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Minireview

Uncovering RNAi mechanisms in plants: Biochemistry enters the foray

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Abstract In plants, the RNA interference (RNAi) machinery responds to a variety of triggers including viral infection, transgenes, repeated elements and transposons. All of these triggers lead to silencing outcomes ranging from mRNA degradation to translational repression to chromatin remodeling. Thus, plants offer us a potentially unique opportunity to understand the full range of RNAi effector mechanisms. In this review, we discuss the recent developments in our understanding of plant RNAi mechanisms from a biochemical perspective.
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1. Introduction

RNA silencing is a general phenomenon in eukaryotic organisms and plays important roles in diverse biological processes including developmental regulation, antiviral defense and chromatin remodeling [1–5]. The key features of RNA silencing include the production of ~21–25 nt small RNAs by Dicer [6] and the formation of Argonaute (AGO)-containing RNA-induced silencing complexes (RISCs) that directly carry out gene silencing at the transcriptional or post-transcriptional level [7–9].

In plants, there are three RNA silencing pathways [2]. The first comprises post-transcriptional gene silencing (PTGS) mediated by ~21 nt small interfering RNAs (siRNAs) that are processed from double-stranded RNAs (dsRNAs). The source of dsRNAs includes replication intermediates of plant RNA viruses, transgenic inverted repeats, and products of RNA-dependent RNA polymerases (RdRps). The second pathway involves a class of endogenous small RNAs, microRNAs (miRNAs). MiRNAs are generated by Dicer-like 1 (DCL1) from miRNA precursors that are transcribed from miRNA genes. MiRNAs downregulate gene expression through base-pairing to target mRNAs, leading to either the degradation of mRNAs or the inhibition of translation or both. The third pathway is transcriptional gene silencing (TGS) that is associated with siRNA-directed chromatin modifications including DNA and histone methylation.

Our understanding of RNA interference (RNAi) pathways in plants has mainly derived from genetic studies and from extrapolating biochemical properties of RNAi components in animals. However, efforts are being made to bring genetic and biochemical approaches together in plants [10–14]. In this review, we summarize the recent findings related to RNAi mechanisms in plants, and discuss the roles of major protein families in RNAi pathways from a biochemical perspective.

2. Plant Dicer-like proteins and biogenesis of small RNAs

The *SIN1/SUS1/CAF* gene (now renamed *Dicer-like 1*, *DCL1*) was isolated through independent genetic screens for *Arabidopsis* mutants with abnormal embryo, ovule and flower development [15]. *SIN1/SUS1/CAF* was shown to encode a conserved multidomain protein containing two RNase III domains in 1999 [16]. In the same year, small RNAs were detected in various plant PTGS systems [17]. However, we remained blind to the connection between DCL1 and small RNA production until the discovery that its homologue in *Drosophila*, Dicer-1, processes dsRNA substrates into ~22 nt small RNAs [6]. The Chen and Bartel labs subsequently cloned miRNAs from *Arabidopsis* and showed that their accumulation was greatly reduced in *caf* (or *dcl1-9*) mutants, suggesting a role for DCL1 in miRNA metabolism [18,19]. However, in the same mutant, neither PTGS nor siRNA production from dsRNA was blocked [20]. This outcome implied that *Arabidopsis* might have distinct DCLs that are responsible for the production of miRNAs and siRNAs.

The idea that a plant might contain multiple Dicer activities was first indicated by the observation of discrete size classes of small RNAs. Hamilton et al. showed that two size-classes (21–22 and 24–26 nt) siRNAs were produced from a transgene whereas only long siRNAs were generated from endogenous retroelements. The short siRNAs correlated with sequence-specific mRNA degradation while the long siRNAs were dispensable for mRNA degradation but correlated with methylation of homologous DNA [21]. Subsequent biochemical studies showed that wheat germ and cauliflower extracts

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Abbreviations: RNAi, RNA interference; DCL, Dicer-like protein; AGO, Argonaute; RISC, RNA-induced silencing complex; PTGS, Post-transcriptional gene silencing; dsRNA, Double-stranded RNA; siRNA, Small interfering RNA; miRNA, MicroRNA; dsRBD, dsRNA-binding domain

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contain Dicer-like enzymes that convert dsRNA into these same two size-classes of siRNAs [11]. *Arabidopsis* extracts also contain two major activities producing 21 and 24 nt siRNAs [12]. Biochemical fractionation suggested that the two products are created by two distinct enzymatic complexes. The *Arabidopsis* genome encodes four Dicer-like proteins, i.e., DCL1–4 [15]. Immunoaffinity purified DCL1 and DCL3 processed a dsRNA substrate into 21 and 24 nt siRNAs, indicating that DCL1 and DCL3 are directly responsible for the production of the two size-classes of small RNAs, respectively. A *dcl2-1* mutant still maintained both activities, suggesting that DCL2 is not the major enzyme that produces either 21 or 24 nt siRNAs. However, no one as yet has characterized the biochemical activity of DCL2. *dcl3-1* extracts contained 21 nt siRNA-generating activity, but lacked 24 nt siRNA-generating activity, indicating that DCL3 is either the sole or the predominant enzyme that produces 24 nt siRNAs [12]. This biochemical property of DCL3 is in full agreement with the genetic data showing that accumulation of ∼24 nt siRNAs in vivo was significantly decreased in *dcl3-1*, but not in *dcl1-7* and *dcl2-1* mutants [22]. Intriguingly, in the *dcl1-7* mutant, the accumulation of 21 nt miRNAs was dramatically decreased [22]. However, DCL1 immunoprecipitates from the mutant maintained the small RNA-generating activity [12]. This suggests that the P415S substitution in the helicase domain of DCL1 does not abolish the small RNA processing activity, but may interfere with the other functions of DCL1 in RNAi pathway. In *Drosophila*, besides their involvement in small RNA biogenesis, Dicers are also required for RISC formation [23–25]. Size exclusion chromatography suggested that DCL1 and DCL3 reside in >660 and ∼440 kDa complexes, respectively [12]. Identification of the components of the complexes may reveal if plant DCLs also have dual functions in both initiation and execution stages of RNAi.

The biochemical mechanism by which *Arabidopsis* DCL1 and DCL3 measure and process dsRNA into two discrete size-classes of small RNAs remains unclear. Dicer and AGO share a conserved PAZ domain. Biochemical and crystal structural analysis have indicated that the PAZ domain in AGOs has a preference for single-stranded RNAs or double-stranded RNAs with 3′ overhangs [26–28]. It was shown that human Dicer preferentially processes siRNAs from the ends of dsRNAs [29]. In accord with the hypothesis that Dicer is an end-recognizing nuclease, mutations within the PAZ domain of Dicer inhibit its activity, and inhibition is more pronounced for dsRNAs with 3′ overhangs than those with blunt ends [30]. This suggests that Dicer recognizes the 3′ overhang of a dsRNA substrate through its PAZ domain. Zhang et al. suggested a model for human Dicer in which a single dsRNA cleavage center is formed through intramolecular dimerization of its two RNase III domains. Dicer would then cleave the dsRNA ∼20 nt from its terminus, making this measurement through recognition of 3′ overhang by the PAZ domain [30]. Thus, the difference in size between DCL1 and DCL3 products may arise from intrinsic structural characteristics of these enzymes. Considering that DCL1 and DCL3 share common domains including a PAZ domain and two RNase III domains [15], the distinct sizes of their products may be attributed to subtle difference in conformation of the two DCLs. It also remains possible that the size of products may be determined by factors that associate with DCL1 and DCL3.

3. AGO1: an *Arabidopsis* Slicer

In animals, once generated by Dicer, siRNAs are loaded into AGO-containing RISC to cleave their target mRNAs. In mammals, through genetic, biochemical and structural analyses, it was shown that Ago2 is the catalytic engine (Slicer) of RISC, directly responsible for mRNA cleavage [26,31]. This occurs through an intrinsic RNase motif formed by the PIWI domain. PIWI bears close resemblance to two classes of nucleases, namely the RNase H family and the transposases. Recently, Rivas et al. showed that a characteristic Asp-Asp-His (DDH) motif is essential for Slicer activity [32]. Unlike animals where the majority of microRNAs operate through translational repression, the majority of the characterized plant miRNAs regulate their target genes through cleavage of target mRNAs [33]. It was shown that wheat germ and *Arabidopsis* extracts have RISC complexes that are competent in mRNA cleavage [11,12]. However, the identity of the plant Slicer was not known.

Arabidopsis contains at least 10 AGO proteins as candidates for Slicer. However, genetic studies have elevated AGO1 as an excellent Slicer candidate. As the founding member of the AGO family [34], AGO1 was isolated through the genetic screening for mutants with aberrant leaf morphology and named after the small squid-like appearance of the mutant plant [35]. AGO1 is required for PTGS [36]. In *ago1* null mutants, accumulation of miRNAs is decreased, which is accompanied by increased levels of miRNA target genes; while in hypomorphic mutants, miRNA accumulation is not substantially changed but target mRNA cleavage is comprised [37]. Recently, we showed that, in *Arabidopsis*, one trans-acting siRNA (ta-siRNA) and three miRNAs could be detected in AGO1 immunoprecipitates, indicating that these ta-siRNA and miRNAs and AGO1 associate in vivo. Such complexes are competent for cleaving the target mRNAs of the ta-siRNA and miRNAs in vitro [12]. Similar results were also obtained using an *Arabidopsis* line expressing a tagged AGO1 [10]. Furthermore, a cleavage-competent RISC can also be reconstituted with immunopurified AGO1 and an exogenous single-stranded siRNA in vitro. In combination with data from genetic studies, these biochemical analyses strongly suggest that AGO1 is a key component of *Arabidopsis* RISC and is at least one of the *Arabidopsis* Slicers [10,12].

Intriguingly, AGO1 did not associate with any of three ∼24 nt siRNAs in vivo [10,12]; however, in the in vitro reconstitution system 24 nt siRNAs could be loaded into AGO1 immunoprecipitates to form cleavage-competent RISC [12]. This suggests that distinctions within pathways in *Arabidopsis* RNA silencing probably occur via formation of interactions between complexes and not simply because specific AGOs in effector complexes are unable to accept siRNAs of inappropriate sizes. Attempts to assemble RISC with exogenous siRNA duplexes have not been successful [11,12,14], most probably due to the lack of an siRNA unwinding activity in plant extracts [14].

Though most characterized plant miRNAs cleave their target mRNAs, it was suggested that one miRNA, miR172, functions predominantly as a translational repressor [38,39]. However, a study using an *Arabidopsis* line overexpressing miR172 suggests that mRNA cleavage and translational repression are similarly important in miR172 function [40]. Recently, we showed that miR172 is associated with AGO1 and

directs the cleavage of its target mRNA in vitro, albeit weakly [12]. There is a G:U wobble at position 7 (counting from the 5′ end of the miRNA). A change from G:U wobble to A:U pair to create perfect complementarity within the targeting region significantly increased the efficiency of the cleavage (Qi and Hannon, unpublished result), suggesting that the low efficiency of the mRNA cleavage mediated by miR172 may be attributed to its imperfect basepairing to its target mRNA at this position. This is consistent to the notion from in vivo studies that mismatches at 5′ region of miRNAs have a substantial impact on target mRNA cleavage [40,41]. However, it still remains possible that miR172 also joins another distinct RISC to carry out its translational repression function. It is noteworthy that the effect of other plant miRNAs on translation has not been evaluated, and the question is still open as to how general it is that a plant miRNA regulates gene expression by both cleaving mRNA and repressing translation.

4. Other roles of AGO proteins in *Arabidopsis*

S. pombe has one AGO that is involved in both PTGS and TGS [42]. However, animals have multiple AGO proteins that seem to have distinct functions. For instance, in *Drosophila*, it was shown that Ago1, but not Ago2 is required for miRNA accumulation, whereas Ago2, but not Ago1 is required for siRNA function [43]. In humans, miRNAs and siRNAs associate indiscriminately with Ago1–Ago4, however, only Ago2 has the ability to cleave mRNA [31,44].

Arabidopsis has 10 AGO proteins [36,45], providing a great potential for functional diversification. An alignment of *Arabidopsis* AGOs with human Ago2 revealed that eight *Arabidopsis* AGOs (i.e., AGO1, AGO4, AGO5, AGO6, AGO7/ZIP, AGO8, AGO9 and ZLL/PNH) have the DDH motif that characterizes the catalytic active site of a Slicer [12,32]. This suggests that *Arabidopsis* might have multiple other Slicers besides AGO1. The large number of AGOs in *Arabidopsis* also suggests that different AGOs might interact with different subsets of miRNAs or other small RNAs to regulate genes in specialized tissues or at particular developmental stages. Supporting this idea, AGO7/ZIP is involved in the regulation of vegetative developmental timing [46]; while ZLL/PNH has a role in the regulation of central shoot meristem cell fate during embryogenesis [47,48].

In plants, besides its role in post-transcriptional gene regulation, RNAi can also direct chromatin modification in a sequence-specific manner. Two *Arabidopsis* AGOs have been implicated in chromatin remodeling. AGO4 has been shown to be required for maintenance of DNA methylation at several endogenous loci: for de novo methylation at the *FWA* gene, and for siRNA accumulation of a subset of endogenous loci [49–51]. Besides its function in miRNA-directed mRNA cleavage, AGO1 also acts at chromatin level to regulate gene expression [45,52,53]. It remains elusive precisely how small RNAs guide chromatin modification at homologous sequences in plants. Two models have been proposed for how small RNAs recognize their target sites: direct RNA–DNA pairing (DNA-recognition model) or pairing of small RNAs and a nascent transcript from the target locus (RNA-recognition model) [54,55]. Recent studies in fission yeast indicated that RNAi-directed chromatin modification is coupled to RNA polymerase II transcription [56,57], supporting the RNA-recognition model. *Arabisopsis* has evolved a distinct polymerase, RNA polymerase IV, that is required for siRNA accumulation and DNA methylation at some loci [58,59]. However, the precise role of RNA polymerase IV in the siRNA–DNA methylation loop remains unclear. In *Arabidopsis*, an earlier report showed that miRNA binding sites in *PHB* and *PHV* mRNAs are required for the methylation of their chromosomal loci [60]. Given that *PHB* and *PHB* mRNAs are cleaved by miR165/166, and that AGO4 contains a DDH motif that might enable it to cleave, it is of great interest to test if a cleavage event is involved in small RNA-mediated chromatin modification.

5. The role of dsRBD proteins

The dsRNA-binding domain (dsRBD) [also known as dsRNA-binding motif (dsRBM)] is responsible for many interactions between proteins and RNA duplexes, and proteins containing dsRBD have diverse functions [61].

DsRNAs are the triggers of RNAi, and many dsRBD proteins have demonstrated roles in RNAi pathways. DsRBD domains in Dicer and Drosha are probably involved in binding to their dsRNA substrates [6,62], as supported by the fact that deletion of dsRBD in human Dicer attenuates its activity [30]. RDE-4, a dsRBD protein in *Caenorhabditis elegans*, interacts with RDE-1 and DCR-1 and functions in the initial steps of RNAi [63]. A potential RDE-4 homologue in *Drosophila*, R2D2 interacts with DCR-2, and is the sensor for siRNA asymmetry [64,65]. Another dsRBD protein, Pasha in *Drosophila* or DGCR8 in humans each interacts with Drosha to form the Microprocessor complex, which processes primary miRNA transcripts into miRNA precursors (pre-miRNA) [66,67]. In turn, Loquacious in *Drosophila* and TRBP in humans form complexes with Dicer and at least in flies Loquacious appears specifically tasked for pre-miRNA processing [68–70]. Thus, in humans and flies, the function of each Dicer and Drosha is facilitated by a particular dsRBD protein. If this holds true in *Arabidopsis*, dsRBD proteins may also be involved in RNAi through interacting with DCLs.

Besides DCLs, there are 16 dsRBD proteins in *Arabidopsis*, two of which have demonstrated roles in RNAi. HYL1 is a potential homologue of RDE-4 in *C. elegans* and R2D2 in *Drosophila*. However, HYL might function differently from these proteins. While RDE-4 and R2D2 play roles in the siRNA pathway through interacting with Dicers that are localized in cytoplasm [63–65], HYL1 is a nuclear dsRBD protein required for the accumulation of miRNA but dispensable for post-transcriptional transgene silencing (S-PTGS) [71,72]. HYL1 resides in a ~300 kDa complex in vivo [71], but its interacting partners await identification. In vitro, HYL1 can interact with DCL1 [73], suggesting that HYL1 might assist in miRNA biogenesis. Another dsRBD protein, HEN1, is also required for the miRNA accumulation but is additionally involved in S-PTGS [18,74]. Recently, HEN1 was biochemically characterized and shown to possess a methyltransferase activity [13]. In vitro, HEN1 methylates miRNA/miRNA* duplex at either the 2′ or the 3′-*O*-ribose position of the last nucleotide. It was further shown that endogenous miRNAs are methylated in *Arabidopsis* [13]. This distinct feature of *Arabidopsis* miRNAs raised the question whether the methyl group facilitates the recognition

of miRNAs by AGO in the process of RISC assembly. However, in the in vitro RISC reconstitution system, non-methylated single-stranded siRNA can be efficiently bound to *Arabidopsis* AGO1 and form a cleavage-competent RISC, and the methylation of siRNAs does not have a positive effect on the RISC activity [12]. So, the impact of methylation on the miRNA function probably occurs at other steps during miRNA biogenesis and RISC assembly in vivo. It remains to be tested whether HEN1 interacts with DCLs and has functions other than its methyltransferase activity.

The possible roles of other *Arabidopsis* dsRBD proteins in RNAi pathways have not been evaluated. By analogy with *Drosophila*, it is reasonable to speculate that each DCL might interact with a distinct dsRBD protein to carry out its function.

6. Outlook

The RNAi machinery in plants responds to a variety of RNAi triggers to produce a full spectrum of RNAi outcomes through distinct pathways. Many key players in *Arabidopsis* RNAi pathways have been identified through forward genetic screens and reverse genetic analysis. However, our understanding of the biochemical mechanisms of plant RNA silencing pathways is still in its infancy. We have thus far been limited to fitting genetic observations into biochemical models derived from data from other organisms. Although plants seem to share with other organisms many common aspects of the RNAi machinery, plant RNAi has numerous distinctive features, including transitivity, signal amplification, short- and long-range signaling and TGS responses. Therefore, biochemical characterization of RNAi components, especially those unique to plants, is necessary to attain a fully elaborated model of plant RNA silencing.

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