FG011	ZmAGO1b	ZmAGO1c ⁹
Glyma01g06370		GmAGO7a [†]	GRMZM2G039455	ZmAGO1c	ZmAGO1b ⁹
Glyma02g12430		GmAGO7b [†]	GRMZM2G361518	ZmAGO1d	ZmAGO1f ⁹
Glyma06g47230		GmAGO9 [†]	GRMZM2G007791	ZmAGO2a	ZmAGO2 ⁸
Glyma20g28970		GmAGO10a [†]	GRMZM2G354867	ZmAGO2b	ZmAGO7 ⁸
Glyma10g38770		GmAGO10b [†]	GRMZM2G589579		ZmAGO4 [†]
Glyma02g00510		GmAGO10c [†]	GRMZM2G461936	ZmAGO5a	ZmAGO5d ⁹
Glyma06g23920		GmAGO10d [†]	GRMZM2G059033	ZmAGO5b	ZmAGO5a ⁹
Glyma05g08170		GmAGO10e [†]	GRMZM2G123063	ZmAGO5c	ZmAGO5b ⁹
Glyma17g12850		GmAGO10f [†]	GRMZM2G347402	ZmAGO6	ZmAGO5c ⁹
Glyma04g21450		GmAGO10g [†]	GRMZM5G892991		ZmAGO7 [†]
Glyma10g00527		GmAGO10h [‡]	GRMZM2G141818	ZmAGO9	AGO104 ⁴⁵ ZmAGO4d ⁸
			AC189879.3_FG003		ZmAGO10a [†]
			GRMZM2G079080		ZmAGO10b [†]
			GRMZM2G105250		ZmAGO18a [†]
			GRMZM2G457370	ZmAGO18b	ZmAGO18c ⁸

* Our review of the literature may not be complete.

† Indicates previous and proposed names are identical; numbers indicate the reference, where previous names are used.

‡ Newly identified in this work.

Figure Legends

Figure 1. Phylogenetic tree of AGO proteins in plants

Representative plant species were selected for the illustration of AGO protein evolution in plants. Full length protein sequences were aligned using Clustal Omega (<http://www.clustal.org/omega/>) and the tree was constructed using FastTree with default settings. AGOs from different lineages or species were coded with various shapes of distinct colors. Abbreviations for AGOs from basal non-flowering plants were as follows, SMO: *Selaginella moellendorffii*; Phpat: *Physcomitrella patens*; Cre: *Chlamydomonas reinhardtii*; Voca: *Volvox carteri*; CSU: *Coccomyxa subellipsoidea*; MPURCC: *Micromonas pusilla* CCMP1545.