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REVIEW ARTICLE

Interplay and transition between small RNA-directed posttranscriptional and transcriptional gene silencing in plants

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Abstract RNA silencing refers to small RNA (sRNA)-directed, sequence-specific regulatory mechanisms operating in diverse eukaryotic organisms. Plants possess several genetically distinct sRNA-directed regulatory pathways that are known to operate either posttranscriptionally or transcriptionally. Over the years, the sRNA-directed posttranscriptional gene silencing (PTGS) and sRNA-directed DNA methylation (RdDM), which can lead to epigenetic transcriptional gene silencing (TGS), were considered distinct pathways with little or no interactions. Recent studies have uncovered an expression-dependent pathway termed RDR6-RdDM which involves the nuclear RNA polymerase II (Pol II), RNA-dependent RNA polymerase 6 (RDR6), and Pol V, but not Pol IV and RDR2, key components of the canonical Pol IV-, RDR2-, and DCL3-dependent RdDM (Pol IV-RdDM). The RDR6-RdDM pathway provides a mechanistic link between sRNA-directed PTGS and TGS, revealing previously unappreciated dynamic features of sRNA-directed regulation in plants. This 21- and 22-nucleotide (nt) sRNA-directed RdDM may represent a general transition step during de novo establishment of TGS through the canonical Pol IV-RdDM in plants.

Keywords Small RNA (sRNA) · microRNA (miRNA) · Small interfering RNA (siRNA) · RNA-directed DNA Methylation (RdDM) · Posttranscriptional Gene Silencing (PTGS) · Transcriptional Gene Silencing (TGS)

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Introduction

Small RNAs (sRNA) are an essential regulatory component in diverse eukaryotic organisms. In plants, the sRNA-directed regulatory mechanisms are known to operate either posttranscriptionally or transcriptionally, depending on the "type" of cellular sRNAs as defined by their biogenesis pathways. Extensive studies over the past decades, especially those on the model plant *Arabidopsis thaliana* have established the frameworks for genetically distinct posttranscriptional (PTGS) and transcriptional gene silencing (TGS) pathways in plants, which involve members of several highly conserved protein families that constitute the core of RNA silencing machinery. Intriguingly, recently studies have uncovered evidence for an interplay and transition between PTGS and TGS, revealing previously unappreciated dynamic features of sRNA-directed regulation in plants. In this short review, we provide a brief presentation on the canonical PTGS and TGS pathways in plants, followed by the recently discovered novel RDR6-RdDM pathway which provides a mechanistic link between the two. We conclude with a discussion on the potential biological significance of the discovery, and new questions it elicits.

sRNA-directed PTGS in plants

Plant sRNAs including microRNAs (miRNA) and small interfering RNAs (siRNAs) are typically of 21- to 24-nucleotide (nt) in size depending on the biogenesis

pathways in which they are produced. Works from the model plant *A. thaliana* have uncovered several genetically distinct pathways for sRNA biogenesis in plants (Axtell 2013; Bologna and Voinnet 2014). In general, key components for each of the sRNA biogenesis pathways include conserved protein machinery responsible for transcription (DNA-dependent RNA polymerases, or Pol), formation of double-stranded RNA (dsRNA) precursors (RNA-dependent RNA polymerases, or RDR), processing of dsRNA into sRNAs (Dicer-like RNase III ribonucleases, or DCL), and formation of sRNA effector complexes (Argonaute, or AGO), respectively. Specifically, of the three functional RDRs, four DCLs, and ten AGOs encoded in the Arabidopsis genome, most have been placed in one of the genetically defined pathways for sRNA biogenesis and function.

miRNA-directed PTGS

miRNAs are a class of endogenous sRNAs processed from non-coding precursor transcripts capable of forming the characteristic fold-back stem-loop structure. The primary transcripts of a miRNA (pri-miRNA) typically arise from a defined *MIRNA* locus by Pol II transcription (Xie et al. 2005). DCL1-dependent processing of a pri-miRNA gives rise to a hairpin-shaped miRNA precursor (pre-miRNA) which is further processed into a sRNA duplex consisting of a mature miRNA and a passenger strand known as a miRNA*. Since the hairpin-containing miRNA precursors represent a species of intra-molecular dsRNA with numerous mismatched bulges, miRNA biogenesis therefore bypasses the need for an RDR activity which catalyzes dsRNA formation from a single-stranded RNA (ssRNA) template. It is generally true in plants that one *MIRNA* locus defines one pre-miRNA which gives rise to one mature miRNA (Jones-Rhoades et al. 2006). Mature miRNAs in plants are typically of 21-nt in size with uridine as the most common 5′-terminal nucleotide (Jones-Rhoades et al. 2006).

The regulatory function of a miRNA is achieved through sequence-specific interactions between the miRNA and its target in an AGO-containing effector complex. It has been well established that plant miRNAs exert their regulatory functions through extensive, near-perfect base-paring with their target RNAs, typically leading to cleavage of the targets at the middle of the base-paired region (Llave et al. 2002; Kasschau et al. 2003), although other mode of interaction such as translational repression has also been reported (Aukerman and Sakai 2003; Chen 2004; Gandikota et al. 2007; Brodersen et al. 2008; Lanet et al. 2009). Of note, in a majority of known miRNA targets that encode proteins, a miRNA-interacting site is found within the open reading frame (ORF), a scenario that differs from animal miRNAs which predominately target 3′ untranslated region (UTR) of mRNAs.

How mature miRNAs are sorted into distinct AGO-containing ribonucleoprotein complexes is not well understood, although several factors are known to affect the formation of specific miRNA-AGO complexes. For instance, AGO1 has been shown to preferably associate with miRNAs with a 5′-terminal uridine whereas AGO7 has a bias to miRNAs with a 5′-terminal adenosine (Mi et al. 2008; Montgomery et al. 2008; Takeda et al. 2008). Tissue-specific co-expression of an AGO family member and miRNA may also affect the formation of specific miRNA-AGO complexes, as has been shown for miR390-AGO7 complex in Arabidopsis (Montgomery et al. 2008).

An essential role of miRNA-directed PTGS in plant development has been well established genetically, as null mutations in key components of miRNA biogenesis or function are embryonic lethal (Lynn et al. 1999; Golden et al. 2002; Kidner and Martienssen 2005) [also reviewed in (Schauer et al. 2002; Vaucheret 2008)]. Moreover, perturbation in expression of a single miRNA either in genetic mutants or through transgenic manipulation often results in profound phenotypic alteration, as has been demonstrated in multiple plant species (Jones-Rhoades et al. 2006; Voinnet 2009). This is perhaps not surprising given the fact that a large proportion of known plant miRNAs target mRNAs encoding a diverse set of transcription factors (TFs), of which many serve as key developmental regulators (Jones-Rhoades et al. 2006). Plant miRNAs also function as important regulators for responses to environmental changes, as revealed through several conserved miRNA families that function to regulate acquisition or cellular homeostasis of numerous macro- and micro-nutrients (Sunkar et al. 2007; Yamasaki et al. 2007; Hsieh et al. 2009; Lin et al. 2010, 2013; Vidal et al. 2010; Kant et al. 2011; Park et al. 2014).

siRNA-directed PTGS

Aside from miRNAs, plants also produce several classes of siRNAs from dsRNA precursors which typically form through the activity of an RDR. At least two well-established, functionally distinct siRNA biogenesis pathways exist in plants. One of these, known as the *trans*-acting siRNA (ta-siRNA) pathway, produces a phased array of 21-nt sRNAs from miRNA-targeted non-coding transcripts (termed *TAS* for *TRANS-ACTING SMALL INTERFERING RNA*), in an RDR6- and DCL4-dependent manner (Axtell 2013; Bologna and Voinnet 2014). Because initiation of ta-siRNA biogenesis from a *TAS* transcript requires a miRNA-directed cleavage, which also serves to set the register, ta-siRNAs are therefore also DCL1-dependent and may be considered as secondary sRNAs derived from a miRNA target (Fig. 1A).

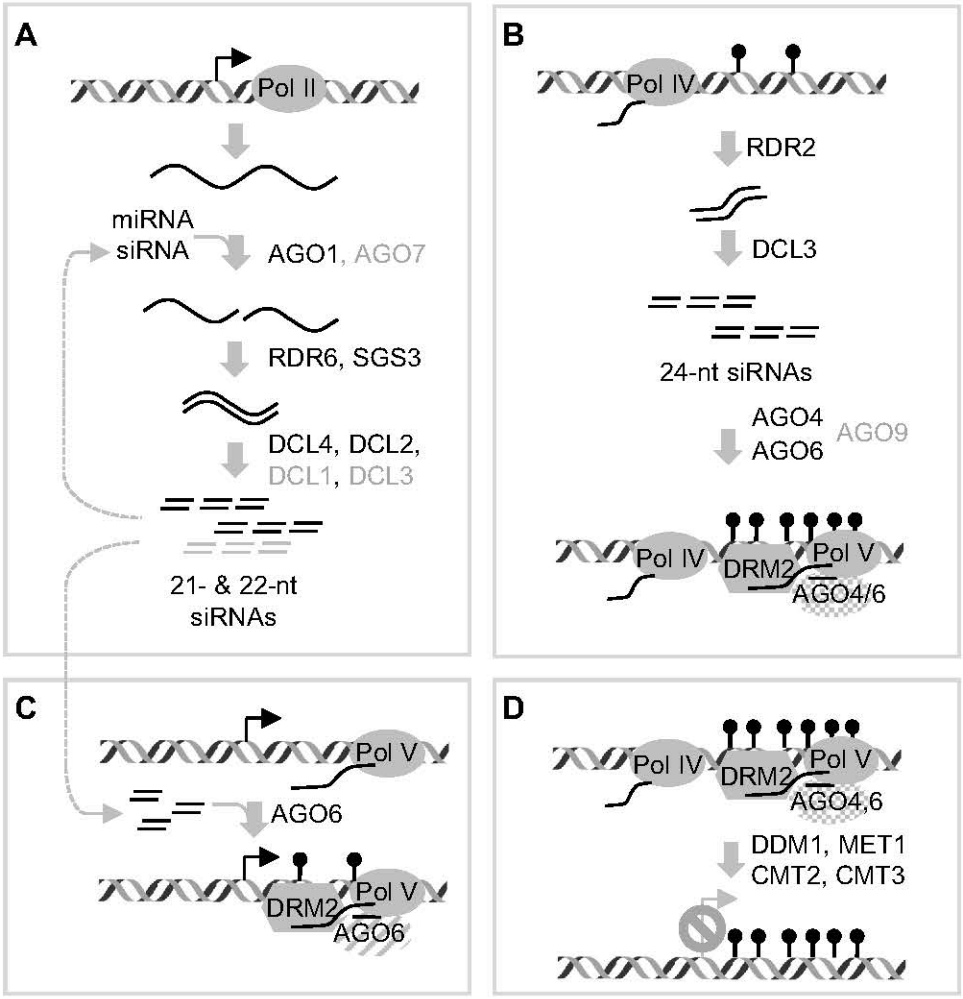

Fig. 1 Interplay and transition between sRNA-directed PTGS and TGS in Plants. **A**. PTGS directed by miRNAs and siRNAs. sRNA-directed cleavage of a target transcript by AGO1 may trigger RDR6-, SGS3-, and DCL4-dependent production of secondary sRNAs, which may in turn direct AGO1-dependent PTGS of homologous targets in *trans*. AGO7 has been shown to be specifically involved in miR390-directed ta-siRNA biogenesis at *TAS3* loci. Involvement of redundant DCL activities can be inferred from the presence of siRNAs of distinct size classes. **B** Canonical Pol IV-, RDR2-, and DCL3-dependent RdDM represents a self-reinforcing mechanism of DNA methylation directed by 24-nt siRNAs, in a process known as Pol IV-RdDM which also requires the activity of AGO4 and AGO6 (may also involve AGO9), Pol V, and DRM2. RDR6-RdDM (see below) may help attract Pol IV activity to the target chromatin with partially methylated DNA to establish DNA methylation at cytosine residues in all sequence contexts (i.e. CG, CHG, and CHH, where H represents either A, C, or T). **C** RDR6-dependent 21- and 22-nt siRNAs may also form sRNA-AGO6 complexes and initiate expression-dependent de novo DNA methylation at the target locus, in a process known as RDR6-RdDM which is also Pol V- and DRM2-dependent. **D** Pol IV-RdDM may lead to epigenetic TGS status at the target chromatin, which may be maintained through the activities of chromatin remodeler DDM1, and DNA methyltransferases MET1, CMT2, and CMT3 independent of 24-nt siRNAs. Many additional known components in the depicted PTGS and TGS pathways are omitted in the illustration for clarity. An horizontal arrowhead above the DNA molecule represents a transcription start site (TSS) that undergoes active Pol II transcription. A lollipop-shapped attachment to the DNA molecule represents a 5-methylcytosine (5mC) in the DNA. Please refer to the main text for a full definition of each abbreviation

The mechanism by which a miRNA-directed targeting triggers ta-siRNA production from the cleaved *TAS* transcripts is not well understood. Nonetheless, several molecular features that are required to route a cleaved miRNA target into the RDR6- and DCL4-dependent siRNA generation pathway have been discovered. For instance, cleavage directed by a 22-nt miRNA derived from an asymmetrically bulged stem-loop precursor appears to be sufficient to trigger ta-siRNA biogenesis (Chen et al. 2010; Cuperus et al. 2010). Alternatively, dual targeting by a 21-nt miRNA at two separate locations on the same transcript also serves as a good trigger (Axtell et al. 2006; Howell et al. 2007). A "two-hit" model was therefore proposed for miRNA-triggered siRNA production (Axtell et al. 2006). Emerging evidence seems to suggest that the "two-hit" mode of miRNA-initiated ta-siRNA biogenesis may have originated from a "one-hit" mode, reflecting an evolutionary refinement in secondary siRNA production

for enhanced processing accuracy and targeting precision (de Felippes et al. 2017). AGO1 appears to play an important role in recruitment of RDR6 and SUPPRESSOR OF SILENCING3 (SGS3), two proteins known to be required for ta-siRNA production (Peragine et al. 2004; Vazquez et al. 2004), to the miRNA-targeted *TAS* transcripts (Fig. 1A). Curiously, the slicer activity of AGO1, although important for phasing, turned out to be dispensable for initiation of ta-siRNA production at *TAS* loci, as RDR6- and SGS3-dependent production of unphased siRNAs was shown to persist in a slicer-deficient *ago1* mutant (Arribas-Hernandez et al. 2016; de Felippes et al. 2017).

Remarkably, miRNA-initiated, ta-siRNA-like secondary siRNA production does not seem to be limited to Pol II transcripts arising from a few non-coding *TAS* loci. Recent works on diverse plant species have shown that a subset of miRNA-targeted protein-coding transcripts can also give rise to ta-siRNA-like phased secondary siRNAs termed phasiRNAs (Fei et al. 2013; Howell et al. 2007). The above-mentioned "rules" established from ta-siRNA biogenesis also seem to apply for routing a miRNA-targeted mRNA into the phasiRNA production pathway (Fei et al. 2013). Quite intriguingly, recent studies in Arabidopsis using density gradient fractionation approaches provided evidence for phased siRNA biogenesis on membrane-bound polysomes (MBPs) (S. Li et al. 2016), a fraction presumably representing polysomes that are attached to the endoplasmic reticulum (ER), or rough ER (Li et al. 2013, 2016). An association of miRNA targets including *TAS* and *PHAS* transcripts with rough ER is rather surprising, as such an association would be expected only for mRNAs encoding either transmembrane proteins or proteins targeted for the secretory pathway. However, previously reported association of AGO7, as well as SGS3, with cytoplasmic membrane structure appears to be consistent with phased siRNA biogenesis on MBPs (Jouannet et al. 2012). Furthermore, current data collectively point to an engagement of ribosomes at the scene of miRNA-triggered phased siRNA production, with the small ORFs within a *TAS* or *PHAS* transcript likely playing an important role in setting the patterns of ribosome occupancy, which could in turn define boundaries of the targeted fragment for RDR6- and SGS3-dependent processing (Zhang et al. 2012; Hou et al. 2016; Li et al. 2016; Yoshikawa et al. 2016).

Once produced, ta-siRNAs or phasiRNAs are thought to exert their regulatory function through guiding target RNA cleavage, in much the same way as miRNAs do. Because Pol II is responsible for transcription at a *TAS* or *PHAS* locus, this siRNA-directed PTGS could be designated as a Pol II-, RDR6-, and DCL4-dependent PTGS pathway (Fig. 1A). What then are the regulatory advantages of phasiRNA-mediated PTGS when compared with miRNA-mediated PTGS? Although the exact answer to this question remains unclear, the following observations may offer some clues. First, the phasiRNA-mediated PTGS allows a rapid clearance of homologous mRNAs that are closely related to a miRNA-targeted mRNA but would otherwise not subject to a direct PTGS by the miRNA (Chen et al. 2007; Howell et al. 2007; Fei et al. 2013). Secondly, miRNAs and phasiRNAs appear to differ in their mobility in vivo. In contrast to miRNAs which appear to be cell-autonomous, the miRNA-triggered phased secondary siRNAs can act non-cell-autonomously (Chitwood et al. 2009; Melnyk et al. 2011; Pyott and Molnar 2015). Such characteristics of a phasiRNA allow formation of a siRNA gradient across multiple layers of cells in a tissue, which could be critical in pattern formation, as has been shown for *TAS3*-derived tasiR-ARF in Arabidopsis (Chitwood et al. 2009). Several conserved *TAS/PHAS* loci, as well as their trigger miRNAs have been identified from multiple plant species [see (Howell et al. 2007) and a recent review (Fei et al. 2013)]. Whether ta-siRNA- or phasiRNA-directed PTGS elicits DNA methylation at targeted loci remains to be investigated.

sRNA-directed TGS in plants

In addition to the above described Pol II-, RDR6-, and DCL4-dependent siRNA biogenesis, plants operate another siRNA pathway which produces 24-nt heterochromatin-associated siRNAs (hc-siRNAs) in an RDR2- and DCL3-dependent manner, typically from genomic regions harboring repetitive sequences such as transposable elements (TEs) and ribosomal RNA genes (rDNA), among others (Law and Jacobsen 2010; Matzke et al. 2015). Initiation of this hc-siRNA pathway involves transcription by a dedicated RNA polymerase known as Pol IV (or NRPD for nuclear RNA polymerase D) which is evolutionarily related to Pol II (B. Zheng et al. 2009; Haag and Pikaard 2011; Li et al. 2015; Zhai et al. 2015) (Fig. 1B). Cellular hc-siRNAs are found to form sRNA-AGO complexes preferably with AGO4 in Arabidopsis, an AGO family member that has been shown to preferably recruit 24-nt siRNAs with a 5′-terminal adenosine (Qi et al. 2006; Ye et al. 2012; Wang and Axtell 2017). Arabidopsis AGO6 and AGO9, two other AGO family members that are closely related to AGO4 based on phylogenetic analysis, are believed to function redundantly with AGO4 although both AGO6 and AGO9 exhibit much restricted expression domains both spatially and temporarily (Mallory and Vaucheret 2010; Poulsen et al. 2013) (Fig. 1B).

While both miRNAs and phasiRNAs direct PTGS, the 24-nt hc-siRNAs function in the nucleus to epigenetically repress transcription of TEs and other genomic repetitive sequences through a process known as RNA-directed DNA

methylation (RdDM) (Law and Jacobsen 2010; Matzke et al. 2015). The downstream steps of RdDM involve targeting of the siRNA-AGO4 complexes to chromatin and further recruitment of chromatin remodeling proteins to the target chromatin. Pol V (or NPRDE for nuclear RNA polymerase E), yet another Pol II-related RNA polymerase, is required for RdDM at the target chromatin (Haag and Pikaard 2011) (Fig. 1B). The role of Pol V in RdDM appears to be twofold. First, the C-terminal domain (CTD) of NRPE1, the largest subunit of Pol V, specifically interacts with AGO4 through its tryptophan-glycine (WG)/GW-rich motifs (El-Shami et al. 2007; Wendte et al. 2017), facilitating the recruitment of siRNA-loaded AGO4 complexes to Pol V-occupied chromatin. Secondly, Pol V transcription at the target chromatin gives rise to long, non-coding transcripts (lncRNAs) that serve as scaffold RNAs to which multiprotein chromatin remodeler complexes may form. The sequence homology between a Pol V transcript and AGO4-associated hc-siRNA is thought to further facilitate the targeting of siRNA-loaded AGO4 to chromatin. This Pol V-AGO4-mediated nucleation is thought to ultimately recruit chromatin remodelers including DRM2 (for DOMAINS REARRANGED METHYLTRANSFERASES2) to the scene for de novo RdDM (Law and Jacobsen 2010) (Fig. 1B). Certain histone modifiers may also be subsequently recruited to further facilitate the formation of repressive chromatin. This Pol IV-, RDR2-, and DCL3-dependent RdDM pathway is further supported by the largely parallel expression patterns observed for AGO4 and DRM2 (Xie et al. 2012).

RDR6-mediated RdDM links siRNA-directed PTGS and TGS in plants

The above described PTGS which targets protein-coding mRNAs, and TGS which targets genomic repetitive sequences, represent sRNA-directed canonical silencing pathways in plants. Until recently, sRNA-directed PTGS and RdDM were considered genetically distinct pathways with little or no interactions, although documented observations exist in literature that appear to be consistent with a likely connection between the two.

Evidence for an interplay between sRNA-directed PTGS and DNA methylation

RDR6 was among the first key components to be identified genetically as required for transgene PTGS (Dalmay et al. 2000; Mourrain et al. 2000). Interestingly, disruption of PTGS of the transgene (i.e. a green fluorescent protein [GFP] or β-glucuronidase [GUS] reporter) in the RDR6 loss-of-function mutants (*sde1* and *sgs2*, respectively) was found to be associated with a reduced DNA methylation in the coding region of the transgenes (Dalmay et al. 2000; Mourrain et al. 2000). These may represent some of the early observations suggesting a link between RDR6-mediated PTGS and DNA methylation. Surprisingly, in some cases sRNA-directed PTGS of endogenous targets not known to involve RDR6 has also been associated with DNA methylation, as has been shown for miR166-regulated Arabidopsis *PHABULOSA (PHB)* and *PHAVOLUTA (PHV)* which encode class III homeodomain leucine zipper (HD-ZIP) TFs (Bao et al. 2004). Methylation at the *PHB* and *PHV* genomic loci appears to be dependent on miR166-target interaction, as heavy methylation was found downstream of the miRNA complementary site in wild type plants but substantially reduced in the dominant *phb-1d* and *phv-1d* mutants in which miR166-target interaction is abolished (Bao et al. 2004). Additional lines of evidence for an interplay between sRNA-directed regulatory pathways came from genetic interactions observed in Arabidopsis. Genetic analysis on Arabidopsis *dcl* mutants revealed partial functional redundancy (Xie et al. 2004; Gasciolli et al. 2005; Yoshikawa et al. 2005; Bouche et al. 2006; Deleris et al. 2006; Henderson et al. 2006) as well as antagonistic effects (Bouche et al. 2006) among the four DCLs. Moreover, a genetic screen identified lesions in NRPD1 (the largest subunit of Pol IV), NRPD2 (the second largest subunit shared by Pol IV and Pol V), and RDR2, key components of the canonical RdDM pathway as suppressors of a partial loss-of-function mutation in HEN1 (Yu et al. 2010) which is a sRNA methyltransferase required for maturation of all functional sRNAs in plants (Yu et al. 2005; Yang et al. 2006). Furthermore, in the context of transgene-induced PTGS, an antagonistic effect of RDR2 on RDR6-mediated siRNA generation has been observed (Jauvion et al. 2012). These observations collectively point to the existence of an intrinsic interplay between sRNA-directed PTGS and DNA methylation.

RDR6-RdDM links RDR6-dependent PTGS and Pol IV-dependent RdDM

Key evidence that has led to the proposed RDR6-dependent RdDM (RDR6-RdDM) first came from an analysis on sRNA and DNA methylation profiles at several *TAS* loci in Arabidopsis (Wu et al. 2012). Prompted by the intriguing finding of high cytosine DNA methylation at multiple ta-siRNA-generating loci, a comprehensive genetic dissection was done to assess the role of each of the known PTGS and RdDM components in DNA methylation at the *TAS* loci. Surprisingly, DNA methylation at the *TAS* loci was found to be dependent on RDR6, SGS3 and Pol V, but not Pol IV and RDR2, key components of the canonical Pol IV-dependent RdDM (Wu et al. 2012; Kanno et al. 2013).

Although AGO1 (and AGO7 in the case of miR390-directed ta-siRNA biogenesis from *TAS3* transcripts) is known to catalyze miRNA-directed cleavage of *TAS* transcripts and initiate ta-siRNA biogenesis, AGO4 and AGO6 rather than AGO1 were shown to be required for DNA methylation at the *TAS* loci (Wu et al. 2012). Also unexpected was the finding that DCL1 appears to be the only DCL protein required for DNA methylation at the *TAS* loci, although a combinatory role for all four DCLs was observed. Of note, previous analyses have implicated DCL1 in siRNA biogenesis as well as DNA methylation associated with silencing of certain TEs in Arabidopsis (Bouche et al. 2006; Laubinger et al. 2010). How DCL1 directly contributes to processing of *TAS* transcripts into siRNAs to direct DNA methylation remains unclear. Based on these observations, a Pol II-, RDR6-, DCL1-, and Pol V-dependent RdDM (RDR6-RdDM) pathway was proposed (Wu et al. 2012) (Fig. 1A, C). Interestingly, data from an independent study indicate that a similar RDR6-dependent, 21-nt siRNA-directed DNA methylation appears to be involved in silencing of endogenous genomic sequences including two TE loci (Pontier et al. 2012). It is also worth noting that an elevated DNA methylation at *PHAS* loci has been observed in isolated male meiocytes in maize (Dukowic-Schulze et al. 2016), although in such case an involvement of RDR6-RdDM remains unknown.

Further insights into operation of RDR6-RdDM came from a series of investigations on silencing of epigenetically activated TEs in Arabidopsis. The Arabidopsis gene *Decrease in DNA Methylation (DDM1)* encodes a SWI/SNF family chromatin remodeler ATPase which functions through coordinating compaction of linker histone chromatin (Zemach et al. 2013; Vongs et al. 1993). DDM1 plays an important role in maintaining an epigenetically silenced status of TEs and other highly repetitive sequences in the genome. The viable loss-of-function Arabidopsis *ddm1* mutants therefore offer a unique system for monitoring the silencing of epigenetically activated TEs. Analysis on sRNA and DNA methylation profiles in *ddm1* and *ddm1rdr6* double mutants has led to important findings that provide insights into the role of RDR6 in progression of TE silencing. Upon release of epigenetic silencing in *ddm1* genetic background, Pol II transcripts arising from activated TE loci were shown to undergo active PTGS, producing exclusively RDR6-dependent siRNAs which are predominantly 21- and 22-nt species (Nuthikattu et al. 2013). Initiation of RDR6-dependent production of TE-derived siRNAs termed "easiRNAs" (for epigenetically activated small interfering RNAs) in *ddm1* mutant appears to involve miRNA-directed cleavage of thousands of TE transcripts, followed by RDR6 processing (Creasey et al. 2014). This RDR6-dependent PTGS of TE transcripts is reminiscent of miRNA-triggered ta-siRNA biogenesis. The 21- and 22-nt TE-derived siRNAs were shown to be incorporated into AGO6, which helps direct the siRNA-AGO6 complexes to TE chromatin to initiate DNA methylation in a Pol V- and DRM2-dependent manner (Nuthikattu et al. 2013; McCue et al. 2015) (Fig. 1C). The limited expression domains (root, shoot, and floral meristems) of AGO6 may therefore restrict the spatial and temporal operation of RDR6-RdDM (Zheng et al. 2007; Havecker et al. 2010; Eun et al. 2011). It is important to note that this RDR6-RdDM may not affect the Pol II transcription of the TE locus, as is the case of *TAS* loci targeted by RDR6-RdDM (Wu et al. 2012). Instead, the RDR6-RdDM may serve as an expression-dependent mechanism to "mark" the actively transcribing TEs for subsequent reestablishment of epigenetic silencing (Nuthikattu et al. 2013). Consistent observations on de novo TE silencing were also made from a different system in which a single-copy invasive endogenous retrotransposon *Evadé (EVD)* derived from epigenetic recombinant inbred lines (epiRILs) was tracked for multiple generations (Mari-Ordonez et al. 2013).

A likely important role of RDR6-RdDM is therefore to facilitate the recruitment of Pol IV to target chromatin with partially methylated DNA to establish TGS through the canonical Pol IV-RdDM. This notion is not only consistent with the observation that Pol IV transcription preferably uses methylated genomic template (Zhai et al. 2015), but also explains how Pol IV is originally recruited to chromatin to initiate the self-reinforcing Pol IV-RdDM (Fig. 1B, C). It is worth noting that both RDR6-RdDM and the canonical Pol IV-RdDM involve DRM2, a DNA methyltransferase known to catalyze de novo DNA methylation in all sequence contexts (i.e. CG, CHG, and CHH, where H represents either A, C, or T) (Law and Jacobsen 2010) (Fig. 1C). Once cytosine methylation is established at TE loci, the TGS status may be perpetuated and maintained by the DNA methyltransferases MET1 (at CG sites), CMT3 (at CHG sites), and CMT2 (at CHH sites) independent of active Pol IV-RdDM (Law and Jacobsen 2010; Stroud et al. 2014) (Fig. 1D). Although much of the above described mechanistic insights were obtained from a system focusing on TE silencing, a role for RDR6-RdDM in the initiation of TGS is not limited to TEs. Transformation of Arabidopsis with the *FLOWERING WAGENINGEN (FWA)* gene is often used as an assay for de novo DNA methylation and establishment of TGS (Soppe et al. 2000; Chan et al. 2004). When an active *FWA* epi-allele was introduced into Arabidopsis *fwa-d* epi-mutant by virus-induced gene silencing (VIGS) using tobacco rattle virus (TRV) as a viral vector, the de novo establishment of inheritable transgene silencing was also shown to involve RDR6-RdDM as a transition state from PTGS to TGS (Bond and Baulcombe 2015). Thus RDR6-RdDM may

serve as a general transition step during de novo establishment of TGS in plants (Fig. 1A–D).

Concluding remarks and prospects

Plants provide an excellent system for studying the genetic and functional diversification of sRNA-directed regulatory mechanisms. Powered by the next generation DNA sequencing (NGS) technology in combination with the excellent genetic and genomic resources, recent studies have continued to reveal the surprising complexity of the sRNA-directed regulation in plants. The recently discovered RDR6-RdDM branch of RNA silencing represents an important mechanistic link between the canonical siRNA-directed PTGS and TGS. Although much of the mechanistic insights into the operation of RDR6-RdDM were uncovered through analyses on TE silencing in a genetic background that allows massive transcriptional activation, they also appear to be relevant to other genomic targets of RNA silencing. In the context of TE silencing, RDR6-RdDM would explain how TE-derived sRNAs could serve as a component of cellular epigenetic memory in corrective DNA methylation (Teixeira et al. 2009). During normal development of plants, RDR6-RdDM may also serve as a latent mechanism operating specifically in the gamete precursor cells such as the vegetative nucleus of pollen where massive 21- to 22-nt TE-derived sRNA production has been observed (Slotkin et al. 2009). Significantly, the distinct sRNA species associated with PTGS, RDR6-RdDM, and Pol IV-RdDM of genes, TEs, and other genomic targets will allow us to better interpret the regulatory sRNA component in the increasing number of sequenced genomes (Panda et al. 2016; Fultz and Slotkin 2017).

The discovery of RDR6-RdDM also elicits several new questions. First, do RDR6-RdDM and RDR6-dependent PTGS use the same pool of RDR6-dependent cellular siRNAs? If so, a mechanism for coordinated loading of siRNAs into distinct AGO (i.e. AGO1 and AGO6) complexes would be required for a self-sustained operation. While current data appear to suggest rough ER as the likely site for RDR6-dependet biogenesis of phased siRNAs, as discussed above, the reported RDR6 localization in both the nucleus and cytoplasmic sRNA bodies does not rule out the possibility of RDR6 activity to exist in more than one subcellular locations (Luo and Chen 2007; Martinez de Alba et al. 2015). Secondly, what triggers the transition from RDR6-RdDM to Pol IV-RdDM? Although de novo DNA methylation created by RDR6-RdDM could presumably contribute to the recruitment of Pol IV to the target chromatin, it does not always lead to Pol IV-RdDM or establishment of TGS, as is the case for the Arabidopsis *TAS* loci (Wu et al. 2012). A study in which the silencing of an active retrotransposon was monitored over multiple generations showed that effective silencing of the TE occurred when the TE has reached a copy number of approximately 40 (Mari-Ordonez et al. 2013), which may hint at the existence of a cellular threshold for RDR6-RdDM to trigger the onset of Pol IV-RdDM. Additionally, also poorly understood is the mechanism by which Pol V is directed to the target chromatin, although both RDR6-RdDM and canonical Pol IV-RdDM require Pol V. Using chromatin immunoprecipitation coupled with sequencing (ChIP-seq), previous studies have revealed an enrichment of Pol V occupancy at promoter regions and evolutionarily young TEs in the Arabidopsis genome (Zhong et al. 2012; Maumus and Quesneville 2014). However, it remains unclear if certain *cis*-elements constitute an effective Pol V promoter. Answers to these questions are also relevant to improve the efficiency of transgenic manipulation in genetic engineering of plants.

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