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Subunit Compositions of the RNA-Silencing Enzymes Pol IV and Pol V Reveal Their Origins as Specialized Forms of RNA Polymerase II

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SUMMARY

In addition to RNA polymerases I, II, and III, the essential RNA polymerases present in all eukaryotes, plants have two additional nuclear RNA polymerases, abbreviated as Pol IV and Pol V, that play nonredundant roles in siRNA-directed DNA methylation and gene silencing. We show that *Arabidopsis* Pol IV and Pol V are composed of subunits that are paralogous or identical to the 12 subunits of Pol II. Four subunits of Pol IV are distinct from their Pol II paralogs, six subunits of Pol V are distinct from their Pol II paralogs, and four subunits differ between Pol IV and Pol V. Importantly, the subunit differences occur in key positions relative to the template entry and RNA exit paths. Our findings support the hypothesis that Pol IV and Pol V are Pol II-like enzymes that evolved specialized roles in the production of noncoding transcripts for RNA silencing and genome defense.

INTRODUCTION

In bacteria and Archaea, a single multisubunit RNA polymerase transcribes genomic DNA into RNA. By contrast, eukaryotes have three essential nuclear DNA-dependent RNA polymerases that perform distinct functions. For instance, 45S ribosomal RNA (rRNA) genes are transcribed by RNA polymerase I (Pol I), mRNAs are transcribed by RNA polymerase II (Pol II), and tRNAs and 5S rRNA are transcribed by RNA polymerase III (Pol III) (Grummt, 2003; Schramm and Hernandez, 2002; Woychik and Hampsey, 2002).

Bacterial DNA-dependent RNA polymerase (RNAP) is composed of only four different proteins (β′, β, ω, α; with two molecules of α in the core enzyme), but archaeal RNAP and eukaryotic Pol I, II, and III are more complex (Cramer et al., 2001; Darst et al., 1998; Hirata et al., 2008). Archaea have a fundamental subunit number of 10, with the caveat that the two largest subunits are generally split into two genes (Werner, 2007). Pol I, II, and III have 12–17 subunits that include homologs of archaeal polymerase subunits, suggesting their functional diversification from an archaeal progenitor. The crystal structures of bacterial, archaeal, and

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eukaryotic Pol II are fundamentally similar (Cramer et al., 2001; Darst et al., 1998; Hirata et al., 2008). In each case, the largest and second-largest subunits, corresponding to the β′ and β subunits of *E. coli* RNAP, respectively, are the catalytic subunits that interact to form the DNA entry and exit channels, the active site, and the RNA exit channel.

Sequencing of the *Arabidopsis thaliana* genome revealed genes for the expected catalytic subunits of Pol I, II, and III but unexpectedly revealed two atypical largest subunit genes and two atypical second-largest subunit genes (reviewed in Pikaard et al., 2008). Moreover, five subunits of Pol I, II, and III that are typically encoded by single genes in yeast and mammals, namely *RPB5, RPB6, RPB8, RPB10,* and *RPB12* (named according to their discovery as Pol II subunits; aka *R*NA Polymerase *B*) (Cramer, 2002; Werner, 2007), are encoded by multigene families in *Arabidopsis*, as are the Pol II-specific subunits *RPB3, RPB4, RPB7,* and *RPB9*. The functional significance of the extensive subunit diversity in plants is unclear.

The genes encoding the atypical largest and second-largest polymerase subunits in *Arabidopsis* are not essential for viability (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005), unlike their Pol I, II, or III counter-parts (Onodera et al., 2008). However, the atypical catalytic subunits are nuclear proteins (Onodera et al., 2005; Pontes et al., 2006) required for siRNA-directed DNA methylation and silencing of retrotransposons, endogenous repeats, and transgenes (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005). The atypical catalytic subunit genes also play roles in the short-range or long-distance spread of RNA-silencing signals, responses to biotic and abiotic stresses, and the control of flowering time (Borsani et al., 2005; Brosnan et al., 2007; Dunoyer et al., 2007; Katiyar-Agarwal et al., 2007; Pontier et al., 2005; Smith et al., 2007). The atypical largest subunit genes are *NRPD1* and *NRPE1*. NRPD1 (formerly NRPD1a) is the largest subunit of Nuclear RNA polymerase IV (Pol IV; formerly Pol IVa) (Herr et al., 2005; Onodera et al., 2005), whereas NRPE1 (formerly NRPD1b) is the largest subunit of Pol V (formerly Pol IVb) (Kanno et al., 2005; Pontier et al., 2005). The second-largest subunits of Pol IV and Pol V are encoded by the same gene, designated by the synonymous names *NRPD2a* (*NRPD2* for simplicity) or *NRPE2* (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005). Pol IV and Pol V are functionally distinct, with Pol IV required for siRNA production and Pol V generating noncoding transcripts at target loci (Wierzbicki et al., 2008). Our current model is that siRNAs bind to Pol V nascent transcripts to bring the silencing machinery to the vicinity of the chromatin at target loci (Wierzbicki et al., 2008).

Aside from their largest and second-largest subunits, the subunit compositions of Pol IV and Pol V are unknown. Here, we show that Pol IV and Pol V have subunit compositions characteristic of Pol II but make differential use of RPB3, RPB4, RPB5, and RPB7 family variants in addition to having distinct catalytic subunits. Collectively, our results support the hypothesis that Pol IV and Pol V are RNA Pol II derivatives whose molecular niche is the production of noncoding transcripts for RNA-mediated silencing.

RESULTS

Identification of Pol IV, V, and II Subunits Using LC-MS/MS

To affinity purify Pol IV and Pol V from *Arabidopsis thaliana*, we engineered full-length *NRPD1 (NRPD1a)* and *NRPE1 (NRPD1b)* genomic clones, including their promoter regions and complete sets of introns and exons, adding a FLAG epitope tag to the protein's C terminus. The transgenes rescue the loss of RNA-directed DNA methylation in their respective null mutants (*nrpd1a-3* or *nrpd1b-11*), indicating that the recombinant proteins are functional (Pontes et al., 2006). NRPD1-FLAG and NRPE1-FLAG, and their respective associated subunits, were affinity purified on anti-FLAG resin, and tryptic peptides were identified by using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). For both

Pol IV and Pol V, their two known catalytic subunits were detected, as expected. However, in each case, ten additional previously unknown subunits were identified, corresponding to the ten noncatalytic subunits of yeast RNA Pol II: RPB3, RPB4, RPB5, RPB6, RPB7, RPB8, RPB9, RPB10, RPB11, and RPB12 (Figure 1; see Table S1 and Figures S1 and S2, available online). The pairs of catalytic subunits specific to RNA Pol I, II, or III were not detected in Pol IV or Pol V samples, ruling out copurification of these polymerases as an explanation for the noncatalytic subunits detected in affinity-purified Pol IV or Pol V. Likewise, coimmunoprecipitation (colP) data show that Pol IV and Pol V do not associate with each other or with Pol I, II, or III (Figure 2A).

For Pol V, peptide sequence data typically allowed unambiguous identification of subunits that are members of protein families (see Figure S1 for peptide coverage maps and Figures S4–S12 for family alignments). An exception was the RPB8 family, for which the sole peptide identified matched both variants, which are 96% identical. Two RPB3-related variants that are 88% identical are present in *Arabidopsis*, and both proteins are detected in Pol V, resulting in their designation as NRPE3a and NRPE3b (Figure 1› Figure 3A). The single RPB11 subunit encoded by the *Arabidopsis* genome was also detected; hence we refer to this protein as NRPE11 (Figure 1). Of six homologs of RPB5 in the genome, only one (NRPE5) is detected in Pol V (Figure 1› Figure S5). Two RPB9-like subunits were identified in Pol V (Figures 1 and 2D). These proteins, designated NRPE9a and NHPE9b, are 92% identical. There are four RPB7 homologs in *Arabidopsis*, only one of which is detected in Pol V, NRPE7. One of two RPB4-like subunits (NRPE4), one of two RPB10-like subunits (NRPE10), one of two RPB12-like subunits (NRPE12), and one of two RPB6-like subunits (NRPE6a) were also detected in Pol V (Figure 1).

Analysis of Pol IV's subunit composition revealed similarities and differences compared to Pol V (Figure 1, Figure S2). As with Pol V, peptides for the single RPB11-like subunit were identified. In the context of Pol IV, we refer to this protein as NRPD11; in the context of Pol V, we refer to this same protein as NRPE11. Similar nomenclature rules were adopted for other subunits shared by more than one polymerase (see Figure 1 for synonyms). NRPD4, NRPD6a, NRPD8b, and NRPD10 subunits were unambiguously identified (Figure 1). Similar to Pol V, both RPB3-like variants were detected in Pol IV, but one is predominant (NRPD3; see Figure 1). Interestingly, the RPB5-like subunit of Pol IV, NRPD5, is identical to the previously identified NRPB5 subunit of Pol II but differs from the NRPE5 subunit of Pol V (Figure 1) (Larkin et al., 1999). The major NRPD7 subunit detected in Pol IV is 62% identical to the Pol V NRPE7 subunit, but low-level peptide sequence coverage for the NRPE7 subunit was detected as well. The Pol IV NRPD9b subunit corresponds to NRPE9b detected in Pol V (Figures 1 and 2D).

The significant number of Pol II-like subunits in Pol IV and Pol V raised questions concerning the relative similarities of Pol II, Pol IV, and Pol V. Therefore, we affinity purified *Arabidopsis* Pol II by exploiting epitope-tagged NRPB2 (*NRPB2-FLAG*) expressed from a transgene that rescues the *nrpb2-1* null mutant (Onodera et al., 2008). LC/MS-MS revealed 12 subunits orthologous to their 12 yeast Pol II counterparts, with no contaminating subunits specific to Pol I, III, IV, or V (Figure 1, Figure S3). The same RPB10, RPB11, and RPB12 family subunits found in Pol IV and/or Pol V are present in Pol II (Figure 1). Sequenced peptide coverage for the RPB6, RPB8, and RPB9-like subunits in the Pol II dataset revealed that each of the two genes for these subunits encodes a subunit incorporated into Pol II (Figure S3), suggesting that the genes are redundant. A single RPB3-like subunit, NRPB3, is predominant

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, three tables, and 18 figures and can be found with this article online at http://www.molecule.org/supplemental/S1097-2765(08)00858-7.

in Pol II, consistent with a previous report (Ulmasov et al., 1996). However, peptides corresponding to the NRPE3b subunit were also detected at low frequency. The single RPB5 subunit identified in Pol II corresponds to the expected subunit based on a previous study (Larkin et al., 1999) and is identical to the NRPD5 subunit of Pol IV but distinct from the NRPE5 subunit of Pol V. Pol II also makes use of RPB4 and RPB7 variants that are distinct from the corresponding Pol IV and Pol V subunits. These NRPB4 and NRPB7 subunits correspond to subunits previously shown to associate with Pol II (Larkin and Guilfoyle, 1998).

Immunological Confirmation of Subunit Associations

To test subunit associations with all five nuclear RNA polymerases, we exploited *Arabidopsis* lines expressing FLAG-tagged Pol I, II, and III second-largest subunits (NRPA2-FLAG, NRPB2-FLAG, or NRPC2-FLAG) or FLAG-tagged Pol IV and Pol V largest subunits (NRPD1-FLAG, NRPE1-FLAG), each expressed from trangenes that rescue corresponding null mutants (Onodera et al., 2008; Pontes et al., 2006). Plants expressing FLAG-tagged genomic clones of *NRPE6a*, *NRPE8b*, *NRPE10*, or *NRPE11* or an *NRPE5* cDNA were also engineered. Each recombinant protein could be immunoprecipitated from transgenic plants and detected by immunoblotting using anti-FLAG antibody (Figure 2A). Probing immunoblots with antibodies for NRPE1 and NRPE2 (Onodera et al., 2005) revealed that these Pol V catalytic subunits are present in NRPE1, NRPE6a, NRPE8b, NRPE10, NRPE11, and NRPE5 immunoprecipitates (Figure 2A; see also the anti-NRPE1 specificity control in Figure 2B), consistent with the detection of all of these subunits in Pol V (Figure 1). Controls show that NRPE2 and NRPE1 do not coimmunoprecipitate with Pol I, II, or III; that NRPE1 does not coimmunoprecipitate with Pol IV; and that NRPE2/NRPD2 is present in Pol IV and Pol V, as expected. The anti-NRPE1 antibody consistently reveals multiple NRPE1 isoforms (Figures 2A and 2B); whether these are degradation, posttranslational modification, or alternative splicing products is unclear.

To test whether NRPE5, NRPE6a, NRPE8b, NRPE10a, and NRPE11 subunits are shared by Pol I, II, and/or III, we used an anti-peptide antibody recognizing an invariant sequence in the Pol I, II, and III second-largest subunits (Onodera et al., 2005); this antibody fails to crossreact with NRPE2/NRPD2 due to a single amino acid substitution. In NRPE6a, NRPE8b, NRPE10, and NRPE11 immunoprecipitated fractions, Pol I, II, or III second-largest subunits are detected, consistent with the LC-MS/MS analysis of Pol II (Figures 1 and 2A). In yeast, RPB6, RPB8, and RPB10 are common to Pol I, II, and III, but RPB11 is Pol II specific. Second-largest subunits of Pol I, II, or III do not coimmunoprecipitate with FLAG-NRPE5, showing that NRPE5 is not a subunit of the essential polymerases (Figure 2A).

The LC-MS/MS data indicate that either of the two RPB8 homologs associate with Pol V. ColP analysis confirms that NRPE8a or NRPE8b will coimmunoprecipitate with the Pol V catalytic subunits (Figures 2A and 2E). Although LC-MS/MS identified only one RPB6 variant (NRPE6a), its paralog (NRPE6b) can also associate with Pol V in vivo (Figure 2C). Both Pol II clade RPB9-like subunits (Figure 2D) were detected in Pol V by LC-MS/MS. ColP analysis confirms that FLAG-NRPE9a associates with the Pol V NRPE1 and NRPE2 catalytic subunits in vivo (Figures 2C and 2D). NRPE6b and NRPE9a also coimmunoprecipitate the second-largest subunits of Pol I, II, or III (Figure 2C).

LC-MS/MS analysis of Pol V identified both potential RPB3 variants (Figure 3A). In confirmation of this result, HA-tagged NRPE3a and NRPE3b both coimmunoprecipitate the Pol V catalytic subunits (Figure 3B). NRPE3a, but not NRPE3b, also coimmunoprecipitates a subunit recognized by the antibody specific for Pol I, II, or III second subunits (Figure 3B); we deduce this to be the Pol II NRPB2 subunit because Pol I and Pol III use third-largest

subunits distinct from RPB3. Moreover, the gene encoding NRPE3a was previously shown to encode a NRPB3 (see Figure 1) subunit present in purified Pol II (Ulmasov et al., 1996).

NRPE11, NRPE6a, NRPE8b, NRPE10, and NRPE9a all coimmunoprecipitate with the Pol IV and Pol II largest subunits (Figures 1 and 4A). Upon immunoprecipitation of NRPE3b, no Pol II is detected in the immunoprecipitated fraction using an antibody recognizing the C-terminal domain (CTD) of the largest subunit. Likewise, Pol IV is detected in only trace amounts using the anti-NRPD1 antibody. We conclude that NRPE3b is used almost exclusively by Pol V (Figures 1 and 4A). In contrast, NRPB3, NRPD3, and NRPE3a are encoded by the same gene. Controls show that the NRPD1 subunit of Pol IV does not coimmunoprecipitate with Pol I, II, III, or V (Figure 4A). Likewise, the NRPB1 subunit of Pol II does not coimmunoprecipitate with Pol I, III, IV, or V (Figure 4A).

Using antibodies specific for NRPB5/NRPD5 or NRPE5 (Larkin et al., 1999), we tested their associations with FLAG-tagged Pol I, II, III, IV, or V (Figures 4B and 4C). Controls show that the NRPD2/NRPE2 subunit common to both Pol IV and Pol V is detected in NRPD1 and NRPE1 IPs, as expected, but not in Pol I, II, or III IPs (Figures 4B and 4C). NRPE5 was detected only in the NRPE1-FLAG immunoprecipitated fraction (Figure 4B), confirming that this subunit is unique to Pol V. By contrast, the NRP85/NRPD5 subunit is detected in Pol I, II, III, and IV fractions, but not in Pol V (Figure 4C), in agreement with the LC-MS/MS data and previous studies showing that NRPB5/NRPD5 copurifies with Pol I, II, and III (Larkin et al., 1999) (Saez-Vasquez and Pikaard, 1997).

We affinity purified FLAG-tagged NRPE5 expressed in the *nrpe5* mutant background and identified the associated RNA polymerase subunits using LC-MS/MS. The results confirmed association of NRPE5 with all Pol V subunits except NRPE7 (Table S2, Figure S18), which most likely escaped detection in this experiment due to insufficient sample mass.

Collectively, the immunological tests of Figures 2–4 confirm the Pol V association of the NRPE1, NRPE2, NRPE3a, NRPE3b, NRPE5, NRPE6a, NRPE8, NRPE9a, NRPE10, and NRPE11 subunits detected by LC-MS/MS. Likewise, the immunological tests confirm the Pol IV associations of NRPD1, NRPD2, NRPD3, NRPD5, NRPD6a, NRPD8b, NRPD9a, NRPD10, and NRPD11. Pol IV and Pol V subunits that are shared with Pol II were also confirmed immunologically.

NRPE5 Is Required for DNA Methylation, siRNA Accumulation, and Gene Silencing at Pol V-Regulated Loci

Of the five full-length homologs of yeast *RPB5* in *Arabidopsis*, RT-PCR analysis shows that only *NRPB5/NRPD5* and *NRPE5* are constitutively expressed; other family members show organ-specific expression patterns (Figure 5A, Figures S5 and S13). Homozygous *nrpe5-1* mutants resulting from a T-DNA insertion (Figure 5B) are viable, as are Pol V *nrpe1* and *nrpe2* mutants. In contrast, homozygous *nrpd5-1/nrpb5-1* T-DNA insertion mutants were not recoverable due to female gametophyte lethality, as shown by reciprocal genetic crosses (Figures S14A and S14B). Female gametophyte lethality is a characteristic of Pol I, II, and III mutants, as demonstrated previously for *nrpa2*, *nrpb2*, *nrpc2*, and *nrpb12* (Onodera et al., 2008). A homozygous *nrpe11* T-DNA insertion mutant was also unrecoverable, consistent with this gene also encoding the Pol II subunit, NRPB11 (Figures S14A and S14B).

Like Pol IV and Pol V catalytic subunit mutants, *nrpe5-1* mutants lack obvious morphological phenotypes but flower later than wild-type plants under short-day conditions (Figure 5C), similar to mutants disrupting the 24 nt siRNA-directed DNA methylation pathway, including *RNA-DEPENDENT RNA POLYMERASE 2 (RDR2)* and *DICER-LIKE 3 (DCL3)* mutants (Chan et al., 2004; Liu et al., 2007; Pontier et al., 2005). Comparison of *nrpe5* and wild-type

individuals suggests that the delay in flowering is stochastic, with some individuals showing substantial delays and others flowering at the same time as wild-type plants (Figure S15).

We tested *nrpe5-1* mutants for Pol V-dependent molecular phenotypes, including DNA hypermethylation at 5S rRNA gene clusters and at *AtSN1* and *AtSN2* retroelements. In *nrpd1* (*nrpd1a-3*),*nrpe1* (*nrpd1b-11*), and *nrpd2/nrpe2* mutants, loss of methylation at 5S rDNA repeats results in increased digestion by the methylation-sensitive restriction endonucleases *Hpa*II and *Hae*III compared to wild-type plants (Figure 5D). In the *nrpe5* mutant, methylation at 5S rRNA genes is reduced compared to wild-type, but to a lesser extent than in *nrpe1* or *nrpd2/nrpe2* mutants (Figure 5D). Transformation of the *nrpe5-1* mutant with a *35S:FLAG-NRPE5* transgene restores methylation to wild-type levels, as shown in three independent transgenic lines (Figure 5D).

To test whether *nrpe5* affects DNA methylation at other Pol V-dependent loci, we examined the SINE retrotransposon families, *AtSN1* and *AtSN2* (Myouga et al., 2001). In wild-type plants, *AtSN1* and *AtSN2* elements are heavily methylated such that their DNA is not cut by *Hae*III and a PCR product can be obtained (Figures 5E and 5F). In *nrpe1* and *nrpe2/nrpd2* mutants, however, methylation is lost such that *Hae*III cuts and PCR amplification fails (Figures 5E and 5F). In *nrpe5-1*, decreased *AtSN1* and *AtSN2* methylation occurs, but not as severely as in *nrpe1* or *nrpe2/nrpd2* mutants. Nonetheless, the decreased methylation in *nrpe5-l* plants is rescued by a *35S:FLAG-NRPE5* transgene (Figures 5E and 5F).

RNA-directed DNA methylation silences *AtSN1* retroelements in wild-type plants such that loss of methylation correlates with increased *AtSN1* transcription (Hamilton et al., 2002; Herr et al., 2005; Kanno et al., 2005). *AtSN1* transcripts are barely detectable in wild-type plants but are abundant in *nrpe5* mutants, as in *nrpe1* or *nrpe2/nrpd2* mutants (Figure 5G). In the *nrpe5-1* genetic background, the *35S:FLAG-NRPE5* transgene restores *AtSN1* silencing (Figure 5G). Collectively, these results demonstrate that *NRPE5* is important for DNA methylation and silencing of *AtSN1* elements.

In the RNA-directed DNA methylation pathway, Pol IV is required for 24 nt siRNA production (Herr et al., 2005; Onodera et al., 2005) such that siRNAs are eliminated in *nrpd1* and *nrpd2* mutants (Figure 5H). In contrast, siRNAs in *nrpe1* mutants are reduced but not eliminated at 5S rRNA genes and *COP1A* elements (Figure 5H). Consistent with a Pol V mutant phenotype, siRNAs are reduced in *nrpe5* mutants relative to wild-type and are restored by the *35S:FLAG-NRPE5* transgene (Figure 5H). MicroRNA and *trans*-acting siRNA levels are unaffected in *nrpe5*, *nrpd1*, or *nrpe1* mutants, consistent with the lack of Pol IV or Pol V involvement in these pathways.

Crystallographic studies indicate that yeast RPB5 is composed of an N-terminal jaw domain and a C-terminal assembly domain separated by a short linker (Figures S5, S16, and S17A). These domains appear to be conserved in nearly all plant RPB5 homologs (Figure S16). A feature of *Arabidopsis* NRPE5, and its presumptive orthologs in other plants, is a short N-terminal extension compared to NRPB5 (Figure S16 and S17A). To test the functional significance of this N-terminal extension, we created a *35S:FLAG-ΔN-NRPE5* construct in which the extension was deleted (Figure S17A). This transgene fails to rescue *nrpe5-1* mutant phenotypes (Figures S17B–S17D). Surprisingly, immunoprecipitation of equal volumes of soluble extracts revealed that the FLAG-ΔN-NRPE5 protein is present at very low levels relative to full-length FLAG-NRPE5, despite similar transcript levels (Figure S17E). These data suggest that the N-terminal extension is important for the stability of the NRPE5 protein in vivo, possibly because the extended sequence facilitates Pol V-specific subunit interactions.

DISCUSSION

Origins of Pol V

Pol IV and Pol V are plant-specific enzymes that appear to have originated in an algal progenitor of land plants several hundred million years ago (Luo and Hall, 2007). Their specific involvement in siRNA-mediated transcriptional gene silencing, which also occurs in other metazoans and fission yeast, has begged the question as to which polymerases accomplish the functions of Pol IV and Pol V in other eukaryotes. In fission yeast, Pol II transcripts traverse silenced loci, serving as binding sites for siRNAs and as templates for the sole RNA-dependent RNA polymerase, thereby generating precursors for further siRNA biogenesis (Buhler and Moazed, 2007; Buhler et al., 2006; Grewal and Elgin, 2007; Irvine et al., 2006). Several nonlethal mutations that disrupt siRNA-mediated silencing and/or siRNA accumulation in *S. pombe* have been mapped to the RPB1, RPB2, and RPB7 subunits of Pol II (Djupedal et al., 2005; Kato et al., 2005; Schramke et al., 2005). Our finding that Pol IV and V have Pol II-like subunit compositions fits the hypothesis that Pol IV and Pol V are derivatives of Pol II that evolved specialized roles in RNA silencing but no longer perform Pol II functions essential for viability, in contrast to fission yeast Pol II, which appears to accomplish all of these tasks. Presumably, the subunits of Pol IV/V that are not shared by Pol II, including NRPD1, NRPE1, NRPD2/NRPE2, NRPE3b, NRPD4/NRPE4, NRPE5, NRPD7, and NRPE7, account for Pol IV- or Pol V-specific activities. It is intriguing that most of these subunits occupy key positions with regard to the template channel and RNA exit paths (Figures 6A and 6B).

Previous analyses of Pol IV and Pol V catalytic subunits had pointed to a Pol II connection. In our initial study of Pol IV, we noted that the NRPD2/NRPE2 subunit is more closely related to the second-largest subunit of Pol II than to the corresponding subunits of Pol I or Pol III (Onodera et al., 2005). Moreover, five out of eight intron positions in the beginning of *NRPD1* and *NRPE1* match the intron positions in *NRPB1*, encoding the largest subunit of Pol II (Luo and Hall, 2007). Based on phylogenetic analyses, Luo and Hall proposed that Pol IV came into existence following a duplication of the *NRPB1* gene that generated the *NRPD1* gene. A subsequent duplication of *NRPD1* to generate *NRPE1* is proposed to have led to the evolution of Pol V after the emergence of land plants but prior to the divergence of angiosperms (flowering plants). Our finding that Pol IV utilizes the same RPB5-family subunit as Pol I, II, and III whereas Pol V uses a distinct variant (NRPE5) is consistent with the hypothesis that Pol V is more distantly related to Pol II than is Pol IV.

The fact that Pol IV and Pol V share numerous small subunits with Pol II, including NRPB3, NRPB6, NRPB8, NRPB9, NRPB10, NRPB11, and NRPB12 family subunits, can explain why alleles for these genes have not been identified in genetic screens; loss-of-function mutations in the subunits of essential polymerases cause female gametophyte lethality (Figure S14) (Onodera et al., 2008). Likewise, the use of more than one NRPE3, NRPE6, NRPE8, or NRPE9 variant by Pol IV or Pol V (Figures 6C and 1) can be expected to make identification of mutations in these genes problematic due to functional redundancies (Figure 6C).

Functions for Mystery Subunits

A number of observations in our study fill in gaps concerning the functions of RNA polymerase subunit families in *Arabidopsis*. For instance, Ulmasov et al. reported the existence of two *RPB3*-like genes in *Arabidopsis*, which they named *AtRPB36a* and *AtRPB36b* based on their predicted sizes of ~36 kD (Ulmasov et al., 1996). AtRPB36a was found in highly purified Pol II fractions (Ulmasov et al., 1996), but AtRPB36b was not, making the function of the latter variant unclear. Our study reveals that AtRPB36b is the NRPE3b subunit of Pol V. AtRPB36a (now NRPB3) and NRPB11 (formerly AtRPB13.6) in Pol II are the homologs and functional equivalents of the two α subunits (α and α′) of *E. coli* RNA polymerase. Previous studies

demonstrated that NRPB3 and NRPB11 copurify with Pol II in vivo and physically interact in yeast two-hybrid assays (Ulmasov et al., 1996). Interestingly, AtRPB36b/NRPE3b also interacted with NRPB11 in yeast two-hybrid assays (Ulmasov et al., 1996), which is likely to be meaningful, occurring in the context of Pol V in a manner equivalent to the interaction of NRPB3 and NRPB11 in Pol II. Interestingly, the AtRPB36a variant also associates with Pol V in vivo; therefore, this protein serves as the NRPB3 subunit of Pol II, the NRPD3 subunit of Pol IV, and one of two alternative Pol V NRPE3 subunits (NRPE3a). How these highly similar RPB3-like subunits are differentially assembled into Pol II, IV, or V is a question deserving further study.

Although peptide coverage for the NRPD4/NRPE4 subunit was low in our study, the Jian-Kang Zhu laboratory identified the *nrpd4/nrpe4* gene in a screen for defective RNA-directed DNA methylation and confirmed the Pol IV and Pol V association of the encoded protein (He, X.-J., Hsu, Y.-F., Pontes, O., Zhu, J., Lu, J., Bressan, R.A., Pikaard, C., Wang, C.-S., and Zhu, J.-K., unpublished data). In budding yeast, RPB4 forms a subcomplex with RPB7 that can be dissociated from the ten subunit Pol II core enzyme without abolishing Pol II catalytic activity in vitro (Cramer, 2004), although the subcomplex appears to be more stable in Pol II from plants (Larkin and Guilfoyle, 1998). In vivo, RPB7 is an essential protein in yeast, whereas RPB4 deletion mutants are temperature sensitive (McKune et al., 1993; Woychik and Young, 1989) and are impaired in transcription elongation and mRNA 3′ end processing (Runner et al., 2008; Verma-Gaur et al., 2008). It is intriguing that Pol II, IV, and V have unique RPB7-like subunits and that the NRPB4 subunit of Pol II is different from the NRPD4/NRPE4 subunits of Pol IV and Pol V. Given that the RPB4/RPB7 complex is thought to interact with the nascent RNA transcript (see Figure 6), these differences are likely to contribute to the unique functions of Pol II, IV, and V.

Previous studies had shown that one of the two consitutively expressed RPB5 family proteins is a subunit by Pol I, II, and III (Larkin et al., 1999; Saez-Vasquez and Pikaard, 1997). The function of the other variant, formerly designated AtRPB5b or AtRPB23.7, was unknown. Our study reveals that the latter protein is the NRPE5 subunit of Pol V. By contrast, the NRPD5 subunit of Pol IV is encoded by the same gene that encodes the Pol II NRPB5 subunit and the equivalent subunits of Pol I and III. As we have shown, *nrpe5-1* mutants display defects in DNA methylation, retroelement silencing, siRNA accumulation, and flowering time, similar to *nrpe1* mutants (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005). However, *nrpe5-1* mutant phenotypes are typically less severe than *nrpe1* or *nrpe2/nrpd2* mutants. Because the T-DNA insertion is near the 3′ end of the gene, *nrpe5-1* may be a partially functional allele. It is also possible that other members of the multigene family are partially redundant with *NRPE5*, particularly *At2g41340*, which shares 70% identity with NRPE5, including the N-terminal extension that is missing in the NRPB5/NRPD5 subunit (Figure 5A and Figure S5). Consistent with this hypothesis, preliminary evidence suggests that a *nrpe5-1 At2g41340* double mutant has a more severe loss of DNA methylation phenotype than does *nrpe5-1* (data not shown). A third possibility is that NRPE5 may not be absolutely required for Pol V transcription. The failure to identify *nrpe5* alleles in genetic screens to date may stem from one or more of these reasons.

The fact that Pol V is unique in using the NRPE5 variant of the RPB5 family is likely to have functional significance. Crystal structures of yeast Pol II reveal that RPB5 Interacts with RPB1 and RPB6 to form a mobile “shelf” module that stabilizes the template DNA as it enters the polymerase (Cramer et al., 2001; Gnatt et al., 2001). RPB5 also interacts with hepatitis B transcriptional activator protein X (HBx); the general transcription factor TFIIB; TIP120, a protein which facilitates recruitment of Pol II to the preinitiation complex (Cheong et al., 1995; Lin et al., 1997; Makino et al., 1999); and the yeast chromatin remodeling complex, RSC (Soutourina et al., 2006). Therefore, the differential use of the NRPD5 or NRPE5 subunits in

the context of Pol IV or Pol V could mediate different template specificity, locus targeting, or transcriptional activation processes.

EXPERIMENTAL PROCEDURES

Plant Materials

A. thaliana nrpd1 (allele *nrpd1a-3*), *nrpe1* (allele *nrpd1b-11*), and *nrpd2/nrpe2* (*nrpd2a-2 nrpd2b-1*) have been described (Pontes et al., 2006). *nrpe11-1* (*nrpb11-1/nrpd11-1*) is from T-DNA line SALK_100563 (Alonso et al., 2003), *nrpd5-1/nrpb5-1* from T-DNA line SAIL_786_E02 (Sessions et al., 2002), and *nrpe5-1* from GABI-KAT T-DNA line 237A08 (Rosso et al., 2003). Primers for *nrpe11-1*, *nrpd5-1*, and *nrpe5-1* genotyping are listed in Table S3. Callus cultures were induced by germinating sterilized seeds on MS media containing Gamborg's vitamins (Sigma), 5% agargel (Sigma), 0.02 mg/L kinetin (Sigma), and 2 mg/L 2,4-dichlorophenoxyacetic acid (Sigma). Plates were incubated at 23°C. Callus frozen in liquid N_2 was stored at −80°C.

Affinity Purification of Pol IV, V, and II

Frozen callus (115–150 g) expressing FLAG-tagged *NRPE1* or *NRPD1* was ground in extraction buffer (300 mM NaCl, 20 mM Tris [pH 7.5], 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, and 1:100 plant protease inhibitor cocktail [Sigma]) at 4°C, filtered through two layers of Miracloth (Calbiochem), and centrifuged twice at 10,000 g, 15 min, 4°C. Pol II and NRPE5 were purified with the same protocol from 150 g of leaf tissue expressing FLAG-tagged *NRPB2* or *NRPE5*, respectively. Supernatants were incubated with anti-FLAG-M2 resin for 2–3 hr in a 15 ml tube using 30 µl of resin per 14 ml of extract. Resin was pelleted at 1000 rpm for 2 min and the supernatant incubated with fresh resin for 2–3 hr. Pooled resin was washed five times in 14 ml of extraction buffer containing 0.4% NP-40 (Sigma). Aliquots (125 µl) of resin were then mixed 2 min with 125 µl Ag/Ab Elution Buffer (Pierce) at 4°C. Resin was pelleted, and the eluted complex was pooled. Two ~500 µl batches of pooled complex were concentrated in YM-10 centricon columns (Millipore) at 4°C and desalted using Pierce 500 µl desalting columns. The final elute of ~70 µl containing ~10–50 µg of protein was subjected to LC-MS/MS.

Mass Spectrometry

Samples adjusted to 50% (v/v) 2,2,2-Trifluoroethanol (TFE) (Sigma) were sonicated 1 min at 0°C and then incubated 2 hr at 60°C with shaking at 300 rpm. Proteins were reduced with 2 mM DTT at 37°C for 1 hr, then diluted 5-fold with 50 mM ammonium bicarbonate. $CaCl_2$ (1 mM) and sequencing-grade modified porcine trypsin (Promega) was added at a 1:50 trypsin-to-protein mass ratio. After 3 hr at 37°C, samples were concentrated to ~30 µl and subjected to reversed-phase liquid chromatography (RPLC) coupled to an electrospray ionization source and LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific). Tandem mass spectra were searched against *A. thaliana* proteins using SEQUEST and filtering criteria, which provided a false discovery rate (FDR) <5%. See the Supplemental Data for details.

Cloning, Vectors, and Transgenic Lines

NRPD1 and *NRPE1* genomic clones (Pontes et al., 2006) were cloned into a Gateway-compatible vector (A.W. and C.S.P., unpublished data) that adds a C-terminal FLAG tag, 3C protease cleavage site, and biotin ligase recognition peptide. *NRPE5*, *NRPE6a*, *NRPE6b*, *NRPE8a*, *NRPE9a*, *NRPB7*, *NRPE3a*, and *NRPE3b* cDNAs were amplified by RT-PCR from poly-T primed cDNA cloned into pENTR-D-TOPO or pENTR-TEV-TOPO. cDNAs were recombined into pEarleyGate 201 (HA tag) or 202 (FLAG tag) (Earley et al., 2006). Genomic *NRPE8b*, *NRPE10*, *NRPE11* and *NRPE6a* clones were similarly amplified by PCR and cloned

into pEarleyGate 302 (FLAG tag). *NRPD1-FLAG, NRPE1-FLAG, NRPA2-FLAG, NRPB2-FLAG, and NRPC2-FLAG* transgenes were previously described (Onodera et al., 2008; Pontes et al., 2006).

Methylation Assays

5S rDNA Southern blot methylation assays and *AtSN1* PCR assays were performed using 250 ng−1 μg of DNA as in Onodera et al. (2005).

RT-PCR Analysis of *AtSN1*

For *AtSN1* transcripts, high-molecular-weight RNA was isolated from 300 mg of leaves using a miRVANA (Ambion) kit, and strand-specific RT-PCR was performed as described (Wierzbicki et al., 2008).

Small RNA Northern Blots

Inflorescence small RNA (7.5 μg) was analyzed by northern blot hybridization using *COPIA*, siR1003 (5S rRNA), 45S rRNA, miR173, and tasiR255 probes as described previously (Allen et al., 2005; Onodera et al., 2005; Pontes et al., 2006; Xie et al., 2004). Blots stripped twice with 50% formamide, 0.1 × SSC, and 1% SDS at 65°C for 2 hr were reprobed to generate multiple figure panels.

Antibodies

Anti-NRPE2/NRPD2, anti-NRPB5/NRPD5, and anti-NRPE5 have been described (Larkin et al., 1999; Onodera et al., 2005). Anti-FLAG antibodies were from Sigma. Anti-NRPB1-CTD (8WG16) was purchased from Abcam. NRPE1 antibodies (Covance) recognize peptide N-CDKKNSETESDAAAWG-C. NRPD1 antibodies (Covance) recognize peptide N-CLKNGTLESGGFSENP-C. Anti-NRPA2/NRPB2/NRPC2 antibodies (US Biologicals) recognize N-CGDKFSSRHGQKG-C. Antibodies were affinity purified using immobilized peptides.

Immunoprecipitation and Immunoblotting

Leaves (2–4 g) were ground in extraction buffer (Baumberger and Baulcombe, 2005), filtered through Miracloth, and centrifuged at 10,000 g for 15 min. Supernatants were incubated 3–12 hr at 4°C with 30 μl of anti-FLAG-M2 resin (Sigma). Beads were washed three times in extraction buffer + 0.5% NP-40 (Sigma) and eluted with two bed volumes of 2× SDS sample buffer, and 5–20 μl was subjected to SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore). Blots were incubated with antibodies in TBST + 5% (w/v) nonfat dried milk. Antibody dilutions were as follows: 1:250 (NRPE1), 1:500 (NRPD1), 1:2000 (NRPB1-CTD), 1:750 (NRPB5/NRPD5), 1:750 (NRPE5), 1:250 (NRPD2/NRPE2), 1:500 (anti-Pol I, II, and/or III) and 1:2000–1:10,000 (FLAG-HRP). The secondary antibody was anti-rabbit-HRP, diluted 1:5000–1:20,000; or anti-mouse-HRP, diluted 1:5000 (GE Healthcare, Sigma). Blots were washed four times for 4 min in TBST and visualized by chemiluminescence (GE Healthcare). Blots were stripped for 35 min in 25 mM glycine (pH 2.0), 1% SDS: re-equilibrated in TBST; and probed with additional antibodies.

Alignments

Sequences were aligned using ClustalW and highlighted using BOXSHADE. Construction of phylogenetic trees was performed using MegAlign. Trees are based on ClustalW alignments of full-length proteins, and bootstrap values are based on 10,000 replicates. Dotted lines represent negative branch lengths.

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Function	Bacteria	Archaea	Sc Pol II	At Homologs	At Pol II	At Pol IV	At Pol V	Names/Synonyms
Catalytic	ß'	RPOA' RPOA"	RPB1	At4g35800	59			NRPB1
				At1g63020		58		NRPD1
				At2g40030			74	NRPE1
	ß	RPOB' RPOB"	RPB2	At4g21710	63			NRPB2
				At3g23780		18	37	NRPD2/NRPE2
Assembly	α	RPOD	RPB3	At2g15430	57	28	45	NRPB3/NRPD3/NRPE3a
				At2g15400	4	4	41	NRPE3b
	α	RPOL	RPB11	At3g52090	75	56	68	NRPB11/NRPD11/NRPE11
		RPON	RPB10	At1g11475	55	54	55	NRPB10/NRPD10/NRPE10
				At1g61700				NRPB10-like
		RPOP	RPB12	At5g41010	16	16	16	NRPB12/NRPD12/NRPE12
				At1g53690				NRPB12-like
Auxillary	ω	RPOK	RPB6	At5g51940	15	15	15	NRPB6a/NRPD6a/NRPE6a
				At2g04630	15	*	*	NRPB6b/NRPE6b
		RPOG	RPB8	At1g54250	30	*	*	NRPB8a/NRPE8a
				At3g59600	30	18	*	NRPB8b/NRPD8b/NRPE8b
		RPOH	RPB5	At3g22320	63	15		NRPB5/NRPD5
				At3g57080			39	NRPE5
				At5g57980				NRPB5-like
				At2g41340				NRPE5-like
				At3g54490				NRPE5-like
		RPOF	RPB4	At5g09920	61			NRPB4
				At4g15950		13	8	NRPD4/NRPE4
		RPOE	RPB7	At5g59180	51			NRPB7
				At4g14660		9	33	NRPE7
				At3g22900		52		NRPD7
				At4g14520				NRPB7-like
		TFS/RPOX	RPB9	At3g16980	22		22	NRPB9a/NRPE9a
				At4g16265	28	22	22	NRPB9b/NRPD9b/NRPE9b

Figure 1. Relationships of *Arabidopsis* Pol II, IV, and V Subunits to *E. coli*, Archaeai, and Yeast RNA Pol II Subunits

Numbers indicate percent protein coverage represented by peptides unique to that protein. "*" indicates that all peptides match both closely related proteins. Unshaded numbers represent alternate subunits detected at trace levels relative to the predominant subunit.

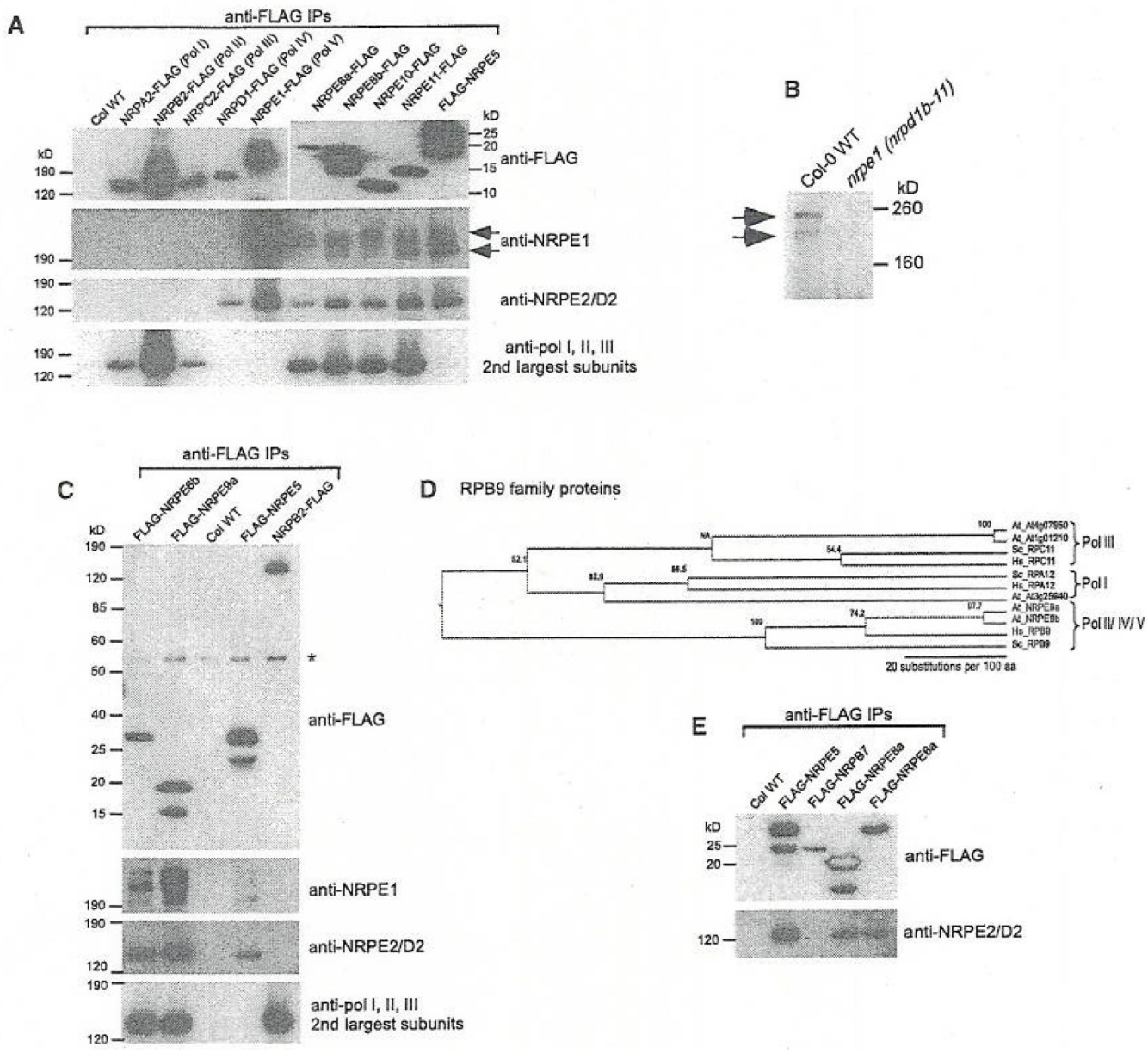

Figure 2. Verification of Pol V Subunit Associations

(A) Pol V includes subunits shared with other polymerases as well as a unique RPB5 family variant. Pol I, II, III, IV, and V were immunoprecipitated by virtue of FLAG-tagged catalytic subunits alongside NRPE6a, NRPE8b, NRPE10, NRPE11, and NRPE5 FLAG-tagged subunits. Duplicate immunoblots were probed with anti-FLAG, anti-NRPE1, anti-NRPE2/ NRPD2 (abbreviated anti-NRPE2/D2), or an antibody recognizing the second-largest subunits of Pol I, II, or III. The two panels in the top row are from the same blot but focus on different size ranges.

(B) Control immunoblot showing that the multiple high-molecular-mass bands characteristic of NRPE1 are lost in an *nrpe1* null mutant (allele *nrpd1b-11*), indicating that the antibody is specific for NRPE1.

(C) NRPE6b and NRPE9a are subunits of Pol V as well as Pol I, II, or III. Immunoprecipitation and immunoblot detection was as in (A). NRPE5 and NRPB2 immunoprecipitations serve as

controls for Pol V and Pol II, respectively. "*" denotes a nonspecific band detected by the anti-FLAG antibody.

(D) Phylogenetic tree based on a CLUSTALW alignment of *Arabidopsis* RPB9-like proteins with the RPB9 (Pol II), RPC11 (Pol III), and RPA12 (Pol I) subunit equivalents of yeast.

(E) NRPE8a and NRPE6a associate with Pol V. Immunoprecipitation and immunoblot detection was as in (A). NRPE5 and NRPB7 serve as controls for Pol V and Pol II, respectively.

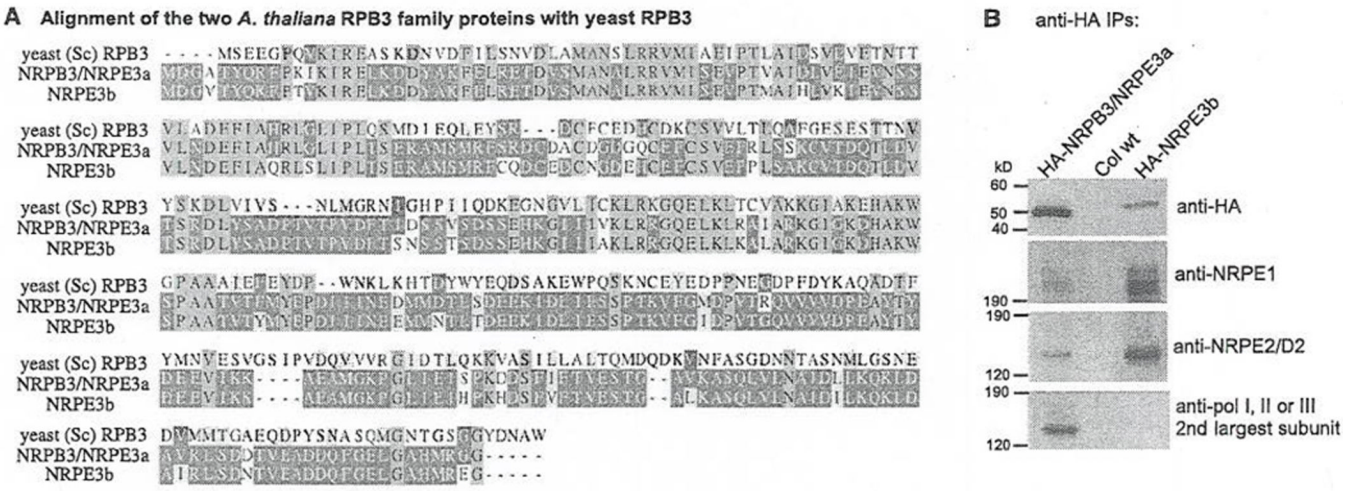

Figure 3. Pol V Utilizes a Distinct RPB3 Variant, NRPE3b, as well as an NRPE3a Variant Corresponding to the Pol II NRPB3 Subunit

(A) Alignment of the two *Arabidopsis* RPB3 family proteins with yeast RPB3.

(B) HA-tagged NRPE3a/NRPB3 and NRPE3b were Immunoprecipitated and resulting immunoblots were probed using the indicated antibodies.

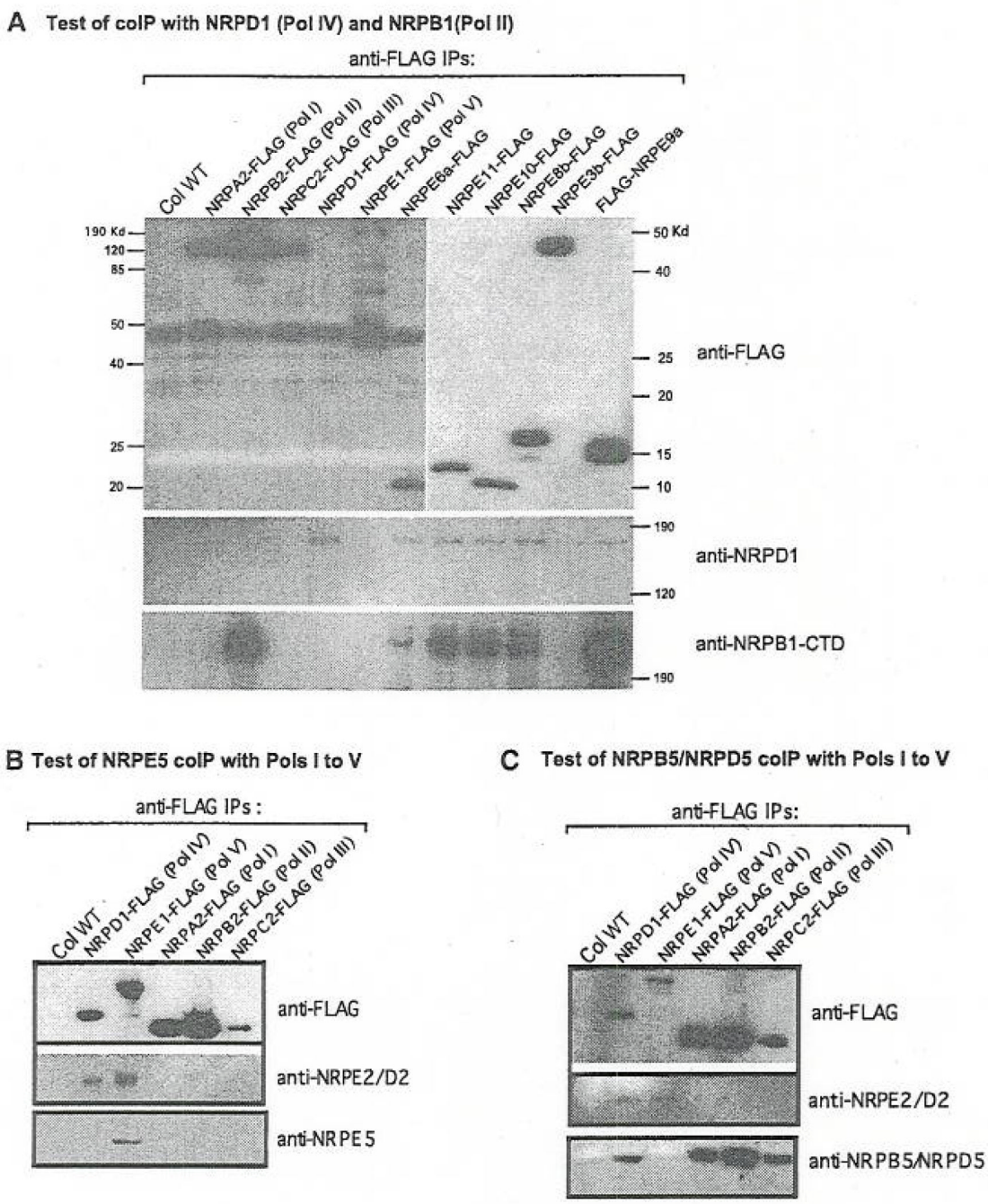

Figure 4. CoIP Tests of Pol V, IV, and II Subunit Associations

(A) Pol I, II, III, IV, and V were immunoprecipitated by virtue of FLAG-tagged catalytic subunits along-side immunoprecipitated NRPE6a, NRPE8b, NRPE9a, NRPE10, NRPE11, and NRPE3b FLAG-tagged subunits. Duplicate immunoblots were probed with anti-FLAG, anti-NRPD1 (Pol IV), or anti-NRPB1-CTD (Pol II). The two panels in the top row show different exposures of the same blot, focused on different size ranges.

(B) Pol I, II, III, IV, and V were immunoprecipitated using the indicated FLAG-tagged subunits and probed with anti-FLAG, anti-NRPE5, or anti-NRPE2/NRPD2.

(C) Immunoprecipitation and immunoblotting using the indicated antibodies were as in (B).

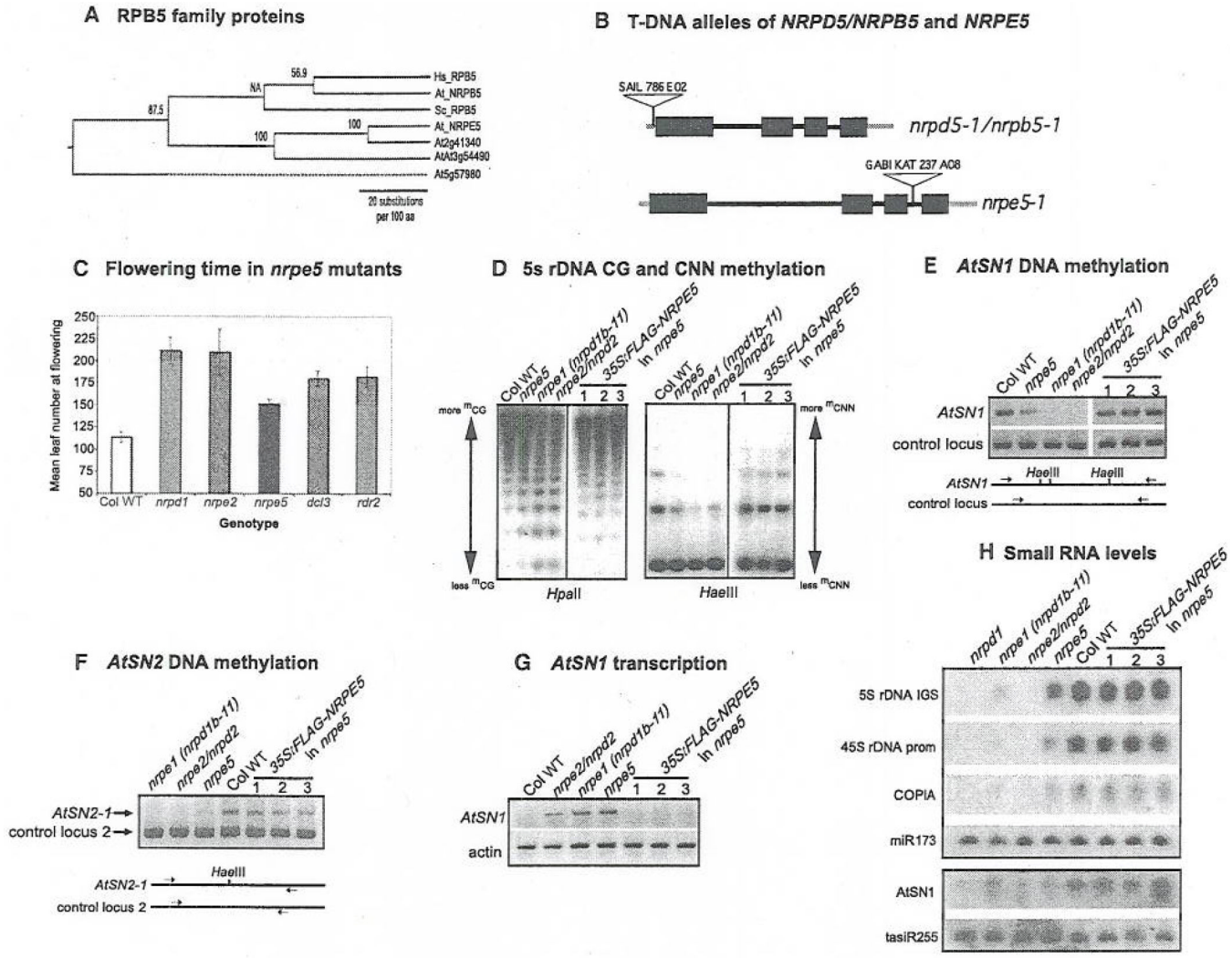

Figure 5. *nrpe5* Mutants Are Defective in RNA-Directed DNA Methylation and Retrotransposon Silencing

(A) Phylogenetic tree based on a CLUSTALW alignment of the five full-length RPB5-like proteins in *Arabidopsis* with the RPB5 subunits of yeast and human.

(B) Locations of T-DNA insertions in the *nrpb5-1/nrpe5-1* and *nrpe5-1* alleles. Black boxes represent exons, black bars represent introns, and gray bars represent 5′ and 3′UTRs.

(C) *nrpe5-1* homozygous mutant plants display a delay in flowering under short-day conditions (8 hr light, 16 hr dark). The mean (±SEM) number of rosette leaves when the floral bolt reached 10 cm is graphed. All mutants are significantly different from wild-type based on a Student's t test ($p < 0.05$).

(D) Methylation-sensitive Southern blot analyses of wild-type, *nrpe1*, *nrpd2/nrpd2*, and *nrpe5* mutants and three different *nrpe5, 35S:FLAG-NRPE5* transgenic lines. Genomic DNA was digested with either *Hpa*II (left, reports on ^{me}CG) or *Hae*III (right, reports on ^{me}CNN) and probed for 5S rDNA repeats. Images for the *Hpa*II or *Hae*III digests are from the same exposures of the same Southern blots; the black vertical lines separate groups of lanes whose order was rearranged for clarity of presentation.

(E and F) PCR-based methylation assay of *AtSN1* and *AtSN2* family retroelements. Genomic DNA was digested with *Hae*III and subjected to PCR using *AtSN1*, *AtSN2-1*, or control primers that amplify sequences lacking *Hae*III sites (*At2g19920* in the case of [B], and an *AtSN2* family

element lacking *Hae*III sites in the case of [C]). Diagrams show the relative positions of the primers flanking the *Hae*III sites.
(G) RT-PCR detection of *AtSN1* and actin transcripts.
(H) Small RNA blot analysis. Blots were probed for siRNAs corresponding to 45S or 5S rRNA genes, Copia or *AtSN1* transposons, and miRNA 173 or *trans*-acting siRNA 255.

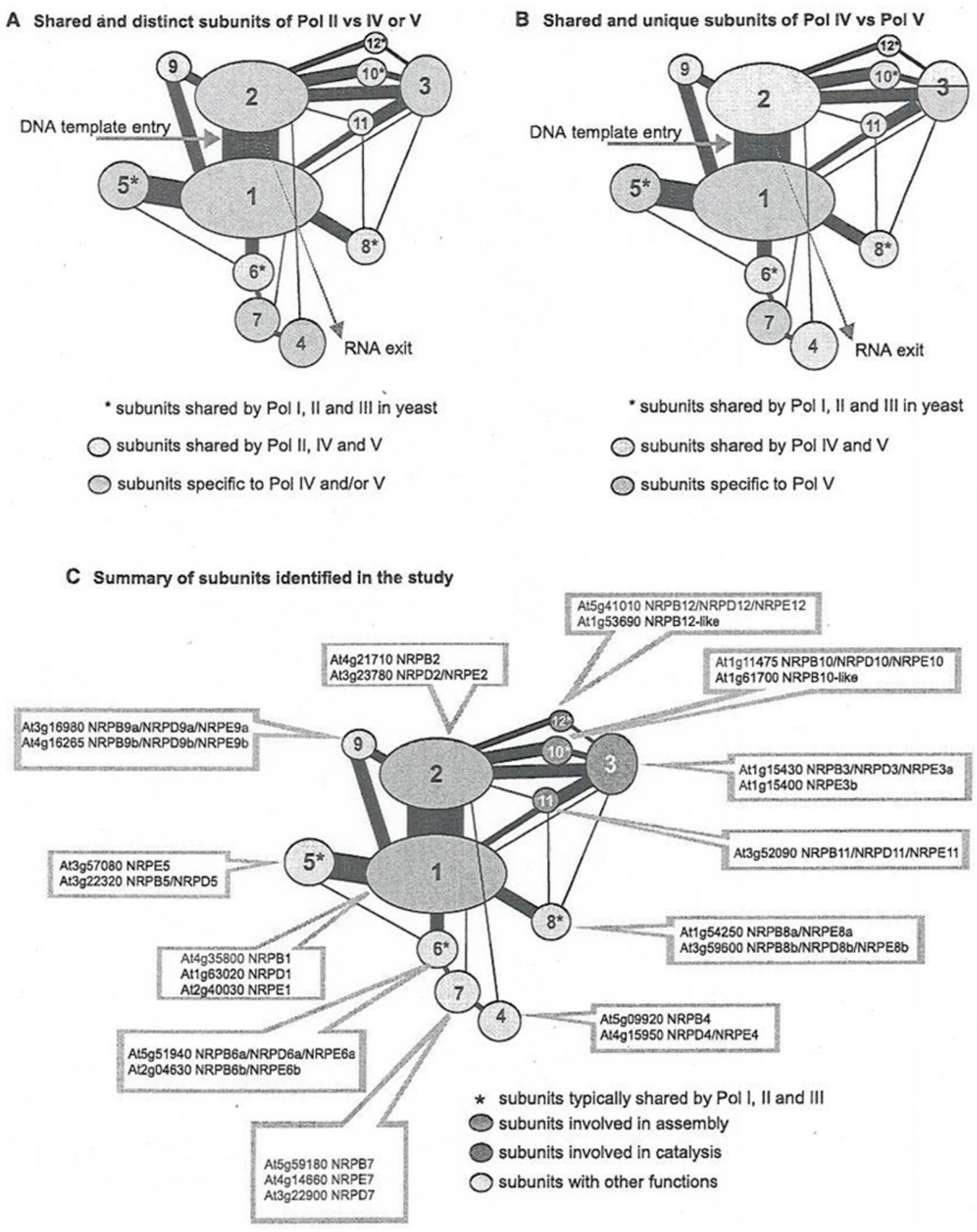

Figure 6. Comparison of RNA Polymerase Subunits in Pol II, IV, and V

(A) Subunits that are unique to Pol IV and/or Pol V compared to Pol II are shown in blue. Subunits common to Pol II, IV, and V are shown in green. The subunit interaction model is based on the yeast Pol II crystal structure (Armache et al., 2005; Cramer et al., 2001; Sampath et al., 2008). The thickness of lines connecting the subunits is proportional to the number of contacts.

(B) Subunits that are unique to Pol V are shown in blue. Subunits common to Pol IV and Pol V are shown in green. The half-blue, half-green shading of the third-largest subunit reflects the fact that Pol V uses the NRPE3b variant that is not used appreciably by Pol IV in addition to the NRPE3a/NRPD3 variant that predominates in Pol IV.

(C) Summary of the *Arabidopsis* genes that encode Pol II, IV, or V subunits.