# Chapter 13
# Plant Multisubunit RNA Polymerases IV and V

**Thomas S. Ream, Jeremy R. Haag, and Craig S. Pikaard**

**Abstract** Plants are unique among eukaryotes in having five nuclear multisubunit RNA polymerases. These include RNA polymerases I, II, and III (Pols I, II, and III), which are ubiquitous among eukaryotes, plus two plant-specific RNA polymerases, Pol IV and Pol V, that are 12-subunit enzymes that evolved as specialized forms of Pol II. Pols IV and V are nonessential for viability but play important roles in RNA-mediated gene silencing pathways that tame transposons, defend against invading viruses, mediate cross talk among alleles, and influence development. Numerous amino acids that are invariant in the catalytic subunits of Pols I, II, and III are substituted, or even absent, in Pols IV or V, implying that Pols IV and V have fewer functional constraints on their evolution than other multisubunit RNA polymerases. In vitro, Pol IV and Pol V can extend an RNA primer hybridized to a DNA template, but the templates transcribed by Pol IV and Pol V in vivo are unclear. Likewise, the boundaries of Pol IV and Pol V transcription units and the characteristics of their primary transcripts remain undefined. In this chapter, the state of our understanding of Pol IV and Pol V subunit compositions and functions is discussed.

**Keywords** Pol IV • Pol V • *Arabidopsis* • Noncoding RNA • RNA silencing • siRNA • Heterochromatin • RNA-directed DNA methylation • Transcription

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## 13.1 Introduction and Overview

All known eukaryotes require nuclear DNA-dependent RNA polymerases I, II, and III (Pols I, II, and III) for cell viability (see Chap. 12). In all but trypanosomes, Pol I specializes in the transcription of a single gene sequence, repeated in hundreds of copies, that encodes a primary transcript that is then processed to form the three largest RNAs of ribosomes, the protein synthetic machines of the cell. Pol II transcribes thousands of genes, including the genes that encode pre-messenger RNAs (mRNAs), pre-microRNAs (miRNAs), and a variety of small structural and regulatory RNAs. Pol III transcribes hundreds of genes, including those encoding pre-tRNAs, 5S ribosomal RNA, short interspersed nuclear elements (SINEs), and a collection of other relatively short (<400 nt) catalytic or regulatory RNAs.

Given the expectation of three, and only three, nuclear multisubunit RNA polymerases in plants, as in all other known eukaryotes, completion of the *Arabidopsis thaliana* genome sequence in 2000 (The Arabidopsis Genome Initiative 2000) revealed a surprise that plants have catalytic subunits for two additional nuclear RNA polymerases, now known as Pol IV and Pol V (Fig. 13.1a, b). The two genes encoding the largest subunits of nuclear Pols IV and V, *NRPD1* and *NRPE1*, respectively, are quite different in sequence compared to other multisubunit RNA polymerase largest subunits but have recognizable similarity to their conserved domains, including core sequences of the catalytic site (Fig. 13.1a, c) (Herr et al. 2005; Haag et al. 2009; Onodera et al. 2005; Pontier et al. 2005). Multiple intron–exon positions in the first half of *NRPD1* and *NRPE1* are identical to their positions in *NRPB1*, the gene encoding the largest subunit of Pol II in *Arabidopsis* (Luo and Hall 2007). However, the C-terminal domains (CTDs) of the NRPB1, NRPD1, and NRPE1 proteins are unrelated, with the heptad repeats typical of the Pol II largest subunit CTD missing. A domain related to the *DEFECTIVE CHLOROPLASTS AND LEAVES* gene implicated in chloroplast rRNA processing (Bellaoui et al. 2003) is present in the CTDs of both NRPD1 and NRPE1 (Fig. 13.1a). The CTD of the Pol V largest subunit, NRPE1, also contains a region composed of ten imperfect copies of a 16 amino acid sequence and a domain of repeating glutamine and serine amino acids, neither of which are present in the Pol II largest subunit, NRPB1 (Fig. 13.1a, e). Collectively, these observations suggest that duplication of the Pol II *NRPB1* gene, combined with rearrangements involving unrelated genes, gave rise to the *NRPD1* gene in an ancestor common to modern day algae and land plants (Luo and Hall 2007). Subsequent duplication of the *NRPD1* gene, occurring prior to the divergence of moss from vascular plants, is then thought to have given rise to the ancestral Pol V largest subunit gene (Luo and Hall 2007; Tucker et al. 2011).

In addition to *NRPD1* and *NRPE1* genes, the *Arabidopsis* genome sequence revealed two nearly identical genes (one of which is a nonfunctional pseudogene) encoding an atypical second-largest subunit, NRP(D/E)2 (Fig. 13.1b). The NRP (D/E)2 amino acid sequence is more similar to the NRPB2 subunit of Pol II than to

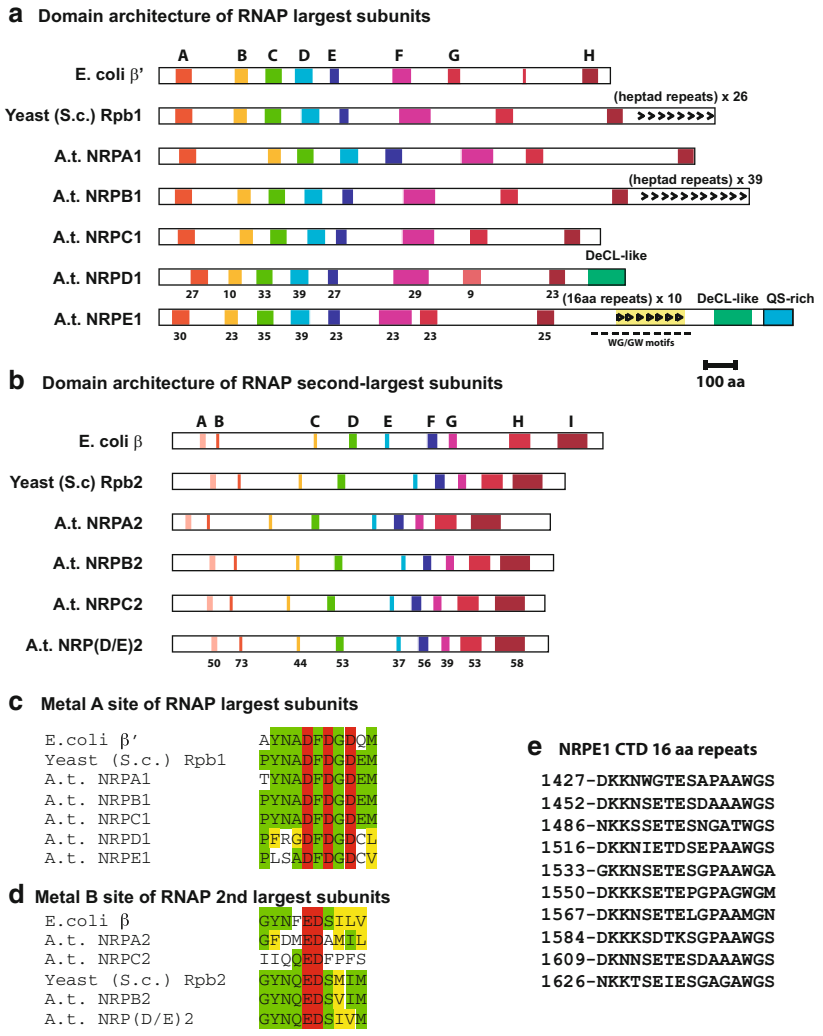


**Fig. 13.1** Comparison of the catalytic subunits *Arabidopsis* nuclear RNA polymerases to those of *E. coli* RNA polymerase and yeast Pol II. (**a**) Diagrams for the largest subunits, with conserved domains A–H shown in different colors. Heptad repeats in the Pol II CTDs refer to tandemly arrayed seven amino acid sequences that are conserved in sequence, but not number, throughout eukaryotes. The DeCL-like domain of the Pol IV and Pol V subunit CTDs shows sequence similarity to the DEFECTIVE CHLOROPLASTS AND LEAVES protein in *Arabidopsis*. The QS rich domain is composed primarily of alternating glutamines and serine amino acids. The number below each of the Pol IV or Pol V domains shows the percent identity of that domain to the corresponding domain of the *Arabidopsis* Pol II subunit. (**b**) Diagrams for the second-largest subunits, with conserved domains A–I highlighted in different colors. The number below each of the Pol IV or Pol V domains is the percent identity between those domains and the corresponding domain of the *Arabidopsis* Pol II subunit. (**c**) Sequences surrounding the invariant aspartates of the magnesium-binding Metal A sites within conserved domain D of the largest subunits shown in panel **a**. (**d**) Sequences surrounding the invariant aspartate-glutamate duo of the magnesium-binding Metal B sites within the second-largest

the equivalent subunits of Pols I and III (Onodera et al. 2005), including sequences in the vicinity of the catalytic site (Fig. 13.1d). Genetic evidence indicated that *NRP(D/E)2* encodes the second subunit of both Pol IV and Pol V (Herr et al. 2005; Kanno et al. 2005; Onodera et al. 2005; Pontier et al. 2005).

The first insights into the functions of Pols IV and V (initially known as Pol IVa and Pol IVb) were revealed in 2005, based on molecular phenotypes of *nrpd1*, *nrpe1*, and *nrp(d/e)2* mutants (Herr et al. 2005; Kanno et al. 2005; Onodera et al. 2005; Pontier et al. 2005). These studies showed that Pols IV and V play nonredundant roles in a transcriptional silencing process known as RNA-directed DNA methylation (RdDM) that is important for silencing transposable elements, endogenous repeats, and transgenes (Zhang and Zhu 2011; Lahmy et al. 2010; Matzke et al. 2009). Roles for Pols IV and V in additional RNA-mediated silencing pathways have since been revealed. These include inter-allelic interactions responsible for the epigenetic phenomenon paramutation, pathogen resistance, and the spread of RNA silencing signals from cell to cell and via the vascular system (for reviews see Haag and Pikaard 2011; Molnar et al. 2011; Erhard and Hollick 2011; Arteaga-Vazquez and Chandler 2010; Brosnan and Voinnet 2011).

Subunit compositions of affinity-purified *Arabidopsis* Pols II, IV, and V, determined in 2009, showed that Pols IV and V are 12-subunit enzymes (Ream et al. 2009) that include subunits shared by Pol II (Ream et al. 2009; Huang et al. 2009; Lahmy et al. 2009; He et al. 2009a) and unique subunits that arose via stepwise duplication and sub-functionalization of Pol II subunit genes (Tucker et al. 2011; Ream et al. 2009) (Fig. 13.2). However, it remains unclear whether Pol IV and Pol V transcription is initiated in a Pol II-like fashion, making use of general transcription factors, activators, or coactivators that recognize promoters or enhancer sequences. Likewise, few details are known concerning Pol IV and Pol V activities as enzymes, including their templates in vivo, their modes of recruitment to specific loci, their transcription initiation or termination sites, the sizes of their primary transcripts, their processivity, or their fidelity.

Multiple subunits that are common to Pols II, IV, and V are encoded by two or more genes (Ream et al. 2009; Law et al. 2011), and one case study shows that alternative subunits can generate functionally distinct polymerase subtypes (Tan et al. 2012). New insights into the roles of the different subunits of Pols IV and V, and by inference, Pol II, may come from such studies, aided by the fact that null mutations eliminating Pol IV- or Pol V-specific subunits are not lethal. The recent demonstration that Pols IV and V can carry out RNA-primed transcription of DNA templates in vitro (Haag et al. 2012) also provides a starting point for learning more about their enzymatic capabilities and, in conjunction with genetic studies, the roles of catalytic versus non-catalytic subunits. These issues of general relevance to multisubunit RNA polymerase functions are explored in more detail in the subsequent sections of this chapter.

**Fig. 13.1** (continued) subunits shown in panel **a**. (**e**) Sequence of the ten 16aa tandem repeats in the CTD of NRPE1. Numbers refer to amino acid positions within the full-length protein

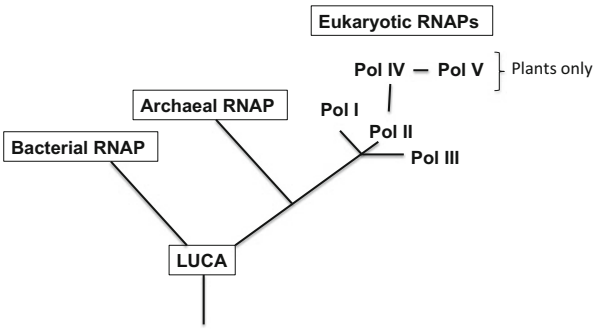


**Fig. 13.2** Evolution of multisubunit RNA polymerases. LUCA refers to the last universal common ancestor of extant bacteria, archaea, and eukaryotes. The three essential polymerases of eukaryotes, Pols I, II, and III, must have existed in the common ancestor of all eukaryotes to explain their ubiquity

## 13.2 Pols IV and V Are Nonessential for Viability but Important for Development

In plants, as in all eukaryotes, Pols I, II, and III are essential. Heterozygous plants that have one wild-type allele and one null mutant allele for Pol I, II, or III catalytic subunits are viable, producing haploid gametophytes that are 50 % normal and 50 % mutant. Haploid female gametophytes that carry the mutant allele arrest early in development, failing to complete the three post-meiotic rounds of mitosis that are needed to form a mature eight-celled gametophyte that includes an egg cell. Thus, no transmission of the mutant alleles takes place through the female (Onodera et al. 2008). In contrast, haploid three-celled male gametophytes (pollen) bearing null alleles for essential Pol I, II, or III subunits are still able to complete development thanks to parentally supplied polymerase subunits (or holoenzymes) that persist from the post-meiotic pollen mother cell into the mature pollen. This parental contribution of Pols I, II, and III is sufficient to allow pollen germination upon landing on the stigma of a receptive female floral organ. There is also just enough parental Pol I, II, or III to allow for the growth of short pollen tubes and fertilization of the nearest (wild-type) ovules but not ovules deeper in the flower. In this way, some paternal transmission of mutant alleles to the next generation can take place, albeit at a low frequency (Onodera et al. 2008).

In contrast to null alleles for essential Pol I, II, or III subunits, null alleles of Pol IV or Pol V catalytic subunit genes (*nrpd1*, *nrpe1*, or *nrp(d/e)2*) are both male and female transmissible and homozygous mutants are viable and fertile. In *Arabidopsis thaliana*, the developmental consequences of being a Pol IV or Pol V mutant are subtle, at least under laboratory or greenhouse conditions; plants are normal in appearance but slower to flower, especially under short-day (long night) conditions. However, in maize, Pol IV null mutants display a number of developmental abnormalities, including altered cell fate in vegetative organs, stem outgrowths,

and partial feminization of male floral organs, in addition to late flowering (Erhard et al. 2009; Parkinson et al. 2007). Collectively, these observations show that although Pols IV and V are nonessential for viability, they are nonetheless important for development. The differing severities of Pol IV mutant phenotypes in maize and *Arabidopsis* may be related to the different transposon contents of their genomes, with ~85 % of the maize genome composed of transposons, as opposed to ~15 % in *Arabidopsis*. Failure to tame transposons through Pol IV- and Pol V-mediated transcriptional silencing may lead to misregulation of adjacent genes.

## 13.3 Roles of Pols IV and V in RNA Silencing

In a wide variety of eukaryotes, small RNAs known as short-interfering RNA (siRNAs) or piRNAs direct the transcriptional silencing of homologous genes by bringing about DNA methylation and/or repressive histone modifications (Ishizu et al. 2012; Chen 2012; He et al. 2011; Law and Jacobsen 2010). In plants, this transcriptional silencing function is carried out by a specific class of siRNAs that are 24 nt in length and produced by DCL3, one of four Dicer endonucleases encoded by the *Arabidopsis thaliana* genome. The 24 nt siRNAs guide DNA methylation and heterochromatin formation to homologous loci, mostly retrotransposons or other repetitive nuclear elements, resulting in transcriptional silencing if promoters are methylated. This process, known as RdDM (Matzke et al. 2009; Zhang and Zhu 2011), is the process for which Pol IV and Pol V functions are best understood (Haag and Pikaard 2011). An abbreviated version of the RdDM process, emphasizing the steps involving Pols IV and V, is shown in Fig. 13.3. More complicated representations of the pathway involving additional activities can be found in recent reviews (Law and Jacobsen 2010; Haag and Pikaard 2011; Zhang and Zhu 2011).

RdDM has two major phases: the first involved in the biogenesis of 24 nt siRNAs and the second encompassing siRNA-programmed chromatin modifications. Pol IV is required in the first phase, and Pol V is critical for the second phase (Fig. 13.3). Mutations that knock out the catalytic subunits of Pol IV (*nrpd1* or *nrp(d/e)2*) essentially eliminate 24 nt siRNAs, as do mutations eliminating RDR2, one of six RNA-dependent RNA polymerases in *Arabidopsis*. These data provided early genetic evidence that Pol IV and RDR2 collaborate in the production of double-stranded RNA (dsRNA) precursors. Immunolocalization experiments showed that Pol IV colocalizes with repetitive loci that give rise to abundant 24 nt siRNAs and that RDR2 becomes mislocalized in *pol IV* mutants; in contrast, Pol IV localization is unaffected in *rdr2* mutants (Pontes et al. 2006). These observations suggested that Pol IV acts upstream of RDR2. More recently, affinity-purified Pol IV complexes analyzed by mass spectrometry were found to contain RDR2, with reciprocal immunoprecipitation experiments confirming that Pol IV and RDR2 associate in vivo (Haag et al. 2012; Law et al. 2011). The Pol IV–RDR2 interaction is dispensable for Pol IV transcription in vitro but appears to be required for RDR2

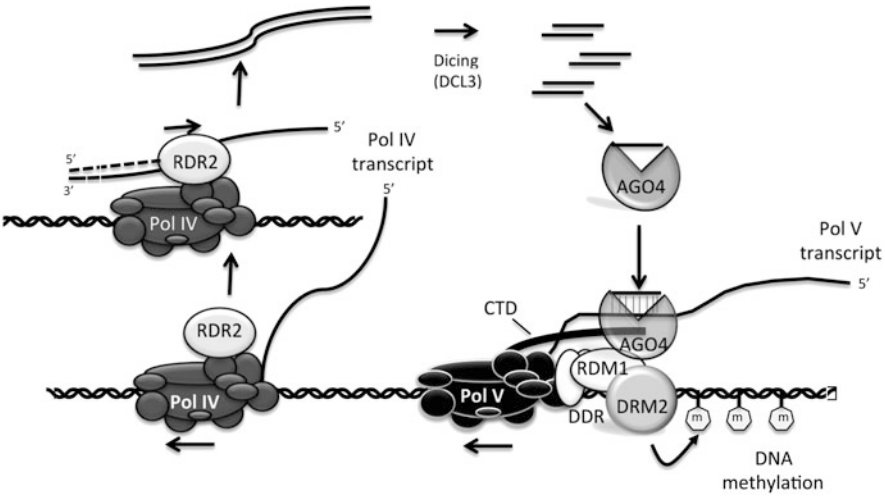


**Fig. 13.3** Roles of Pols IV and V in RNA-directed DNA methylation in *Arabidopsis*. The diagram shows a simplified version of the pathway, focused on the roles of Pols IV and V. Double-stranded RNAs made by the Pol IV–RDR2 enzyme complex are diced by DCL3 and loaded into ARGONAUTE 4 (AGO4). The AGO4–siRNA complex is then recruited to target sites through binding to Pol V transcripts, and by physical interactions between AGO4 and the CTD of the Pol V largest subunit. The DDR complex assists in Pol V transcription and may also mediate interactions between AGO4 and the de novo DNA methyltransferase, DRM2

activity (Haag et al. 2012). This Pol IV-dependence of RDR2 activity suggests a mechanism in which Pol IV and RDR2 activities are coupled, with single-stranded Pol IV transcripts handed off to RDR2 to be used as templates, thus producing and channeling dsRNAs into the RdDM pathway (Fig. 13.3).

dsRNA products produced via Pol IV and RDR2 are diced by DCL3, and resulting 24 nt single-stranded siRNAs are loaded into the Argonaute family protein, AGO4, or its closest relatives, AGO6 or AGO9 (Zheng et al. 2007; Zilberman et al. 2003; Qi et al. 2006; Havecker et al. 2010). dsRNA synthesis, dicing, and AGO loading may be spatially coordinated given that RDR2, DCL3, and AGO4 partially colocalize, along with siRNAs and Pol V, within a nucleolus-associated Cajal body (Li et al. 2006; Pontes et al. 2006). Pol V has been shown to physically interact with AGO4 via WG or GW amino acid motifs (known as "AGO hooks") present in the Pol V CTD (see Fig. 13.1a) (El-Shami et al. 2007). Taken together, these observations suggested that Pol V might be guided to its sites of action by associating with AGO4–siRNA complexes within the Cajal bodies and then using the siRNAs to guide the complex to complementary target sites through base-pairing interactions (Li et al. 2006; Pontes et al. 2006). However, subsequent studies have argued against this hypothesis by showing that Pol V transcription at target loci is unaffected in *pol IV*, *rdr2*, or *dcl3* mutants defective for 24 nt siRNA biogenesis and is also unaffected in *ago4* mutants (Wierzbicki et al. 2008). Instead, siRNA binding to Pol V transcripts appears to be what recruits AGO4 to target loci.

Key evidence is that AGO4 can be chemically cross-linked to Pol V transcripts and that AGO4 associates with target loci (as shown using chromatin immunoprecipitation, or ChIP) in a Pol V-dependent manner (Wierzbicki et al. 2009). Moreover, AGO4's association with chromatin is abrogated in Pol V mutants bearing point mutations in the NRPE1 active site (Wierzbicki et al. 2009). These point mutations abolish Pol V transcriptional activity in vitro (Haag et al. 2012) and all known biological functions attributable to Pol V in vivo (Haag et al. 2009). Collectively, these findings indicate that AGO4 is primarily recruited to Pol V-transcribed loci via siRNA-mediated interactions with Pol V transcripts. AGO4 interactions with the Pol V CTD may further stabilize the complex or mediate downstream events (El-Shami et al. 2007). The functional significance of the Cajal bodies containing RDR2, DCL3, AGO4, siRNAs, and Pol V is not clear, but roles in RNA processing, chromatin modification, transport, or storage are possibilities (Li et al. 2008; Pontes and Pikaard 2008).

Several activities identified in genetic screens for disrupted RdDM are important for Pol V activity. Among these are DRD1, a putative SWI2/SNF2-family chromatin remodeling ATPase (Kanno et al. 2004); DMS3, a protein related to the hinge domains of cohesins and condensins (Kanno et al. 2008); and RDM1, a protein that binds methylated single-stranded DNA in vitro (Gao et al. 2010). Wierzbicki et al. showed that DRD1 and DMS3 are required for the production of Pol V transcripts and for stable Pol V association with chromatin (Wierzbicki et al. 2008, 2009). Subsequent studies showed that DRD1 and DMS3 associate with RDM1 to form a so-called DDR complex, named for the first initials of the three proteins (Law et al. 2010). Mass spectrometric analyses also identified Pol V subunits in affinity-purified DDR samples, suggesting that DDR and Pol V can physically associate (Law et al. 2010). The RDM1 protein of the DDR complex has also been shown to colocalize with AGO4 and the de novo DNA methyltransferase DRM2 (Gao et al. 2010), suggesting that RDM1 may serve as a bridge for recruitment of DRM2 to AGO4-siRNA- and Pol V-engaged loci (Zhang and Zhu 2011).

An SPT5-LIKE, KOW-domain transcription factor, SPT5L/KTF1, is involved in the downstream phase of the RdDM pathway, such that *ktf1*/*spt5l* mutants display a reduction in DNA methylation and reduced levels of siRNAs, similar to the effects of mutating the Pol V-specific subunit, NRPE5 (Bies-Etheve et al. 2009; Huang et al. 2009; Ream et al. 2009). In yeast and humans, Spt5 interacts with Spt4 to form the DSIF elongation factor complex, which is involved in mRNA capping, Pol II elongation, and prevention of premature transcription termination (Yamaguchi et al. 2013). One might expect SPT5L/KTF1 to similarly act as a Pol V elongation factor, consistent with the identification of SPT5L/KTF1 peptides in affinity-purified Pol V (Huang et al. 2009). However, Pol V transcripts are not decreased in abundance in a *spt5l*/*ktf1* loss-of-function mutant, but actually increase slightly (He et al. 2009c). A similar increase in Pol V transcript abundance is observed in *ago4* mutants (Wierzbicki et al. 2009) and SPT5L/KTF1 and AGO4 interact, via WG/GW "AGO-hook" motifs within SPT5L/KTF1 (He et al. 2009c; Bies-Etheve et al. 2009). These and other data have led to an alternative hypothesis that SPT5L/

KTF1 acts downstream of Pol V transcription possibly by helping AGO4 associate with Pol V transcripts or associated chromatin (Rowley et al. 2011). The increased abundance of Pol V transcripts observed in *ago4* and *ktf1* mutants may be a consequence of decreased slicing of Pol V transcripts by AGO4.

## 13.4 Subunit Compositions of *Arabidopsis* RNA Polymerases II, IV, and V

### *13.4.1 Subunits Common to Pols II, IV, and V*

Affinity purification of *Arabidopsis* Pols II, IV, and V, followed by trypsin digestion and analysis of their peptides by LC-MS/MS mass spectrometry, revealed that Pols IV and V are composed of twelve subunits that are identical or homologous to the 12 core subunits of Pol II (Ream et al. 2009). Seven of the twelve subunits present in *Arabidopsis* Pols II, IV, and V are encoded by the same genes: the subunits homologous to yeast Rpb3, Rpb6, Rpb8, Rpb9, Rpb10, Rpb11, and Rpb12.

For all of the Pol II, IV, or V common subunits, except the 11th subunit (NRP(B/D/E)11), there are two genes in *Arabidopsis*. Mass spec analyses have revealed that the different variants can often be detected within the purified polymerases, sometimes equally and sometimes with only one variant being the major form (Ream et al. 2009; Law et al. 2011). Due to the high degree of similarity among most of these alternative subunits (~90 %, or higher, identity), one might expect the proteins to be functionally equivalent. However, a recent analysis of single or double mutants disrupting the two alternative forms of the ninth subunit [NRP(B/D/E)9a and NRP(B/D/E)9b] that are utilized by Pols II, IV, or V has forced a reconsideration of this assumption. This study revealed that the alternative ninth subunit proteins (and/or their genes) are only partially redundant with respect to Pol II functions and are nonredundant for Pol V-specific functions despite being 92 % identical (Tan et al. 2012). These data suggest that different Pol II, IV, and V subtypes exist in plants, resulting from their assembly using alternative subunits in various permutations. Consistent with this hypothesis, maize has three genes potentially encoding alternative NRP(D/E)2 subunits for Pol IV and/or V. These genes are not completely redundant, given that mutations in only one of the three genes disrupt the inter-allelic gene silencing phenomenon known as paramutation (Stonaker et al. 2009; Sidorenko et al. 2009). However, the developmental phenotypes of these mutants are not as severe as for mutants defective for the Pol IV largest subunit, NRPD1. Given that largest and second-largest subunits are equally important for forming the polymerase catalytic center, these observations suggest that there must be partial redundancy among the three second subunit variants with respect to functions affecting development (Pikaard and Tucker 2009).

### 13.4.2 Pol V Makes Use of a Distinct 5th Subunit

Rpb5, Rpb6, Rpb8, Rpb10, and Rpb12 are subunits common to Pols I, II, and III in yeast and humans; thus one might expect these subunits to be common to Pols I through V in *Arabidopsis*, as well. This is mostly true (as discussed above), but not entirely—although Pol IV uses the same fifth subunit as Pols I, II, and III, Pol V makes use of a distinct fifth subunit encoded by the *NRPE5* gene (Lahmy et al. 2009; Ream et al. 2009; Huang et al. 2009). Three additional Rpb5-like genes are also present in the *Arabidopsis* genome, at least one of which is used as an alternative minor subunit of Pol IV (Law et al. 2011). NRPE5 is distinguished from other eukaryotic Rpb5 subunits by the presence of an N-terminal extension, required for the protein's stability in vivo, and by divergence in its C-terminal assembly domain (Lahmy et al. 2009; Ream et al. 2009). In *nrpe5* mutants, normally silenced elements (such as retrotransposons) are derepressed as in *nrpe1* mutants, indicating that NRPE5 and the catalytic subunits are equally required for Pol V-dependent gene silencing (Huang et al. 2009; Lahmy et al. 2009; Ream et al. 2009; Douet et al. 2009). However, DNA methylation and siRNA abundance are less affected in *nrpe5* than *nrpe1* mutants (Ream et al. 2009; Lahmy et al. 2009), which is also the case for *nrp(d/e)4* mutants (He et al. 2009a). These results suggest that non-catalytic subunits of Pols IV and/or V may play roles in RNA silencing that are separable from the RNA synthesis function(s) of the catalytic subunits.

### 13.4.3 Arabidopsis *Pols II, IV, and V Have Unique Subunit 4/7 Sub-complexes*

In addition to their second-largest subunit, NRP(D/E)2, which is encoded by the same gene, Pol IV and Pol V have in common their fourth subunit, NRP(D/E)4, which is distinct from the NRPB4 subunit of Pol II (Ream et al. 2009). NRPD4 does not rescue a yeast *rpb4* mutation, whereas NRPB4 does (He et al. 2009a). Rpb4 and Rpb7 subunits interact, forming a dissociable sub-complex that in yeast has been shown to interact with the RNA transcripts of Pol II and regulate RNA processing, trafficking, and even translation (Choder 2004; Ujvari and Luse 2006; Runner et al. 2008; Harel-Sharvit et al. 2010). Interestingly, Pol II, Pol IV, and Pol V utilize distinct proteins as their major NRPB7, NPRD7, and NRPE7 subunits in *Arabidopsis* (Ream et al. 2009), with NRPE7 also serving as a minor form of the seventh subunit in Pol IV (Ream et al. 2009; Law et al. 2011). The *NRPD7* and *NRPE7* genes lack introns, suggesting that retrotransposition of an NRPB7 cDNA was the duplication event early in Pol IV/V evolution that gave rise to their seventh subunit genes (Tucker et al. 2011). The functional significance of having unique subunit-4/7 sub-complexes in Pols II, IV, and V is unclear but is likely to be important.

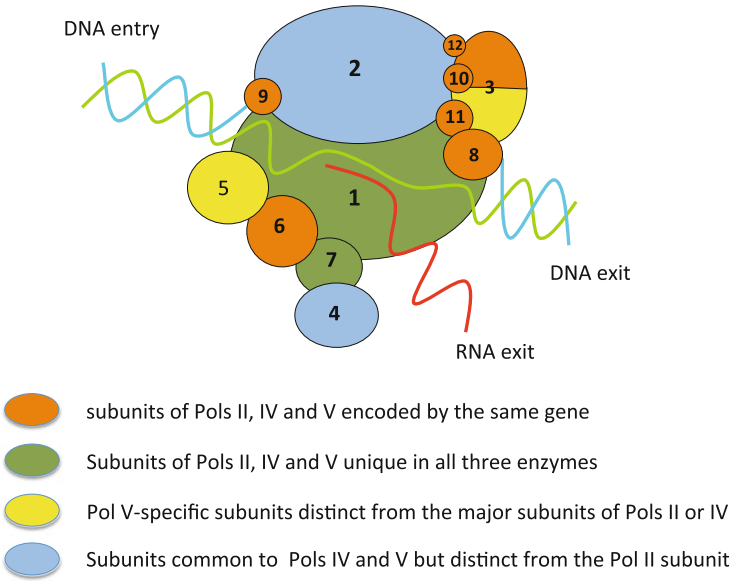


**Fig. 13.4** Shared and unique subunits of *Arabidopsis* RNA polymerases II, IV, and V

To summarize, Pols II, IV, and V differ from one another in a subset of their subunits (Fig. 13.4). Pol IV differs from Pol II in four subunits (NRPD1, NRP(D/E)2, NRP(D/E)4, and NRPD7). Pol V differs from Pol II in five subunits (NRPE1, NRP(D/E)2, NRP(D/E)4, NRPE5, and NRPD7) and also makes nearly equal use of two alternative forms of the 3rd subunit, only one of which is primarily used by Pol II (or Pol IV). Pols IV and V differ in subunits 1, 5, and 7, and in the frequency of their use of the two alternative third subunits, with the major form of the Pol IV 3rd subunit being the same as for Pol II. Understanding how the different subunits contribute to the distinct functions of the three polymerases is a goal of ongoing research.

## 13.5 Pol V and Pol IV Largest Subunit CTD Functions Are Largely Unknown

As discussed previously, the Pol V largest subunit, NRPE1, has a long CTD that lacks the heptad repeats present in the Pol II CTD but has several unique domains, including a QS-rich domain, DeCL domain, and 10 imperfect tandem repeats of a 16 amino acid sequence (see Fig. 13.1a). Embedded within and adjacent to these 16 amino acid repeats are 18 WG/GW AGO-hook motifs implicated in interactions with AGO4 (El-Shami et al. 2007; He et al. 2009c). To date, this is the only function ascribed to the Pol V CTD. Unlike the Pol II largest subunit CTD, whose variable phosphorylation patterns confer regulatory meaning, nothing is known about

potential posttranslation modifications of the NRPD1 or NRPE1 CTDs or their regulatory significance.

## 13.6 Rules Governing Locus-Specific Transcription by Pols IV and V Are Unknown

It is not clear how Pols IV or V are recruited to the thousands of loci where Pol IV-dependent siRNAs are generated or where RdDM occurs. Recent studies identified genomic sites of Pol V occupancy using ChIP-seq (Wierzbicki et al. 2012; Zhong et al. 2012), but motif finding algorithms failed to identify specific DNA sequences, such as putative or known promoter elements or transcription factor binding sites, that could explain the occurrence of Pol V in these regions. Pol IV ChIP-seq studies have not yet been reported nor have Pol IV primary transcripts been identified, but sites of 24 nt siRNA production are signatures of Pol IV (see Fig. 13.3), and motif finding at these siRNA loci has also failed to identify consensus sequences thus far.

Understanding more about the DDR complex may tell us more about Pol V recruitment or transcriptional regulation. In mutants such as *drd1* or *dms3* that disrupt the DDR complex, Pol V does not stably associate with chromatin, as shown by ChIP, and Pol V transcripts are no longer produced (Wierzbicki et al. 2008, 2009; Zhong et al. 2012). Whether the DDR complex plays a role in Pol V recruitment and initiation, or Pol V elongation and processivity is not yet known, but ChIP analyses of the DDR complex may reveal whether DDR localizes at specific subregions within Pol V-associated loci, perhaps revealing potential start sites.

Only two proteins with a known connection to Pol II regulatory mechanisms have emerged thus far in genetic screens for defects in RdDM. These are the SPT5-like protein, KTF1 discussed previously, and an *Arabidopsis* homolog of yeast IWR1, a protein which facilitates the nuclear import of assembled Pol II from the cytoplasm to the nucleus (Czeko et al. 2011), that was identified in two independent genetic screens (Kanno et al. 2010; He et al. 2009b). Mutant plants (*dms4*/*rdm4*) defective for the IWR1 homolog display pleiotropic phenotypic abnormalities in addition to disrupted RdDM, consistent with impaired functions of Pol II in addition to Pols IV and V.

## 13.7 RNAs Synthesized by Pols IV and V In Vivo

To date, no RNA corresponding to a primary transcript of Pol IV has been cloned and sequenced or detected in vivo by RT-PCR or other molecular assays. Most likely, this is due to the fleeting existence of these transcripts before being

converted into dsRNA through the coupling of Pol IV and RDR2 activities, followed by dicing into siRNAs. As a result, nothing is known about Pol IV transcription start sites, termination sites (or regions), or potential transcript modifications, such as the possible addition of a 7-methylguanosine cap on the 5′ end. However, preliminary studies suggest that Pol V transcripts are not polyadenylated.

Unlike Pol IV transcripts, Pol V transcripts have been identified in vivo. Wierzbicki et al. examined genomic regions where no gene models existed and no significant transcription had been detected using DNA microarrays, yet where deep sequencing identified small RNAs that must have had precursors (Wierzbicki et al. 2008). This approach led to the identification of multiple loci where low-abundance transcripts detectable by RT-PCR are absent in *nrpe1* or *nrp(d/e)2* mutants, yet still detectable in *nrpd1* (Pol IV) mutants (Wierzbicki et al. 2008). ChIP analyses using anti-NRPE1 antibodies showed that Pol V physically associates with these loci. For several loci examined, transcripts appeared to initiate at multiple sites, based on 5′-RACE, were enriched among poly-A$^-$ RNA and had either 5′ caps or triphosphates—characteristic of newly initiated RNAs, as opposed to processed RNAs (Wierzbicki et al. 2008). Production of Pol V-dependent transcripts in vivo also requires the conserved aspartates of the NRPE1 Metal A site (see Fig. 13.1c), and these transcripts can be chemically cross-linked to NRPE1, allowing their immunoprecipitation using anti-NRPE1 antibodies. Collectively, these assays strongly suggested that these RNAs are Pol V transcripts.

## 13.8 Pol IV and Pol V Transcription In Vitro

RNA polymerase activity assays typically make use of sheared genomic DNA, or double-stranded DNA having 3′ overhangs, as a source of templates for promoter-independent transcription. Interestingly, Pols IV and V show no activity in such assays, unlike Pols I, II, or III (Onodera et al. 2005), thwarting early attempts by several laboratories, including ours, to detect Pol IV or Pol V biochemical activities. These negative results suggested that Pols IV and V might transcribe unconventional templates, or might even lack RNA polymerase activity altogether, consistent with the divergence, or absence, of more than 160 amino acids that are invariant in the catalytic subunits of Pols I, II, or III (Haag et al. 2009; Landick 2009). These substituted amino acids are clustered in the vicinity of the Metal A and Metal B sites of the catalytic center; in the bridge helix, trigger loop, cleft, and funnel domains of the largest subunits; and within the hybrid-binding domain of NRPD2 (Fig. 13.5; see also Fig. 13.1c, d). A number of these amino acids correspond to positions that interact with the incoming nucleotide triphosphate and the 3′ end of the growing RNA chain and are thus thought to be critical for catalysis. Among these are amino acids at the tip of the trigger loop and in the bridge helix that are not simply substituted but missing altogether in Pols IV and V (Landick 2009).

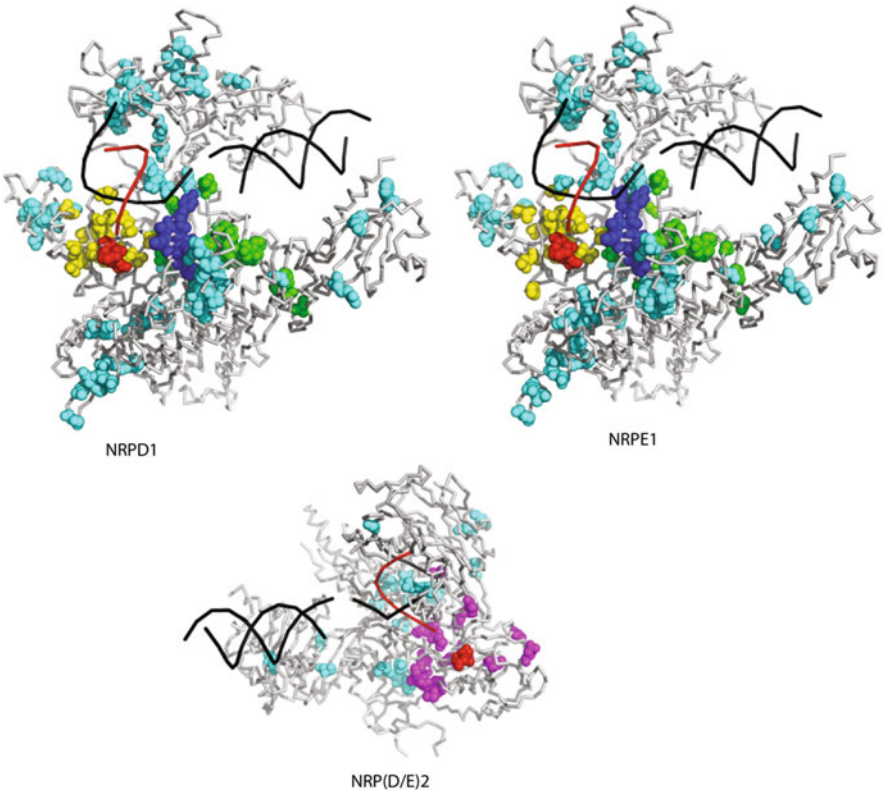


**Fig. 13.5** Sequence divergence in Pols IV and V at amino acid positions that are invariant in Pols I, II, and III. The three images show these substituted positions as space-filling spheres mapped onto the Rpb1 and Rbp2 subunit structures of a yeast Pol II elongation complex determined in the Kornberg laboratory (Protein Data Bank structure 1R9T). The dsDNA substrate is shown in *black* and the RNA product in *red*. Amino acids colored *red mark* the positions of the invariant Metal A and Metal B sites in the largest and second-largest subunits, respectively. Substituted amino acids in the cleft, bridge helix, and active site domains of the largest subunits are colored *green*, *blue*, and *yellow*, respectively. Substituted amino acids in the hybrid-binding domain of the second-largest subunit are colored *magenta*. Substituted amino acids in the largest and second-largest subunits that are located outside of these domains are colored *cyan*. These images are reprinted from the open access article by Haag et al. (2009), as permitted under the Creative Commons Attribution License

Despite their lack of activity using conventional DNA templates, and their divergent amino acid sequences, *Arabidopsis* Pols IV and V are indeed functional RNA polymerases in vitro. Using templates in which an RNA primer is annealed to a DNA template oligonucleotide, thus forming an 8 bp RNA–DNA hybrid, Pols IV and V will elongate the RNA in a templated fashion (Haag et al. 2012), as shown previously for Pols I and II (Kuhn et al. 2007; Lehmann et al. 2007). Unlike Pol II, Pols IV and V are insensitive to the fungal toxin, alpha-amanitin, consistent with the divergence of multiple amino acids within the alpha-amanitin binding pocket of

Pol II (Haag et al. 2012). If double-stranded DNA is present downstream of the template DNA–RNA hybrid region, Pols II and IV are able to generate transcripts, but Pol V cannot, suggesting that Pol V cannot displace a non-template strand during transcription (Haag et al. 2012).

Our inability thus far to coax affinity-purified Pols IV and V to initiate transcription on DNA templates in the absence of an RNA primer may simply be due to the absence of unidentified helper proteins that do not copurify with the polymerases. However, cytological experiments have shown that Pols IV and V become mislocalized in RNase A-treated nuclei, unlike Pol II (Pontes et al. 2006), consistent with some form of RNA involvement, possibly as a template or primer.

## 13.9 Missing Pieces

Pols IV and V are remarkable enzymes given their evolution as specialized forms of Pol II but their substantial divergence at amino acid positions that are otherwise invariant among Pols I, II, and III. Approximately 120 of these normally invariant amino acids are substituted, or missing, in the largest subunits of *Arabidopsis* Pols IV and V, and another 40 are altered in the second-largest subunit (see Fig. 13.5). Moreover, comparison of the largest subunit sequences of Pols IV and V in a variety of plants reveals that they are evolving rapidly, with amino acid substitution rates occurring at 20 times the rate observed for the largest subunits of Pols I, II, or III (Luo and Hall 2007). Collectively, these observations indicate that there are fewer functional constraints on the evolution of Pols IV and V than for other polymerases.

DNA-dependent RNA polymerases perform a variety of functions in addition to RNA synthesis. These include pausing at specific sequences, backtracking along the template, RNA cleavage, and proofreading. These activities allow a polymerase to overcome barriers in the template or correct misincorporated nucleotides (Sydow and Cramer 2009). It is unknown if Pols IV and V possess any of these activities in vivo or in vitro. Given their involvement in noncoding RNA-mediated silencing processes, perhaps Pols IV and V do not need to be high-fidelity enzymes with proofreading or backtracking capabilities. In fact, error-prone transcription might actually be advantageous in the production of siRNAs that participate in the silencing of large transposon families, whose sequences are similar, but not identical. Perhaps by making use of RNA primers derived from Pol II (or other polymerase) transcripts, the catalytic centers of Pols IV and V have evolved to accommodate binding to DNA–RNA hybrid templates and only need to elongate RNAs rather than initiate their synthesis de novo from nucleoside triphosphates. This might also remove constraints on the evolution of the Pol IV and Pol V catalytic centers. Likewise, the possibility that Pols IV and V recognize methylated DNA, or chromatin templates bearing specific histone modifications, may contribute to their different amino acid sequences compared to Pol II. Such speculations should be possible to test upon further development of in vitro assays.

In vivo, identification of full-length Pol V or Pol IV transcripts through strand-specific RNA deep sequencing in appropriate wild-type and mutant backgrounds (coupled with comparative bioinformatics analyses) has not yet been accomplished. However, this basic information is critically important. Knowing where Pol IV and Pol V transcripts begin and end will define their transcription units and allow for more focused analyses of potential consensus motifs in the vicinity of these transcription units, possibly revealing promoters or other regulatory elements that can be tested. Moreover, knowing if Pol IV or Pol V transcripts are co- or post-transcriptionally modified or processed may reveal insights into their channeling, trafficking, and stability.

Understanding the functions of subunits that differ between Pols II, IV, and V is also likely to yield important new insights. Evidence that multiple structurally distinct subtypes of the different polymerases exist, based on alternative choices for many of their subunits, is intriguing. By exploiting the non-lethality of Pol IV- or Pol V-specific subunit mutations, the roles of different subunits in DNA methylation and chromatin modification in vivo, and transcription in vitro, can be explored, potentially revealing new insights, particularly into the functions of non-catalytic subunits. Last but not least, understanding the functions of the unique CTDs of the Pol IV and Pol V largest subunits should also be illuminating, potentially revealing mechanisms that couple transcription with noncoding RNA processing and chromatin modification.

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