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RNA Pol IV and V in Gene Silencing: Rebel Polymerases Evolving Away From Pol II's Rules

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Abstract

Noncoding RNAs regulate gene expression at both the transcriptional and post-transcriptional levels, and play critical roles in development, imprinting and the maintenance of genome integrity in eukaryotic organisms [1–3]. Therefore, it is important to understand how the production of such RNAs are controlled. In addition to the three canonical DNA dependent RNA polymerases (Pol) Pol I, II and III, two non-redundant plant-specific RNA polymerases, Pol IV and Pol V, have been identified and shown to generate noncoding RNAs that are required for transcriptional gene silencing via the RNA-directed DNA methylation (RdDM) pathway. Thus, somewhat paradoxically, transcription is required for gene silencing. This paradox extends beyond plants, as silencing pathways in yeast, fungi, flies, worms, and mammals also require transcriptional machinery [4,5]. As plants have evolved specialized RNA polymerases to carry out gene silencing in a manner that is separate from the essential roles of Pol II, their characterization offers unique insight into how RNA polymerases facilitate gene silencing. In this review, we focus on the mechanisms of Pol IV and Pol V function, including their compositions, their transcripts, and their modes of recruitment to chromatin.

Introduction

In *Arabidopsis*, DNA methylation is established and maintained via several pathways that together shape the DNA methylation landscape and repress the expression of transposable elements and some neighboring genes [6–9]. Here, we focus on an RNA polymerase-centric view of the RNA-directed DNA methylation (RdDM) pathway (Figure 1), and refer those interested in a more comprehensive picture to several recent reviews [6,8]. At its core, the RdDM pathway requires two types of noncoding RNAs, Pol IV-dependent 24-nucleotide (nt) small interfering RNAs (siRNA) [10–15] and Pol V-dependent intergenic noncoding (IGN) RNAs [16], that mediate cytosine methylation in all sequence contexts (CG, CHG and CHH, where H=A, C or T) by the *de novo* methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) [17] (Figure 1).

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In RdDM, Pol IV is proposed to generate long single-strand RNAs (ssRNAs) that are rapidly converted into double-strand RNAs (dsRNAs) by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) [18]. This process also involves CLASSY 1 (CLSY1) [19], but the precise role of this putative chromatin remodeling factor remains unknown. These dsRNAs are then processed into 24-nt siRNAs by DICER-LIKE 3 (DCL3) [18], stabilized by methylation at their 3′end by HUA ENHANCER 1 (HEN1) [20] and loaded into ARGONAUTE (AGO) effector proteins [21], specifically, AGO4 [22,23], AGO6 [23–26] or AGO9 [23,27,28]. Unexpectedly, at least for AGO4, this loading occurs in the cytoplasm and the siRNA-bound AGO4 proteins are then re-imported into the nucleus [29] to target cytosine methylation at cognate DNA sequences within the genome. This downstream methylation-targeting portion of the pathway requires a second RNA polymerase, Pol V [12,30], which generates IGN transcripts [16] and is required for the recruitment of RdDM components including AGO4 and DRM2 to chromatin [31–35]. This Pol V-dependent recruitment is likely via a combination of both protein-protein interactions, mediated by the GW rich “AGO hook” [36] interaction domain within the Carboxy-Terminal Domain (CTD) of Pol V [31,32], as well as nucleic acid interactions involving the AGO-bound 24-nt siRNAs [34,35,37–39]. However, the relative contributions of these protein-protein and nucleic acid interactions remain unclear, as does the nature of the nucleic acid interactions (siRNA:IGN transcript vs siRNA:DNA) [8,16].

Given the central roles of Pol IV and Pol V in the RdDM pathway, recent efforts have focused on gaining a better understanding of their functions. Here we present current insights into the mechanisms governing Pol IV and Pol V activity, focusing on (1) the composition and evolution of these plant specific polymerases, (2) their enzymatic activity and the nature of their transcripts, and (3) the mechanisms through which these polymerases associate with chromatin.

Composition and evolution of Pol IV and Pol V

RNA polymerases are large holoenzymes that contain both catalytic and regulatory/non-catalytic subunits [40,41]. The roles of these subunits are best characterized for Pol II, with the first and second largest subunits forming the catalytic center, and many of the other subunits governing important aspects of polymerase function that remain poorly defined or completely unknown in RNA Pol IV and Pol V, including template selection, initiation, elongation, transcriptional fidelity, termination, and RNA processing [40,41] (Figure 2A). Thus, defining the composition and diversity of RNA Pol IV and Pol V subunits is a critical step in understanding how they function.

Consistent with the different functions of Pol II, Pol IV and Pol V, and with the different types of RNA transcripts they produce (detailed in the next section), affinity purifications of Pol IV and Pol V from *Arabidopsis thaliana* [42–44], cauliflower [45], and maize [46] have revealed significant differences in their composition (Figure 2). These polymerases, designated Nuclear RNA Polymerase (NRP) B, D, or E in reference to Pols II, IV, and V, respectively, are comprised of 12 core subunits and approximately half of these subunits are shared by all three polymerases [42,46] (Figure 2). Current data suggests that the unique subunits of Pol IV and Pol V arose from their Pol II counterparts via many independent

duplication events, starting prior to the evolution of land plants, followed by "Escape from Adaptive Conflict" sub-functionalization [47–50]. Indeed, contrary to previous reports [47,48], all land plants have specialized Pol IV and Pol V complexes [49,50] with unique catalytic cores and 7th subunits [48–50]. On the other hand, additional 4th and 5th subunits appear to be unique to flowering [48–50] and seed plants [49], respectively. Within flowering plants, more recent events have generated even greater diversity between Pols II, IV and V [48–50], giving rise to the unique 7th subunits in *Arabidopsis* Pol IV and Pol V as well as the additional 2nd and 9th subunits shared in the maize Pol IV and Pol V subtypes [42,46] (Figure 2). While further characterization of these divergent polymerase subunits is required to begin assigning specific functions, they are likely playing key roles in shaping the functions of Pol IV and Pol V. Ongoing efforts to understand the diversity, evolution, and function of these RNA polymerases are described below.

Arabidopsis Pol IV and V catalytic and non-catalytic subunits

In *Arabidopsis*, the catalytic centers of Pol IV and Pol V are unique compared to each other and to Pol II [42]. Pol IV and Pol V share a common second largest subunit (NRPD/E2), but they have distinct largest subunits, NRPD1 and NRPE1, respectively [42]. Consistent with the identification of Pol IV [15] and Pol V [16] dependent transcripts, these polymerases (as well as maize Pol IV and Pol V) have maintained key residues associated with catalysis [47,51], and these residues are required for RdDM [52,53]. However, their sequences have diverged at many highly conserved locations invariant in Pols-I, -II, and -III [47,51], suggesting that Pol IV and Pol V are not experiencing the same evolutionary constraints as their essential counterparts [54].

In addition to forming half of the catalytic center, the largest subunit of eukaryotic Pol II polymerases (NRPB1) possesses a highly conserved CTD with a repeating heptad motif that regulates Pol II function [55]. The largest subunits of Pol IV and Pol V completely lack these repeats [54] and, despite sharing a common ancestor [47], they have limited homology with each other outside a single conserved domain of unknown function, termed Defective Chloroplasts and Leaves (DeCLs) [49,54], that was likely acquired via an ancient gene fusion [50]. Perhaps the key functional difference between the CTDs of Pol IV and Pol V is due to the presence of a Pol V-specific GW-rich AGO-hook motif that interacts with AGO4 [31,32] and aids in the targeting of DNA methylation (Figure 1). Interestingly, although the presence of an AGO-hook in the NRPE1 CTD is conserved across land plants [49], the sequences of the GW-repeats and surrounding regions are rapidly evolving and very little sequence conservation is observed among a broad survey of plant species [32,46,49]. While retention of an AGO-hook motif is likely driven by a requirement for AGO interactions, factors driving the rapid evolution of the CTD as a whole remain unclear. One intriguing hypothesis suggests it might be driven by host-pathogen interactions, as several viral suppressor proteins with AGO-hook motifs have been identified [36,56,57]. However, there is currently no direct evidence that this phenomenon extends to AGOs that influence the RdDM pathway.

Non-catalytic and regulatory subunits

Of the 10 non-catalytic polymerase subunits, four subunits of Pol IV and Pol V are distinct from Pol II and half of these differ between Pol IV and Pol V (Figure 2B), making these subunits prime candidates for imparting Pol IV and Pol V specific functions. Notably, the subunits in question (the 3rd, 4th, 5th, and 7th) along with the unique catalytic subunits (1st and 2nd) of Pol IV and Pol V are predicted to cluster mainly on the "leading face" of the polymerase holoenzyme, surrounding functionally important areas including the catalytic center as well as the DNA entry and RNA exit channels [46]. Specifically, the 4th and 7th subunits form a sub-complex termed the "Stalk" [40] that is positioned near the RNA exit channel and is unique in Pol II, Pol IV, and Pol V (Figure 2). The Stalk is implicated in multiple steps of the Pol II transcription cycle [40] (Figure 2), and mutations in the 7th subunit of yeast Pol II specifically affect its role in small RNA biogenesis, demonstrating a precedence for the involvement of this sub-complex in gene silencing [58]. In Pol II, the 5th and 9th subunits interact with the downstream DNA duplex and basal transcription factors, respectively, and form the "Jaw", which is implicated in several features poorly understood with respect for Pol IV and Pol V function—template selection, elongation and polymerase fidelity [40,59,60].

Notably, viable mutants in almost all of the aforementioned Pol IV and Pol V subunits have been identified. Initial characterization of these mutants reveal roles for NRPD/E4 [61], NRPE5 [42,45,53,62], and NRPB/D/E9b [62,63] but not NRPB/D/E9a [63] in gene silencing. Though this is clearly just the beginning, these studies have already provided insights into the functions of these subunits. First, these studies demonstrated that a short N-terminal extension in NRPE5 is required for its stability [42,53], suggesting a potential role for this region in facilitating incorporation of NRPE5 into Pol V. Second, they found that loss of NRPB/D/E/9b does not affect the accumulation of Pol V-dependent transcripts and speculate that rather than regulating Pol V transcription, this subunit might facilitate interactions with RNA processing components [62,63]. With increased accessibility to next generation sequencing technologies and the *in vitro* recapitulation of Pol IV and Pol V transcription (highlighted below), more details regarding the potential locus specificity and roles of these subunits in polymerase activity are likely to follow.

Maize Pol IV and V catalytic and non-catalytic subunits

Purification of the maize Pol IV and Pol V polymerases revealed subunit compositions that suggest maize and *Arabidopsis* employ different strategies to generate functional diversity within their plant-specific RNA polymerases. While in *Arabidopsis* Pol IV and Pol V differ in both their catalytic and non-catalytic subunits [42], maize Pol IV and Pol V share a common set of non-catalytic components and instead possess an expanded repertoire of catalytic subunits [46]. Specifically, they utilize unique largest subunits (*Zm*NRPD1 and *Zm*NPRE1) that associate with one of three second largest subunits (*Zm*NRPD/E2a, D/E2b and E2c), giving rise to 2 Pol IV subtypes and 3 Pol V subtypes [46] (Figure 2C). Like in *Arabidopsis*, these Pol IV and Pol V complexes utilize forms of the 4th, 5th, and 7th subunits that are unique from Pol II [46]. However, they differ from *Arabidopsis* in their utilization of a specialized 9th subunit [46], demonstrating they have a more divergent Jaw region that likely imparts additional functionality to these polymerases.

As in *Arabidopsis*, viable Pol IV/V mutants are available in maize, and despite the fact that only mutations in two subunits, NRPD1 and NRPD/E2a, have been identified (Figure 2), their phenotypes have already provided compelling evidence for functional diversity within polymerase subtypes. Specifically, maize *nrpd1* [64] and *nrpd/e2a* mutants [65–67], which have defects in a silencing phenomenon termed paramutation [68,69] and display a myriad of developmental defects not observed in *Arabidopsis* [70], fail to phenocopy each other [65–67]. These findings strongly suggest that NRPD/E2a and 2b, as well as the polymerase subtypes they associate with [46], act in a non-redundant fashion. In addition to potential differences in activities of these subtypes, functional diversity is likely also mediated by interactions with accessory factors, for example MOP1 [71] (homolog of RDR2 in *Arabidopsis*) appears to specifically associate with the NRPD/E2a containing Pol IV subtype [46].

Pol IV- and Pol V-dependent transcripts

The existence of Pol IV- and Pol V-dependent transcripts, which started as just a hypothesis, has now been borne out with the identification of these RNAs *in vivo* [15,16] and the recapitulation of minimal transcriptional activity using purified Pol IV and Pol V *in vitro* [46,72]. Below we summarize how these transcripts were identified, what is known about the physical nature and function of these RNAs, and what template features are required for polymerase activity *in vitro*.

Pol V-dependent transcripts

Given the roles of long noncoding RNAs in targeting chromatin modifications in other systems [73], it was hypothesized that Pol V might act analogously in RdDM. To identify such transcripts, Wierzbicki et al. [16] searched a well characterized region of the genome that displays DNA methylation at intergenic loci largely devoid of known Pol I, II, or III transcripts. This search identified a set of six IGN transcripts that are present at low levels in wild-type plants but are undetectable or reduced in *pol v* mutants [16]. These IGN transcripts range in size, but can be up to 200-nt in length, placing them in the long noncoding RNA category [16]. They can initiate from multiple adjacent sites, lack poly-A tails and their 5′ ends appear to be a mixture of capped/5′ triphosphates and 5′ monophosphates, suggesting the presence of primary Pol V-dependent transcripts and processed Pol V-dependent transcripts, respectively [16] (Figure 1). Attesting to the importance of these IGN transcripts in facilitating RdDM (and further supporting a role for Pol V in their generation) catalytically dead versions of Pol V failed to produce IGN transcripts and failed to silence adjacent genes [16].

Based on the identification and initial characterization of Pol V-dependent transcripts, a new model was proposed [16] in which Pol V and its transcripts serve as a platform for the recruitment of additional RdDM factors, ultimately leading to the establishment of DNA methylation (Figure 1). As outlined in the introduction, this model is continuing to evolve as additional RdDM factors and IGN transcripts are identified [34,35,37–39,74,75]. However, our knowledge of Pol V-dependent transcripts remains limited to a handful of examples and thus likely represents just the tip of the iceberg. We will have to await a global identification

and analysis of Pol V-dependent transcripts to more fully understand the nature and function of these noncoding RNAs.

Pol IV- and RDR2-dependent transcripts

Pol IV is required for the production of >90% of the *Arabidopsis* 24-nt siRNAs [13,14] and prevailing models suggest this polymerase generates long ssRNAs that act as substrates for siRNA production (Figure 1). Yet, until recently, no such transcripts had been identified. To detect these elusive transcripts, Li et al. [15] devised an elaborate genetic setup coupled with next generation sequencing that allowed Pol IV- and RDR2-dependent transcripts to be inferred by looking at the differences between the transcriptome profiles of *dcl234* triple mutants, which accumulates Pol IV-dependent transcripts, and *dcl234 nrpd1* (or *rdr2)* quadruple mutants, which fail to accumulate these transcripts. All told, Pol IV/RDR2-dependent transcripts corresponding to ~50% of known siRNA generating loci were recovered [15] and, like Pol II transcribed genic regions, these loci are flanked by A/T-rich sequences and depleted in nucleosomes, suggesting these features of Pol II function may be conserved in Pol IV [15]. The absence of detectable Pol IV/RDR2-dependent transcripts at many of the remaining loci can likely be attributed, at least in part, to low levels of siRNA production and/or DNA methylation that arise naturally (*i.e*. in a wild-type background) or artificially (*i.e*. in the *dcl234* triple mutant) and thus are more difficult to detect [15].

Characterization of the nature of these Pol IV/RDR2-dependent RNAs, and the genetic requirements for their production, support some but not all aspects of the current model for siRNA biogenesis, suggesting the model should be revisited. Consistent with the model, Pol IV/RDR2-dependent transcripts are generated from both strands and, like Pol V-dependent transcripts [16], they lack a polyA-tail [15]. Unexpectedly, they also lack a 5′ cap, suggesting that they may represent processed forms of Pol IV/RDR2-dependent transcripts [15]. Alternatively, Pol IV might prime off an unusual RNA substrate that lacks a 5′ triphosphate [15]. Indeed Pol IV can extend off an RNA primer with a 5′ monophosphate *in vitro* [72], but the existence of such RNAs *in vivo* remains unknown. In either case, these findings suggest the involvement of specialized RNAs or unknown RNA processing events as an early step associated with Pol IV function. This notion is supported by a much earlier observation that Pol IV localization by immunofluorescence is disrupted upon RNase A treatment (which degrades ssRNA) [76]. Further highlighting our incomplete understanding of how siRNAs are generated, the Pol IV-dependent transcripts identified were equally dependent on RDR2 for their production [15]. This contradicts the current model in which RDR2 acts downstream of Pol IV function and is at odds with *in vitro* data showing Pol IV activity is unaffected in *rdr2* mutants [72]. What role, if any, RDR2 plays in the aforementioned RNA processing events or in other aspects of Pol IV function, and what causes Pol IV to display different dependencies on RDR2 *in vitro* and *in vivo*, await further investigation. Finally, it warrants mentioning that the techniques used to identify Pol IV-dependent transcripts do not preserve information regarding the size of these RNAs [15]. Thus, while *in silico* assembly of these transcripts provides a useful means of assessing the genomic distribution of Pol IV-dependent transcripts, their length remains unclear. Taken together, the identification of Pol IV-dependent transcripts has perhaps raised more

questions than answers, suggesting we are on the cusp of a new phase of discovery regarding the transcriptional activity of Pol IV and the biogenesis of 24-nt siRNAs.

Pol IV and V *in vitro* activity

Using affinity purified Pol IV and Pol V complexes from *Arabidopsis* and maize, Haag et al. [46,72] have demonstrated Pol IV and Pol V transcriptional activity *in vitro*. These experiments revealed several unusual template features, including the requirement of an RNA primer, that undoubtedly contributed to the delay in the identification of *in vitro* polymerase activity [72]. Like Pol II, Pol IV will transcribe a tripartite substrate that includes an RNA primer and mimics a transcription bubble, but its activity is more robust using a bipartite substrate, suggesting that Pol IV does not efficiently displace the non-template DNA strand [72]. In addition to these DNA-templated Pol IV-dependent products, shorter RDR2-dependent products were also observed [72], which is consistent with data showing that RDR2 co-purifies with Pol IV [43,44,46,72]. Further investigation into the connections between Pol IV and RDR2 activities revealed that Pol IV transcription is independent of RDR2, while RDR2 activity requires the presence of Pol IV, but not its polymerase activity [72]. As discussed above, this one-way dependence *in vitro*, is at odds with what appears to be a complete interdependency of these activities *in vivo* [15]. Intriguingly, Pol IV is also able to transcribe an all-RNA substrate, demonstrating that it is not a strictly DNA-dependent RNA polymerase [72]. In comparison with Pol IV, the template requirements for Pol V are even more restrictive; Pol V is only active using a bipartite substrate, suggesting this polymerase is even less efficient at displacing the non-template DNA strand, and revealing a more strict dependence on a DNA template [72]. Notably, the observed activities for both Pol IV and Pol V are weak relative to Pol II, which could be an intrinsic feature of these polymerases as they show poor conservation within two regions (the trigger loop and the bridge helix) that are required for efficient nucleotide incorporation [77], or could indicate sub-optimal template features or missing accessory components.

Despite great progress in identifying Pol IV and Pol V-dependent transcripts *in vivo* and establishing *in vitro* transcription assays, it remains unclear what templates these polymerases utilize *in vivo* and it is also unclear how their transcription is regulated to prevent the production of aberrant noncoding RNAs. Regulation of transcription occurs at all stages of the transcription cycle (initiation, elongation, termination) and controls important aspects of polymerase activity including, template selection, fidelity and processivity [40,54,78]. It is unclear how Pol IV and Pol V initiation occurs, but if they do indeed require an RNA primer *in vivo*, they may completely bypass this step by hijacking RNAs produced by other polymerases, like Pol II, and proceed directly to elongation [54]. The elongation phase involves displacement of the non-template DNA strand and extension of the RNA transcript. Potential roles for the components of the Pol V-associated DDR complex (Figure 1) in template strand unwinding are discussed in Pikaard et al. [78]. Regarding termination, we are completely in the dark. However, armed with new biochemical assays and available genetic mutants, the field is now primed to address these open questions in both *Arabidopsis* and maize using a combination of *in vitro* and *in vivo* studies. As additional parameters are revealed, it will be of great interest to understand the

extents to which specialized polymerase subunits influence these features of Pol IV and Pol V activity.

Association of Pol IV and Pol V with chromatin

Given the lack of defined promoter or other conserved sequence features at known targets of the RdDM pathway, the question of how Pol IV and Pol V are specifically recruited to these genomic loci has been a topic of intense interest over the last decade. Recently, several studies employing genetic, genomic and biochemical techniques have begun shedding light on this question and, although they have not revealed any common sequence determinants, they have revealed a dependence on specific chromatin modifications, establishing a series of self-reinforcing loops between DNA and histone modifications (Figure 1).

Pol V association with chromatin requires the DDR complex and two methyl-DNA binding proteins

The first insights into the recruitment of Pol V to chromatin were nearly coincident with the discovery of Pol V-dependent transcripts [16]. Two known RdDM components, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) [79] and DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) [80,81], were shown to be required for both the accumulation of Pol V-dependent transcripts and Pol V occupancy at several IGN producing loci [16,35]. Shortly after these discoveries, a third component, RNA-DIRECTED DNA METHYLATION 1 (RDM1), was found to stably associate with both DRD1 and DMS3 [82] and to be required for the production of IGN transcripts [82,83]. This protein complex, termed DDR (after its three main components) also associates Pol V [82], suggesting that the DDR complex facilitates Pol V association with chromatin via a direct interaction [82] (Figure 1).

Until recently it was unclear whether the DDR complex functioned in a locus specific or a more global manner, nor was it clear how this complex itself was recruited to chromatin. The path towards answering these questions began with the identification of Pol V binding sites on a genome-wide scale, which, consistent with the role of Pol V in RdDM, are highly correlated with DNA methylation, siRNAs, and IGN transcripts [74,75]. In *drd1*, *dms3*, or *rdm1* single mutants, Pol V enrichment at binding sites identified in wild-type plants were lost, demonstrating that the DDR complex acts as a global regulator of Pol V chromatin association [75]. In addition to the DDR complex, two methyl-DNA binding proteins, SU(VAR)3-9 HOMOLOG 2 (SUVH2) and SUVH9 [84], that function in RdDM in a partly redundant manner [84,85] and interact with components of the DDR complex [86,87], were also shown to be required for Pol V-dependent transcripts and Pol V chromatin association [86,87]. This finding suggested a link between DNA methylation and Pol V occupancy and indeed, Pol V enrichment at chromatin is nearly abolished in a *met1* mutant [86], which causes global decreases in DNA methylation [88]. Thus, a self-reinforcing loop model has emerged wherein Pol V recruitment depends on pre-existing DNA methylation. In this model, DNA methylation is bound by SUVH2 and SUVH9, which in turn interacts with the DDR complex, thus promoting the association of Pol V with chromatin and leading to the generation of noncoding RNAs that facilitate additional DNA methylation (Figure 1).

In addition to the aforementioned major players that regulate Pol V recruitment, several other RdDM components have also been shown to play more modest roles in association with Pol V function, including SU(VAR)3-9-RELATED PROTEIN 2 (SUVR2) [89,90], DOMAINS REARRANGED METHYLTRANSFERASE 3 (DRM3) [91–93], CHROMATIN REMODELING 27 (CHR27) and CHR28 (a.k.a. SNF2-RING-HELICASE LIKE 1 (FRG1) and FRG2) [89,94]. While the precise functions of these factors remain to be elucidated, they likely function to fine-tune rather than globally regulate the activity and distribution of Pol V.

Pol IV association with chromatin requires SHH1, an H3K9me reader

In addition to the core Pol IV subunits, affinity purification of this polymerase have identified several other accessory factors [43,44,46,72], and characterization of one such component, SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) [43] (a.k.a. DNA-BINDING TRANSCRIPTION FACTOR 1 (DTF1) [95]) has provided mechanistic insight into the association of Pol IV with chromatin [96]. Initial characterization of *shh1* mutants demonstrated defects in gene silencing, DNA methylation, and siRNA accumulation [43,95], but not in the production of Pol V-dependent transcripts, suggesting a specific role for SHH1 in connection with Pol IV [43]. Subsequent genome-wide characterization of *shh1* mutants revealed this factor acts in a highly locus specific manner, phenocopying *pol iv* mutants at approximately half of the siRNA producing loci genome-wide, but having no significant affect at the remaining loci [44,96]. Mechanistically, the observed defects in *shh1* stem from loss of Pol IV occupancy specifically at SHH1-regulated genomic loci [96]. This role of SHH1 requires its SAWADEE domain [96], which recognizes H3K9 methylation [44,96] and adopts a unique tandem-tudor domain fold that interrogates the methylation status of both the H3K4 and H3K9 positions [96]. In *Arabidopsis*, H3K9 methylation maps to regions of the genome that harbor DNA methylation, including those targeted by the RdDM pathway, and is redundantly controlled by a family of histone methyltransferases, SUVH4, SUVH5 and SUVH6 [97–102]. Thus, SHH1 connects the DNA methylation machinery with H3K9 methylation, establishing yet another self-reinforcing loop between repressive chromatin modifications. Alas, several key questions remain unanswered, including how Pol IV recruitment to the remaining RdDM targets is controlled and how SHH1 exerts its affects only at a subset of RdDM targets, as H3K9me is a general feature of RdDM targets.

In regards to Pol IV and Pol V targeting, two additional topics warrant a brief discussion. First, purification of Pol IV and Pol V from maize revealed these polymerases associate with components involved in the recruitment of *Arabidopsis* Pol IV and Pol V to chromatin, namely, ZmSHH2a as well as ZmDMS3 and ZmCHR127 (related to AtDRD1), respectively [46]. Thus, while functional studies have not yet been completed, it appears that similar mechanisms are likely in place to facilitate the recruitment of maize Pol IV and Pol V to chromatin. Second, while updated models of Pol IV and Pol V targeting have provided key sights into the pathway (Figure 1), the built in reinforcing loops raises the question of how DNA methylation is initially established at a naïve locus. It is still early days, but genetic evidence suggests a role for Pol II in DNA methylation pathways [90,103–107] and several

groups have begun characterizing non-canonical RdDM pathways that are excellent candidates for establishing DNA methylation at naïve loci [108–112].

Conclusion and outlook

Plants encode specialized RNA polymerases that act non-redundantly in the *de novo* DNA methylation pathway, differ in their subunit compositions and in the types of noncoding RNAs they generate, and employ unique machinery for their recruitment to chromatin. Recent advances in our understanding of the evolution and composition of these polymerases has confirmed the existence of specialized Pol IV and Pol V machinery across land plants, and has revealed functional diversity both between and within Pol IV and Pol V subtypes. The identification of Pol IV and Pol V transcripts *in vitro* and *in vivo* has provided the first clues into the activities of these polymerases and suggests that they are not playing by the same rules governing Pol II activity. Finally, a quite detailed view of the machinery required to facilitate recruitment of these polymerases to chromatin has emerged, revealing a dependence on previously established chromatin modifications as part of an intricate network of self-reinforcing loops.

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Paper Highlights

- Plants encode extra RNA polymerases (Pol IV and V) that facilitate DNA methylation
- Pol IV and V subunits evolved from Pol II subunits, and are continuing to diversify
- Pol IV and V differ in their composition and in the noncoding RNAs they produce
- *In vitro*, Pol IV and V utilize non-canonical templates and require an RNA primer
- Pol IV and V employ unique machinery to facilitate their recruitment to chromatin

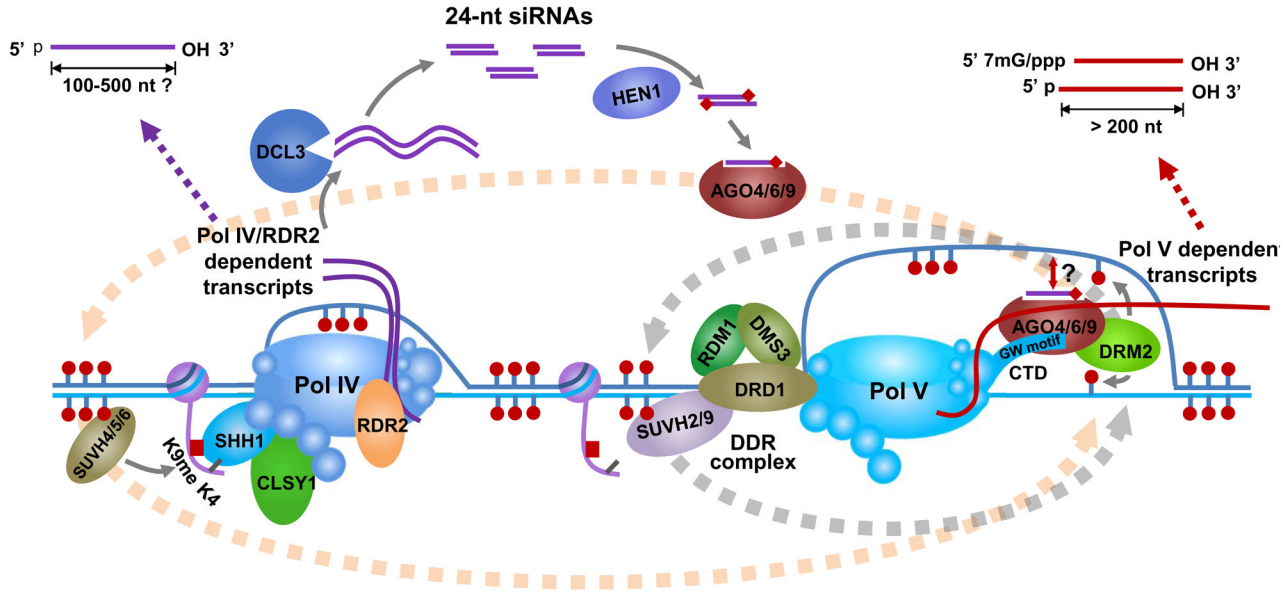

Figure 1. An RNA polymerase-centric view of the RNA-directed DNA methylation (RdDM) pathway highlighting several self-reinforcing loops

Biogenesis of the methylation-targeting siRNAs is initiated by the activity of RNA polymerase IV (Pol IV). Pol IV transcripts are processed by the activities of RDR2, DCL3, and HEN1 into 24-nt siRNAs, which are loaded into argonaute (AGO) effector proteins. Independent of Pol IV activity, another RNA polymerase, Pol V, generates long intergenic noncoding (IGN) transcripts. Both Pol V itself, as well as the transcripts it produces, facilitated the recruitment of siRNA-loaded AGO proteins (and additional components not shown) to chromatin, ultimately leading to recruitment of the *de novo* DNA methyltransferase DRM2. Investigation into the mechanisms for Pol IV and Pol V recruitment to RdDM targets have identified several necessary proteins (and protein complexes) and uncovered interconnected and self-reinforcing loops between DNA and histone methylation as detailed below. The association of Pol IV at many genomic loci depends on SHH1 and its H3K9me binding activity, and H3K9 methylation requires a family of SET domain histone methyltransferases (SUVH4, SUVH5, and SUVH6) that also function as methyl-DNA binding proteins. Together these factors generate a self-reinforcing loop of DNA and histone methylation (peach dashed oval) wherein DNA methylation mediates the association of SUVH4/5/6 with chromatin, leading to the deposition H3K9 methylation that is bound by SHH1, facilitating Pol IV recruitment and siRNA production and ultimately leading to the establish DNA methylation to complete the loop. The association of Pol V at chromatin depends on members of the DDR complex and two associated methyl-DNA binding proteins, SUVH9 and SUVH2, generating a self-reinforcing loop in which DNA methylation deposited via the RdDM pathway is required for Pol V localization and the establishment of additional DNA methylation (grey dashed oval). Currently defined features of Pol IV and Pol V dependent transcripts are indicated in cartoon format above the dashed arrows in purple and in red, respectively. (■) Histone Methylation, (●) DNA methylation, (◆) RNA methylation.

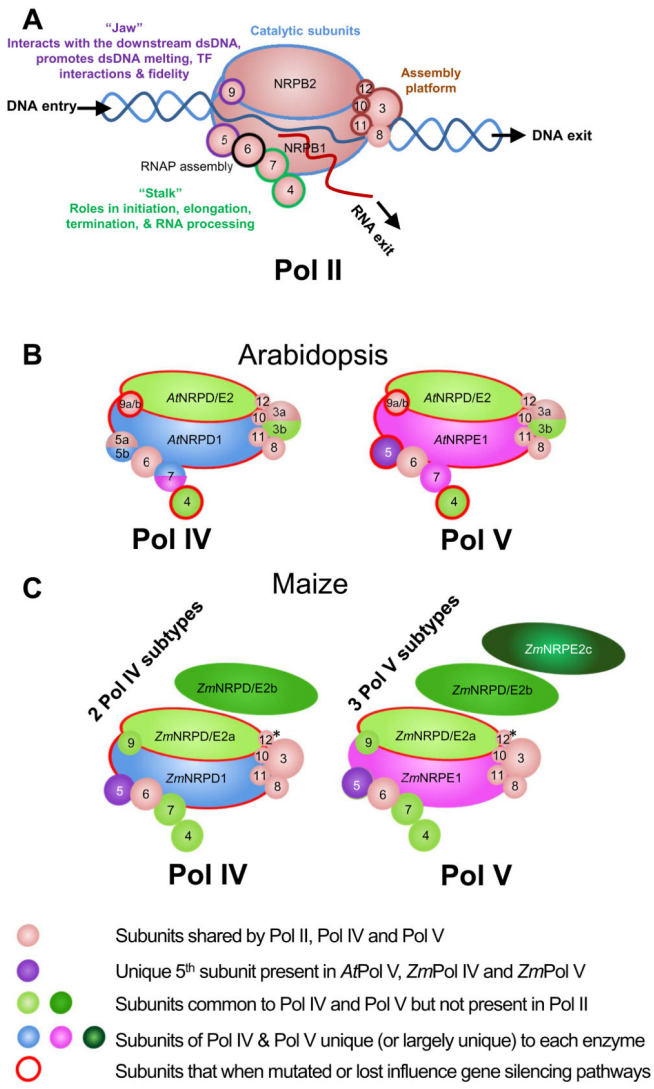

Figure 2. Subunit composition summary for Pol IV and Pol V in Arabidopsis and maize

(A) Cartoon representation of RNA polymerase II, showing the 12 core subunits (1–12) along with the DNA entry, DNA exit and RNA exit channels. Polymerase features and associated functions in yeast Pol II are labeled much as described in [50]. **(B and C)** Cartoon representations of RNA Pol IV and Pol V in *Arabidopsis* (*At*) and maize (*Zm*), respectively, modeled after diagrams in [51]. Subunit compositions are based on the totality of mass spectrometry data from [42–46] and likely represent multiple polymerase subtypes with different combinations of subunits. The individual polymerase subunits are color coded as indicated in the legend. *Subunit 12: Although subunit 12 is conserved in Pol I, II, and III as well as in *At*Pol IV and *At*Pol V, no peptides were identified from the maize affinity purifications thus it remains unclear if this subunit is truly absent or was not detectable for technical reasons [46].