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Ek Han Tan
Washington University School of Medicine in St. Louis

Todd Blevins
Indiana University - Bloomington

Thomas S. Ream
Washington University School of Medicine in St. Louis

Craig S. Pikaard
Indiana University - Bloomington

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Functional Consequences of Subunit Diversity in RNA Polymerases II and V

Ek Han Tan,1,2 Todd Blevins,1 Thomas S. Ream,3,4 and Craig S. Pikaard1,2,∗

1Department of Biology and Department of Molecular and Cellular Biochemistry
2Howard Hughes Medical Institute
Indiana University, Bloomington, IN 47405, USA
3Division of Biology and Biomedical Sciences, Washington University, St. Louis, MO 63110, USA
4Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA
∗Correspondence: cpikaard@indiana.edu
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SUMMARY

Multisubunit RNA polymerases IV and V (Pol IV and Pol V) evolved as specialized forms of Pol II that mediate RNA-directed DNA methylation (RdDM) and transcriptional silencing of transposons, viruses, and endogenous repeats in plants. Among the subunits common to Arabidopsis thaliana Pols II, IV, and V are 93% identical alternative ninth subunits, NRP(B/D/E)9a and NRP(B/D/E)9b. The 9a and 9b subunit variants are incompletely redundant with respect to Pol II; whereas double mutants are embryonic lethal, single mutants are viable, yet phenotypically distinct. Likewise, 9a or 9b can associate with Pols IV or V but RNA-directed DNA methylation is impaired only in 9b mutants. Based on genetic and molecular tests, we attribute the defect in RdDM to impaired Pol V function. Collectively, our results reveal a role for the ninth subunit in RNA silencing and demonstrate that subunit diversity generates functionally distinct subtypes of RNA polymerases II and V.

INTRODUCTION

Eukaryotes decode their genomes using three essential nuclear DNA-dependent RNA polymerases, RNA Polymerases I, II, and III (Cramer et al., 2008; Werner and Grohmann, 2011). In plants, two additional multisubunit RNA polymerases, Pol IV and Pol V, are not strictly required for viability but are important for development, transposon taming, antiviral and antibacterial defense, and interallelic communications mediating paramutation (Haag and Pikaard, 2011).

Pol IV and Pol V functions are best understood with respect to RNA-directed DNA methylation (Haag and Pikaard, 2011; Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005), a process in which 24 nt short interfering RNAs (siRNAs) direct the cytosine methylation, and silencing, of complementary DNA sequences. Pol IV acts early in the pathway, working in partnership with RNA-DEPENDENT RNA POLYMERASE 2 to produce double-stranded RNAs that are diced into siRNAs and loaded (primarily) into ARGONAUTE 4 (AGO4) (Law and Jacobsen, 2010). Independent of siRNA biogenesis, Pol V generates RNA transcripts at loci that undergo RdDM (Wierzbicki et al., 2008) and AGO4 binds these Pol V transcripts (Wierzbicki et al., 2009) as well as Pol V itself (El-Shami et al., 2007). Chromatin modifying activities are subsequently recruited, resulting in de novo cytosine methylation and establishment of repressive histone modifications (Haag and Pikaard, 2011; Law and Jacobsen, 2010).

Arabidopsis thaliana Pols II, IV, and V each have 12 core subunits (Ream et al., 2009). Pol II, IV, and V largest subunits are encoded by unique genes: NRPB1, NRPD1, and NRPE1, respectively (“NRP” denotes “Nuclear RNA Polymerase”; “B, D, and E,” as the second, fourth, and fifth letters of the alphabet, denote Pols II, IV, or V; the numeral 1 indicates the largest subunit). Pol IV and V second-largest subunits are encoded by the same gene, NRPD(E)/2, which is distinct from the corresponding Pol II subunit gene, NRPB2 (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005). The two largest subunits interact to form the catalytic center for RNA synthesis, with noncatalytic subunits playing structural and regulatory roles for initiation, elongation, termination, or RNA processing (Cramer et al., 2008; Werner and Grohmann, 2011). Most of the noncatalytic subunits of Pols II, IV, and V are encoded by the same genes (Ream et al., 2009).

In yeast, the 12 Pol II subunits are each encoded by unique genes, ten of which are essential. One of the nonessential genes encodes the ninth-largest subunit, Rpb9. rpb9 deletion strains are viable, but temperature-sensitive (Woychik et al., 1991). Rpb9 is implicated in multiple aspects of polymerase II function, including initiation (Hull et al., 1995; Sun et al., 1996), processivity (Awrey et al., 1997; Hemming et al., 2000), transcriptional fidelity, proofreading (Koyama et al., 2007; Nesser et al., 2006; Walmacq et al., 2006), and transcription-coupled DNA repair (Li et al., 2006).

Unlike yeast and metazoans, Arabidopsis thaliana and Populus trichocarpa (poplar) each have two genes orthologous to RBP9, and maize and rice have three (Figure 1 and Figure S1). Orthologs of yeast Rpa12 and Rpc11, the Rpb9-like subunits of Pols I and III, respectively, form separate clades (Figure 1). Both Arabidopsis Rpb9 orthologs copurify with affinity-purified RNA polymerases II, IV, or V (Law et al., 2011; Ream et al., 2009),
such that their comprehensive names are NRP(B/D/E)9a and NRP(B/D/E)9b. Despite the fact that the two proteins differ at only 8 of their 114 amino acids, we show here that these ninth subunit variants are incompletely redundant for Pol II and nonredundant for Pol V functions.

RESULTS

Subunits 9a and 9b Are Redundant for Viability

Arabidopsis NRP(B/D/E)9a (At3g16980) and NRP(B/D/E)9b (At4g16265) genes have similar intron/exon structures (Figure 2A). T-DNA insertion alleles, designated nrp(b/d/e)9a-1 (Salk_032670) and nrp(b/d/e)9b-1 (Salk_031043), are disrupted within introns 2 or 1, respectively (Figure 2A). Transcripts of the 9a and 9b (abbreviated for brevity) genes are readily detected in wild-type plants (Figure 2B) but not in 9a-1 or 9b-1 mutants (Figure 2C).

Wild-type (ecotype Col-0) and 9a-1 mutant plants are indistinguishable, but leaves of 9b-1 mutants are more ovate, have shorter petioles and display less downward edge curling (Figure 2C). Other 9b-1 phenotypes include smaller trichomes on the first true leaves, more prominent leaf midveins, changes in the cuticular wax coating on the leaves, and shorter siliques. The morphological differences presumably result from altered Pol II-dependent gene expression given that null mutations eliminating Pol IV or Pol V largest subunits (nrpd1-3 or nrpe1-11, respectively) do not induce similar phenotypes. Moreover, 9b-1 phenotypes are neither suppressed nor enhanced in 9b-1 nrpd1-3 or 9b-1 nrpe1-11 double mutants.

To test 9a and 9b redundancy, homozygous 9a-1 and 9b-1 mutants were crossed, resulting F1 plants were selfed, and their progeny genotyped. In siliques of F2 plants homozygous for 9a-1 and heterozygous for 9b-1, in which 25% of the F3 seeds are expected to be homozygous 9a-1 9b-1 double mutants, 30% (55/181 analyzed) of the seeds arrested in development and 70% developed normally (Figure 2D). Similar results were observed for the progeny of plants homozygous for 9b-1 but heterozygous for 9a-1. In arrested seeds, which are translucent, embryos failed to develop past the globular stage (Figure 2D). Among plants germinated from seeds collected from siliques of plants that were homozygous for either 9a-1 or 9b-1 and heterozygous for the other mutation, no 9a-1 9b-1 double mutants were...
identified (Figure 2E). Because lethality is a consequence of lost Pol II function (Onodera et al., 2008), but not disrupted Pol IV or Pol V function (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005; Ream et al., 2009), we conclude that the 9a and 9b subunits are mostly redundant with respect to Pol II functions required for viability and development, such that only the double mutant is embryo lethal.

Among the progeny of heterozygotes carrying a recessive allele of an essential gene, heterozygotes should outnumber homozygotes 2:1. However, we observed a nearly 1:1 ratio of heterozygotes to homozygous wild-type plants (Figure 2E), suggesting a defect in male or female (or both) transmission of the mutant alleles. Reciprocal crosses showed reduced transmission of 

\[ \text{NRP(B/D/E)}9a \text{ or } \text{NRP(B/D/E)}9b \text{ alleles through both the male and female gametophytes (Figure S2).} \]

This allele transmission behavior differs from null mutations eliminating catalytic subunits of Pol I, II, or III, which show zero transmission via the egg donor (Onodera et al., 2008).

**Subunit 9b Is Required for RNA Silencing**

Loci silenced by Pol IV and Pol V include soloLTR and AtSN1 retroelements, whose expression is undetectable in wild-type plants (Col-0) but prevalent in 

\[ \text{nrpd1-3} \text{ or } \text{nrpe1-11} \text{ mutants.} \]

Retrotransposon silencing correlates with Pol IV and Pol V-dependent RNA-directed DNA methylation (RdDM), rendering methylation-sensitive AluI sites within soloLTR elements and HaeIII sites within AtSN1 elements resistant to AluI or HaeIII digestion. Consequently, PCR using primers that flank the restriction sites amplifies the interval in wild-type (Col-0) plants (Figure 3B). However, in 

\[ \text{nrpd1-3} \text{ or } \text{nrpe1-11} \text{ mutants, loss of RdDM allows AluI and HaeIII to cleave the DNA, and PCR fails (Figure 3B).} \]

Methylation is similarly lost in 

\[ \text{9b-1} \text{ mutants, but not in } \text{9a-1} \text{ mutants (Figure 3B).} \]

Tandemly repeated 5S ribosomal RNA genes are also subject to RdDM. Southern blots of genomic DNA digested with HaeIII and hybridized to a 5S RNA gene probe show a ladder of bands in wild-type Col-0 plants (Figure 3C), with larger bands reflecting increased methylation among adjacent genes. HaeIII methylation is reduced in 

\[ \text{nrpd1-3} \text{, } \text{nrpe1-1} \text{, and } \text{9b-1} \text{ mutants, but not in } \text{9a-1} \text{ mutants.} \]

A complete 

\[ \text{NRP(B/D/E)}9b \text{ transgene, transcribed from its native promoter and containing all its introns, rescues all } \text{9b-1} \text{ mutants.} \]
mutant phenotypes, restoring wild-type leaf morphologies (Figure S3A), NRP(B/D/E)9b mRNA production (Figure S3B), AtSN1 and soloLTR silencing (Figure S3B), and cytosine methylation (Figure S3C). We conclude that 9b-1 mutant phenotypes are due solely to mutation of the NRP(B/D/E)9b gene.

**Pol IV Function Is Not Impaired in 9b-1 Mutants**

Pol IV is required for the biogenesis of ~95% of all 24 nt siRNAs (Herr et al., 2005; Kanno et al., 2005; Mosher et al., 2008; Onodera et al., 2005; Pontier et al., 2005; Zhang et al., 2007). For instance, 24 nt siRNAs corresponding to 5S rRNA genes are severely depleted in nrd1-3 mutants (Figure 3D) (Onodera et al., 2005). In 9a-1 or 9b-1 mutants, siRNAs are detected at wild-type levels, suggesting that Pol IV activity is not impaired. Interestingly, reductions in siRNA levels observed in nrpe1 mutants are also not apparent in 9a-1 or 9b-1 mutants, suggesting that RNA synthesis by Pol V is also unimpaired.

**MRD1** is representative of a small set of Arabidopsis loci at which RdDM and silencing requires Pol IV but not Pol V. Thus, **MRD1** is expressed at low levels in Pol V mutants (nrpe1-11), as in wild-type plants, but is substantially derepressed in Pol IV mutants (nrd1-3), unlike AtSN1 or soloLTR elements that require both Pol IV and Pol V for silencing (Figure 4A). In 9a-1 or 9b-1 mutants, **MRD1** is not derepressed, suggesting that Pol IV function is not impaired in either single mutant (Figure 4A, lanes 4 and 5).

**Pol V Transcripts Are Produced in 9b-1 Mutants**

Pol V transcripts can be detected at specific intergenic loci such as **IGN5** (Law et al., 2010; Wierzbicki et al., 2008; Wierzbicki et al., 2009), therefore we examined whether 9b-1 mutants are impaired for Pol V transcription. **IGN5** transcripts are readily detected in wild-type plants or Pol IV mutants (nrd1) but are substantially reduced in nrpe1 mutants (Figure 4B). **IGN5** transcript abundance is not affected in the 9b-1 mutant (Figure 4B), suggesting that Pol V’s ability to synthesize RNA is not impaired and that a step downstream of RNA synthesis might be impaired, instead.

**DISCUSSION**

In mutants lacking the 9b subunit, Pol IV-dependent siRNA biogenesis is not impaired, nor is silencing of **MRD1**, a locus whose RdDM and repression is dependent on Pol IV but not Pol V. However, loci that require both Pol IV and Pol V for silencing are derepressed in 9b-1 mutants. Based on these observations, we deduce that loss of silencing in 9b-1 mutants is due to a defect in Pol V function. Interestingly, Pol V transcription does not appear to be impaired in 9b-1 mutants, based on **IGN5** transcript production and siRNA abundance at loci where siRNA levels depend, in part, on Pol V activity. Therefore, we reason that the impairment of Pol V function in 9b mutants is not due to a decreased ability of Pol V to synthesize RNA, but an impairment of a regulatory function, possibly mediated by interactions with other proteins. Consistent with this hypothesis, the eight amino acids that are different in the 9a and 9b subunits are predicted to be exposed on the surface of the proteins, based on their homology to yeast Rpb9 (Figure 4C), whose structure is known. Figure 4D shows a space filling model in which the predicted positions of the polymorphic amino acids of 9a and 9b are mapped onto the corresponding amino acid positions of Rpb9 within a yeast Pol II elongation complex (PDB:1Y1W) (Kettenberger et al., 2004).
Rpb9 has two zinc finger domains, Zn1 and Zn2, which are located near the N and C termini of the protein. The Zn2 domain shares homology with the elongation/transcript cleavage factor, TFIIS and is thought to catalyze transcript cleavage events in partnership with TFIIS. Transcript cleavage is important for RNA 3'–end processing, transcription termination, and polymerase backtracking that allows for correction of misincorporated nucleotides, escape from an arrested state, or DNA repair at damaged sites (Awrey et al., 1997; Hemming et al., 2000; Koyama et al., 2007; Nesser et al., 2006; Walmacq et al., 2009). The Rpb9-paralogous subunits of Pols I and III, Rpa12, and Rpc11, also possess transcript cleavage activity and are stronger endonucleases than Rpb9, suggesting that Rpb9 has evolved to be regulated by TFIIS or other factors (Ruan et al., 2011). Several of the amino acid differences between NRBP(B/D/E)9a and NRBP(B/D/E)9b occur within the Zn2 domain (amino acids 77, 82, and 109). Arabidopsis has multiple genes encoding TFIIS-like proteins, leading us to speculate that the eight amino acids that differ between the 9a and 9b subunits might specify interactions with different TFIIS-like proteins, or with proteins that mediate chromatin modifications at Pol V-transcribed loci.

The single multisubunit RNA polymerases used by archaea closely resemble eukaryotic RNA Polymerase II except that they lack an Rpb9-like subunit (Werner and Grohmann, 2011); likewise, yeast strains deleted for the RPB9 gene are viable (Woychik et al., 1991). These observations have suggested that Rpb9 is an important, but nonessential, regulatory subunit in eukaryotes. However, our results show that NRBP9 function is essential in Arabidopsis. Either NRBP9a or NRBP9b (the subunits named in the context of Pol II) is sufficient for viability, but embryogenesis cannot be completed in nrpb9a nrpb9b double mutants. It is noteworthy that nrpb9a nrpb9b mutants develop further than do null mutants for catalytic subunits of Pols I, II, and III, in which female gametophytes fail to develop and are never fertilized, such that no embryogenesis takes place (Onodera et al., 2008). Therefore, NRBP9-mediated functions may be partially dispensable in plants, as in yeast and archaea, specifically at the haploid gametophytic stage of the plant life cycle. However, NRBP9 function is essential during the diploid sporophyte stage of the plant life cycle, at one or more steps required for embryo development, beginning at the globular embryo stage. NRBP9 functions must also affect later vegetative development in order to explain the distinct phenotypes of nrpb9b mutants.

Our data suggest that Pol IV functions are not impaired in 9a-1 or 9b-1 mutants. One possibility is that the ninth subunit is.
dispensable for Pol IV function. Alternatively, the 9a and 9b subunits might be redundant in the context of Pol IV, as they are for most Pol II functions. Unfortunately, the lethality of the 9a-1 or 9b-1 double mutant precludes an easy test of whether a functional ninth subunit is required for Pol IV activity.

Spectral counts of peptides detected upon mass spectrometric analyses of affinity purified Pol V V suggest that the NRPE9a and NRPE9b-containing Pol V subtypes coexist in similar abundance. Our study elucidates a functional requirement for the NRPE9b-containing Pol V subtype (Pol V9b), but not the NRPE9a-containing form of Pol V (Pol V9a). Interestingly, affinity purification of the DDR complex (DRD1, DMS3, and RDM1), which is required for Pol V transcription at IGN5 and other loci (Law et al., 2010; Wierzbicki et al., 2009), resulted in copurification of Pol V subunits that included NRPE9a (Law et al., 2010). One possibility is that Pol V9a functions only at specific loci. Alternatively, Pol V9a and Pol V9b may both be engaged at most, or all loci subjected to RdDM. If so, the genetics indicate that Pol V9a is comprehensive in its functions, explaining the dispensability of the 9a gene (and Pol V9a), whereas Pol V9b has more limited functionality. By the same logic, Pol II9b must have broader functionality than Pol II9a in order to explain the mutant vegetative phenotypes of 9b-1 mutants, the wild-type phenotypes of 9a-1 mutants, but the lethality of the double mutant.

**EXPERIMENTAL PROCEDURES**

**Plant Material**

nrp(b/d/e)9a-1 (Salk_032670) and nrp(b/d/e)9b-1 (Salk_031043) seeds were obtained from the Arabidopsis Biological Resource Center. Plants were grown on soil using an 18 h light/6 h dark cycle. Genotyping PCR primers flanking the T-DNA insertions (Salk_032670 LP: 5’ CAGACAAGAACAGTGCACTTCC, Salk_032670 RP: 5’TCTTGAAATGCACCTCTCTG, Salk_031043 LP: 5’GATA TAAAGGTGCATGGGGATATGC, Salk_031043 RP: 5’TAAACCTATTAAATCTCCCTTTG) were used in conjunction with a T-DNA left border primer (LBa1: 5’TGGTTCAGCAGTGGGCACTCG).

**RT-PCR Assays**

Total RNA was purified from leaves of 3-week old plant using Trizol reagent (Invitrogen). RQ1 DNase-treated RNA (100 ng) (Promega) was reverse transcribed using SuperScriptIII (Invitrogen). RQ1 DNase-treated RNA (100 ng) (Promega) was reverse transcribed using SuperScriptIII (Invitrogen). Primers for 9aPromF: CACCGCACTTCAACAACCCAATTACA and 9bRev: TTCCTCCAGGGTCACTAAGTT. PCR products captured in pENTR D/TOPO (Invitrogen) were recombined into pEARLEYGATE 302 (Earley et al., 2006) using LR CLonase II (Invitrogen) and transformed into nrp(b/d/e)9b-1 homozygous mutants using the floral dip method for Agrobacterium-mediated gene transfer (Clough and Bent, 1998).

**Cloning and Complementation**

A genomic clone of NRP(B/D/E)9b was obtained by PCR amplification of A. thaliana genomic DNA using Pfu Ultra DNA polymerase (Stratagene) and primers: 9bPromF: CACCGCACTTCAACAACCCAATTACA and 9bRev: TTCCTCCAGGGTCACTAAGTT. PCR products captured in pENTR D/TOPO (Invitrogen) were recombined into pEARLEYGATE 302 (Earley et al., 2006) using LR CLonase II (Invitrogen) and transformed into nrp(b/d/e)9b-1 homozygous mutants using the floral dip method for Agrobacterium-mediated gene transfer (Clough and Bent, 1998).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.celrep.2012.01.004.

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