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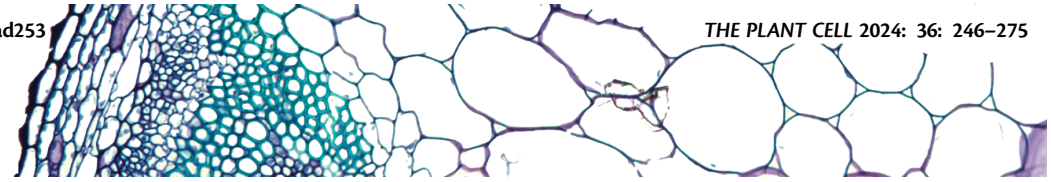

Review

The plant siRNA landscape

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Abstract

Whereas micro (mi)RNAs are considered the clean, noble side of the small RNA world, small interfering (si)RNAs are often seen as a noisy set of molecules whose barbarian acronyms reflect a large diversity of often elusive origins and functions. Twenty-five years after their discovery in plants, however, new classes of siRNAs are still being identified, sometimes in discrete tissues or at particular developmental stages, making the plant siRNA world substantially more complex and subtle than originally anticipated. Focusing primarily on the model Arabidopsis, we review here the plant siRNA landscape, including transposable elements (TE)-derived siRNAs, a vast array of non–TE-derived endogenous siRNAs, as well as exogenous siRNAs produced in response to invading nucleic acids such as viruses or transgenes. We primarily emphasize the extraordinary sophistication and diversity of their biogenesis and, secondarily, the variety of their known or presumed functions, including via non-cell autonomous activities, in the sporophyte, gametophyte, and shortly after fertilization.

How are siRNAs defined?

Eukaryotic 20- to 30-nt silencing small (s)RNAs mostly consist in micro (mi)RNAs and short interfering (si)RNAs derived from long double-stranded (ds)RNA by the action of Dicer/Dicer-like (DCL) RNase-III enzymes. Bona fide Dicer products' signatures include double-strandedness, 2-nt 3′overhangs, and 5′monophosphates licensing their loading into ARGONAUTE (AGO) effector-proteins. Within RNA-induced silencing complexes, AGOs execute RNA-silencing of sequence-complementary RNA/DNA using one selected sRNA strand as a guide (Svoboda 2020). DCLs exist in animals, plants, ciliates, and fungi with exceptions, including, for example, *Saccharomyces cerevisiae*. Dicer-independent sRNAs include Piwi-interacting (pi)RNAs in animals' germlines (Ozata et al. 2019) and plant DCL-independent RNAs likely trimmed from longer single-stranded (ss)RNA (Ye et al. 2016).

Plant miRNAs and siRNAs were originally distinguished on the following assumptions:

- miRNAs derive from ssRNA adopting bulged stem-loop structures whose nuclear processing, generally by DCL1 (out of 4 Arabidopsis DCLs, DCL1→4), yields a single, nonperfectly paired sRNA duplex (Fig. 1A);
- siRNAs derive from perfectly paired bimolecular dsRNAs formed by overlapping convergent/readthrough transcription or upon ssRNA→dsRNA conversion by 1 of 6 Arabidopsis RNA-dependent RNA polymerases (RDR1→6; (Bologna et al. 2018). Through multiple distributive/processive cuts, Dicers process these dsRNAs into populations of perfectly paired sRNA duplexes (Fig. 1B). Arabidopsis DCL4, DCL2, and DCL3, respectively, produce 21-nt, 22-nt, and 24-nt siRNAs presumably reflecting slightly different "ruler" domains separating the

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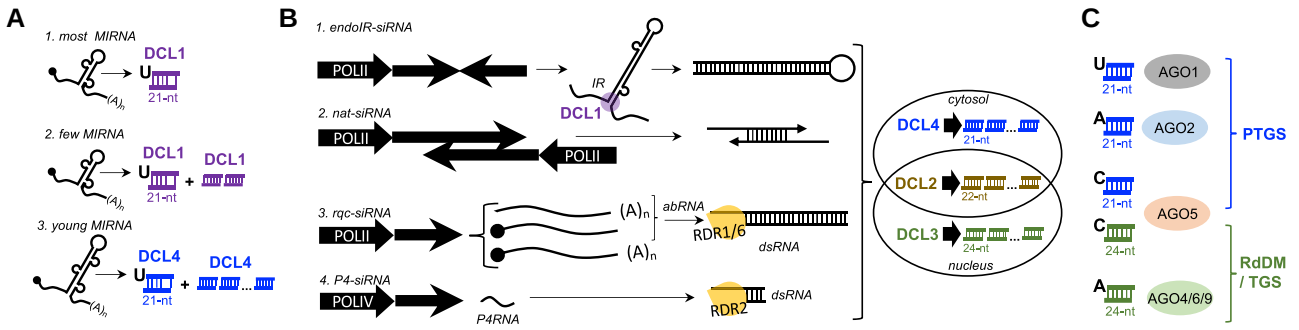

Figure 1. RNAi sources and associated machineries. **A)** Sources of miRNAs. miRNAs derive from the of POLII-transcribed RNAs with imperfect foldback stem-loop structures. 1. Most *MIR*s produce a single miRNA via DCL1, but 2. some *MIR*s can produce up to 3 miRNAs. 3. Young *MIR*s produce multiple miRNAs via DCL4, of which one is considered the major product. **B)** Sources of primary siRNAs. siRNAs derive from perfectly paired dsRNA with 4 possible origins: 1. POLII-transcribed long inverted repeats produce RNAs with foldback stem-loop structures similar to, but longer than, those derive from young *MIR* genes. 2. POLII-transcribed genes arranged in convergent orientation on opposite DNA strands produce mRNAs complementary on the overlapping region 3. POLII-transcribed genes produce a fraction of abRNAs lacking a cap or a polyA tail, which can be converted to dsRNA by RDRs (mostly RDR1 and RDR6) when they evade RNA quality control. 4. POLIV transcribes short RNAs converted to dsRNA by RDR2. When produced in the nucleus, dsRNA are processed into 24-nt siRNAs by DCL3 or, alternatively, into 22-nt siRNAs by DCL2, whereas cytosolic dsRNAs are processed into 21-nt siRNAs by DCL4 or, alternatively, into 22-nt siRNAs by DCL2. **C)** Argonaute machineries. Depending on their size and 5′-terminal-nucleotide identities, siRNAs are loaded into different AGO proteins, which execute either PTGS or RdDM/TGS. Of note, AGO7 and AGO10 are not depicted in the figure as they have only been reported to load particular miRNAs.

PAZ- (anchoring one long dsRNA extremity) from the RNase-III domain (Svoboda 2020). DCL3 is nuclear, DCL4 mostly cytoplasmic, while nucleo-cytosolic DCL2 can functionally substitute DCL4 or DCL3 (Xie et al. 2004) (Fig. 1B). Hence, 21-nt and 24-nt siRNAs are generally, respectively, cytoplasmic and nuclear, while 22-nt siRNAs may accumulate in either compartment in *dcl3* or *dcl4* mutant Arabidopsis (Bologna et al. 2018).

Continuously increasing sequencing depths identified sRNAs not easily classified as either miRNAs or siRNAs according to the above criteria (Meyers et al. 2008). A now-recognized continuum between miRNA and siRNA biogenesis is reflected by subsequent definitions of additional sRNA subclasses (Axtell and Meyers 2018) as follows:

- Most miRNAs derive from imperfect short hairpins generally processed by DCL1 into a single, imperfect duplex (Voinnet 2009; Bologna et al. 2018) (Fig. 1A), although some are processed by DCL1 into up to 3 duplexes, with the most abundant considered the reference miRNA (Zhang et al. 2010) (Fig. 1A);
- Few miRNAs, usually referred to as young miRNAs, derive from mostly imperfect longer hairpins processed by DCL4 into multiple duplexes, with the most abundant considered the reference miRNA (Rajagopalan et al. 2006) (Fig. 1A);
- *EndoIR*-siRNAs derive from imperfect, often extended hairpins processed primarily by DCL2, DCL3, and/or DCL4 into siRNA populations (Fig. 1B), with DCL1 likely facilitating excision of stem-loop regions within endo- and transgenic *IR*s (Henderson et al. 2006; Dunoyer et al. 2007).
- True siRNAs derive from perfect dsRNAs processed as populations by DCL2/DCL3/DCL4 (Vaucheret 2006) (Fig. 1B).

Adding to this complexity, siRNAs can be classified as either "primary" or "secondary" (Table 1). Primary siRNAs are produced by DCL-mediated processing of hairpin-forming transcripts or single-stranded transcripts converted to dsRNA by an RDR. Secondary siRNAs, by contrast, derive from single-stranded transcripts of which the conversion to dsRNA by RDRs requires their prior targeting by primary siRNAs or miRNAs. Secondary siRNAs amplify the silencing response, which, as this review will illustrate, can empower sophisticated biological outputs, including in defense or multigene-family regulations. Identifying/annotating siRNA precursors, including RDRs' ssRNA substrates and dsRNA products, poses another difficulty. Indeed, inferred RDR6 substrates include cap-/poly-A tail-deficient "aberrant" RNAs normally degraded by RNA quality-control (RQC) (Liu and Chen 2016). This makes their deep-seq-based detection challenging in wild-type (WT) tissues compared with, for example, capped and poly-A^+ primary miRNA transcripts (pri-miRNAs). Nevertheless, the Arabidopsis *dcl2dcl3dcl4* triple mutant allowed characterization of RDR2/6 endogenous substrates, for instance (Rajeswaran et al. 2012; Blevins et al. 2015; Zhai et al. 2015a).

Like miRNAs, siRNAs are loaded into one or several AGOs of which there are 9 functional paralogs in Arabidopsis (AGO1→10), with AGO8 considered a pseudogene. The loading specificity depends primarily on sRNA sizes and 5′-terminal nucleotide identities, although nucleotides 2/6/9/13 also seem influential (Thieme et al. 2012). AGO1, AGO2, and AGO5 prefer 21-nt 5′U, 5′A, and 21-nt/24-nt 5′C cargoes, respectively, while AGO4 and its clade members AGO6/9 prefer 24-nt 5′A cargoes (Fig. 1C). This likely reflects

Table 1. Classes of siRNAs

Nomenclature	Full name	Type	Size (nt)	Cellular origin	Genomic origin	Known factors of their biogenesis () = facultative factor	Figure
ea-siRNA	Epigenetically-activated siRNA	Secondary	21 to 22	Sporophyte (*ddm1*, *met1*)	TE	POLII-(miRNA/AGO1-RDR6)-DCL4	4A
endoIR-siRNA	Endogenous inverted repeat-derived siRNA	Primary	21 to 22 to 24	Sporophyte	IR (non-coding)	(DCL1)-DCL2/3/4	1B
nat-siRNA	Natural antisense siRNA	Primary	21 to 22 to 24	Sporophyte	PCG (overlapping region)	DCL2/3/4	1B, 2D
nat-siRNA	Natural antisense siRNA	Secondary	21 to 22	Sporophyte	PCG (adjacent regions)	RDR6-DCL4	2D
P4-siRNA	POLIV-dependent siRNA	Secondary	24	Sporophyte	TE	POLIV-RDR2-DCL3	3A
pha-siRNA	Phased siRNA	Secondary	21 to 22	Sporophyte	PCG (mRNA)	miRNA/AGO1-RDR6-DCL4	2C
pha-siRNA	Reproductive pre-meiotic phased siRNA	Secondary	21 to 22	Male sporophyte?	PHAS (non-coding)	POLII-RDR6-DCL4	2C
pha-siRNA	Reproductive meiotic siRNA	Secondary	24	Male gametophyte	PHAS (non-coding)	POLII-RDR6-DCL5	2C
pollen-siRNA	Pollen siRNA	Likely secondary	21 to 22	Male gametophyte	TE	POLII-RDR6-DCL4 or POLIV/V-miRNA/AGO1-RDR2-DCL4?	5A
rqc-siRNA	RNA quality control-deficiency siRNA	Primary	21 to 22	Sporophyte	PCG (aberrant RNA)	RDR6-DCL4	1B, 2E
siren-siRNA	siRNA in endosperm	Secondary	24	Female sporophyte, seed coat	TE	POLIV-RDR2-DCL3	5B
ta-siRNA	Trans-acting siRNA	Secondary	21 to 22	Sporophyte	TAS (non-coding)	miRNA/AGO1/7-RDR6-DCL4	2A, 2B
tap-siRNA	Tapetum-enriched siRNA	Primary	24	Male sporophyte	TE	POLIV-RDR2-DCL3	5A
uv-siRNA	UV-induced siRNA	Primary	21	Sporophyte	IGR (non-coding)	POLIV-RDR2-DCL4	2F
v-siRNA	Virus-derived siRNAs	Primary and secondary	21 to 22 to 24	Sporophyte	Viral genome (DNA, RNA)	(RDR1/2/6)-DCL2/3/4	7A
va-siRNA	Virus activated siRNA	Primary	21 to 22	Sporophyte	PCG (aberrant RNA)	RDR6-DCL4	2E

AGO-specific optimal steric accommodation of certain nucleosides within their MID domain (Frank et al. 2010), a process operated in coordination with the PIWI domain (Liu et al. 2022). AGO7 and AGO10 stand apart by showing specificity to only certain miRNAs, of which the duplex structure, among other less understood parameters, appears critical for selection (Zhu et al. 2011; Endo et al. 2013; Xiao and MacRae 2022). Being mostly 21-nt 5′U species, miRNAs mainly load into AGO1, whereas siRNAs' heterogeneity in sizes and 5′nt identities causes a diversity of AGO associations and, hence, activities. Beyond miRNA vs siRNA classification, the issue of distinguishing bona fide siRNAs from longer RNAs' degradation products remains. Indeed, a decisive feature of DCL action is duplex formation, yet deep-sequencing (deep-seq) rarely recovers the 2 strands of at least low-abundant siRNAs. A dedicated "phosphate pocket" in their mid-domains helps AGOs discriminate bona fide cargoes from RNA breakdown products generally exhibiting a 5′OH instead of the Dicer-dependent siRNAs' 5′P (Frank et al. 2010). Thus, AGO-immunoprecipitation (IP)-deep-seq can facilitate siRNA annotation, as do technologies universally separating AGO-loaded from nonloaded sRNAs (Grentzinger et al. 2020). Nonetheless, a 5′P is still insufficient for assertive siRNA annotation. For instance, long RNA 5′→3′ exonucleolysis by XRN enzymes not only requires 5′P substrates but also generates 5′P products potentially loadable into AGOs (Peach et al. 2015). ß-Elimination provides an additional handle in plants where all bona fide sRNAs have periodate-resistant 2′-*O*-methylated 3′ends (Yu et al. 2005), uncommon in RNA breakdown/degradation products.

Classes, biogenesis, and functions of plant siRNAs

siRNAs derived from plant genome–encoded loci distinct from transposable elements

siRNAs derived from endogenous inverted-repeats (endoIRs)

Transcribed *endoIRs* resemble pri-miRNAs, yet, likely due to their size/structure, are processed by DCL2, DCL3, and/or DCL4 albeit with DCL1's assistance (Henderson et al. 2006; Dunoyer et al. 2007). The ∼3-kb dsRNA from Arabidopsis *IR71* (the most studied *endoIR*) is processed into 22- and 24-nt siRNAs, likely nuclearly, by DCL2 and DCL3, a pattern unchanged in *rdr1rdr2rdr6* triple mutants (Henderson et al. 2006; Devers et al. 2020). Being genetically programed to produce siRNAs de facto RDR-independently, some *endoIRs* are thus suspected starting points in *MIR* genes' evolution (Allen et al. 2004). Though vastly understudied, *endoIR*-derived siRNAs might have functions of their own: *IR71* likely modulates SA/JA signaling non-cell autonomously (Devers et al. 2023), while *MuKiller* epigenetically and heritably trans-silences

maize *MuDR* transposons (Slotkin et al. 2003). Hundreds of mostly transposon-derived Arabidopsis *endoIRs* are proximal to protein-coding genes (PCG) such that DNA methylation and changes in short-range chromatin loops induced by their associated 24-nt siRNAs might modulate nearby PCG expression. This potentially contributes to phenotypic variation across accessions among which *endoIRs* are poorly conserved (Arce et al. 2023).

siRNAs derived from TAS non-protein coding genes targeted by miRNAs

Historically, *TAS* were characterized as non-protein coding genes producing 21- and 22-nt siRNAs (Fig. 2A), distinguishing them from transposable elements (TEs)/repeat loci spawning mostly 24-nt siRNAs (see next section). The acronyms "*TAS*" and "trans-acting(ta)-siRNAs" reflect that, like miRNAs, ta-siRNAs trans-regulate genes distinct from their loci of origin. However, unlike miRNA biogenesis, ta-siRNA biogenesis requires DCL2/DCL4, RDR6, and SUPPRESSOR-OF-GENE-SILENCING-3 (SGS3), among other factors (Vaucheret 2006), and entails prior binding of specific miRNAs to *TAS* transcripts. ta-siRNAs were, in fact, the first described example of secondary endogenous siRNAs. Two scenarios of ta-siRNA production exist in Arabidopsis. *TAS1a/b/c-*, *TAS2-*, and *TAS4*-derived siRNA biogenesis involves binding by AGO1-loaded 22-nt miRNAs (miR173 for *TAS1* and *TAS2*; miR828 for *TAS4*) (Allen et al. 2005; Rajagopalan et al. 2006) (Fig. 2A). *TAS3*-derived siRNA biogenesis involves dual binding by AGO7-loaded 21-nt miR390, with only 1 of 2 sites undergoing cleavage (Axtell et al. 2006) (Fig. 2B). Because the miRNA-binding site sets a frame for phased ta-siRNA production, miRNA-guided cleavage was originally thought to liberate an SGS3-protected ssRNA fragment converted by RDR6 into a double-stranded ta-siRNA precursor (Allen et al. 2005; Yoshikawa et al. 2005). This overly simplistic model was later revised because ta-siRNA production was found to initiate from only a limited portion of the sequence-related *TAS1a/b/c* and *TAS2* RNAs following a primary AGO1:miR173-mediated cut. RDR6 then inefficiently converts the ensuing polyadenylated cleavage fragment into dsRNA. The dsRNA is processed into a first set of ta-siRNAs, of which one derived from *TAS1c*, TAS1c3′D6(−), guides AGO1 to cut *TAS1a/b/c* and *TAS2* downstream the miR173-binding site. The liberated polyA-minus fragment being a better RDR6 substrate (Baeg et al. 2017), the bulk of ta-siRNA production becomes circumscribed between the miR173- and TAS1c 3′D6(−)-binding sites (Rajeswaran et al. 2012; Sakurai et al. 2021) (Fig. 2A).

When the Arabidopsis noncleavable *TAS3*-contained miR390 site is artificially rendered cleavable, ta-siRNA production ensues (in fact, *TAS3* exhibits 2 cleavable sites in other species; Axtell et al. 2006) (Fig. 2B), albeit without phasing fidelity (de Felippes et al. 2017). *TAS3* ta-siRNA production is invariably circumscribed between the 2 sites, however. In Arabidopsis, RDR6 initiates ssRNA→dsRNA conversion at nucleotide 3 of the cleaved 3′ end and terminates at the AGO7:miR390-bound 5′ site, likely due to steric hindrance (Fig. 2B). The resulting dsRNA exhibits a 2-nt 3′ overhang and a 220-nt 5′ overhang on, respectively, the cleavable and noncleavable miR390 sides. The former optimizes PAZ domain–mediated DCL4 recruitment because Dicers prefer short overhangs, creating a single-phased *TAS3* ta-siRNA register unlike in species supporting dual cleavage (Rajeswaran and Pooggin 2012) (Fig. 2B "Arabidopsis" vs "other species"). Dual cleavage by miR173 and TAS1c 3′D6(−) also spawns dsRNA of which both ends optimally recruit DCL4, creating 2 distinct, yet opposite, phased registers for *TAS1a/b/c* and *TAS2* ta-siRNAs (Rajeswaran et al. 2012) (Fig. 2A). Despite this caveat, dual-cut ta-siRNA production, more efficient, is preferred for artificial ta-siRNA engineering (Tretter et al. 2008). Transgenic Arabidopsis expressing slicer-defective AGO1 accumulates unphased RDR6- and SGS3-dependent ta-siRNAs, suggesting that AGO1, rather than *TAS* cleaved fragments, recruits RDR6/SGS3 (Arribas-Hernandez et al. 2016; de Felippes et al. 2017). That ta-siRNA–promoting miRNAs are 22 nt instead of 21 nt long (Figs. 2, A to C) might strengthen pairing and hence increase AGO1 dwell-time on target RNAs for better RDR6 recruitment. Furthermore, SGS3 associates with AGO1:miR173 and AGO7:miR390 complexes by binding the protruding 3′ end of the dsRNA formed at the miRNA:*TAS* target site, possibly further facilitating RDR6 recruitment (Rajeswaran and Pooggin 2012; Yoshikawa et al. 2013, 2021; Iwakawa et al. 2021; Sakurai et al. 2021). Finally, SGS3/AGO1:miRNA complexes cause ribosome stalling, and indeed translation of conserved 5′ *TAS* mini-ORFs empowers ta-siRNA production (Yoshikawa et al. 2016; Bazin et al. 2017). Nevertheless, while stalled ribosomes positively regulate ta-siRNA production, it can still occur independently of translational arrest (Iwakawa et al. 2021), so the exact role of ribosome stalling in promoting ta-siRNA production remains unclear.

What advantage(s) might ta-siRNAs confer to gene expression control? Populations of ta-siRNAs with distinct sequences being simultaneously produced from individual miRNA-targeted *TAS* precursors, they could theoretically enlarge the scope-of-action of single miRNAs via coordinated downregulation of multiple mRNA targets. Only 1 or 2 ta-siRNAs usually target mRNAs, however. Indeed, *TAS1-* or *TAS2*-derived ta-siRNAs target only 9 close members of the vast *PPR* multigene family (Howell et al. 2007), while *TAS3* and *TAS4*-derived ta-siRNAs target, respectively, only 3 and 2 of the *ARF* and *MYB* family members (Allen et al. 2005; Rajagopalan et al. 2006). Direct side-by-side comparisons indicate that artificial ta-siRNAs move over greater cell numbers than a single artificial miRNA, likely reflecting *TAS* precursors' amplification by RDR6 (de Felippes et al. 2011). Thus, activation of ta-siRNA production might enhance the spatial activity range (as opposed to intracellular target range) of certain, for example 22-nt-long, miRNAs (Chitwood et al. 2009; Schwab et al. 2009).

siRNAs derived from protein-coding genes (PCGs) targeted by miRNAs

Similarly to ta-siRNA production from noncoding *TAS* genes, some PCGs produce siRNAs coined pha-siRNAs

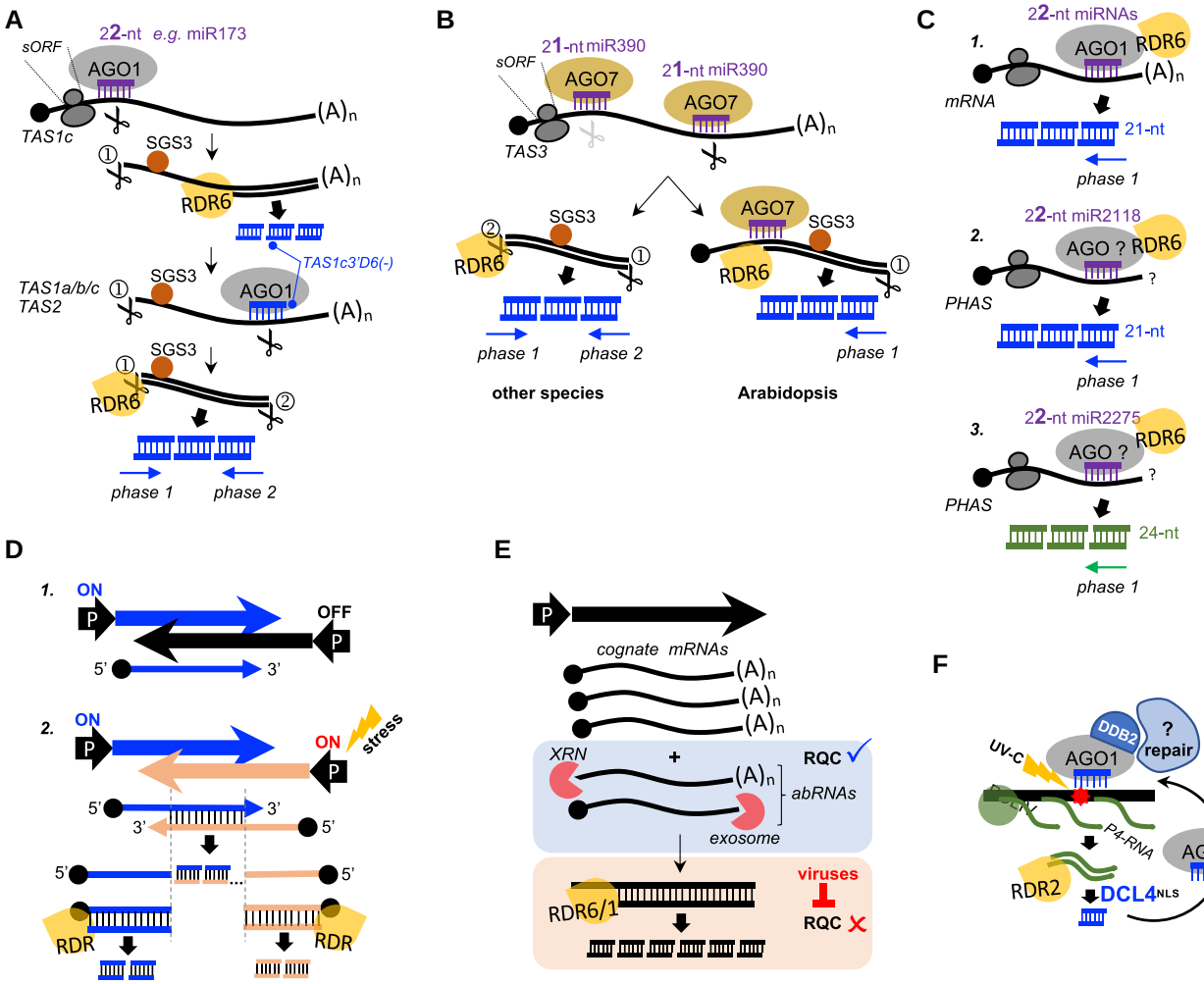

Figure 2. Biogenesis of 21-22-nt siRNAs from non-*TE* loci. **A)** AGO1-dependent ta-siRNAs. Targeting *TAS1a/b/c*/or *TAS2/4* RNAs by the 22-nt miR173/AGO1 complex attracts SGS3 and RDR6 to the cleavage products to spawn a first round of ta-siRNAs, including TAS1c3′D6(−). This ta-siRNA in complex with AGO1 also targets *TAS1a/1b/1c/2* RNAs, resulting in double-cleaved, uncapped, and nonpolyadenylated TAS RNAs that are better substrates for RDR6. Successive dicing by DCL4 generates phased ta-siRNAs from both ends of the dsRNA. **B)** AGO7-dependent ta-siRNAs. Dual targeting of *TAS3* RNAs by the 21-nt miR390/AGO7 complex attracts SGS3 and RDR6 to the cleavage products to produce phased ta-siRNAs from both dsRNA ends. In Arabidopsis, only 1 miR390 site is cleavable, resulting in phased ta-siRNAs from only 1 end of the dsRNA. **C)** AGO-dependent pha-siRNAs. 1. Targeting Arabidopsis protein-coding RNAs with the 22-nt miRNA/AGO1 complex attracts SGS3 and RDR6 to the cleavage products to spawn pha-siRNAs similarly to ta-siRNAs from *TAS* RNAs targeted by a single miRNA. 2. In some monocots, noncoding *PHAS* genes are targeted by miR2118 to produce 21-nt premeiotic pha-siRNAs. 3. In the same species, other noncoding *PHAS* genes are targeted by miR2275 to produce 24-nt meiotic pha-siRNAs. **D)** DCL-dependent nat-siRNAs. Protein-coding genes arranged as convergent units often involves a constitutively expressed gene and a stress-inducible gene. Dual expression produces bimolecular dsRNA, which upon DCL cleavage generates nat-siRNAs and 2 cleavage products lacking a polyA tail, which serves as substrates for RDR6. Subsequent processing of RDR6-derived dsRNA generates siRNAs from the 2 RNAs outside of the overlapping regions. **E)** RQC-dependent siRNAs. RQC normally eliminates abRNAs produced from protein-coding genes. When RQC is dysfunctional or impaired during virus infection, abRNAs become substrates for RDR1 or RDR6, resulting in the production of siRNAs from thousands of protein-coding mRNAs. **F)** UV-dependent siRNAs. UV irradiation induces DNA damages prevalently in intergenic regions, resulting in the production of 21-nt siRNA through the action of POLIV, RDR2 and a likely nuclear form of DCL4 ($DCL4^{NLS}$, see also Fig. 6E). The DNA DAMAGE-BINDING PROTEIN 2 (DDB2), AGO1 and 21-nt uv-siRNAs form a chromatin-bound complex possibly facilitating sequence-specific recruitment of DNA repair-recognition factors at damaged sites.

(Howell et al. 2007) reflecting their (1) phasing, (2) reliance on AGO1 association with 22-nt miRNAs, and (3) RDR6/SGS3 dependency (Fig. 2C). Ribosome stalling is not involved, however (Iwakawa et al. 2021), echoing its accessory role in ta-siRNA biogenesis. The 22-nt, unlike 21-nt, ta-siRNAs can also initiate pha-siRNA production (Howell et al. 2007). Thus, RDR6 recruitment is sRNA size dependent rather than class (mi/siRNA) dependent, perhaps via increased AGO1 dwell-time on targets. Compared with the narrow regulatory scope of noncoding *TAS* RNA-derived ta-siRNAs, pha-siRNAs may trans-regulate expression of large gene families since they derive from a member thereof [extensively

reviewed in (Liu et al. 2020)]. In many plant species, pha-siRNA–based regulations occur during abiotic and biotic stress to which they likely enable rapid responses. For instance, *NB-LRR* disease resistance gene families spawn pha-siRNAs (Zhai et al. 2011; Boccara et al. 2014). Constitutive *R* gene expression reduces plant fitness, and bacterial/fungal/viral suppressors of RNA-silencing (B/F/VSRs) inhibit si/miRNA action. Release of constitutive pha-siRNA–mediated silencing by B/F/VSRs would thus elevate the cells' defense levels possibly more rapidly than via *R* genes' de novo transcription.

siRNAs derived from PHAS non–protein-coding genes targeted by miRNAs

Two additional classes of phased siRNAs accumulate specifically in the male germline of monocots and some but not all dicots (e.g. absent in Arabidopsis) (Fei et al. 2015; Zhai et al. 2015b; Kakrana et al. 2018; Xia et al. 2019; or for a recent review, Chow and Mosher 2023). These reproductive pha-siRNAs resemble more ta-siRNAs than PCG-derived pha-siRNAs because they derive from POLII-dependent noncoding transcripts (coined *PHAS*) produced from non-TE/repeat loci. Premeiotic 21-nt siRNAs produced in anthers' epidermis depend on 22-nt miR2118- and DCL4- actions. The larger miR2118/482 superfamily triggers pha-siRNA production from *NB-LRR* resistance genes in vegetative tissues (Zhai et al. 2011; Zhang et al. 2016), suggesting miR2118 specialization in targeting noncoding *PHAS* in certain species' male reproductive tissues. In rice, mutation in *MEIOSIS ARRESTED AT LEPTOTENE* (*MEL1*) (encoding an AGO protein that selectively binds 21-nt pha-siRNAs) causes early meiosis arrest, suggesting that premeiotic pha-siRNAs enable male fertility (Nonomura et al. 2007; Komiya et al. 2014). Meiotic 24-nt siRNAs produced in the somatic tapetum also accumulate in germinal cells and persist in pollen (Zhou et al. 2022b). In monocots, their production depends on 22-nt miR2275 and a DCL3 variant coined DCL5, which sets these 24-nt pha-siRNAs apart from the PolIV-DCL3-dependent 24-nt siRNAs produced from TE/repeats in sporophytic tissues (see the TE-derived siRNAs section below). In maize, loss of the transcription factors required for *PHAS* and *DCL5* expression causes sterility, indicating that 24-nt pha-siRNA also enable male fertility (Nan et al. 2022) via mechanisms awaiting clarification.

siRNAs derived from protein-coding (PCG) or non–PCG arranged as antisense pairs

Pending their simultaneous expression, overlapping genes transcribed oppositely can produce RNAs antisense to each other, coined natural antisense transcripts (*NAT*s). The first reported *NAT* pair involves a constitutively expressed and salt stress–inducible gene pair (Borsani et al. 2005). Under salt stress, phased siRNAs coined nat-siRNAs were spawned from the *NAT* overlapping region—genetically poised to form dsRNA, hence RDR independently—but also from the transcripts' single-stranded flanking portions, this time RDR dependently. Presumably, DCL-mediated cleavage of the paired *NAT* RNAs liberates ssRNA fragments used by SGS3/RDR6 to produce dsRNA as in ta-si/pha-siRNA production (Zhang et al. 2012) (Fig. 2D). Additional *NAT* pairs were characterized in Arabidopsis and rice (Wang et al. 2005; Zhang et al. 2012), involving either PCG/PCG or PCG/non-PCG pairs, of which one partner is usually biotic or abiotic stress induced. Nonetheless, the stress adaptation potential of nat-siRNAs still remains mostly undetermined. Recently, a staggering 35,000 *NAT* pairs were tentatively predicted in Arabidopsis despite challenging annotation criteria due to the expression conditionality and/or uncapped or nonpolyadenylated status (evading conventional RNA-seq analyses) of at least one *NAT* RNA (Jin et al. 2022b). Whether stress-induced nat-siRNAs genuinely differ from PCG-derived siRNAs produced upon RQC dysfunction (see below) remains to be determined.

siRNAs derived from PCGs upon RQC impairment

PCGs sometimes produce dysfunctional RNAs: splicing errors can introduce premature or delete normal stop codons; transcriptional read-through can increase 3′ UTR length. Cells discriminate these aberrant (ab)RNAs via RQC (Moore 2005) including non–sense-mediated decay (NMD). In NMD, abRNA degradation starts with 5′ cap- and 3′ polyA-tail removal by decapping and deadenylase complexes. This is followed by 5′→3′ exonucleolysis by XRNs from unprotected 5′ P ends, and 3′→5′ exonucleolysis by the multimeric exosome from 3′ ends (Liu and Chen 2016). Remarkably, thousands of PCGs spawn siRNAs, coined RQC-deficiency-induced (rqc)-siRNAs or coding transcripts (ct)-siRNAs, in Arabidopsis mutants with compromised RQC (Martinez de Alba et al. 2015; Zhang et al. 2015; Scheer et al. 2021; Krzyszton and Kufel 2022) (Fig. 2E). Other PCG-derived siRNAs called virus-activated (va)-siRNAs accumulate in infected WT Arabidopsis (Cao et al. 2014). The PCG origins of ct/rqc-siRNAs and va-siRNAs largely overlap, suggesting that viruses might suppress RQC perhaps counter-defensively since, for example, NMD restricts phytovirus infections (Garcia et al. 2014). Alternatively, viruses might stimulate abRNA production from PCGs at levels beyond which RQC saturation triggers their RDR-mediated ssRNA→dsRNA conversion. Indeed, most ct/rqc/va-siRNAs derive from both strands in an RDR1- and/or RDR6-dependent manner (Cao et al. 2014; Martinez de Alba et al. 2015). Overall, ∼5000 of the approx. 27,000 Arabidopsis PCGs produce siRNAs upon genetic RQC impairment or infection (Cao et al. 2014; Martinez de Alba et al. 2015; Zhang et al. 2015; Scheer et al. 2021; Krzyszton and Kufel 2022), a likely underestimate given the seedling lethality of many RQC mutants and that virus-infected plants often die before flowering. Plausibly, most PCGs might spawn siRNAs under compromised RQC.

Neither siRNA production nor developmental defects are caused by singly impairing 5′→3′ or 3′→5′ cytoplasmic RQC. These occur only when both are simultaneously compromised in a manner suppressed by *rdr6* and *dcl2* but not *dcl4* mutations (Zhang et al. 2015). Impairing DCL4 alone,

but not RDR6 or DCL2, causes limited siRNA production and mild developmental defects, both suppressed in *rdr6* or *dcl2* (Bouché et al. 2006; Wu et al. 2017). These mild defects are strongly enhanced, however, when either 5′→3′ or 3′→5′ cytoplasmic RQC are additionally compromised. This causes massive 22-nt siRNA production from PCGs that, instead of mRNA degradation, trigger translational repression both gene specifically and globally through mechanisms awaiting clarification (Wu et al. 2020). Environmental stress stimulates accumulation of PCG-derived 22-nt siRNAs in WT plants, suggesting their potential role in plant adaptation to external constraints/cues.

siRNAs derived from PCGs in particular varieties/cultivars

As evoked above, PCGs rarely spawn siRNAs under laboratory growth conditions, that is, without stress-induced RQC impairment/saturation. However, PCG-derived siRNAs accumulate under unstressed conditions in certain plant varieties that nearly invariably exhibit duplications at the siRNA-producing loci, unlike regular varieties (Clough et al. 2004; Della Vedova et al. 2005; Tuteja et al. 2009; Morita et al. 2012). These duplications allow constitutive dsRNA production and, consequently, siRNA biogenesis without overt fitness costs likely because they involve PCGs dispensable to plant growth/reproduction; many such genes indeed encode the anthocyanin-producing CHALCONE SYNTHASE. Thus, most PCGs might be evolutionarily poised to avoid dsRNA/siRNAs production, and essential gene duplications might be lethal unless rapidly compensated by sequence divergence or other mechanisms.

siRNAs induced after UV stress and DNA breaks

In Arabidopsis, UV-C irradiation induces DNA damages prevalently in intergenic regions, where 21-nt siRNAs, coined uv-siRNAs (Schalk et al. 2017; Graindorge et al. 2019), accumulate. uv-siRNA biogenesis requires RDR2 and DCL4 activities and transcription by a plant-specific DNA-dependent RNA polymerase called POLIV (Herr et al 2005; see next section). Upon UV-C irradiation, the DNA DAMAGE-BINDING PROTEIN 2, AGO1, and 21-nt uv-siRNAs form a chromatin-bound complex possibly facilitating sequence-specific recruitment of DNA repair recognition factors at damaged sites (Fig. 2F). Likely involved in uv-siRNA biogenesis, an NLS-containing DCL4 isoform uses POLIV-and RDR2-dependent dsRNA (Pumplin et al. 2016). Whether other genotoxics causing other DNA lesions also induce siRNA production remains undetermined. dsDNA breaks caused by site-specific enzymes or CRISPR-Cas9 activities also spawn 21-nt sRNAs, coined dsDNA break-induced (di)RNAs (Wei et al. 2012; Miki et al. 2017). However, this was only shown at transgenic but not endogenous loci (Miki et al. 2017). Given transgenes' intrinsic proneness to generate siRNAs (see further section), whether diRNAs represent a truly distinct species remains unclear.

siRNA derived from transposable elements (TEs)

Genomic origin and biogenesis of TE-derived siRNAs

The 24-nt siRNAs form the bulk of endogenous siRNAs in healthy plants, with their abundance merely reflecting the multiplicity of their TE/repeat loci of origin. In Arabidopsis sporophytic tissues (inflorescences, leaves), DCL3 predominantly produces these molecules, hence their signature size. However, tissue-dependent changes in TE epigenetic states (e.g. transcriptionally silent vs reactivated, see below) as well as species- or even hybrid-specific changes in DCL usage (Lopez-Gomollon et al. 2022) can considerably alter this picture. POLII-dependent transcription of TE loci rearranged into sense-antisense pairs or *IR*s can spawn 24-nt siRNAs, as illustrated with the *Mu killer IR* that heritably trans-silences *Mutator* TEs in maize (Slotkin et al. 2003). Nonetheless, the bulk of 24-nt siRNAs requires transcription by the aforementioned POLII-derived, plant-specific POLIV (Herr et al. 2005). POLIV's involvement reflects the silent epigenetic steady state of most TEs, characterized by hypermethylated DNA wrapped around compact nucleosomes, where histone 3 tails display the POLII-repressive dimethylated lysin-9 epigenetic mark (H3K9me2). DNA-hypomethylated and transcriptionally active genes, by contrast, display the POLII-permissive H3K4me2 signature of relaxed nucleosomes (Richards and Elgin 2002) (Fig. 3A). By probing simultaneously for unmethylated H3K4 vs methylated H3K9, the dual-lysin reader SAWADEE homeodomain homolog 1 (SHH1) associates preferentially with the compact chromatin of 24-nt siRNA-generating loci (Law et al. 2013). Assisted by CLASSY-family putative chromatin remodelers, SHH1 likely recruits POLIV to transcribe this POLII-refractory, condensed chromatin (Zhou et al. 2018). POLIV noncoding RNAs, coined P4-RNAs, then undergo ssRNA→dsRNA conversion by RDR2 followed by DCL3-dependent processing of 24-nt siRNAs, coined P4-siRNAs (Gallego-Bartolome 2020) (Fig. 3A). How POLIV avoids occupying euchromatin and POLII-dependent PCGs was partly elucidated by the identification of ZMP (for zinc finger, mouse double-minute/switching complex B, Plus-3 protein), a chromatin-associated "junction" protein enriched at regions where chromatin with depleted H3K4 methylation is abutted by chromatin enriched in this mark (Wang et al. 2022) (Fig. 3A).

Use of the *dcl3* or *dcl2dcl3* background revealed that P4-RNAs, initiated at POLII-like transcription start sites (TSS), are only 26 to 45 nt long (Blevins et al. 2015; Zhai et al. 2015a). Their 5′ P ends often display an adenosine, while their 3′ ends have nontemplated nucleotide extensions. These coincide with the template DNA strands' methylated cytosines, suggesting that methylation signals termination (Zhai et al. 2015a). The high density of methyl-cytosines found at P4-siRNA–generating loci, in all sequence contexts (CG/CHG/CHH, where H is A/C/T), therefore helps conceptualize how reiterated transcription initiation-termination events spawn many short P4-RNAs (Fig. 3, A and B). In vitro, POLIV termination along a DNA bubble causes its backtracking, with the reannealing between template-nontemplate

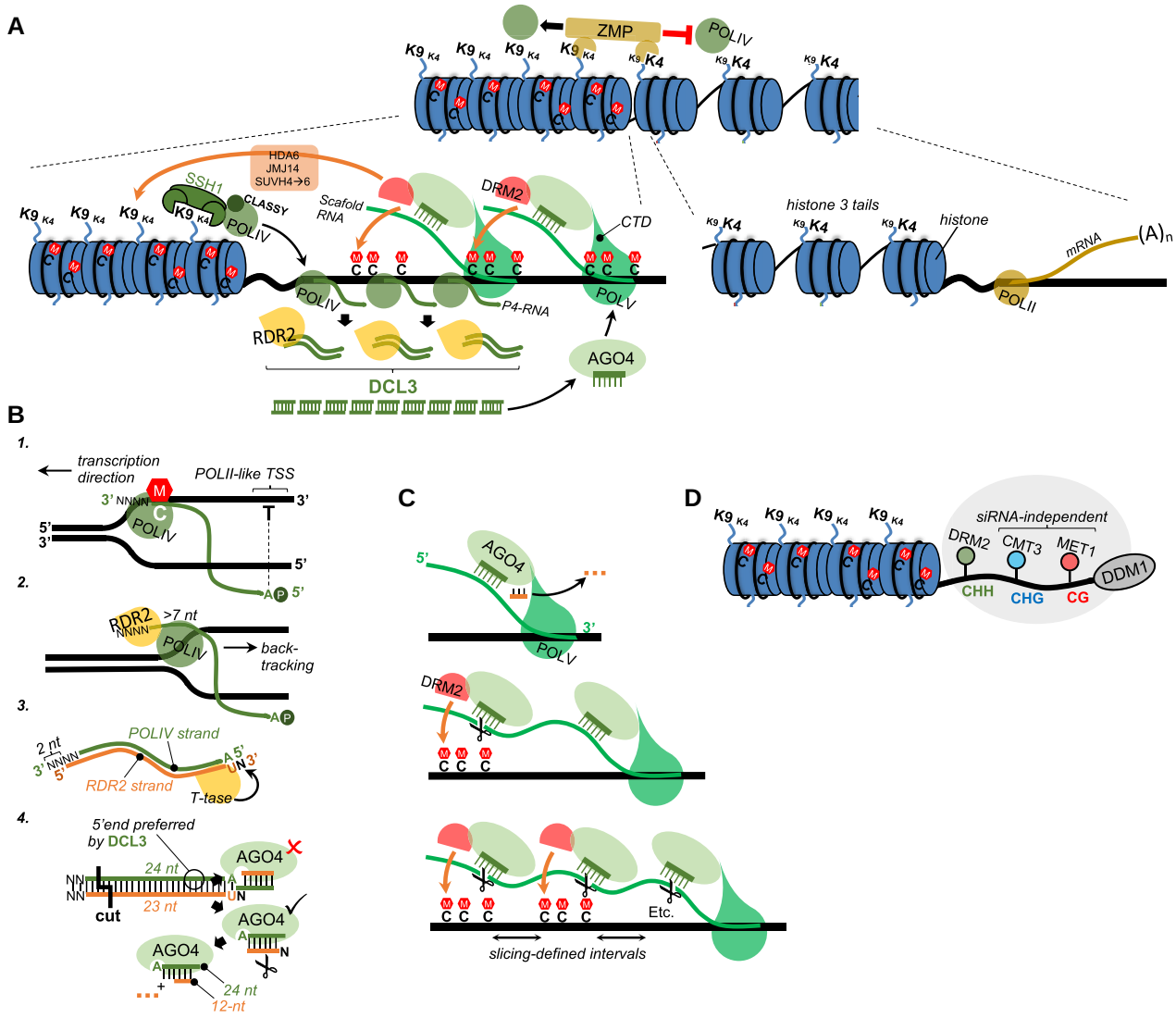

Figure 3. Biogenesis of 24-nt siRNAs RdDM establishment and maintenance. **A)** RdDM pathway. Euchromatin exhibits H3K4me3 marks and is transcribed by POLII, whereas heterochromatin exhibits H3K9me2 marks and cytosine methylation. The former attracts SHH1, which, together with CLSY, likely recruits POLIV. The POLIV-interacting ZMP protein is enriched at hetero/eu- chromatic "junction" regions by presumably monitoring local changes in H3K4 methylation, thus promoting POLIV activity on H3K4-poor chromatin and impeding it in the H3K4-rich regions. POLIV produce short transcripts called P4-RNAs. Converted into dsRNA by RDR2, P4-RNAs are diced into 24-nt siRNAs by DCL3. The ensuing 24-nt siRNA/AGO4 complex interacts with POLV's carboxy-terminal domain enabling its annealing to nascent POLV transcripts. This attracts DRM2 to further methylate DNA, while HISTONE DEACETYLASE 6, JMJ14, and SUPPRESSOR OF VARIEGATION 4,5,6 reinforce the heterochromatic state. **B)** Detailed biogenesis of POLIV-dependent DCL3 substrates. 1. POLIV initiates transcription at POLII-like TSSs and produce 26- to 45-nt P4-RNAs often displaying a 5′ adenosine. 2. Complementary RNA synthesis by RDR2 preferentially starts from the third nucleotide. 3. RDR2's terminal-transferase activity adds an untemplated 3′-nucleotide to the P4-RNA. 4. DCL3 preferentially dices 5′A dsRNA explaining the bias for 5′ A in AGO4 associated 24-nt siRNAs. **C)** Targeting of POLV RNA by AGO4:siRNA complexes. Reiterated slicing events by AGO4 simultaneously enables AGO4-POLV dissociation and tethering of AGO4 to cleaved POLV RNA fragments. Ensuing AGO4:siRNA:ncRNA complexes might extend DRM2 local recruitment and RdDM without impeding POLV progression along the chromatin. **D)** Methylation maintenance. Methylation at CHH sites requires the constant action of DRM2 guided by 24-nt siRNAs. Methylation at CG and CHG sites, while established de novo by 24-nt siRNAs and DRM2, is maintained in a DNA-replication-dependent manner by MET1 at CG sites, and by the self-reinforcing action of CMT3 (among other factors) at CHG sites. All processes require the (hetero)-chromatin remodeler DMM1 presumably relaxing chromatin during DNA replication.

DNA strands extruding the elongating P4-RNA 3′ end (Fukudome et al. 2021) (Fig. 3B). This signals complementary RNA synthesis by RDR2, preferentially from the third nucleotide, similar to RDR6 initiation on cleaved *TAS* 3′ends. RDR2 terminal-transferase activity adds an untemplated 3′-nucleotide to the P4-RNA-complementary (i.e. RDR2-derived) strand (Singh et al. 2019) (Fig. 3B). Because P4-derived dsRNAs are only 26 to 45 bp long, they undergo one dicing event by DCL3, which indeed

prefers small substrates (Nagano et al. 2014). This can theoretically occur from both the POLIV-derived A-terminal strand or RDR2-derived complementary 5′strand baring the untemplated nucleotide. However, DCL3 prefers thermodynamically unstable 5′A/U ends (Nagano et al. 2014). Moreover, AGO4, AGO6, and presumably AGO9 preferentially load 5′ A small RNA (Liu et al. 2022). Thus, the 5′ A status of steady-state P4-siRNAs likely reflects 3 combined biases: (1) 5′ A selection at Pol IV transcription initiation sites, (2) thermodynamically unstable end-selection by DCL3, and (3) active 5′ A selection by AGO4-clade AGOs (Fig. 3B).

P4-siRNAs mediate RNA-directed DNA methylation via AGO4-clade AGOs

Since siRNA strand separation occurs post duplex binding by AGOs—including via the nucleotide-binding pocket probing the 5′ end of each siRNA strand—AGO4 strand selection should be biased. This was effectively revealed in IP-deep-seq of AGO4-bound sRNAs. Indeed, DCL3 measures 24 nt from the 5′ A end of the Pol-IV–derived P4-siRNA strand; its staggered cut leaves a 3′ end overhanging the RDR2-derived strand by 2 nt. Yet, because the RDR2 strand bares a 3′ end untemplated 1-nt extension, a 23-nt—not 24-nt—RNA is generated, yet it is hardly detected in AGO4 IPs (Singh and Pikaard 2019). Thus, the RDR2-mediated 1-nt extension and DCL3's 5′ A preference apparently predetermines AGO4's higher retention of POLIV-derived P4-siRNA strands, with RDR2-derived 23-nt strands being merely labile "passengers" (Singh et al. 2019) (Fig. 3B). Together, the above parameters define a "dicing code" that likely diversifies P4-siRNA production (Loffer et al. 2022). Separation of RDR2- from POLIV-derived strands requires slicing by AGO4, resulting in 11 to 12-nt RDR2-strand fragments (Wang et al. 2023a) (Fig. 3B). The ensuing mature AGO4:P4-siRNA complexes are programmed to bind sequence-complementary RNAs originally conceptualized as "scaffolds" produced from the P4-siRNA loci of origin. These loci's compact chromatin and the immediate ssRNA→dsRNA conversion-dicing of P4-RNA disqualify POLII and POLIV as scaffold providers. This function was ascribed to a second plant-specific, POLII-derived, DNA-dependent RNA polymerase, coined POLV (Wierzbicki et al. 2008). POLV's long ncRNAs initiate on the opposite DNA strand used by POLIV, thereby matching AGO4's prevailing selection of POLIV-derived P4-siRNAs as guide strands. AGO4 interacts with the carboxy-terminal domain of POLV's largest subunit and recruits the de novo methyltransferase DRM2 to locally target RNA-directed DNA methylation (RdDM) onto cytosines in all (i.e. CG/CHG/CHH) contexts (Gallego-Bartolome 2020) (Fig. 3, A and C). In addition to enabling PolIV- vs RDR2-derived strand separation/elimination, slicing also stimulates RdDM. AGO4 appears tethered to cleaved POLV transcripts in a manner that simultaneously (1) facilitates POLV progression along the chromatin owing to AGO4-POLV dissociation, and (2) maintains scaffolded AGO4:ncRNA interactions, thereby furthering DRM2 recruitment for RdDM (Wang et al. 2023a). This likely underpins a previously noted discontinuity in cytosine methylation, whereby AGO4-mediated slicing events define RdDM intervals (Fig. 3C).

Interestingly, (1) histone deacetylation mediated by HISTONE DEACETYLASE 6 (Aufsatz et al. 2002), (2) H3K9 dimethylation mediated by SUPPRESSOR OF VARIEGATION 4,5,6 (Li et al. 2018), and (3) H3K4 demethylation mediated by JumonjiC-domain containing JMJ14 (Wang et al. 2023b) accompany RdDM. This effectively promotes further SHH1 and, hence, POLIV recruitments on target loci, ultimately enabling more P4-siRNA synthesis and DNA methylation (Fig. 3A). This POLIV-POLV–mediated self-enforcing feedback likely confers robustness to the transcriptionally silent state (from a POLII standpoint) of many TEs/repeats, especially since RdDM's primary initiation requires only low P4-siRNAs levels (Wendte et al. 2019). Moreover, RdDM can be maintained independently of P4-siRNAs at CG/CHG- symmetrical sites during DNA replication, for example, in meristems. Indeed, METHYLTRANSFERASE 1 (MET1) reproduces mother-strand's methylated CG sites at daughter-strands' unmethylated GC sites, a role for CHROMOMETHYMASE 3 (CMT3) at CHG sites; nonsymmetrical CHH sites, unmaintainable via DNA replication, require persistent P4-siRNA action (reviewed in Gallego-Bartolome 2020) (Fig. 3D). Hetero chromatin decondensation is required in all the above instances, a function ascribed to the master SWI/SNF remodeler DECREASE IN DNA METHYLATION 1 (DDM1; (Jeddeloh et al. 1998) (Fig. 3D).

RDR6- and AGO1-mediated PTGS rescues silencing of sporophytic, epigenetically activated TEs

Development and stress influence RdDM's efficacy (Slotkin et al. 2009; Yu et al. 2013). In the Arabidopsis *ddm1* mutant sporophyte, where decreased DNA methylation levels mimic RdDM-deficient conditions, POLII transcription resumes at thousands of *TEs*, where abundant 21-nt siRNAs now substitute p4-siRNAs (Teixeira et al. 2009). These were coined "easiRNAs" because DCL4/DCL2 process them from dsRNA in part copied by RDR6 from henceforth "epigenetically activated" TE-derived mRNAs. easiRNAs rescue TE silencing presumably via AGO1-mediated PTGS (Slotkin et al. 2009; Creasey et al. 2014). One scenario for RDR6 recruitment—experimentally validated with a handful of *Gypsy* and *COPIA* family LTR elements—involves miRNAs that are sequence complementary to activated TEs. Their size (22 nt) or double-hit mode of action likely enables RDR6 action as in ta-/pha-siRNA biogenesis (Creasey et al. 2014; Borges et al. 2018) (Fig. 4A). But why would TEs maintain miRNA target sites? Involved in a similar albeit genetically distinct pathway (see next sections), the pollen-specific miR845 provides at least an answer because it targets highly conserved primer-binding sites (PBSs) of certain *Gypsy* and *COPIA* elements (Borges et al. 2018). PBSs are mandatory for *LTR* element reverse-transcription (RT) and hence are unlikely to withstand miRNA-counteracting mutations. Incidentally, RT is not part of DNA element biology, which is easiRNA free

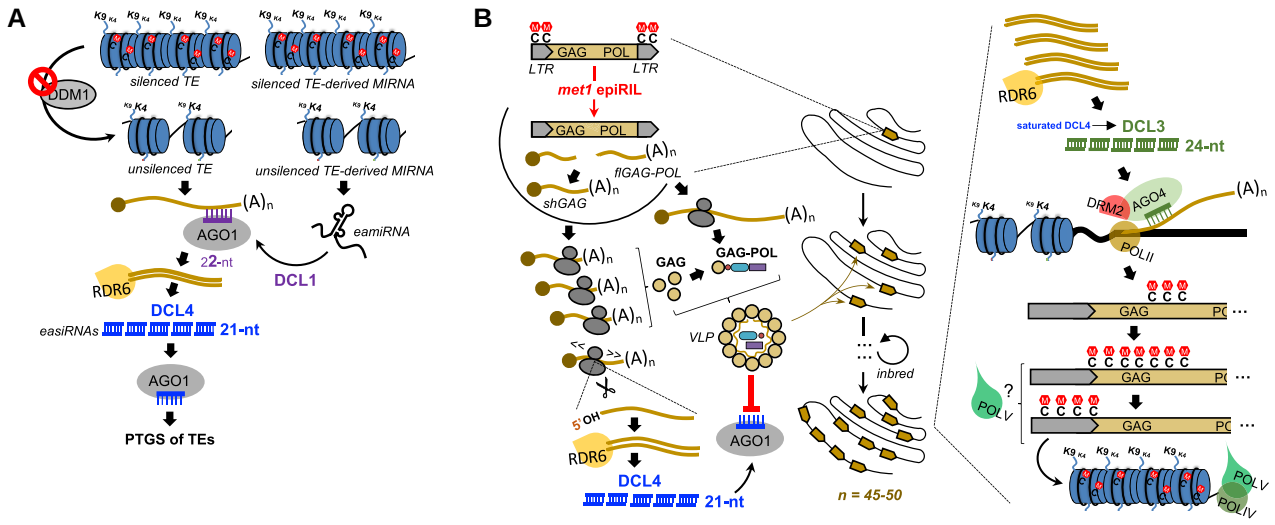

Figure 4. Epigenetic activation of TEs in mutant sporophytes and RdDM onset on *EVD*. **A)** EasiRNA biogenesis in *ddm1* mutant sporophytes. Loss of DDM1 causes chromatin decompaction and gene expression at many *TE* and some *MIRNA* loci. Certain epigenetically activated TEs are targets of these epigenetically-activated miRNAs (eamiRNAs) in an AGO1-dependent manner. Their size (22 nt) or dual-hit mode of action (not shown) promotes RDR6 recruitment to produce TE-derived dsRNA in a DCL4-dependent manner. Presumably loaded into AGO1, these trigger PTGS of sequence-homologous TEs. **B)** PTGS-to-RdDM transitions underpin *EVD* de novo silencing. Upon reactivation in *ddm1* or *met1* epigenetic recombinant-inbred lines, *EVD* transposes despite production of RDR6-DCL4–dependent 21-nt siRNAs derived specifically from the *EVD shGAG* subgenomic RNA upon ribosome stalling and 5′OH RNA production. Its seclusion within VLPs likely protects the full-length *EVD* (*flGAG-POL*) against AGO1-mediated PTGS. When the *EVD* copy number reaches 40 to 50, the large amount of dsRNAs accrued over inbred generations likely saturates the capacity of DCL4, leading to its processing into 24-nt siRNAs by DCL3. Loaded into AGO4, these initiate RdDM, first on the *shGAG*-matching region of the *GAG* open Reading frame, and then onto the LTR on possible antisense transcript by POLV. Self-enforcing POLIV/V-dependent cytosine methylation and chromatin compaction on the LTRs eventually leads to TGS of *EVD*.

(Oberlin et al. 2022). Inverted-repeat copies of the "to-be targeted" TEs likely underlie the henceforth suggested *MIRNA-TE* coevolution (Sarazin and Voinnet 2014), and indeed Arabidopsis *MIR845* is a [5′LTR + PBS]-encompassing truncation inversion (Borges et al. 2018). Being TE-derived and thus often epigenetically silent themselves, such *MIRNA* loci produce miRNAs that mostly accumulate in, for example, *ddm1* and hence were coined "eamiRNAs" (Fig. 4A). Nonetheless and as discussed (Sarazin and Voinnet 2014), the predicted set of ≥50 TE-targeting (ea)miRNAs unlikely underpins, alone, the observed bulk of easiRNA production in *ddm1*. For instance, the evolutionarily young (2 copies) *COPIA* element *ÉVADÉ* (*EVD*; Mirouze et al. 2009) displays no overt miRNA target sites and indeed spawns comparable levels of RDR6-dependent easiRNA in both *ddm1* and *dcl1 dmm1* sporophytes (Oberlin et al. 2022; see section below). Moreover, *TEs* rearranged into *IRs* (Slotkin et al. 2003) likely spawn easiRNAs de facto RDR independently (Fig. 1B). Thus, neither miRNAs nor RDRs are stringent prerequisites of easiRNA production, though they may enable it at certain *TEs*.

Primary onset of RdDM as illustrated with epigenetically activated EVD

Plant TEs are usually studied at steady states where RdDM has been already set and perpetuated, including before their artificial or natural reprogramming. This raises the fundamental question of how RdDM is first established, for instance upon horizontal transfer of a new TE. The RdDM's self-enforcing nature also makes it difficult to tease apart what "primal RdDM" event underlies the first DNA methylation and H3K9me2 waves required for the process's secondary perpetuation. Addressing these intertwined issues requires authentic, that is, genome-resident as opposed to transgene-based TEs because transgenes intrinsically and artificially attract RNA silencing (see next section). Moreover, most Arabidopsis TEs are mere past invasions' nonautonomous remnants onto which long-term TGS has imparted mutational degeneration (Quesneville 2020). Yet primal RdDM is likely triggered by transposition-proficient, genome-invasive TEs of which *EVD*, previously evoked, is indeed a rare, 2-copy example (Mirouze et al. 2009). *EVD*'s LTR being exclusively CG methylated in the reference genome, *EVD* transposition could be re-initiated in *met1* epigenetic recombinant-inbred lines of Arabidopsis (Reinders et al. 2009). An *EVD* copy number increase over successive inbred generations was paralleled by increasing levels of RDR6/DCL4-dependent and AGO1-loaded *EVD*-derived 21-nt siRNAs (Mari-Ordonez et al. 2013) (Fig. 4B). These nearly exclusively map to a subgenomic (sg)mRNA, coined *short GAG* (*shGAG*), produced via splicing-coupled premature cleavage-polyadenylation of the *full-length* genomic *EVD* mRNA (*flGAG-POL*). *shGAG*'s disproportionate translation over *flGAG-POL* presumably yields the GAG nucleocapsid's stochiometric balance necessary for RT and

mobilization (Oberlin et al. 2017, 2022). *flGAG-POL* accumulation remained unaffected by 21-nt *shGAG* siRNAs, however, allowing further copy accretion. Nonetheless, in every *met1* epigenetic recombinant-inbred lines' single-seed descent in which the copy number had reached 45 to 50, abrupt TGS of *EVD* correlated with de novo LTR methylation in all cytosine contexts, the landmark of RdDM (Mari-Ordonez et al. 2013) (Fig. 4B). Underlying this PTGS→RdDM transition was a progressive, albeit much lower, accumulation of DCL3-dependent and AGO4-loaded *sh*GAG-derived 24-nt siRNAs over inbred generations. Being 3 nt longer than their abundant DCL4-dependent 21-nt siRNA counterparts, these 24-nt siRNAs were likely derived from the same RDR6-dependent dsRNA (Mari-Ordonez et al. 2013). Presumably, increasingly higher levels of *shGAG* dsRNA caused by *EVD* copies' transgenerational accretion had saturated DCL4 activity, giving way to DCL3's due to hierarchical surrogacy (Fig. 4B). Forty to 50 copies were likely needed for this saturation to manifest consistently. Before saturation, de novo DNA methylation started precisely over the *shGAG*-matching 5′ region of *EVD* (Mari-Ordonez et al. 2013). Thus, ongoing POLII-dependent *EVD* transcription likely provided primal scaffolds to the neo-formed AGO4-24-nt-siRNA complexes (Sigman et al. 2021) (Fig. 4B), although AGO4-siRNA-DNA interactions cannot be excluded. Regardless, preceding the abrupt TGS event, DNA methylation had spread 5′ from the primally methylated *shGAG* region, toward and within the LTR, due, presumably, to POLV-dependent antisense transcription, ultimately resulting in self-sustained LTR-restricted RdDM (Mari-Ordonez et al. 2013; Sigman et al. 2021) (Fig. 4B). Whether *EVD*'s primal RdDM applies to other TEs remains undetermined, yet it illustrates how PTGS and TGS pathways can form a continuum of action, contrasting with the compartmentalized view often conveyed by mutants' studies.

Ribosome stalling and ensuing translation-dependent silencing as sources of EVD's primal RdDM

With *EVD*, the concomitant RDR6-mediated saturation vs stimulation of DCL4 vs DCL3 activities initiates RdDM. Accordingly, *EVD* copies can accrue well beyond 100 in *rdr6* mutants with ensuing fertility/developmental defects not observed in RdDM-defective backgrounds (Oberlin et al. 2022). *shGAG* siRNA production being miRNA-independent, the specific recruitment of RDR6 on *shGAG* remains puzzling. Unlike the highly translated *shGAG*, *flGAG-POL* is mostly nuclear (Oberlin et al. 2022), explaining—additionally to its seclusion in viral-like particles (Mari-Ordonez et al. 2013) (Fig. 4B)—its resistance to cytosolic RDR6 and AGO1-dependent PTGS directed by *shGAG* siRNAs. Sensitive, by contrast, to both processes, cytosolic *shGAG* ranks among the top 4% to 2% (WT-*rdr6*) of Arabidopsis transcripts with the most intense ribosome stalling. The prominent *shGAG* stalling site coincides with the 5′ end of truncated *EVD* RNAs. Because these uncapped mRNAs exhibit an unconventional 5′ OH, they evade RQC by XRN enzymes, likely explaining their conversion into *shGAG* siRNAs by RDR6-DCL4 (Oberlin et al. 2022) (Fig. 4B). Unlike inferred by Kim et al. (2021), most ORF-debilitated TE remnants populating the Arabidopsis genome evade translation-dependent silencing, consistent with their poor, if any, polysome association (Oberlin et al. 2022). Translation-dependent silencing may thus form a primary defense against de novo invading TEs not having sufficiently coevolved with their hosts to enable TE-derived miRNA production and/or being sufficiently sequence divergent to escape recognition by host-resident, TE-derived siRNAs.

Tapetal TE-derived 24-nt siRNAs protect pollen precursor cells and refine the paternal methylome and gene expression landscape

In the male gametophyte, somatic tapetum nurse cells assist the development of meiocytes, the gametes' diploid precursors. Upon meiosis, meiocytes yield 4 haploid microspores each undergoing 2 cell divisions. In the resulting pollen, a large vegetative cell (VC) encases 2 haploid sperm cells (SCs) (Fig. 5A). Contributing to a unique meiocyte's methylome inherited in sperm, ∼700 hyper-methylated genes (MetGenes) influence paternal-specific gene expression and meiosis (Walker et al. 2018). How RdDM initiates germline specifically at these particular loci had remained mysterious until abundant nurse cell P4-siRNAs (coined here tap-siRNAs) derived from ∼800 "HyperTEs" were found to move into meiocytes (Long et al. 2021) (Fig. 5A). There, they not only enforce TE silencing via RdDM but also promote de novo *trans*-methylation at many protein-coding loci (encompassing the MetGene-set) to which they display imperfect nucleotide sequence complementarity, indeed tolerated by RdDM (Fei et al. 2021). Remarkably, *trans*-methylation is absent in somatic nurse cells. Thus, somatic or meiocyte-specific factors might respectively suppress or enable this process in a likely dose-dependent manner because HyperTEs produce up to 1,000 times more P4-siRNAs than other tapetal RdDM-targeted loci (Long et al. 2021). This abundance correlates with their as-yet-unexplained enrichment in tapetal-specific CLASSY3 thought to assist SHH1-mediated POLIV recruitment on RdDM-targeted loci (Long et al. 2021) (Fig. 5A). Although widespread in plants, abundant tapetal-specific 24-nt pha-siRNAs, already evoked before, are conspicuously absent in Brassicaceae (including Arabidopsis) but might be functionally analogous to Arabidopsis tap-siRNAs (Liu et al. 2020). They indeed induce *cis*-RdDM in tapetal nurse cells and undergo tapetum to meiocytes movement (Zhou et al. 2022b), through their *trans*-methylation ability therein remains unknown.

Sperm cells are likely protected by TE-derived 21- to 22-nt siRNAs made elsewhere during gametophytic development

In WT pollen, the VC, unlike the two SCs, does not express DDM1 and undergoes active de novo demethylation by DNA glycosylases, including DEMETER (DME). Both processes likely underpin the long-known highly decondensed state of the VC's chromatin (Slotkin et al. 2009; Ibarra et al. 2012). This causes widespread POLII-dependent transcriptional

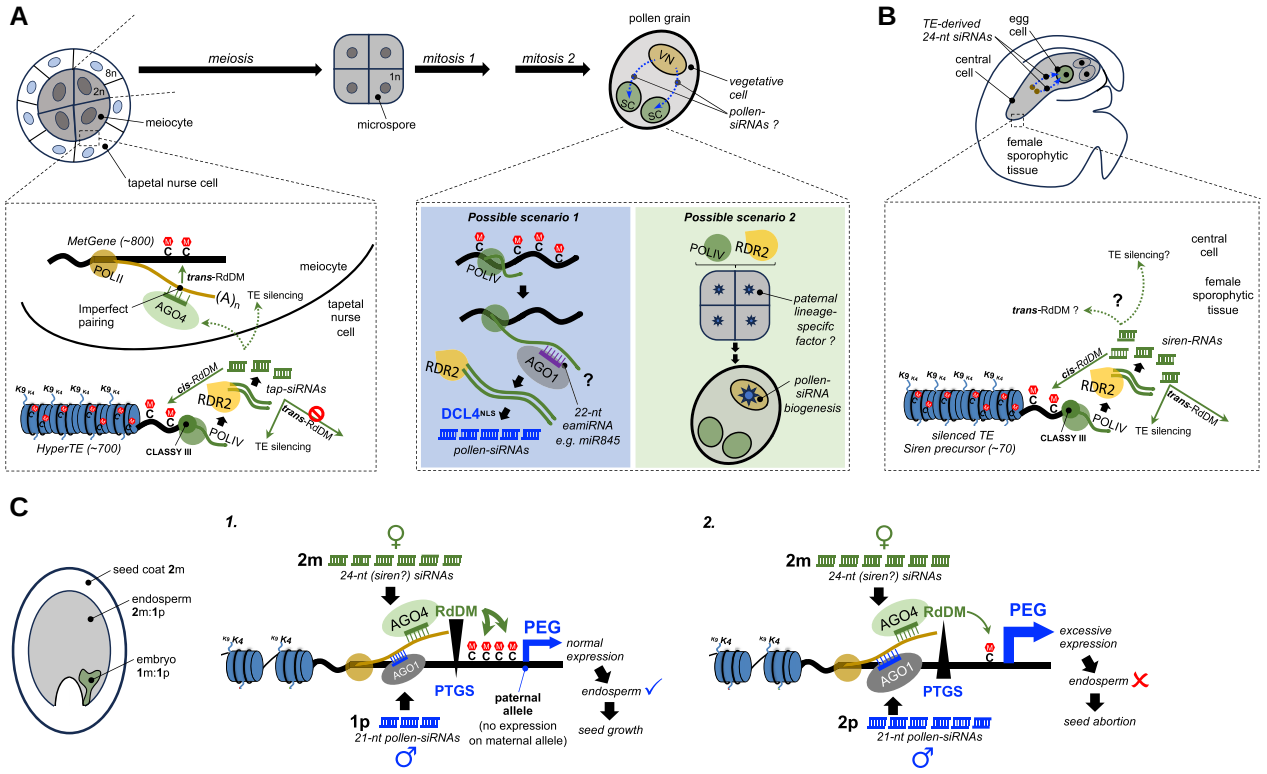

Figure 5. TE-derived siRNAs in gametophytes and seeds. **A)** *TE* methylation reprogramming in the Arabidopsis male germline. Left: at the meiocyte stage, polyploid somatic nurse cells forming the nourishing tapetum produce mobile P4-siRNAs (tap-siRNAs) from a subset of silent "HyperTEs" selectively enriched in CLASSY 3. Upon presumed CLASSY 3-mediated POLIV recruitment, up to 1,000 folds more P4-siRNAs accumulate at these compared with other *TE* loci, mediating *cis-RDRM* and silencing of sequence-related TEs within the tapetum. Upon movement into adjacent meiocytes, HyperTE-derived P4-siRNAs also mediate AGO4-dependent *trans-RdDM* by imperfectly pairing to target DNA/RNA produced at a subclass of MetGenes whose expression defines a paternal identity that persists through meiosis and mitosis into the mature pollen. For unexplained reasons, *trans-methylation* of MetGenes does not occur in tapetal nurse cells. Right: in the mature pollen grain, where a DDM1-deficient and DME-proficient vegetative cell encases 2 sperm cells (SCs), POLIV-dependent TE-derived 21-nt pollen-siRNAs are thought to move from the vegetative cell's nucleus (VN) into the SCs. Pollen-siRNA biogenesis occurs via an as yet-undefined pathway (scenario 1) that might involve longer-than-normal P4-RNAs spawned from hypomethylated DNA, subsequently targeted by TE-derived miRNAs such as the 22-nt-long miR845. Targeting promotes recruitment of RDR2 and/or possibly RDR6 to synthesize dsRNA processed into 21-nt pollen-siRNAs by the nuclear DCL4^{NLS} isoform. Note that this pathway is entirely speculative. Alternatively (Scenario 2), POLIV requirement might be indirect and occur earlier during meiocyte differentiation, with POLIV andRDR2-dependent processes (e.g. tap biogenesis and action) ultimately delineating a paternal lineage-specific gene expression landscape of which a product might be inherited and amplified in the VN or VC where it would specifically activate pollen-siRNA biogenesis. See main text for details. As for scenario 1, scenario 2 is entirely speculative. **B)** *TE* methylation reprogramming in the Arabidopsis female germline. In a manner conceptually analogous to tap-siRNA biogenesis and action in the male germline, 24-nt siren-RNA are produced in the female sporophyte from a discrete number of *TE* loci enriched in CLASSY 3. In the sporophyte, siren-RNA mediate *cis-* and trans-methylation influencing gene expression and TE silencing. siren-RNA are also thought to move into the female gametophyte composed of a large and bi-nucleate central cell encasing an egg cell, among other cell types. As established for tap-siRNAs, siren-RNA might mediate *trans*-methylation and TE silencing therein, though this still requires experimental validation. Additionally, indirect evidence based on methylome comparisons suggests movement of TE-derived 24-nt siRNAs from the central cell's nucleus (which undergoes active demethylation via DME, resulting in TE activation) to the egg. **C)** Model for TB in paternal excess endosperm. Left: seeds consist in a diploid coat of maternal origin, a triploid endosperm with a 2:1 matrigenic/patrigenic ratio, and a diploid zygote with a 1:1 matrigenic/patrigenic ratio. Right: 1. Fertilization of a diploid central cell by haploid pollen brings 21-nt pollen-siRNAs in amounts that are insufficient to offset RdDM and TGS of PEGs by female 24-nt (siren?)-siRNAs. The ensuing adequate PEG expression levels allow normal endosperm and seed development. 2. Fertilization of a diploid central cell by diploid pollen provides an excess of 21-nt pollen-siRNAs, which overcomes RdDM and TGS of PEGs by female 24-nt (siren?)-siRNAs. This results in PEG overexpression, abnormal endosperm development and, ultimately, seed abortion.

activation of TEs correlating with abundant production of DCL4/DCL2-dependent 21- to 22-nt siRNAs (coined here pollen-siRNAs), evoking the *ddm1* sporophyte's situation (Slotkin et al. 2009). Remarkably, these molecules are detected in SCs isolated from pollen grains, suggesting that their VC to SC movement consolidates the integrity of the SC and future zygotic genome by targeting, via PTGS, TEs potentially spuriously expressed therein (Slotkin et al. 2009; Martínez et al. 2016)

(Fig. 5A). Unexpectedly, however, pollen-siRNAs, including those produced via the PBS-targeting miR845, still accumulate in *dcl1* mutant pollen, where miR845 is below detection, implying that miR845 is produced (1) before, (2) during meiosis, or (3) early at the onset of gametogenesis but not in the VC (Fig. 4B). While pollen developmental stage–specific deep-seq pinpoints stages 2 to 3 as likely underlying miR845 action (Oliver et al. 2022), a key role for the VC in pollen 21-nt-siRNA biogenesis remains likely (Fig. 5A). This was recently suggested by elegant VC- and SC-specific siRNA ablation experiments (Pachamuthu et al. 2023) (Fig. 4, C and D) and is further discussed here.

Despite a notable, albeit incomplete, sequence overlap between the 2 molecules' sets (Slotkin et al. 2009; Martinez et al. 2018), the earlier notion that abundant pollen-siRNAs are akin to *ddm1*-dependent sporophytic easiRNAs also has been challenged. Indeed, unlike POLII-dependent easiRNAs, pollen-siRNAs vanish in the pollen of *polIV* mutants (Borges et al. 2018; Martinez et al. 2018), with their biogenesis likely being prominently *RDR2*- not *RDR6*-dependent (Satyaki and Gehring 2019). POLIV dependency could suggest that P4-RNAs are used for pollen-siRNA biogenesis, but the extremely small size (25 to 40 nt) and sequence diversity of P4-RNAs conflict with putative miRNA (e.g. miR845)-mediated initiation events. However, cytosine methylation (the presumed signal for P4-RNA termination; Fig. 3B) might be reduced in the elusive cell(s) involved, thereby yielding, perhaps, longer P4-RNAs (Fig. 5A, scenario 1). Additionally, DCL2 is nucleocytosolic and a promoter-hypomethylation-dependent and NLS-containing DCL4 isoform (already evoked in uv-siRNA biogenesis and further later) that uses POLIV-RDR2-, not RDR6-dependent, dsRNA in young siliques, also accumulates in the hypomethylated VC (Borges et al. 2018) (Fig. 5A, scenario 1). Finally, some siRNAs derived from miRNA-target transcripts are lost in *rdr2* (Ronemus et al. 2006).

Alternatively, the POLIV dependency of pollen-siRNAs could be merely indirect. Perhaps POLV's genomic distribution is modified in relevant POLIV-deficient cells, enabling POLV long ncRNAs—possibly more miRNA-accessible—to become RDR templates. Curiously, pollen-siRNA accumulation has not been tested in *polV* mutants so far. Alternatively, VC-based biogenesis of pollen-siRNAs might require prior production and cytosolic inheritance, over 1 meiosis and/or 2 mitosis, of key paternal lineage–specific factors whose expression depends on RDR2/POLIV (Fig. 5A, scenario 2). Strikingly, POLIV-RDR2–dependent mobile tap-siRNAs shape the long-term paternal gene expression landscape alongside other possible meiocyte-resident P4-siRNAs (Long et al. 2021) (Fig. 5A). These paternal lineage–specific factors, possibly in conjunction with miR845-like molecules, might trigger production of discrete primary sRNAs in, for example, microspores, to be subsequently amplified in the VC, with RDR2 (or even RDR6) using epigenetically-activated POLII-dependent TE transcripts as abundant templates (Fig. 5A). The striking inversion in 21-/22-nt vs 24-nt siRNA abundance in microspores (high 24-nt; Wang et al. 2020) vs pollen (high 21/22 nt likely mostly VC-contributed; Martinez et al. 2018) supports this 2-step-amplification scenario. Regardless, given the importance ascribed post fertilization to pollen-21-nt-siRNAs—including in triploid block (TB) regulation (Borges et al. 2018; Martinez et al. 2018; also see further section)—it now appears critical to determine when, where, and how POLIV is required for their biogenesis along the paternal gametophyte's development. Inducible removal of *POLIV* via tapetum-, meiocyte-, microspore-, VC-, or SC-specific CRISPR-Cas is achievable as is cell-type-specific sRNA depletion (Pachamuthu et al. 2023).

Conceptually similar pathways underpinned by TE-derived 24-nt siRNAs are found in the Arabidopsis female gametophyte

Of the 4 haploid megaspores derived from meiosis during female germline development, one undergoes several mitosis. This yields the female gametophyte in which a large binucleate central cell encases one haploid egg cell, among others (Fig. 5B). Their fertilization by each of the 2 haploid sperms eventually yields respectively a triploid endosperm supporting development of the diploid embryo in the seed. Post fertilization, the sporophytic maternal tissue surrounding the central cell and egg becomes the seed coat. Reminiscent of tap-siRNAs during paternal germline development (Fig. 5A), abundant CLASSY3-dependent P4-siRNAs derived from ~70 TE loci accumulate in the maternal sporophyte. There, they trans-methylate protein-coding genes and modulate gene expression (Mosher et al. 2009; Burgess et al. 2022; Zhou et al. 2022a) (Fig. 5B). Whether trans-methylation also occurs in the gametophyte remains unknown. However, indirect evidence suggests that these so called "siren"-RNAs (for siRNAs in endosperm, where they also accumulate post fertilization; Mosher et al. 2009) move into the gametophyte, where they might enforce TE silencing via RdDM (Li et al. 2020; Chow and Mosher 2023) (Fig. 5B). The overlap between tap-siRNA- and siren-RNA–producing loci is very small, suggesting that CLASSY3 recruitment mechanisms on select TEs differ between the female sporophyte and tapetal nurse cells (Chow and Mosher 2023). Cell-specific methylome analyses also suggest that 24-nt siRNAs move from the central cell (whose genome, like the VC's, undergoes DME-mediated active demethylation) into the egg (Ibarra et al. 2012). Therefore, both paternal and maternal plant gametophytes undergo premeiotic (tapetum, female gametophyte) and postmitotic (VC, SC) protection by TE-derived siRNAs produced from epigenetically reprogrammed companion cells not contributing to heredity. In a striking analogy, the Drosophila quiescent oocyte is loaded with AGO-like PIWI proteins, which, together with their TE-derived piRNA cargoes, are produced in surrounding nurse cells (Senti and Brennecke 2010).

TE-derived siRNAs in seeds

Cis- and *trans*-acting 24-nt tap-siRNAs accumulating in meiocytes during male gametophyte development ensure their protection against TEs, as do 24-nt-long siren-RNAs and central cell–derived siRNAs in the female gametophyte, all of which are produced through conventional RdDM. So, why would a noncanonical and complex pathway produce 21-nt,

not 24-nt, pollen-siRNAs to achieve the same protective effects in the SCs? Perhaps pollen-siRNAs play additional roles during and/or shortly after double fertilization, whereby their PTGS-promoting action might antagonize TGS-promoting functions of maternal siRNAs. Reproductive TE-derived siRNAs would thus fit into the parental conflict hypothesis reflecting opposing interests of the maternal and paternal genomes during offsprings' growth (Haig and Westoby 1991), a conflict likely resolved by genomic imprinting enabling parent-of-origin gene expression. How 21-nt pollen-siRNAs, in particular, might help resolve the parental conflict in the endosperm is briefly discussed below, and we refer the reader to (Chow and Mosher 2023) for an extensive review of similar (asserted or speculated) functions of reproductive siRNA in gametophytes, zygotes, and seeds.

Fertilization of the maternal diploid central cell by a haploid sperm cell yields the triploid endosperm, therefore exhibiting a 2m:1p matrigenic(m):patrigenic(p) ratio. Excess dosage from either parent's genome beyond the 2m:1p ratio leads to detrimental overexpression of maternally or paternally expressed (i.e. imprinted) genes (PEGs). This causes high-frequency endosperm failure and, hence, seed abortion. This so-called TB prevents breeding of plant species/varieties with incompatible ploidies and contributes naturally to reproductive isolation. A paternal excess Arabidopsis endosperm can be produced from artificially induced 2n sperm, and its abortion was largely ascribed to overexpression of key PEGs in which individual mutations indeed suffice to bypass the TB (Kradolfer et al. 2013). Intriguingly, this bypass also occurs with 2n pollen derived from *polIV-* (Martinez et al. 2018), and indeed other RdDM mutants (Satyaki and Gehring 2019), in which pollen-siRNA production is abolished. Remarkably, reduced levels of miR845—a proposed trigger for pollen-siRNAs—also reduce the TB's impact (Borges et al. 2018), and several TEs or remnants thereof that spawn pollen-siRNAs are proximal to some of the aforementioned key PEGs (Martinez et al. 2018). It was thus proposed that, by over-targeting, for PTGS, POLII- or POLV- scaffold RNAs derived from these TEs, excess 21-nt pollen-siRNAs from 2n sperm might antagonize RdDM mediated by maternal 24-nt siRNAs (Martinez et al. 2018). RdDM would normally bring PEGs to 2m:1p ratio-compatible expression levels during or shortly after fertilization (Fig. 5C). Supporting this model, loss of maternal POLIV exacerbates seed lethality in paternal excess crosses (Satyaki and Gehring 2022). Furthermore, TE mRNA levels are not further increased in *ddm1* vs *ddm1rdr6* sporophytes (Creasey et al. 2014), as would be nonetheless expected from lack of easiRNA production (and, hence, of TE-targeted PTGS) in double mutants (Fig. 4A). In fact, many *TE*s become hypermethylated in *ddm1rdr6* (Creasey et al. 2014), suggesting that 21-nt easiRNAs—and possibly, by extension, 21-nt pollen-siRNAs—antagonize RdDM mediated by 24-nt siRNAs as proposed in the model in Fig. 5C (Martinez et al. 2018). Nonetheless, this model makes several key, as-yet-untested assumptions, least of which that pollen-siRNAs are indeed deposited in zygotes. It also predicts a large nucleotide sequence overlap between pollen-siRNAs and maternally deposited or seed coat–derived (i.e. post fertilization) 24-nt siRNAs, which, based on our current understanding, should be siren-RNAs (Fig. 5B). Whether lack of pollen-siRNA production solely accounts for the near-complete TB suppression by *polIV* or other RdDM mutants also remains unclear, given that many additional processes might be affected in *polIV*. Curiously, while 21nt pollen-siRNAs are assumed to crucially influence TB, the effect of *dcl4* or *dcl2dcl4* 2n pollen on TB bypass also has not been reported. Other potentially deposited TE-derived siRNAs possibly unrelated to pollen-siRNAs also contribute to TB bypass (Wang et al. 2018), and similar scenarios can be postulated in the embryo itself (Chow and Mosher 2023) in addition to the surrounding endosperm discussed here.

siRNAs derived from transgenes

IR transgenes produce dsRNA processed into 21, 22, and 24 nt by DCL4, DCL2, and DCL3, respectively. AGO1-loaded 21-/22-nt siRNAs can trigger *IR*-PTGS, while 24-nt siRNAs loaded into AGO4-clade AGOs can trigger RdDM and *IR*-TGS of sequence-complementary endogenous RNA/DNA (Dunoyer et al. 2007). Yet non-*IR* (or "sense") transgenes can also spawn 21-22-nt siRNAs, leading to "sense-PTGS" (S-PTGS) as discovered in attempts to overexpress endo-mRNAs from the viral *35S* promoter (*p35S*). Some transgenic lines indeed displayed "co-suppression," that is, the coordinated silencing of the transgene and corresponding endogene(s) (Napoli et al. 1990; van der Krol et al. 1990) (Fig. 6, A to D). *p35S*-driven sense transgenes expressing mRNAs without endogenous sequence counterparts also undergo S-PTGS (Elmayan and Vaucheret 1996), suggesting that, in co-suppression, transgenes are PTGS inducers and endogenes PTGS targets. In Arabidopsis, S-PTGS requires RDR6 and SGS3 (Mourrain et al. 2000), and its efficiency is enhanced under RQC-defective conditions (Martinez de Alba et al. 2015). In a commonly accepted model, S-PTGS occurs when transgenes produce abRNAs escaping complete RQC-mediated degradation, undergoing, instead, RDR6-mediated ssRNA to dsRNA conversion followed by DCL4/DCL2-mediated dicing (Parent et al. 2015). Similarly to *TAS*, transgene mRNAs bound to AGO1:22-nt siRNA complexes can undergo RDR6-mediated dsRNA conversion, with ensuing secondary siRNAs ultimately bolstering mRNA degradation.

Some transgene mRNAs with bacterial/animal-derived sequences can display low GC content (Sidorenko et al. 2017), suboptimal codons promoting ribosome stalling (Kim et al. 2021), and/or 5′OH mRNA breakage as in *EVD shGAG* (Oberlin et al. 2022). These are all plausible RDR6 stimulants. Yet, how do overexpressed plant-derived mRNAs trigger co-suppression/S-PTGS? Perhaps *p35S*-transcribed mRNAs (the prevailing form of transgene expression) are discriminated against endo-mRNAs relative to capping, splicing, nuclear export, translation, or localization. However, endogenous genes overexpressed with a *35S* enhancer upstream of their native promoter can trigger S-PTGS (Vaucheret et al. 1995). Thus, mere overexpression might predispose transgene abRNAs to readily overwhelm RQC, and, indeed, transgene transcription and S-PTGS intensities correlate positively

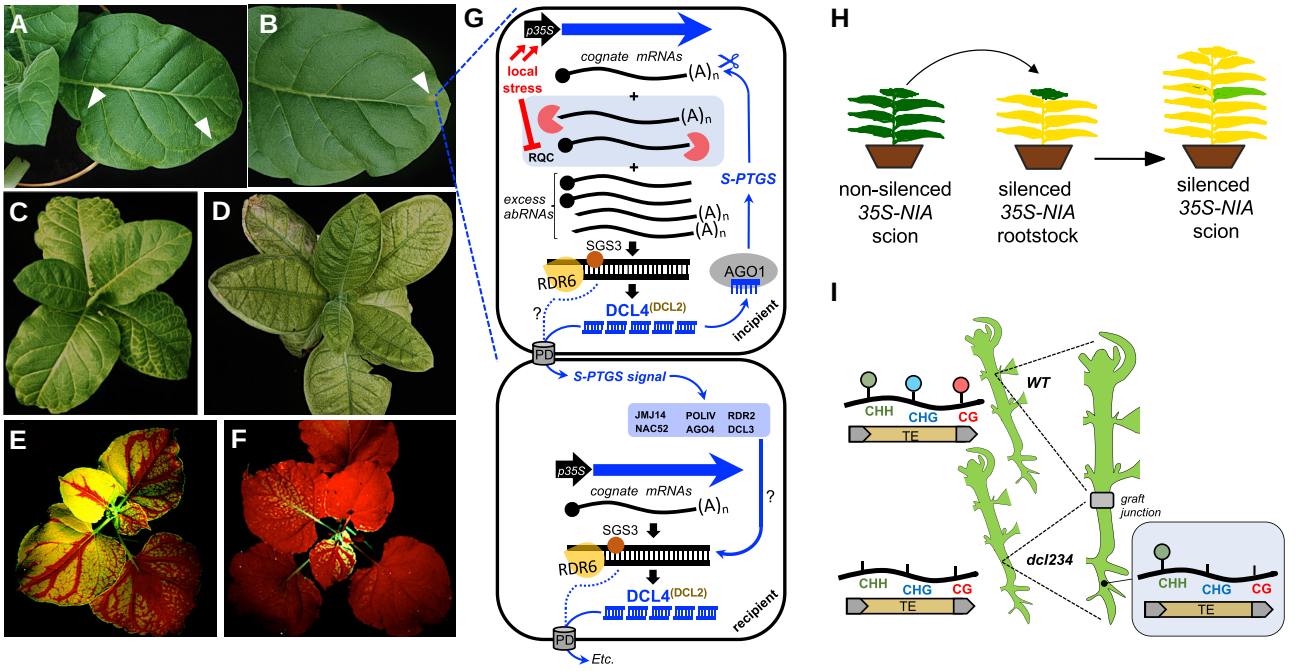

Figure 6. PTGS and RdDM are systemic processes. **A–D)** *NITRATE REDUCTASE* (*NIA*) PTGS initiates locally and spreads throughout transgenic tobacco plants. *35S:NIA2* tobacco lines spontaneously trigger PTGS of both the endogenous *NIA1*/*NIA2* and *35S:NIA2* loci, visible as small chlorotic spots indicated by arrow heads (**A–B**). PTGS subsequently spreads through the veins and progressively invades the entire plants (**C–D**). Age of the plants: A and B: 35 days, C: 50 days, D: 70 days. **E–F)** Transgene GFP PTGS artificially initiated locally spreads throughout transgenic *N.benthamiana* plants. *35S:GFP* lines that do not spontaneously initiate PTGS can be induced to do so by local, transient introduction of extra *35S:GFP* copies in a few leaves. The induced PTGS spreads through the veins **(E)** and progressively invades the entire plants (**F**). Adapted from Voinnet (2005) https://doi.org/10.1016/j.febslet.2005.09.039 **G)** Possible mechanisms for spontaneous initiation and spread. A local stress-induced burst of aberrant (ab)RNAs that saturate RQC or a deficiency thereof allows abRNAs to be converted into dsRNA by RDR6/SGS3 to initiate PTGS via DCL2/4-dependent siRNAs. Movement of siRNA and/or dsRNA precursors thereof from cell-to-cell and over long distances allows PTGS re-initiation in recipient cells. This occurs independently of RQC saturation/deficiency, likely because siRNAs induce epigenetic changes at the transgene loci through the as-yet-understood action of JMJ14, NAC52, NRPD1, RDR2 and DCL3-AGO4. **H)** A PTGS signal moves through graft unions. Grafting non-silenced transgenic scions onto silenced transgenic rootstocks provokes systemic silencing of the homologous transgene in the grafted scion. **I)** Mobile endogenous siRNAs can mediate RdDM at distance. Grafting wildtype scions onto *dcl234* rootstocks triggers mainly CHH methylation in *dcl234* rootstocks at loci targeted by siRNAs (TEs depicted here) produced in the scions.

(Vaucheret et al. 1997). Its constitutive deficiency being lethal (Martinez de Alba et al. 2015; Zhang et al. 2015), dysfunction/saturation of RQC and resultant S-PTGS activation likely occur only in a few cells (Fig. 6, A and B), which would be accommodated by the capacity of S-PTGS to move between cells and organs (Fig. 6, C to F).

S-PTGS is systemic not only because incipient cells produce mobile silencing signals but also because transgenes within as-yet-nonsilenced recipient cells can initiate S-PTGS de novo (Palauqui et al. 1997; Palauqui and Vaucheret 1998; Voinnet et al. 1998) without transient RQC saturation/deficiency. RDR6-produced secondary siRNAs underpin this relay amplification, ultimately causing near-uniform S-PTGS in whole plants (Fig. 6, D and H). Nonetheless, transgene silencing propagation and perception mechanisms remain elusive, not least because genetically dissecting these processes requires physically separating silencing-incipient from silencing-recipient tissues (Fig. 5, H and I). This can be achieved via reciprocal grafting, whereby WT recipient tissues are grafted onto mutant incipient tissues and vice versa. *ago1*, *dcl2*/*dcl4*, *rdr6*, and *sgs3* mutations in either incipient or recipient tissues impair systemic PTGS (Brosnan et al. 2007; Taochy et al. 2019), likely reflecting requirements for intracellular silencing execution. Mutations impeding incipient to recipient tissues transmission per se are lacking so far, contrasting with lesions altering the recipient tissue's capacity to trigger PTGS de novo when grafted onto silencing-incipient tissues. However, roles for the corresponding factors, including POLIV, RDR2, DCL3, AGO4, JMJ14, and its NAC-domain transcription cofactor NAC52, remain elusive (Brosnan et al. 2007; Taochy et al. 2019; Butel et al. 2021). As evoked before for the POLIV/RDR2-dependent biogenesis of pollen-siRNAs (Fig. 5A), POLIV and RDR2 might act indirectly by promoting cell-specific expression of factors required for the incipient to recipient tissues transmission of PTGS. Although required for neither PTGS execution nor silencing signal production, JMJ14 and NAC52 are both mandatory for systemy (Le Masson et al. 2012; Butel et al. 2021) (Fig. 6G). Moreover, their loss-of-function increases DNA methylation at

transgenic, but not endogenous, loci. Transgene-specific hypermethylation likely prevents de novo PTGS in recipient cells because this process is restored if *DRM2* and *CMT3*, encoding de novo and maintenance DNA methyl-transferases (Fig. 3D), are co-inactivated in the *jmj14*-null background (Butel et al. 2021). How JMJ14 specifically attenuates transgene DNA methylation (which it promotes on TE/repeats) and enforces systemic silencing awaits clarification.

siRNA derived from viral genomes

Molecular origins of virus-derived (v)siRNAs

Most phytoviruses have positive-stranded (+)RNA genomes translated upon uncoating. Their replicase copies its own progenitor (+)RNA into complementary (−)RNAs, producing, in turn, many translated or virion-encapsidated progeny (+) RNAs. While perfect (+/−) dsRNA hybrids form, if shortly, during (+)→(−)/(−)→(+)RNA copying, "subgenomic" (sg)RNAs transcribed from (−)RNA-embedded promoters also spawn (+/−)dsRNA hybrids (Fig. 7A). Illustrating how phytoviruses are henceforth intrinsic RNAi inducers, virus-derived (v) siRNAs may represent up to 30% to 40% total cellular si/miRNAs in infected tissues (Donaire et al. 2008, 2009; Garcia-Ruiz et al. 2010, 2015; Aregger et al. 2012; Devers et al. 2020; Annacondia and Martinez 2021). Cauliflower mosaic virus- (CaMV) or geminivirus- (both DNA viruses) infected cells also accumulate v-siRNAs, suggesting alternative v-siRNA sources. These include the ∼600-bp "leader" of the *35S* RNA transcribed nuclearly from CaMV mini-chromosomes, the extensive intramolecular folding of which evokes endo-*IR*s or pri-miRNAs (Blevins et al. 2011). Local (+) or (−)RNA secondary structures also yield v-siRNAs from viral RNA genomes (Molnar et al. 2005; Donaire et al. 2008, 2009), as does the *NAT*-like overlapping sense-antisense transcription of geminiviral genomes (Aregger et al. 2012) (Fig. 7A). That nearly all phytoviruses have evolved VSRs (Pumplin and Voinnet 2013) further underscores that v-siRNA–mediated RNAi is antiviral in plants, as later rediscovered in invertebrates (reviewed in Lecellier and Voinnet 2004; Ding and Voinnet 2007) and some mammalian cells (Maillard et al. 2013).

Hierarchical action and surrogacy of plant DCLs during v-siRNA production

All siRNA-generating Arabidopsis DCLs (DCL2/3/4) can produce v-siRNAs, with their respective involvement largely dictated by subcellular sites of viral genome replication/transcription. Hence, RNA viruses—undergoing both processes cytosolically—are primarily sensitive to cytosolic DCL4 and, secondarily, to its nucleo-cytosolic surrogate, DCL2 (Xie et al. 2004) (Fig. 1B), probably reflecting contrasted availabilities to and/or differential affinities for viral dsRNA. The hierarchical DCL4→DCL2 surrogacy is genetically diagnosed by a dominant 21-nt v-siRNA pool (DCL4 products) accumulating in infected WT plants being replaced by 22-nt v-siRNAs (DCL2 products) in *dcl4* mutants (Bouché et al. 2006; Deleris et al. 2006) (Fig. 7B). In rarer cases—exemplified with turnip crinckle virus (TCV, an RNA carmovirus)–DCL4-dependent 21-nt v-siRNAs accumulating in *dcl2* mutants replace DCL2-dependent 22-nt v-siRNAs detected in WT infections (Deleris et al. 2006). This is not because DCL2 displays stronger affinity than DCL4 for TCV-derived dsRNA but, instead, because the TCV-encoded VSR indirectly inhibits DCL4's primary activity (Deleris et al. 2006; Qu et al. 2008; Azevedo et al. 2010) (Fig. 7B). DCL3 additionally produces 24-nt v-siRNAs during WT plant infections by DNA viruses replicated and transcribed nuclearly (Blevins et al. 2011; Aregger et al. 2012). DCL contributions are not only evident by v-siRNA signature lengths in infected WT tissues but also by enhanced viral titers in corresponding higher-order *dcl* mutants: *dcl2dcl4* for RNA viruses; *dcl2dcl3dcl4* for DNA viruses. Hyper-susceptibility will be most visible, however, with VSR-deficient viruses because VSRs will often confound relevant *dcl* mutation effects (Deleris et al. 2006; Diaz-Pendon et al. 2007; Garcia-Ruiz et al. 2015) (Fig. 7, B and C). Nonetheless, VSR ablation is dispensable for hyper-susceptibility assessment if the VSR under consideration is required temporally and/or in discrete tissues, as is the 16-kDa facilitator of tobacco rattle virus' (TRV; an RNA tobravirus) meristem entry (Martín-Hernández and Baulcombe 2008).

Although likely applicable to most RNA/DNA virus-plant interactions, the above-mentioned DCL activity framework may manifest variably depending on both protagonist-intrinsic factors. Indeed, in infected WT Arabidopsis, distinct sections of TRV's bipartite RNA genome contribute differently to v-siRNA production, whose genomic distribution/polarities vary, moreover, between *N. benthamiana* and Arabidopsis (Donaire et al. 2008). DCLs are neither recruited equally along the TRV genome; DCL2's contribution dominates, for instance, in the RNA1 3′ end. There, 2 sgRNAs initiated internally (Donaire et al. 2008) form (+/−) dsRNA hybrids instead of, for example, imperfect intramolecular folds at other genomic locales. In contrast, DCL4 alone contributes most v-siRNAs and resistance to turnip yellow mosaic virus (an RNA tymovirus; Sehki et al. 2023). Because infections are usually studied in whole tissues averaging disparate infection stages, they provide poor insight into a likely temporal dimension to DCLs' hierarchical surrogacies. Primary antiviral DCLs (e.g. DCL4) may act early and become seconded, or superseded, by surrogates (e.g. DCL2) upon their saturation by excessive viral dsRNA levels, similar to the DCL4→DLC3 transition in *EVD*-derived 21→24-nt siRNA production (Mari-Ordonez et al. 2013) (Fig. 4B).

Studies of dissected infected tissues reveal disproportionate DCL involvements in space as opposed to time. For instance, DCL4-dependent v-siRNAs suffice to prevent initial infections of Arabidopsis leaves by VSR-deficient turnip mosaic virus (TuMV; RNA potyvirus). By contrast, DCL2-dependent v-siRNAs are neither necessary nor sufficient to limit infection by VSR-deficient TuMV in either inoculated or non-inoculated cauline leaves (Garcia-Ruiz et al. 2010). Likewise, DCL4, not DCL2, prevents vascular unloading of VSR-deficient TCV in non-inoculated cauline leaves (Deleris et al. 2006). In *dcl4*

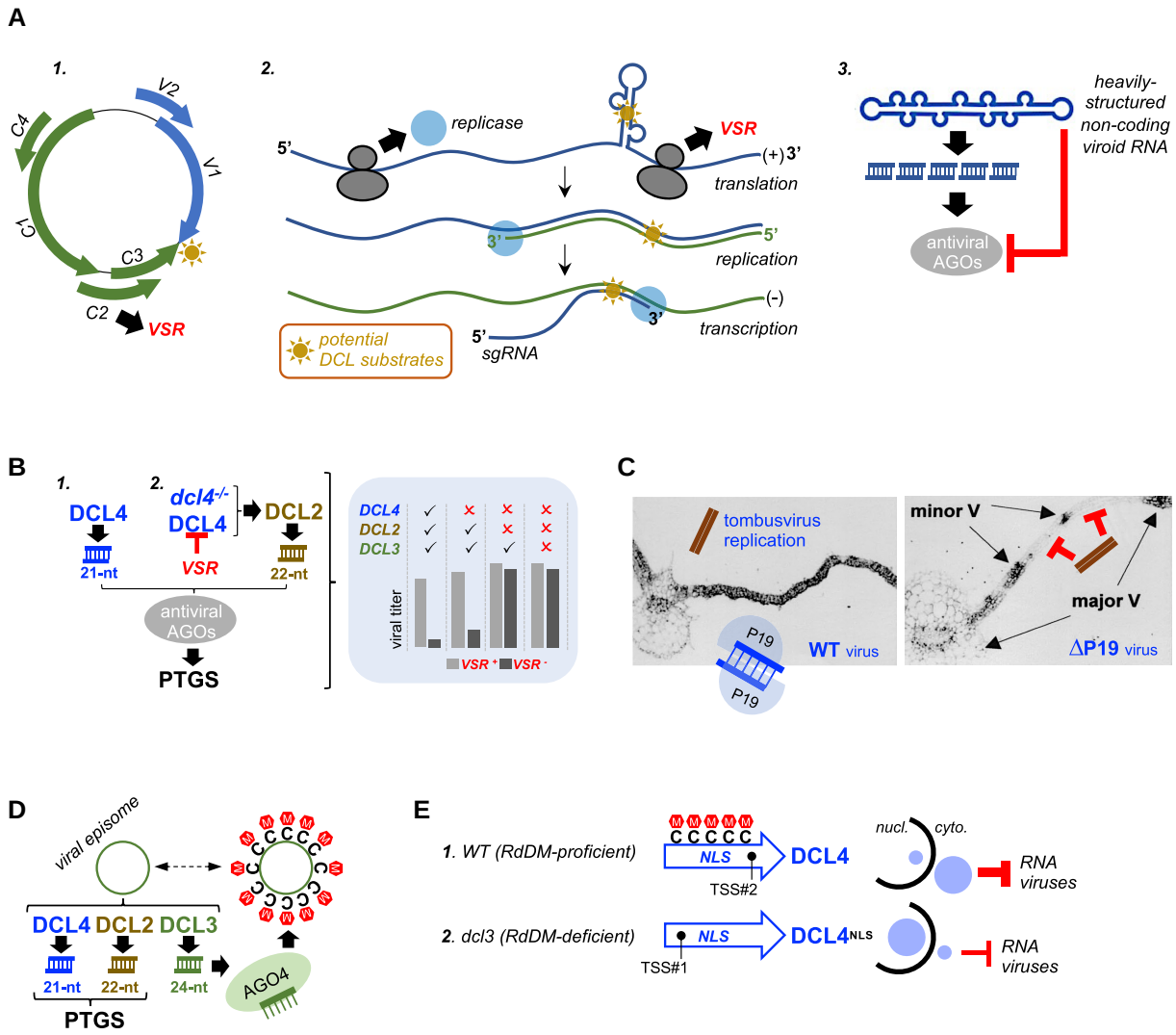

Figure 7. Anti-viral RNAi. **A)** dsRNA sources from diverse plant viruses and viroids. 1. Geminiviruses with a DNA genome can spawn dsRNA via read-through transcription of convergent overlapping ORFs (e.g. C3/V1 or C2/V1) located on opposite DNA strands. 2. Single-stranded RNA from RNA viruses can fold locally into dsRNA structures. dsRNA may also form during replication or transcription of sub-genomic (sg)RNA. Both RNA and DNA viruses combat antiviral RNAi by producing VSRs translated from their genomes, indicated here in red. 3. Viroids produce siRNAs but evade targeting by siRNA/AGO complexes, likely due to the rod-like complementary nature of their RNA. **B)** The affects of antiviral RNAi are mostly evident with VSR-deficient viruses. An illustrative framework for the hierarchical surrogacy linking DCL4 to DCL2 during RNA virus infections. Both 21- (DCL4-dependent) and 22-nt (DCL2-dependent) v-siRNAs promote PTGS upon their loading into antiviral AGOs. DCL4 action usually dominates but may be directly or indirectly inhibited by VSR activities, a circumstance mimicked by use of the *dcl4* mutant background. In either situation, DCL2 action takes over to rescue antiviral PTGS via 22-nt v-siRNAs. Genetically, this translates into VSR-proficient (VSR^+) viral titres remaining mostly unaffected in either the *dcl4* single- or *dcl2dcl4* double- mutant backgrounds due to the genetically-redundant VSR action. The VSR-deficient (VSR^-) viral titres are, by contrast, strongly enhanced yet mostly in the *dcl2dcl4* double mutants due the DCL4-DCL2 surrogacy. Neither the VSR^+ nor VSR^- viral titres are further enhanced in the *dcl2dcl3dcl4* triple mutant background because, unlike DNA viruses (see F), most RNA viruses are insensitive to RdDM mediated by 24-nt v-siRNAs, should they accumulate at all. **C)** P19-mediated sequestration of tombusvirus-derived v-siRNAs prevents sequence-specific immunization of virus-proximal tissues. The images (adapted from Havelda et al. 2003) depict in situ hybridizations of the tombusviral (−)RNA strand (attesting replication). The P19-proficient virus unloads from the vasculature to the adjacent leaf laminal cells. By contrast, the P19-deficient virus only accumulates in the vasculature whereas the adjacent tissues exhibit nucleotide-sequence-specific immunity to secondary tombusvirus challenge, suggesting vascular-to-laminal movement of a virus-derived silencing signal. **D)** DNA viruses activate an RdDM-like response. Additionally to activating antiviral PTGS via 21-22-nt v-siRNAs, DNA viruses spawn DCL3-dependent 24-nt siRNAs in the nucleus, which trigger cytosine methylation of viral episomes/mini-chromosomes. **E)** Possible indirect effects of RdDM-deficient conditions on antiviral PTGS. *DCL4* promoter demethylation in RdDM mutants allows alternative transcription start sites usage (TSSS#1 *versus* TSS#2 in WT, i.e. RdDM-proficient conditions). This enables production of a longer, NLS-containing DCL4 isoform that is more retained in the nucleus, with henceforth reduced activity in the cytosol where RNA viruses replicate.

mutants, however, DCL2 limits inflorescence infection by VSR-deficient TuMV (Garcia-Ruiz et al. 2010). Differential DCL4-vs-DCL2 contributions likewise prevent the access of potato virus X (PVX, an RNA potexvirus) to certain Arabidopsis tissues (Andika et al. 2015). Some DCL activities are also naturally higher in certain non-infected plant tissues, such as DCL3 in apices, where meristematic divisions underpin strong RdDM initiation/maintenance. Thus, the mere choice of sampled tissue will also influence the observed 21-nt-vs-22-nt-vs-24-nt v-siRNA pattern.

Roles for DCL1 and DCL3 in antiviral defense

RNA virus infections of *dcl2dcl3dcl4* triple-mutant Arabidopsis usually yield only very residual 21-nt v-siRNAs (Bouché et al. 2006; Deleris et al. 2006; Diaz-Pendon et al. 2007; Garcia-Ruiz et al. 2010), suggesting that the miRNA-specialized nuclear DCL1 accesses very poorly cytosolically replicated RNA viruses. Deep-seq in a hypomorphic *dcl1* vs WT background indeed shows no overt changes in TuMV-derived v-siRNA genomic distribution or accumulation, abrogated, by contrast, in *dcl2dcl3dcl4* (Garcia-Ruiz et al. 2010). Abundant, as opposed to residual, *35S* leader-derived 21-nt v-siRNAs accumulate in CaMV-infected *dcl2dcl3dcl4* plants (Blevins et al. 2011), presumably reflecting its nuclear transcription and pri-miRNA–like structure. As with endo- and transgenic *IR*s, DCL1 likely facilitates primary stem-loop excisions (Fig. 1B) since leader-derived 21-22-24-nt *35S* v-siRNA levels are substantially decreased in hypomorphic *dcl1* (Blevins et al. 2011). Potent virus-induced gene-silencing from recombinant cabbage leaf curl virus (a DNA geminivirus) engineered with an artificial miRNA confirms that DCL1 efficiently accesses secondary structures from transcribed DNA virus genomes (Tang et al. 2010).

Certain RNA virus infections in WT (e.g. TRV), *dcl4-* (e.g. cucumber mosaic virus [CMV], a tripartite RNA cucumovirus), or *dcl2dcl4* (e.g. TuMV) backgrounds yield abundant DCL3-dependent 24-nt v-siRNAs (Deleris et al. 2006; Garcia-Ruiz et al. 2010; Wang et al. 2010), raising 2 intertwined questions: (1) do 24-nt v-siRNAs—effectively involved in nuclear RdDM against DNA viruses (Fig. 7D)—impact RNA viruses? Generally, *dcl2dcl4* and *dcl2dcl3dcl4* loss-of-function backgrounds accumulate similarly higher RNA virus titers compared with WT. In one case, however, a weak Arabidopsis DCL4 mutant protein (dcl4-1) still retaining dominance over DCL2 became inapt for producing 21-nt v-siRNAs from VSR-deficient CMV and for conferring antiviral defense, but only in the null *dcl3* mutant background (Diaz-Pendon et al. 2007). One mechanism might involve recently discovered nucleo vs cytosolic DCL4 isoforms enabled by alternative TSS usage. Under RdDM-proficient (i.e. normal) conditions, *DCL4* promoter methylation causes production of a short, cytosolic DCL4 isoform likely optimally adapted to target RNA viruses. By contrast, RdDM-defective tissues (e.g. *dcl3* background) accumulate a longer DCL4 isoform with an N-terminal nuclear localization signal (NLS), likely suboptimal to combat RNA viruses (Pumplin et al. 2016) (Fig. 7E). RdDM deficiency might thus decrease the weak dcl4-1 protein's cytosolic availability, possibly explaining why *dcl4-1* loss-of-function effects are only evident in *dcl3*. DCL3/AGO4-dependent DCL4 isoformation could likewise explain why *ago4* mutants appear super-susceptible to certain de facto RdDM-insensitive RNA viruses (reviewed in (Carbonell and Carrington 2015). The second question raised by 24-nt v-siRNA accumulation during RNA virus infections pertains to subcellular localization. Indeed, DCL3 is nuclear in healthy plants, whereas viral RNA genomes are replicated and transcribed cytosolically. Perhaps DCL3 relocalizes cytosolically during infections, an open possibly given the paucity of cell biological data available for plant antiviral RNA-silencing. Generally, if and how DCLs (and cofactors) access RNA virus replication intermediates—almost invariably embedded in virally remodeled endo-membranes called viral factories—also remains largely unknown, circumstantial evidence aside (Clavel et al. 2021).

Involvement of RDRs in v-siRNA biogenesis

An alternative explanation for 24-nt v-siRNA production during RNA virus infections is that host-encoded, as opposed to viral-encoded, replicase-like enzymes convert possible nucleasrly accumulated viral RNA breakdown products into dsRNA. One candidate is RDR2 (already evoked in nuclear P4-siRNA production), which is required for optimal TRV- and TuMV-derived v-siRNA accumulation (Donaire et al. 2008; Garcia-Ruiz et al. 2010). This is mostly evident, however, upon concomitant inactivation of RDR1 and RDR6, which broadly restrict RNA and DNA viruses (reviewed in Qu 2010). Similar roles for RDR3, RDR4, and RDR5 are yet to be established, however, reflecting, perhaps, cell/tissue-restricted effects inaccessible via whole-plant analyses. As in *dcl* mutant studies, VSR activities will also often confound relevant *rdr* mutation effects. Indeed, only with VSR-deficient CMV was a role established for the salicylate-induced RDR1 in CMV-derived v-siRNA accumulation (Diaz-Pendon et al. 2007; Wang et al. 2010), adding to the previously identified RDR6 key contribution (Mourrain et al. 2000). v-siRNA mapping shows that RDR1 preferentially uses RNA derived from the 5′ ends of the 3 CMV RNAs, with RDR6 attacking the remaining sections (Wang et al. 2010). This evokes how distinct DCLs use distinct dsRNA types along, for example, bipartite TRV (Donaire et al. 2008), notwithstanding possibly considerable between-species variations. For instance, the 72-bp loss-of-function insertion in the *RDR1* locus in a *N. benthamiana* laboratory strain possibly underpins its popularity as a broad-spectrum experimental host (Bally et al. 2015).

RDR(s) recruitment onto viral RNA remains mysterious. Abundant 5′/3′ fragments derived from viral RNA slicing by AGO-loaded v-siRNAs might overwhelm RQC and feed RDRs, as in S-PTGS initiation (Figs. 1B, 2E, and 6G). Alternatively, interactions involving 22-nt or imperfect based-paired v-siRNAs (delaying/preventing slicing) might enhance antiviral AGO dwell time and hence recruit RDRs as in ta-siRNA biogenesis (Fig. 2, A and C). Antiviral AGOs would be essential under both circumstances, explaining perhaps why turnip yellows virus (an RNA

polerovirus) accumulation is unchanged in *rdr1rdr2rdr6* triple-mutant Arabidopsis (Devers et al. 2020) because the luteovirus-encoded VSR physically degrades AGOs (Baumberger et al. 2007; Bortolamiol et al. 2007). Thirdly, the viral genomes' foreignness relative to codon usage or GC-quadruplexes, for instance, could underlie ribosome stalling as a trigger of 5′-OH RNA breakage and ensuing RDR action, as with RDR6 recruitment onto *EVD shGAG* mRNA (Oberlin et al. 2022) (Fig. 4B). All 3 scenarios accommodate the apparent patchy RDR recruitment along viral genomes revealed by v-siRNA sequencing-mapping (reviewed in Qu 2010).

AGO effectors of v-siRNA action

Anti-TRV silencing is abrogated in *dcl2dcl4* double-mutant Arabidopsis, which displays high virus titers despite DCL3-dependent 24-nt v-siRNA accumulating comparably with the 21-nt v-siRNAs solely produced in *dcl2dcl3*, where DCL4 activity suffices for antiviral silencing. Indeed, TRV titers remain unchanged therein compared with WT (Deleris et al. 2006). Thus, mere dsRNA dicing—equally potent in *dcl2dcl4* and *dcl2dcl3*—is insufficient to convey antiviral immunity: AGOs are additionally required downstream v-siRNA production (Deleris et al. 2006). TRV-derived 24-nt v-siRNAs were presumably innocuous due to their loading into AGO4-clade AGOs acting at the DNA, not RNA, level. In contrast, 24-nt v-siRNAs mediate RdDM, chromatin compaction, and TGS against DNA virus mini-chromosomes/episomes (Raja et al. 2008), similar to P4-siRNA action against host TEs (Fig. 3A). That AGO1, among all Arabidopsis AGOs, is near ubiquitously and highly expressed likely influenced the results of forward genetic screens for mutants impaired in S-PTGS or miRNA activity. These processes resemble antiviral RNA silencing and are indeed strongly AGO1 reliant. A large array of isolated hypomorphic *ago1* alleles was thus naturally tested for potential hypersusceptibilty, initially to CMV (Morel et al. 2002) and, later, other viruses; most indeed displayed enhanced accumulation. Accordingly, 21-/22-nt v-siRNAs routinely coimmunoprecipitate with AGO1 (Azevedo et al. 2010; Garcia-Ruiz et al. 2015). That AGO1 historically remained the "tree hiding the forest" is likely explained by the narrower/lower spatial patterns/levels of most other Arabidopsis AGOs, all of which have now been implicated in antiviral defense to varying degrees (reviewed in Carbonell and Carrington 2015).

Other hinderances to recognizing AGOs' antiviral roles—also affecting DCL/RDR studies—include genetic redundancy and obfuscation by VSRs. Hence, AGO2's antiviral role was initially only diagnosed if *ago1* hypomorphic Arabidopsis was infected with VSR-defective CMV (Wang et al. 2011), though it was later recognized as a broad-spectrum antiviral effector (Harvey et al. 2011; Garcia-Ruiz et al. 2015; Ma et al. 2015). Other PTGS-related antiviral AGOs include AGO5, AGO7, and AGO10, yet with more spatially restricted effects (reviewed in Carbonell and Carrington 2015). Expression/action of certain AGOs might also be conditioned by silencing unrelated antiviral immunity pathways yet via RNA silencing–regulated mechanisms. Systemic acquired resistance stimulated in Arabidopsis leaves enhances *AGO2* and *AGO3* mRNA accumulation in nontreated leaves by negating a promoter-linked RdDM-like process reducing their transcription (Ando et al. 2021). Because systemic acquired resistance is commonly activated during plant-virus interactions, this regulatory network could prime antiviral AGO expression in yet-to-be-infected tissues, possibly seconding systemic silencing by mobile AGO-free v-siRNAs (see final section). Systemic *AGO5* induction in non-inoculated leaves of VSR-deficient PVX–infected Arabidopsis was likewise reported (Brosseau and Moffett 2015).

IP shows that AGO:v-siRNA associations follow the AGO-sorting principles of endogenous siRNAs yet without guaranteeing antiviral activity. Indeed, v-siRNAs derived from secondary structures within (+)/(−)RNA strands, although efficiently diced, might act poorly if the loaded AGOs fail to access v-siRNA-complementary sites buried inside such structures. Likewise, abundant viroid-derived siRNAs inefficiently target these subviral pathogens' circular, rod-like complementary RNA genomes (Wang et al. 2004) (Fig. 7A), which, devoid of ORFs, might exemplify silencing evasion rather than suppression. The highly structured *35S* RNA leader—the vastly dominant v-siRNA source from CaMV—not only resists v-siRNA action but also constitutes a decoy, whereby *35S* v-siRNA production might divert AGO:v-siRNA complexes away from CaMV promoter- and coding- regions (Blevins et al. 2011). In vitro reconstructed antiviral AGO assays (Schuck et al. 2013) further suggest that individual AGO:v-siRNA complexes mediate antiviral defense unequally. Likewise, v-siRNA quantity might not necessarily equate quality: low-abundant, "elite" v-siRNA species might suffice to target key viral genomes' regions for destruction or RDR recruitment.

v-siRNAs, va-siRNAs, endo-sRNAs, and viral disease symptoms

Quantitative image-based trait analysis of growth and leaf color—2 main disease symptom's outputs—applied to higher-order *ago* mutant Arabidopsis infected with VSR-deficient TCV revealed how distinct AGOs have specialized modular roles in antiviral defense across distinct tissues (Zheng et al. 2019). But to what extent is symptom severity—as opposed to viral titers—indeed commensurate with antiviral silencing potency, often thought to be itself strongly influenced by virulence-conferring VSRs? VSR-deficient CMV induces enhanced symptoms in *dcl2dcl4* Arabidopsis as severe and occurring even earlier than those elicited by WT CMV in WT plants; the CMV-encoded VSR even suppresses symptoms during early infection (Diaz-Pendon et al. 2007). Further complicating the picture are observations made with *e.g* the CMV Y-satellite (Y-Sat) noncoding RNA, long known to induce chlorotic symptoms in its natural host, tobacco, unlike in tomato or Arabidopsis. In tobacco, a single Y-Sat–derived siRNA targets the mRNA for magnesium protoporphyrin chelatase-subunit-I (*ChlI*) involved in chlorophyll biosynthesis (Shimura et al. 2011; Smith et al. 2011).

The experimentally demonstrated v-siRNA target site was found degenerated in the tomato and Arabidopsis *Chll* alleles, but Y-Sat genome engineering to restore v-siRNA-mRNA complementarity was sufficient to induce chlorosis without overt changes in Y-Sat accumulation or propensity to produce all other siRNAs. Thus, while underpinned by RNA-silencing, these symptoms are unrelated to Y-Sat–derived siRNA antiviral effects, if any. A similar rationale applies to the "albino" phenotype of peach latent mosaic viroid (PLMVd)-infected peach leaves, where 2 PLMVd-derived siRNAs target the host chloroplastic *HEAT-SHOCK PROTEIN 90* mRNA (Navarro et al. 2012).

v-siRNAs from many viruses may commonly alter host-gene expression by base-pairing to endogenous mRNAs (Miozzi et al. 2013; Annacondia and Martinez 2021). But is this targeting merely fortuitous or, on the contrary, does it exemplify adaptations underlying symptom development? Likewise, are va-siRNAs produced by virus-mediated RQC saturation (Cao et al. 2014) merely inconsequential infection byproducts or do they contribute to host defense/viral virulence? Finally, metazoan endogenous sRNAs can influence infections by base-pairing with viral RNAs (Lecellier et al. 2005; Henke et al. 2008). If and how plant endo-si/miRNA repertoires and their variations between cells, tissues, organs, or even species similarly impact phytovirus infections remains largely unknown, as is the degree of interconnection between antiviral RNAi and more recently discovered RNA-based viral restriction pathways including NMD (Garcia et al. 2014).

Regulation of siRNA biogenesis/steady-states and AGO turnover

While many factors regulate/fine-tune miRNA biogenesis and steady-state accumulation (reviewed in Li and Yu 2021), little is known, correspondingly, for siRNAs. Forward genetic screens for impaired S-PTGS or RdDM might have mostly yielded mutations in key amplification steps possibly masking fine-tuning at others. Alternatively, a fine-tuning deficit might reflect that S-PTGS or RdDM play, or evolved from, defensive as opposed to gene-regulatory functions. Nonetheless, these and the miRNA pathway share common check-points pertaining to sRNA protection and AGO-sRNA turnover, potentially diagnosed as cross-pathway competitions. For instance, HUA ENHANCER (HEN1) 2′-O-methylates plant mi/siRNA 3′ ends (Yu et al. 2005), likely reflecting the pervasiveness of perfect/near-perfect sRNA:target interactions in plants. This indeed causes sRNA 3′ ends to extrude from AGO PAZ domains (Ameres et al. 2010), thereby exposing them to poly-uridylation and subsequent exonucleolysis, antagonized by HEN1. A suppressor screen for restoration of suboptimal miRNA methylation in hypomorphic *hen1* identified mutants impaired in P4-siRNA production, the most abundant endo-sRNA class (Yu et al. 2010). Thus, plant miRNAs compete with endo-siRNAs under limiting HEN1 activity (Fig. 8A), which may be stress induced. Supporting this notion and further illustrating a HEN1-centered siRNA-*vs*-miRNA nexus, several VSRs

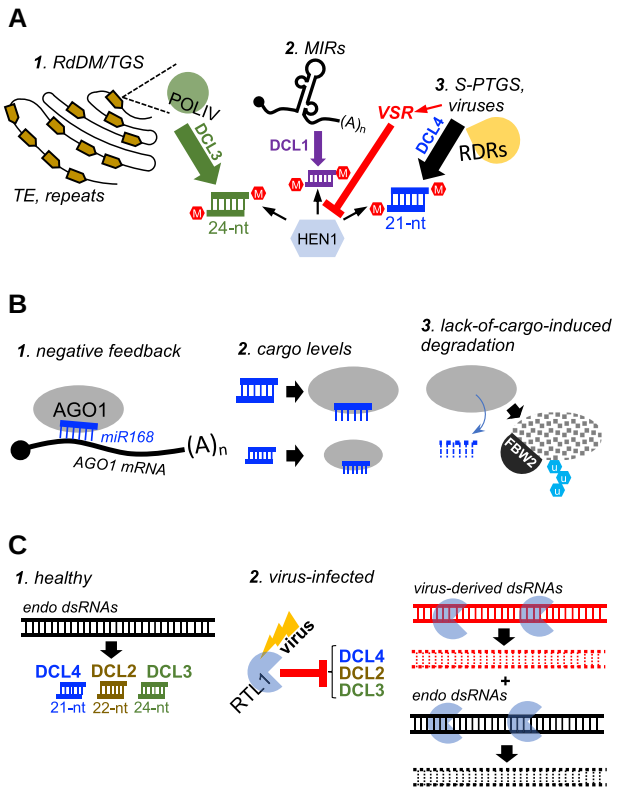

Figure 8. RNAi homeostasis. **A)** Multiple RNAi pathways compete for the same component. HEN1 2′O-methylates miRNAs as well as siRNAs involved in the TGS and PTGS pathways, making its availability for one pathway limited by the others. VSRs can target HEN1, reducing further its availability for these sRNA pathways. **B)** AGO1 homoeostasis involves multiple levels of control. (1) AGO1 mRNA levels are feedback-regulated by a miRNA, miR168, which forms a complex with the AGO1 protein. (2) AGO1 is stabilized by the loading of 21-22-nt miRNAs and siRNAs. As a result, transgenic plants undergoing PTGS or virus-infected plants accumulate more AGO1 protein to accommodate the excess of 21-22-nt molecules. Transgene siRNAs and v-siRNAs may represent up to 30% and 70% of total siRNAs, respectively. (3) Lack of cellular 21-22-nt si/miRNAs (for example in miRNA-deficient mutants) provokes AGO1 degradation, thereby preventing its spurious loading with inappropriate RNA molecules. **C)** DCLs and virus-induced RTL1 compete for long dsRNA. (1) Endogenous long dsRNAs are normally processed by DCL4, DCL2 and DCL3 into signature siRNA products. (2) Upon virus infection, RTL1 is induced and compete with DCL2/3/4 for substrates, including viral-derived dsRNAs, which it degrades.

target HEN1 activity in virus-infected cells; Fig. 8A), causing enhanced turnover of normally HEN1-protected v-siRNAs. Such VSRs also destabilize miRNAs (and presumably ta-si/pha-siRNAs), a potential basis for virus-induced developmental aberrations (Kasschau et al. 2003; Jay et al. 2011).

Competition also exists at the AGO-loading level. For example, most miRNAs are 5′ U and 21 to 22 nt long and thus compete for AGO1 loading with similarly featured siRNAs. This likely explains why impairing miRNA production strongly potentiates transgene S-PTGS, indeed mostly reliant on 21-22-nt siRNA-loaded AGO1 (Martinez de Alba et al. 2011). Nevertheless, several mechanisms help adapt AGO1 levels to the available amounts of loadable

sRNAs in WT plants. First, an AGO1:miR168 complex feedback-regulates *AGO1* mRNA levels negatively (Vaucheret et al. 2004). Secondly, 21- to 22-nt mi/siRNA loading stabilizes the AGO1 protein such that its levels increase when loadable sRNA levels increase, for example during transgene S-PTGS (Vaucheret et al. 2006). Conversely, Arabidopsis miRNA-pathway mutants often accumulate less AGO1 (Hacquard et al. 2022). Lastly, empty AGO1 undergoes proteolysis through the E3 ubiquitin-ligase component FBW2, preventing AGO1's spurious loading with illegitimate sRNAs and ensuing off-targeting effects (Hacquard et al. 2022) (Fig. 8B). Similar to *AGO1*, *AGO2* levels are negatively regulated by miR403 (Allen et al. 2005), and AGO4-clade AGOs are heavily destabilized in *pol-IV*, *rdr2*, or *dcl2dcl3dcl4* mutant Arabidopsis, all lacking otherwise abundant P4-siRNAs (Havecker et al. 2010; Devers et al. 2023). Virus infection can, likewise, impact AGO stability/activity across si/miRNA pathways by saturating cells with v-siRNAs competing with miRNAs for AGO1 loading. This is achieved by deploying AGO-antagonistic or si/miRNA-sequestering VSRs (Lakatos et al. 2004; Azevedo et al. 2010) or by strongly inducing *MIR168* gene expression (Várallyay et al. 2010). Illustrating a possible host counter-defense, the virus-induced rice AGO18 sequesters miR168 away from rice AGO1, thereby enhancing AGO1's antiviral potential (Wu et al. 2015). Some viruses also induce expression of RNase-III-LIKE-1 (RTL1), which acts as an endogenous suppressor of siRNAs, but not miRNAs, by competing with DCL2, DCL3 and DCL4 for processing perfect, long dsRNA including that derived from viral replication (Sehki et al. 2023) (Fig. 8C). While this benefits viruses by dampening v-siRNA production, whether RTL1 regulates endo-siRNA production in healthy plants –as suggested by its evolutionary conservation– remains undetermined.

The post-loading fate of plant siRNAs remains elusive. SMALL RNA DEGRADING NUCLEASE (SDN) family members were shown to degrade mature miRNAs from their 3′ends in vitro, a process unimpeded by 2′O-methylation in vivo; accordingly, *sdn1sdn2sdn3* triple-mutants accumulate higher miRNA levels (Ramachandran and Chen 2008). Other *SDN* family members might likewise use AGO-loaded siRNAs as substrates and indeed an *SDN* paralog degrades v-siRNAs in barley (Jin et al. 2022a). Molecular calipers formed by the tombusviral-encoded homo-dimeric VSR, P19, specifically sequester 21-22-bp sRNA duplexes, including, chiefly, v-siRNAs thereby prevented from antiviral AGO-loading (Lakatos et al. 2004) (Fig. 7C). Intriguingly, si/miRNAs bound to transgenically or virally expressed P19 undergo 1- to 2-nt 3′ end trimming possibly enabling P19 recycling because the ensuing 19–20 bp duplexes dissociate from it (Kontra et al. 2016). While the nuclease involved remains mysterious, P19-induced trimming hints at as-yet-undiscovered endogenous si/miRNA-modifying processes in healthy plants, given the propensity of viruses to hijack host pathways.

Non-cell autonomous siRNA action

Predating the discovery of siRNAs, evidence accumulated in tobacco that S-PTGS/co-suppression might physically move between cells/organs. Erased during seed maturation, systemic PTGS is not transgenerational, however. Instead, progeny transgenic plants trigger S-PTGS/co-suppression at a given frequency at each generation (Palauqui and Vaucheret 1995). In certain systems, this occurs at seed imbibition/early germination, giving an impression of uniform PTGS initiation. Systemic PTGS initiates later during growth in others, enabling a better appreciation of movement, first locally, from trigger-cells (in which RQC is presumably spontaneously impaired/overwhelmed) and then through the vasculature (Palauqui et al. 1996). The eventual whole-plant invasion is now recognized as de novo initiation in possibly every silencing-recipient cell combined with relay-amplification by RDR6 and other co-factors detailed in a previous section (Fig. 6, A to G). Grafting unambiguously demonstrated that silenced transgenic rootstocks transmit a nucleotide sequence-specific signal to initially non-silenced transgenic scions (Palauqui et al. 1997) (Fig. 6H). Noninvasive monitoring (Fig. 6, E and F) showed that movement occurs symplastically, from cell-to-cell, likely via plasmodesmata (PDs) and vascularly, via the PD-connected phloem sieve-elements (SEs; (Voinnet and Baulcombe 1997; Voinnet et al. 1998). The movement pattern and dynamics strikingly evoke that of viruses, also similarly affected by the photoassimilates' source-*vs*-sink organ status, thus suggesting that mobile transgene PTGS approximates the systemic arm of the previously-discussed v-siRNA-mediated antiviral defense.

The "recovery" of certain plants from highly symptomatic infections established in already developed leaves is likely an extreme manifestation of this process. The asymptomatic "recovered" new growth indeed accumulates low viral titers and displays immunity against secondary challenges by the same, but not a sequence-divergent, virus (Ratcliff et al. 1997), as if a sequence-specific virus-derived signal produced in developed, infected tissues had moved into, and immunized, the as-yet-uninfected apical growing points. That recovery, systemic S-PTGS and mobile *IR*-PTGS require a common gene set in Arabidopsis indirectly supports this notion (Kørner et al. 2018) as do observations that some VSRs, or interactors thereof, are partially PD-localized (Rosas-Diaz et al. 2018). More compellingly, accumulation of P19-proficient tombusvirus is widespread in leaves, whereas that of a P19-deficient counterpart is vascular-restricted, yet the surrounding leaf laminal tissue exhibits sequence-specific immunity to secondary viral challenge (Havelda et al. 2003) (Fig. 7C). Likely, their cell-autonomous P19-mediated sequestration normally prevents 21-22-nt v-siRNA duplexes –the established, exclusive P19 homodimers' molecular cargoes (Brioudes et al. 2022)– from moving outside the virus phloem-unloading zones. Without P19, mobile virus-derived silencing immunizes laminal tissues. Implicit to this interpretation is that v-siRNAs, not their longer dsRNA precursors (e.g. partial replication-intermediates, RDR products, neither of which would be P19-bound), move between cells.

Experiments involving 1) an *IR* transgene expressed under the companion-cell (CC)-specific promoter *pSUC2*, 2) the vascularly-expressed *IR71*, and 3) a naturally phloem-restricted luteovirus collectively confirmed that DCL4-,

DCL2- and DCL3-dependent AGO-free siRNA duplexes all move between cells (Devers et al. 2020). However, as they traverse cells, siRNAs are "consumed" by cell-autonomous AGOs (incidentally ruling out mobile AGO:siRNA complexes) based, in part, on their 5′-nt identities. Hence, in leaves, 5′G- and 5′C-siRNAs were more collected in silencing-recipient tissues than 5′U- (AGO1-loaded) or 5′A- (AGO2- and AGO4-loaded) siRNAs likely because AGO5 (loaded with 5′C siRNAs) is absent in leaves and no known plant AGO is 5′G-specific (Devers et al. 2020). Consumption further rules out long dsRNA precursor movement, at least during *IR*-PTGS/anti-luteovirus silencing, which are all RDR-independent processes. Reiterative long dsRNA processing would indeed spawn siRNA with similar 5′-nucleotides across all layers (Devers et al. 2020). Whether dsRNA produced by RDRs involved in e.g. S-PTGS/RdDM move cell-to-cell remains unclear (Fig. 6G), though relay-amplification of virtually infinite 5′-nucleotide contexts would predictably override consumption in this case. Despite their suspected importance, the channels for non-autonomous siRNA action within and between male/female sporophytes/gametophytes are yet-to-be described. Several of the organs/structures involved form isolated symplastic domains –as is well-documented for the developing embryo (Stadler et al. 2005)– across which the involvement of PDs, as opposed to e.g. apoplastic transfer of siRNA- or AGO:siRNA-loaded vesicles (Ruf et al. 2022), is questionable. Cytosolic projections connecting the paternal VC's nucleus to the generative cell (precursor of the two SCs; (McCue et al. 2011)) may also allow movement of AGO-siRNA complexes, which is supported by SC- and VC-specific siRNA-ablation experiments targeting non-loaded duplexes (Pachamuthu et al. 2023).

Experiments involving *IR*-PTGS-transgenic- or WT-Arabidopsis scions grafted onto respectively WT- or siRNA-deficient *dcl2dcl3dcl4* mutant- rootstocks showed that both transgene-derived and endo- siRNAs can move via the vasculature, additionally to cell-to-cell (Molnar et al. 2010; Lewsey et al. 2016) Fig. 5I). Long-distance movement of (at least) 24-nt endo-siRNAs is functional because many TE/repeat loci in *dcl2dcl3dcl4* rootstocks had increased methylation levels at mostly CHH sites (diagnostic of RdDM) compared to non-grafted *dcl2dcl3dcl4* plants (Lewsey et al. 2016) (Fig. 6I). Moreover, de novo CHH-based epiallelism diagnosed by SNPs occurred in rootstocks of grafts involving distinct Arabidopsis accessions (Molnar et al. 2010; Lewsey et al. 2016). dsRNA precursor movement over long-distances –not formally ruled out by the above or other experiments– is unlikely because AGO-mediated consumption (to which mobile long dsRNAs should be *de facto* insensitive) impacts the efficacy of long-distance *IR*-PTGS movement. Influenced by siRNA sizes (21-/22-/24-nt) in addition to 5′-nt identities, this process explains a previously-noted apparent selectivity in siRNA long-distance movement in certain contexts (Devers et al. 2023). Consistent with results obtained with mobile synthetic ta-siRNAs (Cisneros et al. 2022), targeted and inducible callose deposition at PDs in siRNA-emitting cells of *IR-PTGS* transgenic Arabidopsis shows that long-distance spread need not be RDR-amplified to be highly effective over extended periods. This is granted, in part, by little siRNA leakage occurring in transporting stems (Devers et al. 2023). Strikingly, forward genetics of siRNA movement is yet to identify a single factor generically involved in RNA-silencing transmission between cells and organs, reflecting, perhaps, the essential nature or genetic redundancy of the underlying factors. Alternatively, intracellular silencing control, including via AGO-mediated consumption, might suffice to modulate movement (Voinnet 2022).

Concluding remarks and outstanding questions

Despite considerable knowledge-gain on plant siRNAs, many questions/challenges remain. Firstly, given the number of non-PCG loci from which they can originate, and the data paucity concerning such loci's expression under stress, the so far-established endogenous siRNA repertoires are likely vastly incomplete. Secondly, the astonishing complexity and diversity of their biosynthetic pathways strongly suggest that many more, as-yet-undiscovered means of endo-siRNA production exist in plants. For instance, how many types of abRNAs can be used as RDR substrates, and how are they formed? Thirdly, are all siRNAs, in their diversity, actually functional or are some–perhaps most—mere byproducts of competing RNA-based surveillance/turnover pathways? In particular, what are the roles of most non-TE/repeat-derived and often abundant endogenous siRNAs, including endo-*IR*-siRNAs, nat-siRNAs, va-siRNAs? Can they systemically impart epigenetic changes as suggested for graft-transmitted P4-siRNAs? And indeed, can mobile P4-siRNAs reach shoot meristems to induce transgenerational epiallelism, and to what effects, if any, in progenies? The mechanism and channels of the proposed short-range movement of reproductive tap-, siren- and pollen-siRNAs remain to be clarified, as are the paternal deposition and biogenesis of the latter. Fourthly, how is siRNA production-*vs*-degradation regulated, especially during stress, beyond the mere impact of expression-changes imparted at their loci of origin? For instance, 21-/22-nt siRNA-biogenesis-components were shown to reside in cytosolic bodies coined siRNA-bodies. These co-localize with stress-granule markers upon stress-induced translational repression, evoking a possible interplay between siRNAs and stalled mRNAs during stress-induced translation arrest (Jouannet et al. 2012). In addressing these and many still-outstanding questions, one should finally consider that most of our siRNA-related knowledge comes from studies in Arabidopsis whose genome contains few and seldom active TEs compared to the TE-replete tomato, rice, or maize genomes. Unlike in Arabidopsis, siRNA pathway mutants in these species are generally lethal or cause strong pleiotropic developmental effects hampering in-depth analyses, as exemplified by the sterility of rice or maize mutants impaired in the production of reproductive pha-siRNAs (Nonomura et al. 2007; Komiya et al. 2014; Nan et al. 2022). Last but not least, several transgressive traits seen in neither

parents of, for example, tomato hybrids (whereas Arabidopsis is autogamous) correlate with complete changes in hybrid's DCL usage and siRNA size/action at multiple loci (Lopez-Gomollon et al. 2022). Have we thus merely glimpsed the tip of a plant siRNA iceberg?

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