The Diversity, Biogenesis, and Activities of Endogenous Silencing Small RNAs in *Arabidopsis*

Nicolas G. Bologna and Olivier Voinnet

Department of Biology, Swiss Federal Institute of Technology (ETH-Z), 8093 Zurich, Switzerland; email: voinneto@ethz.ch

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Abstract

In eukaryotic RNA silencing, RNase-III classes of enzymes in the Dicer family process double-stranded RNA of cellular or exogenous origin into small-RNA (sRNA) molecules. sRNAs are then loaded into effector proteins known as ARGONAUTEs (AGOs), which, as part of RNA-induced silencing complexes, target complementary RNA or DNA for silencing. Plants have evolved a large variety of pathways over the Dicer–AGO consortium, which most likely underpins part of their phenotypic plasticity. Dicer-like proteins produce all known classes of plant silencing sRNAs, which are invariably stabilized via 2′-O-methylation mediated by HUA ENHANCER 1 (HEN1), potentially amplified by the action of several RNA-dependent RNA polymerases, and function through a variety of AGO proteins. Here, we review the known characteristics and biochemical properties of the core silencing factors found in the model plant *Arabidopsis thaliana*. We also describe how interactions between these core factors and more specialized proteins allow the production of a plethora of silencing sRNAs involved in a large array of biological functions. We emphasize in particular the biogenesis and activities of silencing sRNAs of endogenous origin.

Contents

CORE ENZYMES REQUIRED FOR SMALL-RNA BIOLOGY

DICER-LIKE Proteins

Dicer RNase-III endonucleases process long double-stranded RNA (dsRNA) into small-RNA (sRNA) duplexes bearing 2-nucleotide (nt) 3′ overhangs and 5′ monophosphates. Both metazoan and plant DICER-LIKE (DCL) proteins display DExD-box, helicase-C, domain of unknown function 283 (DUF283), PIWI/ARGONAUTE/ZWILLE (PAZ), RNase-III, and dsRNA-binding domain (dsRBD) domains (74) (**Figure 1**). Studies of the unique human Dicer (hDcr) revealed that distinct dsRNA recognition modes by these domains underlie the accuracy and specificity of sRNA processing from distinct substrates. In the first mode, one end of a near-perfect dsRNA helix is anchored to the PAZ domain, which is connected to the catalytic domain through an α-helix (the "ruler") whose length determines the size of processed sRNAs. In the absence or hindrance of PAZ, a second mode entails nonspecific dsRNA binding via the dsRBD. A third mode involves recognition of imperfect stem-loop substrates [i.e., microRNA precursors (pre-miRNAs)] via the binding of single-stranded loops by the ATPase/helicase domain, acting as the primary RNA sensor for discriminating between pre-miRNA and long dsRNA substrates, even though the NTPase activity per se is dispensable for miRNA biogenesis (57, 104). For instance, compromising NTPase activity in the hDcr or single *Caenorhabditis elegans* DCR-1 does not affect single-cut miRNA release but abrogates small-interfering-RNA (siRNA) processing through sequential cuts. Dicer helicase thus likely functions as an ATP-dependent translocase that provides the energy required for multiple cuts along dsRNA substrates. The same results were obtained with the two specialized forms of Dicer in *Drosophila melanogaster*, where the helicase domain of Dcr-1 facilitates pre-miRNA recognition in an ATP-independent manner, whereas the helicase domain of the siRNA-specialized Dcr-2 catalyzes translocation on long dsRNA substrates in an ATP-dependent manner (15, 117).

Plant *DCL* genes form a monophyletic group spawned after the plant–animal split but before the monocot–dicot divergence 150 million years ago (38). They share structural similarities with their metazoan counterparts, suggesting that the biochemical properties evoked above are also applicable to their varied modes of action, as discussed below.

MicroRNA-generating DCL1. The DCL1 domain architecture comprises a DExD/H-box RNA helicase, DUF283 [which was recently ascribed RNA-binding properties (93)], PAZ, two tandem RNase-III domains, and two tandem dsRBDs (74). Individual mutations in the helicase domain, RNase-III catalytic site, or dsRBDs reduce miRNA accumulation (51, 55, 81, 121), and a

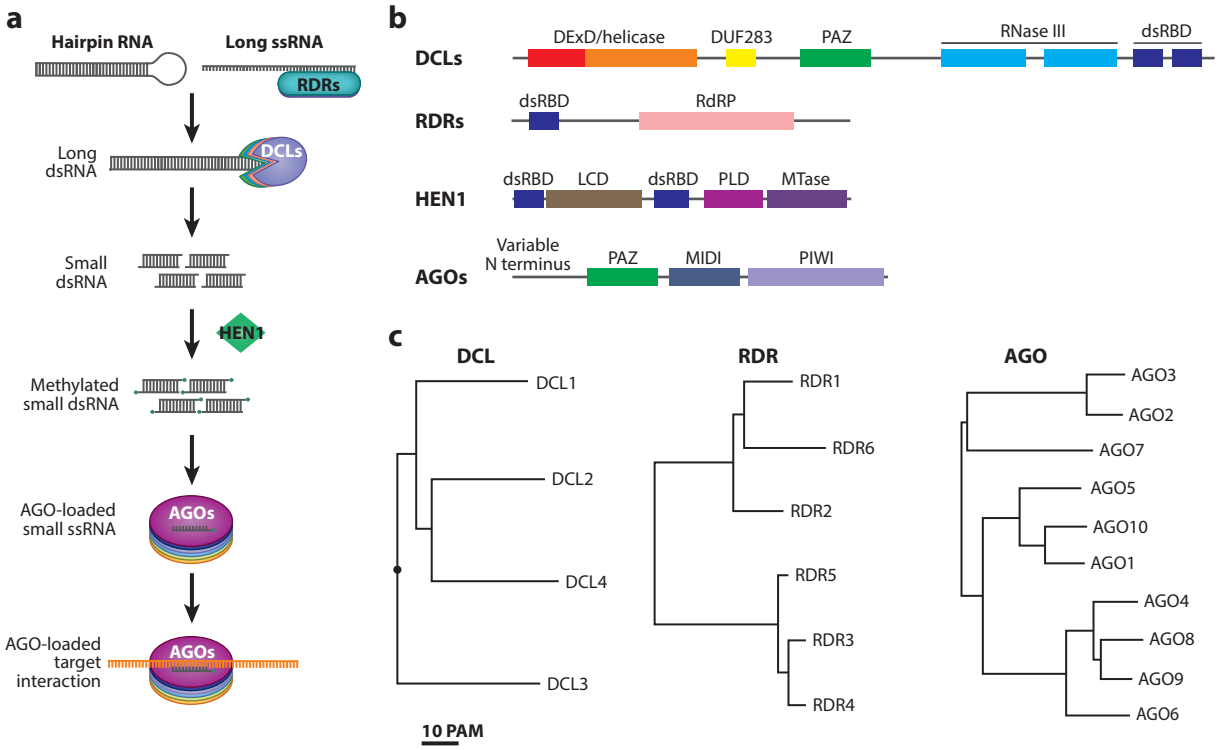


Figure 1

Arabidopsis core silencing proteins. (*a*) Generic functions of DICER-LIKE (DCL), RNA-dependent RNA polymerase (RDR), HUA ENHANCER 1 (HEN1), and ARGONAUTE (AGO) proteins in small-RNA (sRNA) pathways. (*b*) Domain organization showing the conserved regions present in each class of core proteins. (*c*) Phylogenetic classification of *Arabidopsis* DCL, RDR, and AGO proteins. Protein sequences were aligned using MultAlin (**http://multalin.toulouse.inra.fr**). Additional abbreviations: dsRBD, double-stranded-RNA-binding domain; dsRNA, double-stranded RNA; DUF283, domain of unknown function 283; LCD, La-motif-containing domain; MTase, methyltransferase; PAM, point accepted mutation; PAZ, PIWI/ARGONAUTE/ZWILLE; PLD, PPIase-like domain; ssRNA, single-stranded RNA.

functional helicase is required for accurate in vivo processing of at least some primary miRNA (pri-miRNA) by DCL1, which displays ATP dependency in vitro (66, 149). The involvement of at least two catalytic cycles by DCL1 for pri-to-pre-miRNA and pre-to-mature-miRNA processing (compared with the pre-to-mature-miRNA single cut operated by metazoan Dicers) likely explains ATP dependency in plant miRNA biogenesis (7, 55). The second C-terminal dsRBD, also found in DCL3 and DCL4 but absent in DCL2, promotes DCL1 localization into the nucleus, where it congregates with other miRNA biogenesis factors in specialized dicing bodies (12, 30). DCL1 undergoes negative feedback regulation by two of its own miRNA products: miR162 targets the DCL1 mRNA at the junction of exons 12 and 13, whereas the miR838 precursor resides within intron 14, such that its processing compromises DCL1 splicing, generating two nonproductive mRNA fragments (94, 128) (**Figure 2*a***). As with several core miRNA pathway mutants, the *Arabidopsis dcl1* knockout is embryonic lethal, and so most genetic studies are conducted with hypomorphic *dcl1* alleles (107).

Small-interfering-RNA-generating DCL2, DCL3, and DCL4. *Arabidopsis* DCL2, DCL3, and DCL4 process long, near-perfect dsRNA substrates into populations of 22-, 24-, and 21-nt

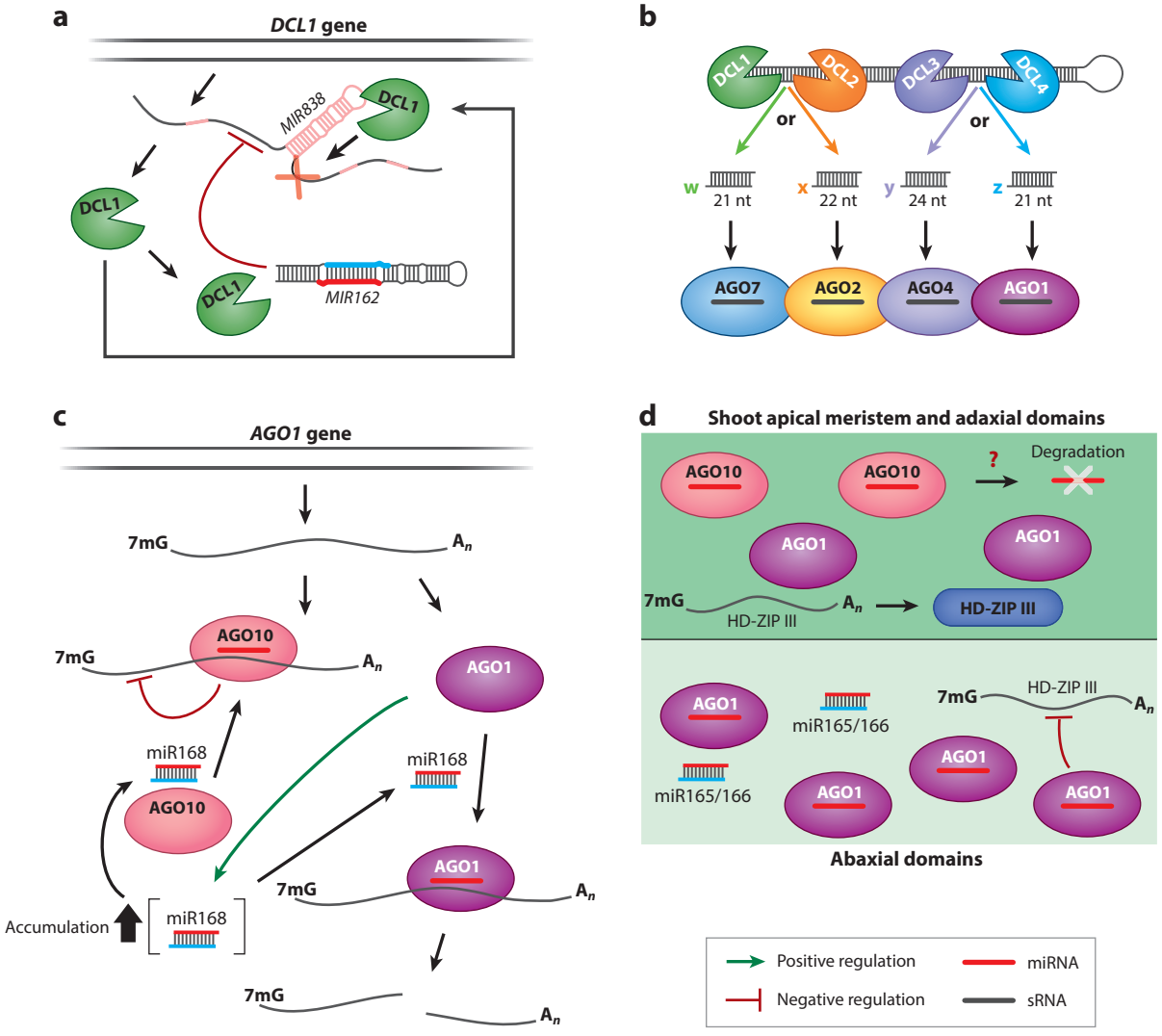


Figure 2

Competition and feedback regulation of silencing core proteins. (*a*) The microRNA (miRNA) pathway performs negative-feedback regulation of DICER-LIKE 1 (DCL1). (*b*) DCL proteins may compete for binding any given long double-stranded-RNA (dsRNA) substrate, generating small RNAs (sRNAs) of various lengths and 5′-end nucleotide identities (depicted here as *w*, *x*, *y*, and *z* letters), ultimately influencing their loading into specific ARGONAUTE (AGO) proteins. (*c*) AGO1 homeostatic regulation by miR168 may occur via AGO1-dependent slicing, AGO10-dependent translational repression, or increased miR168 accumulation in response to elevated AGO1 levels. (*d*) AGO10 and AGO1 compete for miR165/166 binding during shoot apical meristem development. Additional abbreviations: 7mG, 7-methylguanylate cap; A_n, polyadenine tail; HD-ZIP III, class-III homeodomain-leucine zipper; nt, nucleotide.

siRNAs, respectively (38). These size specificities are most likely underpinned by the distance separating the PAZ and catalytic domains of each protein. ATP hydrolysis provided by their conserved helicase domains is likely required for DCL2, DCL3, and DCL4 translocation along their endogenous substrates, constituted mostly by long dsRNA products of host-encoded RNA-dependent RNA polymerases (RDRs). DCL3 action in transcriptional gene silencing (TGS), mediated by

24-nt siRNAs, is generally linked to RDR2 products originating from transposons and repeats undergoing RNA-directed DNA methylation (RdDM) (88). DCL4-dependent posttranscriptional gene silencing (PTGS), mediated by 21-nt siRNAs, initiates from endogenous RDR6 and RDR1 products, including *trans*-acting siRNA (tasiRNA) precursors (see below). DCL2 and its 22-nt siRNA products act redundantly in TGS and PTGS, downstream of (at least) RDR6 and RDR2 (47). Generic substrates for the three DCLs also include intramolecular RNA fold-back transcripts originating from endogenous inverted repeat (IR) loci or their transgenic counterparts; some evolutionary young pre-miRNAs (e.g., pre-miR822) also fold into near-perfect IRs and, as such, are processed by DCL4 instead of DCL1 (94). Other endogenous DCL-specific substrates generate particular siRNA species and are detailed below. Generic exogenous DCL2, DCL3, and DCL4 substrates comprise virus-derived dsRNA produced by the combined action of virus- and host-encoded RDRs, including RDR1/6 (RNA viruses) and RDR2 (DNA viruses) (91).

Studies of single and multiple mutations in *DCL2*, *DCL3*, and *DCL4* have revealed a hierarchical continuum of action between these proteins. In effect, all three DCLs can access any given long dsRNA, but they have distinct affinities that likely depend on the subcellular localization or biosynthetic pathway. The dominant action of a particular DCL on a dsRNA substrate underlies not only the specific size but also the 5′-nucleotide identity of siRNA duplexes, and both of these critically influence their partitioning into a specific ARGONAUTE (AGO) protein and, ultimately, their biological output (**Figure 2*b***). Depending on the availability of particular DCLs in specific tissues or cell types, a given dsRNA may thus be processed into siRNAs with drastically different modes of action. This important feature was instrumental, for instance, in orchestrating the PTGS-to-TGS transition during de novo silencing of a reactivated retrotransposon in *Arabidopsis* (75). The DCL2, DCL3, and DCL4 continuum of action is also likely important for plant physiology, as *dcl2 dcl3 dcl4* triple-mutant *Arabidopsis* plants show more pronounced developmental defects than any *dcl* single or double mutant (38). Whereas *dcl2* and *dcl3* single mutants lack developmental abnormalities, *dcl4* mutant leaves are epinastic owing to defective RDR6-dependent production of endogenous tasiRNAs controlling organ polarity and the juvenile-to-adult phase transition (126, 133). Recent in vitro experiments showed that DCL4 processes long dsRNA without a base-pair preference at the 5′ phosphorylated end, whereas DCL3 preferentially processes shorter dsRNA with 5′ phosphorylated adenosine or uridine (84).

DCL3 and its 24-nt siRNA products, RDR2 and AGO4, congregate into specialized siRNA processing/loading subdomains. These colocalize with perinucleolar Cajal bodies and, curiously, are physically distant from the chromosomal source/target loci of DCL3-dependent 24-nt siRNAs (e.g., transposons and repeats) (88, 127). Thus, subnuclear trafficking might link these various TGS components together. Somewhat less extensive analyses, generally involving transiently expressed fusion proteins, suggest that *Arabidopsis* DCL2 and DCL4 are mostly nuclear, which is at odds with their defensive role against RNA viruses replicating exclusively cytoplasmically as well as with the alleged cytoplasmic distribution of the tasiRNA biogenesis machinery (40, 91, 127) (**Table 1**).

RNA-Dependent RNA Polymerases

RDRs, defined by a conserved catalytic domain required for copying single-stranded RNA (ssRNA) into dsRNA (**Figure 1*a***), are found in RNA viruses, plants, fungi, protists, and *C. elegans* but are notably absent in *Drosophila* and mammals. One member of each of the three eukaryotic RDR clades—RDRα, RDRβ, and RDRγ—was present in the most recent common ancestor of plants, animals, and fungi (150). Whereas *RDRα* genes have been sequenced from all three kingdoms, *RDRβ* and *RDRγ* are unique to animals and fungi and to plants and fungi, respectively (150).

Table 1 Subcellular localization and interaction of small-RNA pathway proteins

Protein[a]	Method[b]	Subcellular localization[c]		Colocalization[a]	Promoter used[d]	Fusion protein position[e]	Fusion protein tag[f]	Expression type[g]	Reference(s)
		Cytosol	Nucleus						
Core proteins									
DCL1	FP	—	NO, NP bodies	HYL1, SE, CPL1, NOT2b	OE, EN	Ct, Nt	GFP, YFP, Venus	T, S	30, 41, 72, 112
	BiFC	—	NP bodies	HYL1, SE, TGH	OE, EN	Ct, Nt	YFP	T	30, 97
	IF	—*	NP bodies (periNO)*	DCL3*, DCL4*, HEN1	EN*	—	—	S	89
DCL2	FP	—	NP	—	OE	Ct	GFP	T	127
	IF	—*	NP bodies (periNO)*	—	EN*	—	—	S	89
DCL3	FP	—	NP	—	OE	Ct	GFP	T	127
	IF	—	NO bodies, NP bodies (periNO)*	AGO4*-RDR2*-NRPE1*-DCLs*-HEN1*	EN*	—	—	S	88, 89
DCL4	FP	—	NP, NO	—	OE	Ct	Venus, GFP	T	40, 54
	IF	—*	NP*, periNO	SGS3*, AGO7*	EN	—	—	S	41, 89
RDR2	FP	—	NO bodies, NP bodies (periNO)*	DCL3*, AGO4*, NRPE1*	EN	Ct	YFP	S	88
RDR6	FP	Cyt bodies	—	SGS3, AGO7	OE	Ct, Nt	GFP, RFP	T	48, 54, 83
	BiFC	Cyt bodies	—	SGS3	OE	Nt	GFP	T	54
	IF	Cyt bodies	NP	SGS3*	EN	Ct	FLAG	S	41, 89
HEN1	FP	Cyt	NP, NP bodies	—	OE, EN	Ct	GFP, YFP	T	30, 127
	IF	—*	PeriNO*	DCL1*, DCL3*, DCL4*	EN	—	—	S	89
AGO1	FP	Cyt bodies, ER mb	NP, NP bodies	HYL1, AGO1	OE, EN	Nt	GFP, RFP, YFP	T, S	21, 30, 64, 102, 113
	BiFC	—	NP bodies	HYL1	EN	Nt	YFP	T	30
	IF	—*	PeriNO*	—	EN	—	—	S	89

AGO4	FP	—	NP	—	OE, EN	Nt	GFP, RFP	T, S	127, 131
	IF	—*	NO bodies, NP bodies (periNO)*	RDR2*, DCL3*, NRPE1*	EN	Nt, Ct	FLAG, MYC	S	62, 88, 89
AGO5	FP	Cyt	—	—	EN	Nt	YFP	S	106
AGO7	FP	Cyt bodies	—	RDR6, SGS3, DCL4*	OE, EN	Nt	GFP, RFP	T, S	48
	IF	Cyt bodies	PeriNO*	RDR6, SGS3, DCL4*	OE, EN	Nt	GFP, HA, FLAG*	S	48, 89
miRNA pathway									
HYL1	FP	—	NP bodies, NP (less extension)	DCL1, SE, SIC, CPL1, MOS2, DRB4	OE, EN	Nt, Ct	GFP, YFP, RFP, Venus	T, S	30, 40, 72, 111, 124
	IF	—	NP bodies	DCL1, SE, AGO1, CPL1, SIC, TGH	OE, EN	Nt, Ct	YFP	T	30, 72, 97, 111
SE	FP	Cyt (less extension)	NP, NP bodies	HYL1, CPL1, RACK1	OE, EN	Nt, Ct	CFP, YFP, Venus	T, S	30, 72, 102
	BiFC	—	NP bodies	DCL1, HYL1, TGH, CPL1, NOT2b, SE	OE	Nt, Ct	YFP	T	30, 72, 97
CBP20	BiFC	—	NP bodies	CBP80, SE, NOT2b	OE	Ct	YFP	T	112
CBP80	BiFC	—	NP bodies	CBP20, NOT2b	OE	Ct	YFP	T	112
TGH	BiFC	—	NP bodies	DCL1, HYL1, SE	OE	Nt	CFP	T	97
SIC	FP	—	NP bodies	HYL1	EN	Nt	GFP	S	141
	IF	—	NP bodies	HYL1	EN	Nt	FLAG	S	141
CPL1	FP	—	NP, NP bodies	HYL1, SE, DCL1	EN	Nt	GFP	T, S	72
	BiFC	—	NP, NP bodies	HYL1, SE	EN	Nt	YFP	T	72
NOT2b	FP	—	NP	DCL1	OE	Ct	RFP	T	112
	BiFC	—	NP bodies	SE, CBP20, CBP80	OE	Ct	YFP	T	112

(*Continued*)

Table 1 (*Continued*)

Protein[a]	Method[b]	Subcellular localization[c]		Colocalization[a]	Promoter used[d]	Fusion protein position[e]	Fusion protein tag[f]	Expression type[g]	Reference(s)
		Cytosol	Nucleus						
RACK1	FP	—	NP	SE, AGO1	EN	Ct	GFP	S	102
	BiFC	—	NP bodies	SE	OE	Nt	YFP	T	102
AMP1	FP	ER mb	—	—	OE	Nt	GFP	T	64
MOS2	FP	—	NP	HYL1	OE	Ct	GFP	T	124
tasiRNA pathway									
SGS3	FP	Cyt bodies	—	RDR6, AGO7	OE	Ct, Nt	GFP, RFP, YFP	T	48, 54, 83
	BiFC	Cyt bodies	—	RDR6	OE	Nt	GFP	T	54
	IF	—*	NP*, periNO*	RDR6*, SGS3*, DCL4*	EN*	Ct*	FLAG*	S	89
DRB4	FP	—	NP, NO	HYL1	OE	Ct	GFP, YFP	T	30, 40
rasiRNA pathway									
DRM2	IF	—*	NP*	—	EN*	Nt*	FLAG*	S	62
Pol IV (NRPD1)	IF	—*	NP bodies*	AGO4*, NRPD2*, NRPE1*	EN*	Ct*	FLAG*	S	62, 88
Pol IV (NRPD2)	IF	—*	NP bodies*	AGO4*, NRPD1*, NRPE1*	EN*	Ct*	FLAG*	S	62, 88
Pol V (NRPE1)	IF	—*	NP, NO bodies*	AGO4*, NRPD2*	EN*	Ct*	FLAG*	S	62, 88
DRD1	IF	—*	NP*	—	EN	—	—	S	62, 88

[a]AGO, ARGONAUTE; AMP1, ALTERED MERISTEM PROGRAM 1; CBP, CAP-BINDING PROTEINS; CPL1, C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1; DCL, DICER-LIKE; DRB4, double-stranded-RNA-binding protein 4; DRD1, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1; DRM2, DOMAINS REARRANGED METHYLTRANSFERASE 1; HEN1, HUA ENHANCER 1; HYL1, HYPONASTIC LEAVES 1; miRNA, microRNA; MOS2, MODIFIER OF SNC1 2; NRPD1 Pol IV subunit NUCLEAR RNA POLYMERASE D1; NRPD2 Pol IV subunit NUCLEAR RNA POLYMERASE D2; NRPE1, Pol V subunit NUCLEAR RNA POLYMERASE E1; Pol, RNA polymerase; RACK1, RECEPTOR FOR ACTIVATED C KINASE 1; rasiRNA, repeat-associated small interfering RNA; RDR, RNA-dependent RNA polymerase; SE, SERRATE; SGS3, SUPPRESSOR OF GENE SILENCING 3; SIC, SICKLE; tasiRNA, *trans*-acting small interfering RNA; TGH, TOUGH.

[b]BiFC, bimolecular fluorescence complementation; FP, fusion protein; IF, immunofluorescence.

[c]Cyt, cytoplasm; ER mb, endoplasmic reticulum membrane; NO, nucleolus; NP, nucleoplasm; *, only isolated nuclei experiments were performed.

[d]EN, endogenous expression; OE, overexpression; *, only isolated nuclei experiments were performed.

[e]Ct, C terminus; Nt, N terminus; *, only isolated nuclei experiments were performed.

[f]CFP, cyan fluorescent protein; GFP, green fluorescent protein; HA, human influenza hemagglutin; RFP, red fluorescent protein; YFP, yellow fluorescent protein; *, only isolated nuclei experiments were performed.

[g]S, stable expression; T, transient expression.

All known functions of plant RDRs are coordinated with the sequential processing of their long dsRNA products by one or several DCLs into so-called secondary siRNAs. These differ from primary siRNAs, which may trigger, directly or indirectly, RDR activity via direct priming or by enabling AGO-directed endonucleolytic cleavage as a starting point for dsRNA synthesis. Accordingly, plant recombinant RDRs act via both primer-dependent and primer-independent mechanisms in vitro (109).

Among the six *Arabidopsis* RDRs, RDR1, RDR2, and RDR6 (RDRα clade) share the canonical C-terminal catalytic DLDGD motif of eukaryotic RDRs (**Figure 1*b*,*c***). They show functional diversification in distinct endogenous silencing pathways by being linked to the action of specific siRNA-processing DCLs, as discussed in the previous section. Loss of RDR1, RDR2, or RDR6 function also enhances plant susceptibility to RNA and DNA viruses (91). RDRα clade members also have indirect roles in defense against nonviral pathogens (e.g., bacteria, oomycetes, and nematodes) and herbivores by producing endogenous regulatory sRNAs, including tasiRNAs and natural antisense transcript siRNAs (nat-siRNAs) (39, 52, 86). The continuum of action linking DCL2, DCL3, and DCL4 on any given dsRNA substrate also likely applies to the RDRα clade members, upstream of siRNA biogenesis. For instance, RDR6-dependent transgene PTGS is exacerbated in *Arabidopsis* plants carrying a mutation in *RDR2*, which is normally involved in TGS, suggesting that the two enzymes compete to convert a limiting pool of transgene-derived ssRNA into dsRNA. Mutations in *RDR1* display no morphological abnormalities and do not enhance those of *rdr6*, ascribed largely to tasiRNA defects that result in aberrant organ polarity, lateral root production, and anthocyanin buildup. *Arabidopsis* RDR2 has developmental roles in the female gametophyte (85). *Arabidopsis* RDR3, RDR4, and RDR5, defining the RDRγ clade, display an atypical catalytic DFDGD motif and have not been assigned any RNA silencing functions (150). Nonetheless, all six RDRs show distinct developmental and stress-responsive expression patterns (120).

Robust studies in planta and in isolated nuclei show that RDR2 localizes around the nucleolus and in Cajal bodies together with DCL3 and AGO4 (62, 88). In heterologous transient overexpression, RDR6 fusion proteins form small, discrete cytoplasmic foci known as siRNA bodies, which are apparently membrane associated and enriched in proteins involved in tasiRNA biogenesis (48, 54, 83). Direct immunofluorescence in *Arabidopsis* isolated nuclei also detects RDR6 in this compartment (41). The RDR1 subcellular localization remains unknown (**Table 1**).

HUA ENHANCER 1

HUA ENHANCER 1 (HEN1) deposits a methyl group onto the 2′-OH of each strand of all plant DCL products characterized to date (63, 136), protecting them from degradation (**Figure 1*a***). *Arabidopsis* HEN1 consists of five structural domains (**Figure 1*b***), four of which directly interact with sRNA substrates: a highly conserved methyltransferase domain phylogenetically unrelated to any known RNA 2′-*O*-methyltransferases, two dsRBDs (dsRBD1 and dsRBD2), and a La-motif-containing domain (LCD) that specifically recognizes the 3′-OH group. The other domain—the PPIase-like domain (PLD)—does not interact with sRNA substrates (42). In vitro methylation assays and structural analysis of the *Arabidopsis* HEN1–dsRNA complex indicate that the two 3′ termini of dsRNA duplexes are separately methylated (42, 63, 136). Monomeric HEN1 likely binds sRNAs through its N-terminal dsRBD1, possibly explaining why HEN1 catalyzes only 2′-O-methylation on double-stranded sRNA. An sRNA end-capping interaction by the LCD is then synergized by dsRBD2, which, together with dsRBD1, forms a strong grip on the duplexed region, helping position the two overhanging 3′-end termini toward the methyltransferase domain. The

preferred length of HEN1 sRNA substrates is likely determined by the distance between the methyltransferase site and the LCD 5′-end-capping site (42).

Because plant miRNAs are stabilized by 2′-O-methylation, only hypomorphic *hen1* mutant alleles are viable in *Arabidopsis*. A classical genetic screen for suppressors of the weak *hen1-2* allele not only allowed the identification of factors underlying enhanced sRNA turnover in *Arabidopsis hen1* mutants, but also uncovered a competition for 2′-O-methylation between miRNAs and highly abundant repeat- and transposon-derived 24-nt siRNAs. Indeed, a mutation in *RDR2* was sufficient to rescue the miRNA defects and developmental phenotype of *hen1-2* plants. Presumably, loss of abundant heterochromatic siRNAs had freed a limiting pool of HEN1 still available in the *hen1-2* background, allowing sufficient 2′-O-methylation of miRNAs to restore their stability and function (134). A second and somewhat more expected class of suppressors is defined by *HEN1 SUPPRESSOR 1* (*HESO1*), encoding a terminal nucleotidyltransferase that adds untemplated uridine to the 3′ end of the sRNA, a process prevented by HEN1-mediated 2′-O-methylation of sRNA duplexes (96, 145). Although a HEN1 reporter gene fusion protein (e.g., HEN1-YFP) is present in both the nucleus and cytoplasm (30, 127), the precise subcellular sites of sRNA methylation remain unknown (**Table 1**).

ARGONAUTE Proteins

AGO and AGO-like proteins are the main RNA silencing effectors across kingdoms, and their gene numbers vary greatly, ranging from 1 in *Schizosaccharomyces pombe* to 27 in *C. elegans*. The *Arabidopsis* genome encodes 10 *AGO* genes, defining three major phylogenetic clades: *AGO1*, *-5*, and *-10*; *AGO2*, *-3*, and -7; and *AGO4*, *-6*, *-8*, and *-9* (71) (**Figure 1*a*,*c***). Canonical eukaryotic AGOs contain four main domains: a variable N-terminal domain and the more highly conserved PAZ, MID, and PIWI domains, which together correctly position sRNAs relative to their targets. PAZ, MID, and PIWI are connected by the L1 and L2 linker regions (**Figure 1*b***). AGOs fold into a bilobal structure displaying a central groove for sRNA binding (111). A nucleotide-specificity loop lining the sRNA-binding pocket in the MID domain recognizes the 5′ nucleotide of sRNAs, and the PAZ domain binds the 3′-terminal end (31). The PIWI domain adopts an RNase-H-like fold and exhibits endonuclease (slicer) activity mediated by an Asp-Asp-His (DDH) catalytic triad (115), although the DDH domain per se is not always sufficient for slicing (67, 79).

Slicing has been experimentally demonstrated for *Arabidopsis* AGO1, -2, -7, and -10 (mediating PTGS) and AGO4 (mediating TGS) (13, 73, 148). Studies of plant AGO immunoprecipitates have revealed that the sRNA size and 5′-terminal nucleotide bias the loading of these proteins (80, 82, 148). Thus, AGO4, -6, and -9 associate mostly with 24-nt siRNA, whereas AGO1, -2, -5, -7 and -10 bind 21–22-nt molecules. AGO7 and -10 are associated almost exclusively with miR390 and miR165/166, respectively, whereas AGO1, -2, and -5 preferentially bind sRNAs exhibiting a 5′-end uridine, adenosine, or cytosine, respectively (80). In addition, AGO4, -6, and -9 associate primarily with 5′-adenosine sRNAs. Although mutational analyses confirmed the importance of the 5′-nucleotide identity in AGO sorting of some sRNAs, further studies also revealed additional, or even different, requirements (including base-pair mismatches or protein interactions) for the sorting of others. Hence, most *MIR165/166* family members contain a 5′-terminal uridine, normally licensing them for AGO1 loading, but these specifically associate with AGO10 instead (148). Likewise, miR390 selectively loads into AGO7 instead of AGO2 despite its 5′-terminal adenosine (82).

Plant, metazoan, and fungal AGOs act as platforms to bind proteins containing Gly-Trp (GW) dipeptides (28, 90, 105). These GW proteins are often essential cofactors in various RNA silencing reactions. In plants, AGO4 binds directly to GW repeats in the large subunit of RNA polymerase

V, a plant-specific RNA polymerase required for RdDM (28). Recently, the helicase SDE3 was found to contain GW motifs required for AGO1 and AGO2 interaction in transposon silencing and antiviral defense (33). In animals, AGO proteins associate with members of the GW182 protein family required for miRNA action. Although a plant GW182 homolog has yet to be identified, SUO, a large protein with two C-terminal GW repeats, localizes to processing bodies, and its loss of function specifically compromises miRNA-mediated translational repression (TR) via AGO1. Nonetheless, whether SUO's GW repeats are necessary for AGO1 interaction in this mode of action remains undetermined (130).

The AGO1, -5, and -10 clade. Central to miRNA function and tasiRNA production/activity (DCL1- and DCL1/DCL4-dependent processes, respectively), AGO1 also mediates antiviral silencing upon loading with 21- and 22-nt virus-derived siRNAs (viRNAs) produced by DCL4 and DCL2, respectively (91). Verified modes of AGO1 action in some of these pathways include slicing as well as TR, possibly coupled to RNA decay. A fraction of AGO1 associates with endomembranes, and AGO1 is itself a peripheral membrane protein. Moreover, specific hypomorphic *ago1* mutant alleles display compromised membrane association, and this association is reduced upon knockdown of *HMG1*, which encodes a regulatory enzyme controlling isoprenoid end-product accumulation; furthermore, *hmg1* mutant plants display defective miRNA-mediated regulation (10). One clearly defined endomembrane association by AGO1 is to the rough endoplasmic reticulum, recently implicated as a prominent site of miRNA-mediated TR (64). Another link between AGO1 and endomembranes is illustrated by the polerovirus-encoded F-box protein P0. P0 ubiquitinates AGO1 and promotes its degradation in a manner sensitive to drugs or mutations that impair autophagy, in which cytosolic material is delivered to lysosomes for degradation. In fact, P0 hijacks a normal physiological process whereby unloaded AGO1 undergoes selective autophagy (21). AGO1 levels are also regulated during its loading with sRNA, which requires HEAT-SHOCK PROTEIN 90 (HSP90) and the *Arabidopsis* cyclophilin-40 ortholog SQUINT (SQN) (43, 44). *sqn* loss-of-function mutants display lower AGO1 levels and morphological phenotypes resembling those of weak *ago1* alleles (100). These defects are suppressed by secondary mutations in the F-box protein gene *FBW2*, suggesting a role for FBW2 in 26S-proteasome-mediated turnover of AGO1 (27). Autophagic degradation of unloaded AGO1 and FBW2-mediated control of AGO1 loading/chaperoning are part of a homeostatic control mechanism that enables AGO1 steady-state levels to remain relatively constant under adverse environmental or stress conditions, including virus infections. Reminiscent of DCL1 control by miR162, AGO1 homeostasis also entails its regulation by miR168 via (*a*) miR168-AGO1-dependent slicing of AGO1 mRNA, (*b*) TR of AGO1 mRNA in a miR168-AGO10-dependent manner, and (*c*) increased miR168 accumulation in response to elevated AGO1 levels. Attesting to the importance of miR168 in AGO1 regulation, miR168-resistant AGO1 causes strong developmental defects (71) (**Figure 2*c***).

Homeostatic AGO1 control also entails production of specific 22-nt miR168 isoforms that, upon cleavage of the AGO1 mRNA, instigate production of RDR6-dependent secondary siRNAs to further strengthen AGO1 downregulation. In fact, this secondary siRNA production, initiated on target 3′-cleavage products, can be triggered by other 22-nt isoforms from other miRNAs that are normally processed by DCL1 as cognate 21-nt species. Additional cases also involve miRNAs produced almost exclusively as 22-nt entities, including miR173, which initiates tasiRNA production. Because engineering the miR173 pre-miRNA to produce 21-nt forms of miR173 abolishes tasiRNA synthesis without altering AGO1 loading (17, 18), miRNA length was proposed to play a key role in this process. However, further investigations suggested that asymmetry in the duplex miRNA and its opposing fragment, called miRNA*, might be important (73), although the molecular underpinnings of RDR6 recruitment on targets remain elusive. Several studies describe

AGO1 as a cytoplasmic factor showing either a uniform distribution or a discrete localization in foci, colocalizing with the processing-body marker DCP1 (21, 129). As explained above, functional AGO1 associations with the endoplasmic reticulum and with lysosomes have also been reported (10); however, other studies have uncovered a nuclear distribution for AGO1, indicating the need for a rigorous assessment of its localization, and indeed that of other plant AGOs as well (30, 89, 113) (**Table 1**).

AGO10 (also known as ZWILLE or PINHEAD), the closest AGO1 homolog in *Arabidopsis*, regulates shoot apical meristem development by specifically binding members of the *MIR165/166* family. These miRNAs also associate with AGO1 to suppress class-III homeodomain-leucine-zipper (HD-ZIP-III) transcription factors required for shoot apical meristem establishment (70). *ago10* mutants display elevated miR165/166 levels and reduced HD-ZIP-III transcript levels, indicating that, unlike AGO1, AGO10 positively regulates HD-ZIP III, a function not involving its slicing activity (68). Whereas AGO1 accumulates ubiquitously in all plant tissues, AGO10 is expressed moderately in the shoot apical meristem, in only the adaxial domains of leaf primordia, and at higher levels in vascular precursors. A current model proposes that AGO10 competes with AGO1 for miR165/166 to protect HD-ZIP-III transcripts from repression in the shoot apical meristem and leaf adaxial domains (**Figure 2*d***). AGO10 also mediates TR of several endogenous miRNA target genes, including *AGO1* (70, 144). Laser-capture microdissection identified significant *AGO5* expression in and/or around developing megaspores during the transition to megagametogenesis. Accordingly, *Arabidopsis* plants with the semidominant *ago5-4* allele show defects in megagametogenesis, as do plants expressing the potyviral silencing suppressor HcPro under the *AGO5* promoter. In male gametophytes, AGO5 localizes preferentially in the sperm cell cytoplasm of mature pollen, where, given its analogy to AGO1, it may direct miRNA- and siRNA-mediated functions required for male gametophyte development or cell type specification (106).

The AGO2, -3, and -7 clade. Although it belongs to a different clade, AGO2 displays both additive and overlapping activity with AGO1. For instance, AGO1 and AGO2 redundantly regulate the plantacyanin mRNA via miR408, and both proteins are required for siRNA-mediated silencing of transcribed, nonconserved intergenic regions, pseudogenes, and evolutionary young transposons in *Arabidopsis* (78). *Pseudomonas* infection in *Arabidopsis* stabilizes the accumulation and loading into AGO2 of several miRNA* strands that are otherwise rapidly turned over in uninfected plants. These include miR393*, which targets the soluble NSF attachment protein receptor (SNARE) MEMB12, a negative regulator of the secretion of the antimicrobial protein PR1 (143). Interestingly, miR393, also induced by *Pseudomonas* but loaded into AGO1, also contributes to antibacterial defense by dampening auxin signaling. Studies of other miRNA/miRNA* pairs similarly induced by infection or abiotic stress should uncover the extent and importance of this AGO1–AGO2 dual-channeling process. Genotoxic stress induced via DNA double-strand breaks also revealed a key AGO2 function in DNA repair (116), which entails its loading with a hitherto unknown class of endogenous sRNAs dubbed double-strand-break-induced RNAs (diRNAs) (discussed below). Recent studies also implicate AGO2 in defense against a broad range of viruses. Like AGO1, AGO2 is induced and loaded with DCL4- and DCL2-dependent viRNAs in virus-infected plants. Also, like hypomorphic *ago1* mutants, *ago2* plants are hypersusceptible to viruses, a phenotype enhanced in *ago1 ago2* double mutants, indicating the additive and nonoverlapping effects of these proteins. Loss of *AGO2* function was also sufficient to allow systemic infection of a virus not normally hosted by *Arabidopsis*, whereas AGO1 had no effect on this host-range determination (91). Echoing the targeting of AGO1 by miR168, AGO2 levels are regulated by miR403 in an AGO1-dependent manner (2). This regulatory network may

tasiRNAs: WHY THE FUSS?

Given that tasiRNA production is triggered primarily by miRNAs, why is *trans*-silencing of unlinked loci not simply achieved directly via the initiator miRNAs? There are at least two non-mutually-exclusive reasons for the apparently superfluous complexity of tasiRNA-mediated regulation. First, tasiRNAs are generated as cohorts, therefore allowing the regulation of a much wider variety of transcripts than would an individual miRNA alone. Indeed, all known *Arabidopsis* tasiRNAs simultaneously target many members of sometimes very large multigene families, including those of pentatricopeptide repeat proteins (regulated by *TAS1* and *TAS2*) as well as ARF and MYB transcription factor families (regulated by *TAS3* and *TAS4*, respectively). A second advantage of tasiRNA regulation is that it is non-cell-autonomous. Hence, the activity range of endogenous plant miRNAs is limited to a few cells at most, consistent with their near-coincident transcription and activity patterns. By contrast, RDR6-amplified siRNAs display extensive cell-to-cell mobility. tasiRNAs therefore constitute an elaborate means to provide an extended activity range to molecules (the tasiRNA-initiating miRNAs) that are normally not mobile or only poorly mobile. *TAS3*-mediated regulation of ARF transcripts, initiated by miR390:AGO7, illustrates this notion well by allowing the establishment of a gradient of ARF expression throughout leaves that is inversely correlated with that of the decreasing concentration of mobile tasiRNAs. Effectively acting as morphogens, the *TAS3* tasiRNAs enable, in this process, abaxial–adaxial leaf polarization. Attesting to the importance of this regulatory network, *TAS3*, miR390, and AGO7 are conserved out to the bryophytes, whereas *TAS1* and *TAS2* are specific to *Arabidopsis*. In fact, many examples of species-specific *TAS*-mediated regulation have now been found, and the system seems to be used extensively in long-lived perennials and plants with clonal reproduction (11, 20).

allow AGO2 to take over antiviral defense when AGO1 levels are themselves dampened by viral silencing suppressors, such as the polerovirus P0 protein mentioned above.

Despite its phylogenetic and genomic proximity to AGO2, AGO3 has not been ascribed any biological roles thus far. As mentioned, the third member of the clade, AGO7 (also known as ZIPPY), is associated almost exclusively with miR390 via mechanisms that require the integrity of the 5′-terminal adenosine and the central region of the miR390/miR390* duplex. Moreover, despite mismatches in the seed and central regions of the duplex, miR390* strand cleavage is required for AGO7–RNA-induced silencing complex (RISC) maturation, unraveling complex and highly specific features of the AGO7–miR390 association (82). miR390-bound AGO7 triggers biogenesis of *TAS3* family tasiRNAs, which regulate AUXIN-RESPONSE FACTOR 3 (ARF3) and ARF4 (3, 82) to ensure proper juvenile-to-adult phase transition and adaxial–abaxial patterning (see sidebar tasiRNAs: Why the Fuss?).

Although the AGO2 and AGO3 subcellular localizations are unknown, transient overexpression of AGO7 shows its congregation in siRNA bodies with other tasiRNA biogenesis factors, including RDR6, the subcellular distribution of which nonetheless remains debated (41, 48) (**Table 1**). Nuclear retention of AGO7 impairs tasiRNA production, suggesting that cytoplasmic localization underlies at least this specific AGO7 function (48).

The AGO4, -6, -8, and -9 clade. AGO4, -6, and -9 bind DCL3-dependent 24-nt siRNAs displaying a 5′-terminal adenosine bias (80). AGO4 is the major effector of RdDM and TGS of transposons and repeats, via a mechanism discussed below. Rescuing an *Arabidopsis ago4* knockout mutant with a catalytic-deficient allele of *AGO4* shows that AGO4-mediated slicing is required for DNA methylation at some loci and dispensable at others (92). In addition, AGO4 loads functionally with rare DCL3-dependent 24-nt miRNAs to direct sequence-specific DNA methylation at the *MIRNA* loci of origin and may also functionally substitute AGO1 and -7 in tasiRNA biogenesis

initiated by miR172 and miR390, respectively (82, 92). AGO4, like AGO1, is widely expressed in most *Arabidopsis* tissues and associates with DCL3, 24-nt siRNAs, and RDR2 into nucleolar Cajal bodies (62, 88, 89). Nonetheless, recent evidence suggests that AGO4 loading with 24-nt siRNAs occurs cytoplasmically to somewhat license AGO4 entry into the nucleus, a possible regulatory point prior to the effector stage of RdDM (131).

A classical genetic screen in a sensitized mutant background identified AGO6 as a TGS suppressor (146). Methylation levels at a few well-established RdDM targets were lower in *ago6* than in wild-type *Arabidopsis*, an effect exacerbated in the *ago4 ago6* double mutant, suggesting partially redundant functions and targets for the two RdDM effectors. Subsequent large-scale analyses showed, however, that the sets of 24-nt siRNAs loaded into AGO4, -6, and -9 have distinct loci of origin. The difference was attenuated when *AGO6* and *-9* were expressed under the *AGO4* promoter, whereas the cognate AGO4 sRNA pattern was only partially recapitulated, suggesting that AGO6 and -9 mediate RdDM at specific loci in specific tissues (37). Indeed, AGO6 expression is confined mostly to the shoot and root growing points and connecting vascular tissue (29). Accordingly, *ago6* (but not *ago4*) was retrieved in a specific TGS suppressor screen in shoot and root apical meristems (29). Also agreeing with the results of *AGO4↔AGO9* promoter swaps, AGO9—expressed in ovules, anthers, and seed coats—controls female gamete formation by restricting the specification of gametophyte precursors, apparently in a dosage-dependent and non-cell-autonomous manner (85). Hence, AGO9 is not expressed in the gamete lineage per se and instead forms cytoplasmic foci in surrounding, somatic companion cells from which sRNA movement may control the gamete cell specification. This may be correlated with the demonstrated function of AGO9 in silencing transposons in female gametes and accessory cells. AGO8 shows low-level expression at all stages of *Arabidopsis* development and in all tissues inspected. It is generally considered a pseudogene and therefore has not been ascribed any function.

THE ENDOGENOUS SMALL-RNA LEGION OF PLANTS

Upon interacting with more specialized proteins, the core silencing factors described above contribute to the production of a plethora of silencing sRNAs that regulate gene expression, genome integrity, or stress responses via either chromatin modification/TGS or PTGS. For the sake of simplicity and clarity, the sRNA species described in the following sections have been categorized according to their two main modes of action.

Posttranscriptional Gene Silencing–Associated Small RNAs

In *Arabidopsis*, distinct PTGS-associated endogenous sRNAs can be subdivided into different groups based on their origins, biogenesis pathways, or functions.

MicroRNAs. Most plant miRNAs are 21 nt long and require a DCL1-clade DCL for biogenesis and an AGO1-clade AGO for function; many belong to multigene families that are sometimes conserved over long evolutionary distances (19, 98). Plant miRNAs often have narrow sets of target transcripts encoding transcription factors; stress-response proteins; or factors controlling cell identity, development, and growth.

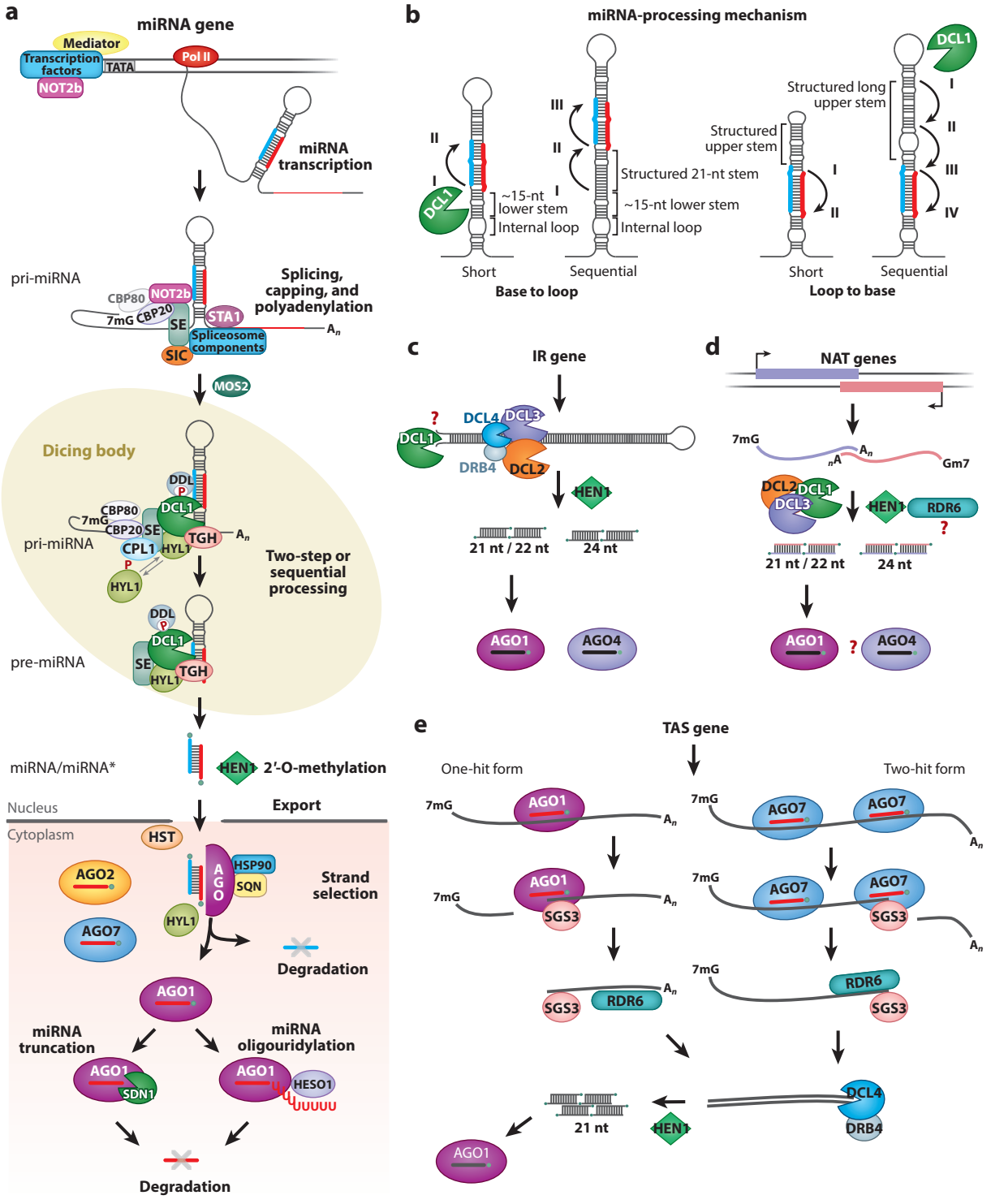
Transcription and maturation of microRNA primary transcripts. Most pri-miRNAs define independent transcription units containing a single miRNA-generating fold-back structure (35), unlike their often multibranched or polycistronic metazoan counterparts. Also, unlike animal miRNAs, which are frequently derived from introns or untranslated regions (14), plant *MIRNA*

loci are rarely nested within protein-coding genes. They are transcribed by RNA polymerase II (Pol II) and core coactivators such as Mediator and NOT2b (53, 112) (**Figure 3*a***). Accordingly, TATA boxes and *cis*-regulatory motifs are overrepresented in plant miRNA promoters, allowing their spatiotemporal or stress-responsive regulation by *trans*-acting factors and differential accumulation of individual miRNA isoforms. For instance, POWERDRESS-dependent recruitment of Pol II regulates only some *MIR172* family members (137). Likewise, locus-specific recruitment of the FUSCA 3 transcription factor underpins differential accumulation of specific *MIR156* family members (110).

Capping and polyadenylation generate pri-miRNAs (125), some of which undergo extensive alternative splicing of introns localized mainly 3′ to miRNA fold-backs, a feature facilitating mature miRNA accumulation (6, 99). The intimacy between splicing and pri-miRNA processing is consistent with colocalization, in DCL1-dicing bodies, of the spliceosomal markers SmD3 and SmB, the serine/arginine splicing factor SR33, and the alternative splicing factor SRp34 (30, 32). Furthermore, generic pre-mRNA splicing factors contribute to pri-miRNA processing, including the C2H2 zinc-finger protein SERRATE (SE) and subunits CAP-BINDING PROTEIN 80 (CBP80) and CBP20 of the cap-binding complex (CBC) (58), which all associate with the pri-miRNA transcriptional coactivator NOT2b (112). *Arabidopsis* plants deficient in STABILIZED 1 (STA1), a homolog of the human U5 small nuclear ribonucleic particle (snRNP)–associated protein PRPF6, display strong intron retention and accumulate unspliced pri-miRNAs (5). Like *se*, *cbp20*, and *cbp80*, *sta1* exhibits reduced mature miRNA levels, probably resulting from combined defects in pri-miRNA and DCL1 mRNA splicing (5). *Arabidopsis* plants lacking the proline-rich protein SICKLE (SIC) also display reduced miRNA and tasiRNA accumulation and accumulate higher levels of unspliced pri-miRNAs (141).

MicroRNA maturation machinery. The stem-loop structure contained within pri-miRNAs defines the pre-miRNA. Structural determinants initiate at least two staggered cleavage events within the pre-miRNA stem, separated by approximately 21 nt, which release the miRNA and its opposing fragment (miRNA*). Of key importance is the first cleavage position, which determines the mature miRNA sequence and therefore its target specificity. The second cut usually proceeds at a fixed distance from the end of the precursor. Pre-miRNA processing is mediated by DCL1 assisted by the dsRNA-binding (DRB) proteins HYPONASTIC LEAVES 1 (HYL1) and SE (58, 108), which in vitro bind the dsRNA section and ssRNA/dsRNA junctions of pri-miRNAs, respectively. Concurrently, the second dsRBD of HYL1 specifically associates with the DCL1 DUF283 domain, whereas both the N-terminal and zinc-finger domains of SE are required for DCL1–SE interaction. Attesting to their coordinated action in vivo, HYL1, SE, and DCL1 congregate with pri-miRNA in dicing bodies. HYL1 and SE improve the efficacy and precision of DCL1-mediated cleavage and, accordingly, strong *se* mutant alleles are embryonic lethal, whereas *hyl1* knockouts cause severe developmental defects (23, 58, 108) (**Table 1**).

Inactivating the RNA-binding proteins TOUGH (TGH) and MODIFIER OF SNC1 2 (MOS2) increases total pri-miRNA levels, whereas these levels are reduced in HYL1 immunoprecipitates, implicating both proteins in pri-miRNA processing. Both effectively bind pri-miRNAs, but whereas TGH associates with DCL1, HYL1, and SE in dicing bodies, MOS2 is uniformly nuclear. *mos2* mutants display fewer HYL1 foci but intact interactions between DCL1, HYL1, and SE. Thus, TGH appears to be integral to the complex modulating DCL1 activity, whereas MOS2 might, as an external cofactor, facilitate the recruitment of pri-miRNAs by this complex (97, 124). The forkhead-associated-domain protein DAWDLE (DDL) also binds pri-miRNAs, but *ddl* mutants, unlike *mos2* and *tgh*, do not overaccumulate pri-miRNAs (135). DDL interacts with DCL1 through a protein segment likely phosphorylated in vivo and might thereby promote the access to,



or recognition of, pri-miRNAs by DCL1. Without DDL, the portion of pri-miRNA not properly channeled to DCL1 is probably degraded, hence the reduced pri-miRNA levels in *ddl* mutants (69). SIC, required for pri-miRNA splicing, also localizes in nuclear HYL1 foci, suggesting its additional involvement in pri-miRNA processing (141).

The ribosomal protein RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1), a direct and specific interactor of SE, localizes in nuclear dicing bodies to modulate processing and transcription/stability of only some pri-miRNAs. RACK1-GFP also coimmunoprecipitates and colocalizes with AGO1 in both the nucleus and cytosol, suggesting that, as part of one or multiple nonribosomal AGO1 complexes, it also acts downstream of miRNA biogenesis (102). C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1 (CPL1) is another direct interactor of SE. Unlike in *hyl1*, *se*, *tgh*, and *mos2*, unprocessed pri-miRNAs do not overaccumulate in *cpl1* mutants, but several miRNAs are misprocessed. A model was proposed in which its interaction with SE recruits CPL1 to the DCL1 complex, where it might license HYL1 activity via dephosphorylation (72) (**Figure 3*a***).

Mature microRNA processing and stabilization. Plant pre-miRNAs are much more variable in length than their ~70-nt metazoan counterparts (ranging from 49 to 900 nt in length) and can undergo four main processing mechanisms, influenced by the sequential processing direction and number of cuts required for miRNA release (**Figure 3*b***):

1. In the short base-to-loop mechanism, an internal loop followed by a ~15-nt lower stem specifies the position of the first cleavage (I); this structure is found in most plant *MIRNA* families (76, 101, 118). Although this stem segment might contain bulges, the transition from internal loop (single strand) to lower stem is rather sharp, and three paired bases usually define the beginning of the precursor's lower stem (8). The second cut proceeds at a fixed distance of ~21 nt from the position of the first one (II).
2. In the sequential base-to-loop processing mechanism (e.g., in the *MIR169* family), the first cut proceeds as above (I), but then two more cuts (II and III) are required for miRNA release, generating, in the process, low levels of additional sRNAs (8).
3. In the short loop-to-base mechanism (e.g., in the *MIR156* and *MIR160* families), processing is guided by an upper stem segment, and two cuts release the mature miRNA; the terminal regions of these precursors have a conserved length (~42 nt) and a small loop (8).
4. In the sequential loop-to-base mechanism, four sequential DCL1 cuts process miRNA precursors that usually display a conserved, long stem segment (e.g., in *MIR319* and *MIR159* families) from which other sRNAs are also generated (1, 7, 8).

Upon miRNA/miRNA* release, the 3′ ends of both strands are 2′-O-methylated by HEN1 before their dissociation, via mechanisms detailed above. Loss of HEN1 function incurs 3′-to-5′ exonucleolysis (truncation) as well as tailing by HESO1, which adds 3′-oligouridylate tails to

Figure 3

Posttranscriptional gene silencing–associated small-RNA (sRNA) pathways. (*a*) The *Arabidopsis* microRNA (miRNA) pathway. (*b*) Various types of miRNA-processing mechanisms. (*c*) The inverted repeat (IR)–derived small-interfering-RNA (siRNA) pathway. (*d*) The natural-antisense-transcript-siRNA (nat-siRNA) pathway. (*e*) The *trans*-acting-siRNA (tasiRNA) pathway. Additional abbreviations: 7mG, 7-methylguanylate cap; AGO, ARGONAUTE; A_n, polyadenine tail; CBP, CAP-BINDING PROTEINS; CPL1, C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1; DCL, DICER-LIKE; DDL, DAWDLE; DRB4, double-stranded-RNA-binding protein 4; HEN1, HUA ENHANCER 1; HESO1, HEN1 SUPPRESSOR 1; HSP90, HEAT-SHOCK PROTEIN 90; HST, HASTY; HYL1, HYPONASTIC LEAVES 1; MOS2, MODIFIER OF SNC1 2; nt, nucleotide; Pol II, RNA polymerase II; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; RDR6, RNA-dependent RNA polymerase 6; SDN1, SMALL RNA DEGRADING NUCLEASE 1; SE, SERRATE; SGS3, SUPPRESSOR OF GENE SILENCING 3; SIC, SICKLE; SQN, SQUINT; STA1, STABILIZED 1; P, phosphate; TGH, TOUGH.

unmethylated miRNAs, leading to their degradation via mechanisms genetically distinct from those mediating truncation (96, 136, 140, 145). Truncation and tailing both require AGO1 but not its slicing activity, and HESO1 colocalizes with AGO1, suggesting that 3′ modifications of unmethylated miRNAs occur after AGO1 loading. Normal miRNA turnover in wild-type plants involves a family of SMALL RNA DEGRADING NUCLEASE (SDN) proteins with 3′–5′ exoribonuclease activity capable of degrading 2′-O-methylated ssRNA (95). Because SDNs are inhibited by 3′-oligouridylation, they are unlikely to contribute to the truncation of 3′-oligouridylated miRNAs accumulating in *hen1* (96, 145).

Mature miRNAs function in the cytoplasm, implying their nuclear export. The role of the plant exportin-5 homolog HASTY (HST) in this process is not as clear as in mammals, where pre-miRNAs (as opposed to mature miRNAs) are experimentally verified exportin-5 cargoes (87, 139). In fact, *hst* mutants show decreased accumulation of some (but not all) miRNAs in both nuclear and cytoplasmic fractions, suggesting HST-mediated control of miRNA accumulation rather than export (87). Defects in the *Arabidopsis* importin-β homolog EMA1 enhance miRNA activity, but neither the accumulation nor the nucleo-cytoplasmic distribution of miRNAs is affected (113). Clearly, the plant miRNA export mechanism requires further investigation.

RNA-induced-silencing-complex assembly and microRNA activity. Guide-strand selection in plant miRNA/miRNA* duplexes is directed in part by the lower thermodynamic stability of the guide strand's 5′ end relative to that of the miRNA*. HYL1 and CPL1 also facilitate this process, suggesting its coupling to miRNA biogenesis, as seen in metazoans (26, 72). Unlike for siRNA duplexes, miRNA passenger-strand removal from AGO1 is slicing independent and instead requires AGO1 disassociation from HSP90 and SQN (43, 44), possibly causing AGO1 conformational changes, as suggested during passenger-strand removal from metazoan AGOs (36, 56). Upon guide-strand selection, the miRNA* is generally degraded, although some miRNA* might be stabilized and functional, including under stress conditions and upon their loading into AGO2 (22). AGO–miRNA complexes recognize target mRNAs via base complementarity, and unlike metazoan miRNAs, most plant miRNAs display extended target complementarity. 5′-RACE (rapid amplification of cDNA ends) and degradome analyses show that decreased target levels resulting from plant miRNA action correlate qualitatively with AGO-mediated slicing between paired positions 10–11; the cytoplasmic exosome and 5′–3′ exoribonuclease XRN4 degrade the 5′- and 3′-cleavage products, respectively (34). Some miRNA targets, however, do not accumulate slicer products at all, and more generally, the extent and contribution of slicing to plant miRNA-mediated silencing remain difficult to measure, as none of the above methods are quantitative by nature. A crucial role for AGO1-mediated slicing is nonetheless suggested by the strong developmental defects of catalytic-deficient alleles of *AGO1* expressed in the *ago1* null background and by their dominant-negative effects in the wild-type background (13).

Despite extended complementarity in most plant miRNA:target pairs, a fraction of AGO1-targeted transcripts evade slicing and instead undergo protein-level repression. This likely involves TR because plant miRNAs associate with polysomes in an AGO1-dependent manner (9, 10). AGO10 also mediates TR, notably by regulating AGO1 protein levels via miR168, but the degree of redundancy/distinction between these and perhaps other AGOs in TR is unknown.

Remarkably, the extent of TR versus slicing varies intrinsically depending on the miRNA/siRNA:target pair under consideration, with no apparent correlation with target site multiplicity, complementarity, or position within messages (9). Owing to the poor resolution of sRNA action in plants, it is unclear whether AGO1-mediated TR and slicing are spatially separated between cells or coincident within single cells. In the former case, the absence/presence of AGO1-slicer antagonists in some but not other cell types might underlie the apparent concurrence of both

processes in whole-tissue analyses. In the latter case, partitioning of AGO1:target RNA pools into specific subcellular domains dedicated to TR or slicing might entail selective AGO1 associations with particular cellular factors. Supporting this view, TR occurs on the rough endoplasmic reticulum and requires the endoplasmic reticulum–transmembrane and AGO1-colocalized protein ALTERED MERISTEM PROGRAM 1 (AMP1) (64). Protein synthesis quantification via pulse labeling indeed provided direct evidence that at least one plant miRNA can inhibit target protein synthesis in an AMP1-dependent manner. Analyses of multiple miRNA:target pairs showed that AMP1 somehow promotes the exclusion of target transcripts from membrane-bound polysomes (64). Although these results suggest an impairment of translation initiation, more experiments are required to address the precise molecular mechanisms of plant miRNA-mediated TR. These mechanisms might also entail dynamic and perhaps transient protein–protein interactions, as suggested by the specific requirement for the microtubule-severing enzyme KATANIN (KTN) in TR in *Arabidopsis* (9).

In metazoans, miRNA-mediated TR is associated with reduced target mRNA levels achieved through slicing-independent mechanisms in cytoplasmic RNA-protein aggregates called processing bodies. Processing bodies are sites of mRNA decay, including via decapping/deadenylation, which downregulates most metazoan miRNA-targeted transcripts. Although pervasive miRNA-mediated slicing in plants confounds the specific contribution, if any, of TR-coupled mRNA decay, VARICOSE (VCS), which is associated in plant processing bodies with the mRNA decay components DECAPPING 2 (DCP2), DCP1, DCP5, and XRN4, promotes miRNA-mediated silencing at a step coinciding with or downstream of TR. Furthermore, a fraction of cytoplasmic AGO1 apparently localizes to processing bodies (129). Recent experiments conducted in plant lysates showed that AtAGO1-RISC has the ability to repress translation without promoting deadenylation or mRNA decay. It was shown that AtAGO1-RISC bound in the 5′ untranslated region or the open reading frame can sterically block the recruitment or movement of ribosomes (45).

Inverted repeat–derived small interfering RNAs. Discrete loci scattered in plant genomes are configured as IRs that produce extensively base-paired RNA hairpins of variable lengths. In particular, endogenous hairpins significantly longer than typical pre-miRNAs often generate endogenous siRNAs (24, 25, 50) (**Figure 3*c***). A detailed study in *Arabidopsis* showed a remarkable overlap between the processing requirements for two such extended hairpins and those of a transgenic IR used for experimental RNA interference. All IRs generated active, HEN1-methylated siRNAs upon their coprocessing by DCL4/2 (21–22-nt siRNAs) and DCL3 (24-nt siRNAs). DCL1 also stimulated siRNA accumulation indirectly, presumably by facilitating separation of the dsRNA stem from the single-stranded section of IR transcripts, as in pri-to-pre-miRNA maturation. However, *hyl1* had only minor or no effects on siRNA levels, as did *drb2*, *drb3*, and *drb5*. By contrast, loss of function in the DCL4-interacting protein DRB4 strongly enhanced the production of DCL3-dependent 24-nt siRNAs, suggesting that DCL3 normally competes with the DRB4–DCL4 complex for access to endogenous and exogenous IRs (24, 25). IR-derived siRNA production required none of the factors involved in RDR-mediated dsRNA synthesis in the PTGS or TGS pathways, consistent with the intramolecular base-pairing of IRs.

Studies of various *Arabidopsis* accessions showed that endogenous IRs formed by gene duplication define a class of fast-evolving genes, some of which are poised to respond to stress transcriptionally, although their abundant endo-siRNA products are yet to be assigned any biological functions. Given their sometimes tissue-specific expression and demonstrated ability to drive non-cell-autonomous silencing at both transcriptional and posttranscriptional levels, some IRs may have adaptive value by integrating temporally and/or spatially restricted stresses or environmental signals at the whole-plant level and perhaps in progenies (24). Regardless, the regulatory

potential of IR-derived siRNAs has been somewhat neglected thus far in plants and metazoans, and robust and comprehensive annotations of non-miRNA hairpins in these genomes are needed.

Natural antisense transcript small interfering RNAs. In contrast to those of plant miRNAs and IR-derived siRNAs, precursors of nat-siRNAs are formed by annealing of two complementary and separately transcribed RNA strands. When the opposite strands originate from the same locus (i.e., in *cis*), the siRNAs are termed *cis*-nat-siRNAs (**Figure 3*d***). *Cis*-NAT pairs are widespread in eukaryotic genomes and occur at an estimated 9% of all *Arabidopsis* genes, most commonly by the overlapping 3′ ends of coding or noncoding RNA pairs (114). *Cis*-NATs regulate gene expression via various mechanisms, including Pol-II collision, chromatin modification, translational control, and nat-siRNA production. However, only 4–6% of *Arabidopsis cis*-NAT pairs can potentially generate *cis*-nat-siRNAs above background levels. In the cases studied so far, one transcript of the *cis*-NAT pair is usually constitutively expressed and the other is stress or developmentally induced, after which downregulation of the constitutive target gene by *cis*-nat-siRNAs confers tolerance to the inductive stress (142). The pathways underlying *cis*-nat-siRNA production are complex and differ widely from one case to another, raising the question of whether nat-siRNAs even form a single class of RNA silencing effectors. However, a requirement for single or multiple RDRs for nat-siRNA biogenesis has been recurrently observed; these RDRs perhaps amplify the process of initial siRNA production triggered by NAT RNA hybridization. Nonetheless, the RDR-dependent siRNAs mapping to *cis*-NAT gene pairs may be simply correlated with, and not caused by, the overlapping transcripts. Further investigation is required to address these various issues.

Trans*-acting small interfering RNAs.** RNAs from the eight recognized *Arabidopsis TAS* loci (*TAS1a–c*, *TAS2*, *TAS3a–c*, and *TAS4*) are transcribed by Pol II, capped, polyadenylated, and then processed to release mature tasiRNAs following two main pathways. In the one-hit pathway, single target sites for the 22-nt-long miR173 and miR828 are found in the TAS1a–c/pri-TAS2 and pri-TAS4 RNAs, respectively (**Figure 3*e). Upon mandatory cleavage by AGO1 and RDR6-mediated dsRNA synthesis, tasiRNAs are processed by DCL4 from the miRNA-cleaved 3′ fragments. The two-hit pathway involves the near-exclusive loading of AGO7 with 21-nt-long miR390, which recognizes two distinct target sites in the pri-TAS3a–c RNAs (**Figure 3*e***). Pri-TAS3a–c are cleaved at the 3′- but not the 5′-miR390 target site, and in this case tasiRNAs are derived from the 5′-cleavage fragment. In both cases, the near-invariable positions defined by miRNA-directed cleavage, before dsRNA conversion by RDR6, lead DCL4 to produce contiguous and phased siRNA species within a specific sequence register (2, 17, 18, 82, 133).

In addition to RDR6 and DCL4, genetic screens for suppressors of sense-transgene silencing and for heteroblasty have identified a suite of *Arabidopsis* proteins whose endogenous functions appear to be linked exclusively to tasiRNA biogenesis/activity. For instance, the coiled-coil domain protein SUPPRESSOR OF GENE SILENCING 3 (SGS3) forms a complex with miR173-containing RISC and TAS2 RNA-cleavage fragments. Either a 1-nt deletion at the 3′ end of miR173 or a mismatch at the 3′ end of the miR173 target site on TAS2 is sufficient to abolish formation of this complex. Because this correlates with degradation of, rather than RDR6 recruitment at, the 3′-cleavage fragment, miR173-AGO1 and SGS3 likely stabilize the miR173-cleavage fragment. SGS3 and RDR6 also colocalize with AGO7 in cytosolic small interfering bodies, suggesting that SGS3 may similarly stabilize interactions between miR390-AGO7 and TAS3 RNA cleavage fragments (132). The role for SILENCING-DEFICIENT 5 (SDE5) (a homolog of the human mRNA export factor TAP) in tasiRNA production has been tentatively determined to occur downstream of pri-TAS cleavage but before dsRNA formation (46); components of the THO/TREX complex, required for mRNA nucleo-cytosolic trafficking, act on

pri-TAS after their transcription but before their miRNA-guided cleavage, through mechanisms that remain unclear (46). In addition, generic silencing components required for miRNA/siRNA stability (HEN1, HESO1) and AGO1 loading (HSP90, SQN, FBW2) also contribute to tasiRNA biogenesis. Intriguingly, DNA-level effects in *cis* have been reported at the *TAS1c* and *TAS3a* loci, where sequences corresponding to the RDR6-amplified cleavage products become de novo methylated, indicating that tasiRNAs or their long dsRNA precursors can guide DNA methylation (122). This methylation is lost in *sgs3* and *rdr6* mutant *Arabidopsis* plants, suggesting that an RdDM-based feedback regulatory loop fine-tunes *TAS* expression, a notion that requires further investigation. Upon loading into AGO1, tasiRNAs posttranscriptionally downregulate protein-coding transcripts from unrelated loci via slicing and, perhaps, TR/mRNA decay. In *Arabidopsis*, these targets include ARF transcripts that acquire a spatially graded accumulation pattern required for organ polarization (see sidebar tasiRNAs: Why the Fuss?).

Transcriptional Gene Silencing– and DNA-Associated Small RNAs

The largest group of *Arabidopsis* sRNA is composed of DNA-associated molecules involved in TGS and, as discovered more recently, DNA repair.

Repeat-associated small interfering RNAs. RdDM by DCL3-dependent 24-nt repeat-associated siRNAs (rasiRNAs) dampens transcription of transposons and repeats through de novo methylation of cytosines in all sequence contexts (CG, CHG, and CHH, where H = A, T, or C), a reaction mediated by DOMAINS REARRANGED METHYLTRANSFERASE 1 (DRM1) and DRM2. De novo established DNA methylation patterns may then be maintained during DNA replication in a rasiRNA-independent manner (77). CHG methylation maintenance involves the coordinated action of CHROMOMETHYLTRANSFERASE 3 (CMT3) and several H3K9 histone methyltransferases, whereas DNA METHYLTRANSFERASE 1 (MET1) maintains CG methylation patterns (49). DDM1, a SWI/SNF chromatin remodeler also required for maintenance, facilitates DNA methyltransferases' access to heterochromatin (138).

In addition to the generic RNA silencing factors detailed above, rasiRNA biogenesis and function require two plant-specific RNA polymerases called Pol IV and Pol V, which act at each end of the RdDM pathway. The current model proposes that Pol IV, with the assistance of the SNF2-domain-containing protein CLASSY 1 (CLSY1), transcribes heterochromatic regions to produce long ssRNA that is then converted into dsRNA by RDR2. Processed by DCL3 and methylated by HEN1, 24-nt rasiRNAs are then loaded into AGO4 clade members. Independent of Pol-IV transcription and siRNA biogenesis, Pol V generates transcripts at RdDM target loci with the assistance of the DDR complex, comprising the putative chromatin-interacting ATPase DRD1, the hinge-domain protein DMS3, and the ssDNA-binding protein RDM1, which displays a preference for methylated DNA (59, 147). AGO4 can be cross-linked to Pol-V transcripts, suggesting that Pol-V noncoding RNAs serve as scaffolds for AGO4–siRNA complexes through RNA–siRNA base-pairing. AGO4 can also interact with the C-terminal domain of the largest Pol-V subunit, presumably stabilizing the complex through protein–protein interactions. Subsequent recruitment of DRM2 and other chromatin-modifying enzymes may be mediated by RDM1 independently of its role in the DDR complex, bridging DRM2 to AGO4–siRNA–Pol-V transcript complexes and thereby facilitating de novo cytosine methylation of Pol-V-transcribed loci (61, 119) (**Figure 4*a***). Depending on the extent of methylation and nature of the targeted sequences (e.g., promoter), RdDM may ultimately result in TGS by impediment of Pol-II-dependent transcription.

Intrinsic to the above self-enforcing RdDM model is the notion that specific features should instruct Pol IV to initiate transcription and rasiRNA synthesis at transposable-element- or

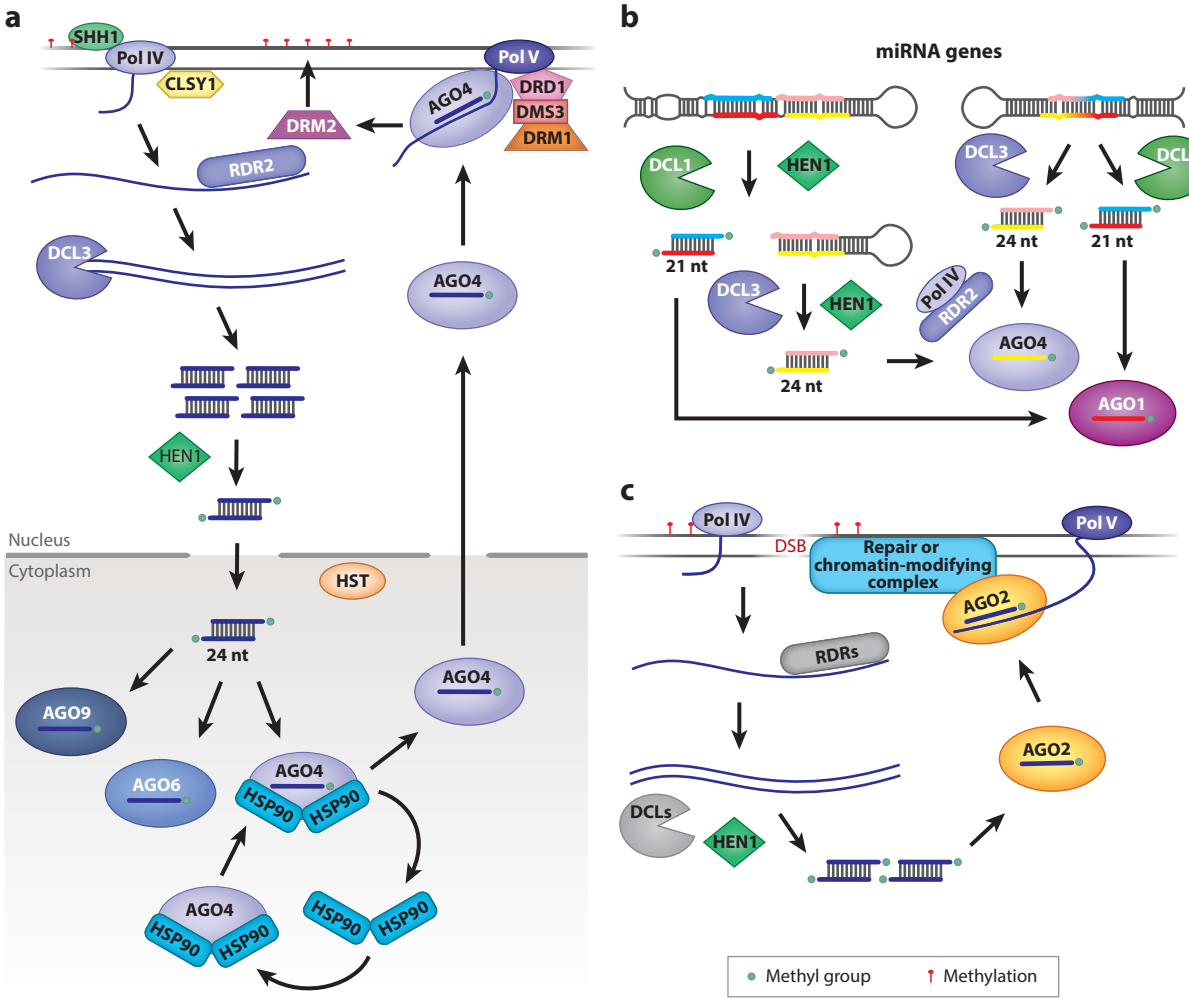


Figure 4

Transcriptional gene silencing– and DNA-associated small-RNA (sRNA) pathways. (*a*) The repeat-associated small-interfering-RNA (rasiRNA) pathway. (*b*) The long-microRNA (lmiRNA) pathway. (*c*) The double-strand-break-induced-RNA (diRNA) pathway. Additional abbreviations: AGO, ARGONAUTE; CLSY1, CLASSY 1; DCL, DICER-LIKE; DMS3, DEFECTIVE IN MERISTEM SILENCING 3; DRD1, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1; DRM, DOMAINS REARRANGED METHYLTRANSFERASE; DSB, double-strand break; HEN1, HUA ENHANCER 1; HSP90, HEAT-SHOCK PROTEIN 90; HST, HASTY; nt, nucleotide; Pol, RNA polymerase; RDR, RNA-dependent RNA polymerase; SHH1, SAWADEE HOMEODOMAIN HOMOLOG 1.

repeat-associated loci but not at other loci. Recently, the Pol-IV-interacting SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) was found to enable Pol-IV recruitment and/or stability at a large subset of the most active *Arabidopsis* RdDM-targeted loci. Accordingly, *shh1* mutant plants display significantly reduced de novo DNA methylation, rasiRNA production, and Pol-IV occupancy at RdDM-targeted loci. SHH1 coordinately binds unmethylated K4 and methylated K9 modifications on histone 3 (H3), which are features of repressed chromatin states typically associated with transposons and repeats (60). These interactions take place through lysine-binding

REFINING RdDM

Recently, whole-genome bisulfite sequencing in *Arabidopsis* enabled an exhaustive analysis of DNA methylation in 86 silencing mutants and their combinations, of which 29 had been previously suggested to affect RdDM (103). An examination of methylation levels at DRM1/2-dependent CHH methylated sites in particular revealed four main classes of RdDM component requirements: (*a*) those where RdDM mutations eliminate DRM1/2-dependent methylation, (*b*) those where mutations reduce methylation, (*c*) those where mutations weakly reduce methylation, and (*d*) those that affect only a very small proportion of sites. Whereas *rdm1*, *ago4*, *drd1*, *nrpe1*, *nrpd1*, *drm1/2*, *dms3*, *rdr2*, and *dms4* mutations were associated with loss of methylation, *ago6* and *dcl2 dcl3 dcl4* mutations resulted in reduced methylation levels. *dcl3* and *clsy1* mutations resulted in weakly reduced methylation levels, and *dcl2*, *dcl4*, *dcl2 dcl4*, and *hen1* mutations resulted in unaltered methylation levels at DRM1/2-dependent CHH sites. These results are consistent with AGO6 being involved in only a specific subset of tissues and affecting a select, discrete number of target loci (as discussed in the main text). They also suggest that, in the absence of DCL3 action, DCL2 and perhaps DCL4 can mediate DNA methylation at most RdDM target sites. The near lack of effect of *hen1* might be linked to the hypomorphic nature of the mutant allele used. Interestingly, of all mutants not involved in canonical RdDM, *rdr6* and the related *rdr1* mutant showed the strongest hypomethylation, affecting all cytosine contexts. RDR6-associated methylation was linked to Pol-II-dependent transcription of active genes rather than Pol-IV- and Pol-V-dependent transcription of transposable-element remnants and/or repeats. This suggests the existence of a hitherto unknown endogenous pathway linking PTGS to RdDM.

pockets within the tandem Tudor-like fold adopted by the SAWADEE domain, which is essential for rasiRNA production and DNA methylation at targeted loci. Pol-IV recruitment at SHH1-independent target loci remains mysterious, as do the chromatin/DNA features required for Pol-V occupancy along the genome. A recent exhaustive analysis of *Arabidopsis* silencing pathway mutants revealed many refinements in the basic RdDM model proposed here along with somewhat unsuspected requirements for PTGS pathway components in this process (103) (see sidebar Refining RdDM).

Long microRNAs. Studies in *Arabidopsis* and rice indicate that several pri-miRNAs can be processed by DCL3 to release 24-nt long miRNAs (lmiRNAs). Like other functional sRNAs, lmiRNAs require HEN1 for their stabilization and are loaded into AGO4 (16, 123). AGO4–lmiRNA complexes can direct localized cytosine methylation both in *cis* at the *MIRNA* locus of origin and in *trans* at the miRNA targeted loci. Recently work in rice suggests that both DCL1 and DCL3 can recognize the same pri-miRNA and either (*a*) compete with each other to release either the miRNA or the lmiRNA or (*b*) work sequentially together to release both functional sRNA species (123) (**Figure 4*b***). However, contrasting results were obtained regarding the requirement of additional factors in lmiRNA biogenesis. Therefore, although rice lmiRNA levels were not reduced in RDR2-knockdown RNA-interference lines (123), production of *Arabidopsis* lmiRNAs required RDR2 and Pol-IV functions (16). A much earlier study also showed how miR165 and miR166 direct DNA methylation downstream of their target sites in the PHABULOSA (PHB) and PHAVOLUTA (PHV) coding regions in a manner not affected in *dcl1* and *ago1* hypomorphic mutants (4). Interestingly, *MIR165* and *MIR166* can give rise to 23- to 26-nt sRNAs associated specifically with AGO4 and AGO7. Even though other examples were subsequently described, the biological significance of chromatin modifications directed by lmiRNAs remains poorly understood (16, 123). However, a case can be made that methylation in *cis* might provide a means of feedback regulation of *MIRNA* transcription.

Double-strand-break-induced RNAs. DNA double-strand breaks cause mutations, genome instability, and cell death. Critical for genome integrity maintenance and cell survival, double-strand-break repair mechanisms in plants and mammals apparently entail the production of previously unknown double-strand-break-induced RNAs (diRNAs). In *Arabidopsis*, 21- and 24-nt diRNAs derive from both sense and antisense strands around double-strand-break sites in a DCL2-, DCL3-, and DCL4-dependent manner, and repair rates in *dcl2 dcl3 dcl4* triple-mutant plants are significantly reduced accordingly (116). Supporting the idea that diRNA biogenesis results from de novo synthesized and amplified dsRNA, diRNA levels were reduced by ~90% in *rdr2* and *rdr6* single mutants. However, repair efficacy was not altered, suggesting that RDR2/RDR6 action is redundant, other RDRs are involved, or low diRNA levels are sufficient for repair. Although repair rates were reduced by 80% and 50% in the *nrpd1* and *nrpe1* mutant backgrounds, diRNA production was greatly compromised in the former but increased in the latter, suggesting that Pol IV and Pol V are distinctively involved in diRNA biogenesis and activity, respectively (116). Further analyses identified AGO2 as one effector of diRNA action and double-strand-break repair (116). A highly speculative model, inspired largely by the RdDM pathway, proposes that Pol IV generates long ssRNA from double-strand-break-proximal DNA regions. Possibly redundant RDR functions then convert the ssRNA into dsRNA, which is subsequently processed into diRNAs by the coordinated actions of DCL2, DCL3, and DCL4. AGO2–diRNA complexes would then be recruited to double-strand-break sites by binding sequence-complementary, Pol-V-dependent scaffold transcripts (**Figure 4*c***). Uncharacterized diRNA-induced chromatin/DNA modification would then recruit the repair machinery, which, alternatively, may be brought to double-strand-break sites upon interaction with the AGO–diRNA complex.

SUMMARY POINTS

1. DICER-LIKE (DCL) proteins, RNA-dependent RNA polymerases (RDRs), HUA ENHANCER 1 (HEN1), and ARGONAUTE (AGO) proteins are the core enzymes responsible for the production and function of all silencing small RNAs (sRNAs) in plants.
2. Plant DCLs, RDRs, and AGOs are encoded by multigene families and belong to conserved clades that are often specialized in the production and function of distinct endogenous sRNA classes.
3. In addition to the core proteins, each sRNA pathway requires the supplementary actions of other factors, some of which may be shared between pathways.
4. A hierarchical continuum of action links many of the core silencing protein family members. Diverse affinities for specific types of RNA substrates and/or distinct subcellular localizations of DCLs, RDRs, and AGOs ultimately determine the biological outputs.
5. Several core silencing proteins are subjected to negative-feedback regulation orchestrated by their own sRNA products. This allows the homeostatic control of cellular silencing machinery.
6. Based on their biogenesis mechanisms, sizes, and 5′-terminal nucleotide compositions, seven classes of plant endogenous sRNAs have been characterized to date. These sRNAs regulate gene expression via mRNA degradation, translation inhibition, or chromatin modifications. A novel class of sRNAs dubbed double-strand-break-induced RNAs (diRNAs) has been implicated in DNA repair.

DISCLOSURE STATEMENT

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Errata

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