OPEN ACCESS
CellPress

Cell Reports
Resource

Functional Diversification of Maize RNA Polymerase IV and V Subtypes via Alternative Catalytic Subunits

Jeremy R. Haag,[1,2] Brent Brower-Toland,[3] Elysia K. Krieger,[3] Lyudmila Sidorenko,[4] Carrie D. Nicora,[5] Angela D. Norbeck,[5] Andre Irsigler,[6,7] Huachun LaRue,[3] Jan Brzeski,[4] Karen McGinnis,[6] Sergey Ivashuta,[3] Ljiljana Pasa-Tolic,[5] Vicki L. Chandler,[4] and Craig S. Pikaard[1,2,*]

[1]Department of Biology and Department of Molecular and Cellular Biochemistry, Indiana University, 915 E. Third Street, Bloomington, IN 47405, USA
[2]Howard Hughes Medical Institute, Indiana University, Bloomington, IN 47405, USA
[3]Monsanto Company, 700 West Chesterfield Parkway, Chesterfield, MO 63017, USA
[4]BIO5 Institute, Department of Plant Sciences, University of Arizona, Tucson, AZ 85719, USA
[5]Pacific Northwest National Laboratory, Richland, WA 99352, USA
[6]Department of Biological Science, Florida State University, Tallahassee, FL 32306, USA
[7]Present address: EMBRAPA Genetic Resources and Biotechnology, Brasilia 70770-917, Brazil
*Correspondence: cpikaard@indiana.edu
http://dx.doi.org/10.1016/j.celrep.2014.08.067

SUMMARY

Unlike nuclear multisubunit RNA polymerases I, II, and III, whose subunit compositions are conserved throughout eukaryotes, plant RNA polymerases IV and V are nonessential, Pol II-related enzymes whose subunit compositions are still evolving. Whereas *Arabidopsis* Pols IV and V differ from Pol II in four or five of their 12 subunits, respectively, and differ from one another in three subunits, proteomic analyses show that maize Pols IV and V differ from Pol II in six subunits but differ from each other only in their largest subunits. Use of alternative catalytic second subunits, which are nonredundant for development and paramutation, yields at least two subtypes of Pol IV and three subtypes of Pol V in maize. Pol IV/Pol V associations with MOP1, RMR1, AGO121, Zm_DRD1/CHR127, SHH2a, and SHH2b extend parallels between paramutation in maize and the RNA-directed DNA methylation pathway in *Arabidopsis*.

INTRODUCTION

Plants are unique in having evolved multisubunit RNA polymerases IV and V in addition to Pols I, II, and III, the three ubiquitous nuclear DNA-dependent RNA polymerases of eukaryotes. Pols IV and V synthesize noncoding RNAs for transcriptional silencing of transposons, repetitive elements, and a subset of genes (Haag and Pikaard, 2011; Herr et al., 2005; Kanno et al., 2005b; Onodera et al., 2005; Pontier et al., 2005; Ream et al., 2014). Both are 12-subunit enzymes (Ream et al., 2009) that evolved as specialized forms of Pol II, as shown by mass spectrometry (Huang et al., 2009; Law et al., 2011; Ream et al., 2009) and phylogenetic analyses (Luo and Hall, 2007; Onodera et al., 2005; Tucker et al., 2010).

Pols IV and V play distinct roles in RNA-directed DNA methylation (RdDM) in *Arabidopsis* (Law and Jacobsen, 2010; Matzke et al., 2009; Pikaard et al., 2012; Wierzbicki, 2012; Zhang and Zhu, 2011). Pol IV acts early in the process, generating transcripts that serve as templates for RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) (Xie et al., 2004), which physically associates with Pol IV (Haag et al., 2012; Law et al., 2011). Other *Arabidopsis* Pol IV-associated proteins include CLSY (a subfamily of SWI2/SNF2-like putative ATP-dependent DNA translocases; Smith and Baulcombe, 2007) and SHH1 (also known as DTF1), a BAH domain protein that binds histone H3 that is dimethylated on lysine 9 and unmethylated on lysine 4, thereby recruiting Pol IV to heterochromatin (Law et al., 2011, 2013; Zhang et al., 2013). Double-stranded RNAs generated by Pol IV and RDR2 are diced by DCL3 into 24 nt small interfering RNAs (siRNAs) that are bound by ARGONAUTE 4 (AGO4), AGO6, or AGO9 (Havecker et al., 2010; Mallory and Vaucheret, 2010). The Argonaute-siRNA complexes bind to Pol V transcripts, whose synthesis requires a multiprotein complex that includes a SWI2/SNF2-family ATP-dependent DNA translocase (DRD1), a protein related to the hinge domains of cohesins and condensins (DMS3), and a single-stranded DNA-binding protein (RDM1) (Gao et al., 2010; Kanno et al., 2005a, 2008; Law et al., 2010; Wierzbicki et al., 2008, 2009). AGO4 can also interact with the C-terminal domain of the Pol V largest subunit, NRPE1 (El-Shami et al., 2007). Pol-V-dependent recruitment of AGO-siRNA complexes enables subsequent recruitment of de novo DNA methylation, histone modification, and chromatin-remodeling machineries, yielding chromatin states refractive to Pol I, II, or III transcription (Wierzbicki, 2012; Wierzbicki et al., 2008, 2009).

Paramutation is an epigenetic phenomenon in which a functional allele can be inactivated upon exposure to a silenced allele of the same gene (Arteaga-Vazquez and Chandler, 2010; Erhard and Hollick, 2011). How alleles communicate in paramutation is unclear, but maize orthologs of *Arabidopsis* RdDM pathway proteins are required. These include MEDIATOR OF PARAMUTATION1 (MOP1), the ortholog of *Arabidopsis* RDR2 (Alleman et al., 2006), and REQUIRED TO MAINTAIN

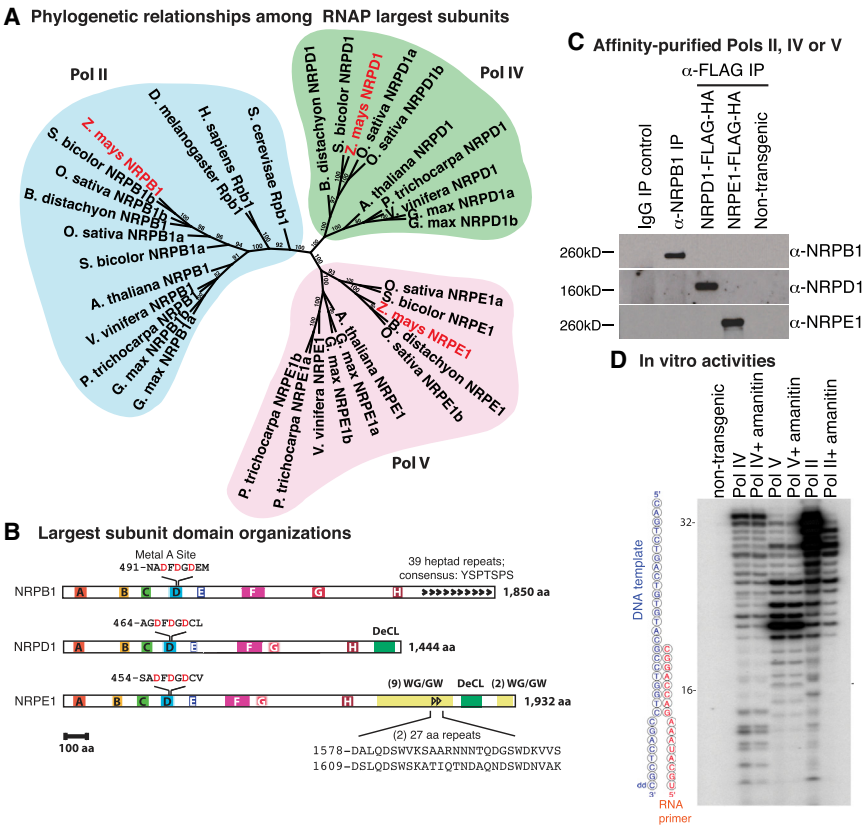

Figure 1. Maize Pol II, IV, and V Largest Subunits

(A) Neighbor-joining tree, with bootstrap values, generated by MUSCLE alignment of full-length RNAP largest subunits. Maize sequences are in red.

(B) Domain features of the maize NRPB1, NRPD1, and NRPE1 proteins, including conserved domains A–H, metal A catalytic centers (invariant aspartates are colored red), NRPB1 C-terminal domain heptad repeats (open arrowheads), DeCL domains of NRPD1 and NRPE1 (green), NRPE1 WG/GW-rich regions (yellow), and 27 aa repeats (closed arrowheads).

(C) Immunoblots of immunoprecipitated NRPB1, FLAG epitope-tagged NRPD1, or NRPE1 using antibodies recognizing native NRPB1, NRPD1, or NRPE1.

(D) Affinity-purified Pols II, IV, and V are functional for transcription in vitro. A 32 nt DNA template annealed to a 16 nt RNA oligonucleotide, yielding an 8 bp DNA-RNA hybrid region (diagrammed at left), was incubated with affinity-purified Pols II, IV, and V and nucleotide triphosphates, including alpha^{32}P-CTP, in the presence or absence of 5 μg/ml alpha-amanitin. Reaction products were subjected to denaturing polyacrylamide gel electrophoresis, transferred to filter paper, dried under vacuum, and visualized by phosphorimaging.

See also Figure S1A and Table S1.

REPRESSION6 (RMR6), which corresponds to the Pol IV largest subunit, NRPD1 (Erhard et al., 2009). Likewise, RMR1 encodes a paralog of *Arabidopsis* CLSY or DRD1 proteins, suggesting possible roles in Pol IV or Pol V transcription (Hale et al., 2009).

The second-largest subunits of Pols IV and V in *Arabidopsis* (ecotype Col-0) are encoded by a single gene, *NRP(D/E)2* (also known as *NRP(D/E)2a*; Herr et al., 2005; Kanno et al., 2005b; Onodera et al., 2005; Pontier et al., 2005). A closely linked paralog, *NRP(D/E)2b*, is nonfunctional. *NRP(D/E)2* is most similar to *NRPB2*, the *Arabidopsis* gene uniquely encoding the second subunit of Pol II (Onodera et al., 2005). Interestingly, maize has three *NRP(D/E)2*-like genes (designated with the suffixes a, b, and c) and two *NRPB2*-like genes. Independent studies have shown that the paramutation mutants *mop2* and *rmr7* correspond to mutant alleles of *NRP(D/E)2*-like$_a$ (Sidorenko et al., 2009; Stonaker et al., 2009), but the affected polymerase(s) are unclear. To address this question, we affinity-purified maize Pol II or epitope-tagged Pol IV, Pol V, or MOP1 and identified their subunits and associated proteins using mass spectrometry. Surprisingly, the fundamental difference between maize Pols IV and V is their use of distinct largest subunits. Moreover, we show that Pols IV and V can assemble using two or all three, respectively, of the NRP(D/E)2 subunit variants (a, b, and c). Alternative forms of Pol II likewise assemble using two NRPB2 subunit variants. Maize Pol IV associates with the RNA-dependent RNA polymerase, MOP1, paralleling Pol IV-RDR2 association in *Arabidopsis*, and with SWI2/SNF2-family proteins RMR1 and CHR167, suggesting that the latter are functionally analogous to *Arabidopsis* CLSY proteins. Maize Pol V likewise associates with paralogs of *Arabidopsis* RdDM pathway proteins, including ARGONAUTE121 (related to *Arabidopsis* AGO6), CHR127 (related to *Arabidopsis* DRD1), and DMS3. Two closely related proteins, SHH2a and SHH2b, associate with both Pols IV and V in maize, suggesting that both enzymes are targeted to heterochromatic regions in the same way, analogous to *Arabidopsis* Pol IV targeting by SHH1/DTF1 (Law et al., 2013; Zhang et al., 2013). Collectively, our proteomic analyses provide evidence for ongoing Pol IV/Pol V functional diversification as well as protein partnerships important for paramutation.

RESULTS

Subunit Compositions of Maize RNA Polymerases II, IV, and V

In the maize genome, single-copy genes encode the largest subunits of RNA polymerases II, IV, and V (Figure 1A; accession numbers for genes used to generate the phylogenetic tree are provided in Table S1). The encoded proteins (NRPB1, NRPD1, and NRPE1, respectively) have magnesium-ion-binding motifs (metal A site) and conserved domains A–H typical of multisubunit RNA polymerase largest subunits (Figure 1B; see also Figure S1a). The C-terminal domain (CTD) of the Pol II largest subunit, NRPB1, consists of repeating seven-amino-acid (heptad) motifs, as in all eukaryotes (Egloff and Murphy, 2008; Hsin and

Manley, 2012), that are absent in NRPD1 and NRPE1 of Pols IV and V, respectively (Haag and Pikaard, 2011). Instead, maize NRPD1, as in *Arabidopsis*, has a short CTD with a so-called DeCL domain, named for its similarity to the DEFECTIVE CHLORLOPLASTS AND LEAVES protein implicated in chloroplast ribosomal RNA processing (Bellaoui and Gruissem, 2004). The CTD of the maize Pol V largest subunit, NRPE1, also has a DeCL domain plus two imperfect repeats of 27 amino acids. Aside from the conserved DeCL domains, maize and *Arabidopsis* Pol V CTDs display little, if any, primary sequence conservation. Whereas the *A. thaliana* (ecotype Col-0) Pol V CTD has ten imperfect repeats of 16-amino-acid sequence and a glutamine/serine-rich domain at the extreme C terminus of the protein, these features are absent in the maize Pol V CTD. However, maize and *Arabidopsis* Pol V CTDs have in common the occurrence of numerous WG or GW amino acid pairs (11 in maize; 18 in *Arabidopsis*). In *Arabidopsis*, these WG/GW "AGO-hook" motifs facilitate Argonaute protein interactions (El-Shami et al., 2007).

Transgenic maize cell cultures expressing full-length NRPD1 or NRPE1 fused at their C termini to tandem FLAG and hemagglutinin (HA) epitope tags were used for affinity capture of Pol IV or Pol V via anti-FLAG immunoprecipitation (IP). Maize Pol II was IPed using an antibody recognizing the CTD heptad repeats of NRPB1. Immunoblot analyses using antibodies specific for Pol II, IV, or V largest subunits (NRPB1, NRPD1, or NRPE1, respectively) revealed that each polymerase had been isolated free of cross-contamination (Figure 1C). Affinity-purified Pol II, IV, and V complexes are functional for transcription, as shown by their ability to synthesize radioactively labeled RNA transcripts in vitro (Figure 1D). As in *Arabidopsis* (Haag et al., 2012), transcription by maize Pols IV and V is insensitive to the fungal toxin, alpha-amanitin, whereas Pol II activity is inhibited (Figure 1D).

Tryptic peptides of affinity-purified maize Pol II, IV, and V were analyzed by liquid chromatography paired with tandem mass spectrometry (LC-MS/MS). Peptides of NRPB1, NRPD1, or NRPE1 were detected only in Pol II, Pol IV, or Pol V samples, respectively (summarized in Table 1; for additional details see Tables S2–S4), consistent with the immunoblotting results (Figure 1C). Eleven of the expected twelve subunits were identified for each polymerase, but no peptides corresponding to any of three 12^{th}-subunit paralogs were detected (Table 1). The 12^{th} subunit is one of five subunits common to RNA polymerases I, II, and III in eukaryotes and is also common to *Arabidopsis* Pols IV and V (Ream et al., 2009). Our failure to detect 12^{th}-subunit peptides may be a consequence of the small size of the proteins (~52 amino acids), thus limiting the number of potential tryptic fragments amenable to ionization and detection.

Multiple Subtypes of Pols II, IV, and V Assemble Using Alternative Second Subunits

Maize has five genes encoding proteins similar to the yeast Pol II second subunit, Rpb2. Two group with the *Arabidopsis* Pol II second-subunit gene, *NRPB2* (Figure 2A; Table 1; see also Figure S1b). Both of these proteins, NRPB2a and NRPB2b, are detected in affinity-purified Pol II (Table 1; Figure 2A; see also Table S2), indicating that either can assemble into Pol II. The remaining three maize Rpb2 paralogs are similar to the *Arabidopsis NRP(D/E)2* gene that encodes the second subunits of Pols IV and V but segregate into two monocot-specific clades (Figure 2A). The protein encoded by *NRP(D/E)2-like*$_a$, the gene disrupted in *mop2* and *rmr7* mutants, is present in both Pol IV and Pol V, as is its closest paralog, NRP(D/E)2-like$_b$ (Table 1; Figure 2A; see also Tables S3 and S4). We have thus renamed the proteins NRP(D/E)2a and NRP(D/E)2b to reflect their confirmed associations with both Pol IV and Pol V. Peptides unique to NRP(D/E)2-like$_c$ were identified only in Pol V; the protein is thus renamed NRPE2c (Table 1; Figure 2A; see also Table S4).

As an independent test of NRP(D/E)2a associations with Pols IV and V, a cell-free extract of transgenic maize expressing NRP(D/E)2a fused to tandem, C-terminal FLAG and HA epitope tags was immunoprecipitated using anti-FLAG resin. The affinity-purified proteins were then analyzed by immunoblotting, using antibodies recognizing the largest subunits of native Pol IV (NRPD1) or Pol V (NRPE1) or the HA tag on the recombinant NRP(D/E)2a-FLAG-HA protein (Figure 2B), as well as by mass spectrometry. NRPD1 and NRPE1 both copurify with NRP(D/E)2a, as shown by immunoblotting (Figure 2B) and by mass spectrometry (Tables 2 and S5). Interestingly, a different engineered form of the protein, FLAG-NRP(D/E)2a, which has the FLAG epitope fused to the amino terminus of the protein, copurified only with Pol V (Tables 2 and S6), suggesting that the N-terminal epitope tag interferes with Pol IV assembly.

Subunits Indicative of Pol II/Pol IV/Pol V Divergence

Multiple subunits of maize Pols II, IV, and V are encoded by the same genes, namely the third, sixth, eighth, tenth, and eleventh subunits (Table 1; Figure 2C; see also Figures S1C, S1F, and S1H–S1K). The 12^{th} subunit is also expected to be common to all three polymerases (as well as to Pols I and III) but was undetected, as discussed previously. The third and eleventh subunits of Pols II, IV, and V interact to form a subcomplex (Ulmasov et al., 1996) and are homologs of the two alpha subunits of bacterial RNA polymerase, which mediate activator-dependent transcription initiation (Ebright and Busby, 1995). Subunits six, eight, ten, and twelve are common to all eukaryotic RNA polymerases examined thus far and are thought to be important for RNA polymerase assembly (Werner and Grohmann, 2011).

Six subunits define the evolutionary split between Pol II and Pols IV and V in maize: subunits one, two, four, five, seven, and nine (see Table 1; Figure 2D), revealing similarities and important differences compared to *Arabidopsis*. As in *Arabidopsis*, the maize Pol IV and Pol V fourth subunits are encoded by the same gene, *NRP(D/E)4*, which is distinct from the Pol II fourth subunit gene, *NRPB4* (Table 1; see also Figure S1D). In *Arabidopsis*, the fifth subunits of Pols I, II, III, and IV are encoded by the same gene, NRP(A/B/C/D)5, but the corresponding subunit of Pol V, NRPE5, is encoded by a distinct gene (Lahmy et al., 2009; Ream et al., 2009). Interestingly, maize has a clear ortholog of *Arabidopsis NRPE5* (GRMZM2G469969), which surprisingly encodes the fifth subunit of both Pols IV and V (Tables 1, S3, and S4; Figures 3A and S1E). We thus designate this gene *NRP(D/E)5*. The seventh subunits of Pols IV and V are encoded by different genes in *Arabidopsis*, yet the same gene encodes

Table 1. Proteins Identified by Mass Spectrometry in Affinity-Purified Pols II, IV, or V

		Affinity-Purified Protein			
Yeast Protein	Maize Homologs	NRPB1 (Pol II)	NRPD1 (Pol IV)	NRPE1 (Pol V)	Names/Synonyms
DNA-Dependent RNA Polymerase Subunits					
Rpb1	GRMZM2G044306	26			NRPB1
	GRMZM2G007681		50	2	NRPD1/RMR6
	GRMZM2G153797			59	NRPE1
Rpb2	GRMZM2G084891	6			NRPB2a
	GRMZM2G113928	2			NRPB2b
	GRMZM2G054225		14	28	NRP(D/E)2a/MOP2/RMR7
	GRMZM2G427031		3	16	NRP(D/E)2b
	GRMZM2G133512		[a]	4	NRPE2c
Rpb3	GRMZM2G402295	58	50	63	NRP(B/D/E)3
Rpb4	GRMZM2G119393	60			NRPB4
	GRMZM2G453424		49	51	NRP(D/E)4
Rpb5	GRMZM2G476009	47			NRPB5a
	GRMZM2G099183	38			NRPB5b
	GRMZM2G469969		44	48	NRP(D/E)5
Rpb6	GRMZM2G013600	[a]	[a]	[a]	NRP(B/D/E)6a
	GRMZM2G086904	[a]	[a]	[a]	NRP(B/D/E)6b
Rpb7	GRMZM2G179346	23			NRPB7
	GRMZM2G040702		26	42	NRP(D/E)7
Rpb8	GRMZM2G034326	61	78	77	NRP(B/D/E)8
	GRMZM2G347789				NRPB8-like
Rpb9	GRMZM2G046061	37			NRPB9a
	GRMZM2G023028	37			NRPB9b
	GRMZM5G898768		18	33	NRP(D/E)9
Rpb10	NP_001152395	35	42	59	NRP(B/D/E)10a
	GRMZM5G803992	[a]	[a]	42[b]	NRP(D/E)10b
	GRMZM5G834335	[a]	[a]	42[b]	NRP(D/E)10c
Rpb11	GRMZM2G130207	32	48	48	NRP(B/D/E)11
	GRMZM2G043461	[a]	[a]	[a]	NRPB11-like
Rpb12	GRMZM2G146331				NRPB12a
	GRMZM2G322661				NRPB12b
	GRMZM2G540834				NRPB12c
Polymerase-Associated Proteins					
RNA-dep RNAP	GRMZM2G042443		29		MOP1/RDR101
	GRMZM2G145201		[a]		RDR102
Swi2/Snf2 family	GRMZM2G154946		4		RMR1
	GRMZM2G178435		15		CHR167 RMR1-like
	GRMZM5G574858			8	CHR127/Zm_DRD1a
	GRMZM2G393742			[a]	CHR156/Zm_DRD1b
-	NP_001132336			5	Zm_DMS3
-	GRMZM2G111204			5	Zm_SHH2a
	GRMZM2G126170			2	Zm_SHH2b
Iwr1	GRMZM2G098603	9	25	8	IWR1/Zm_DMS4/Zm_RDM4
Ago1	GRMZM2G589579			[a]	AGO105
	GRMZM2G089743			[a]	AGO119
	GRMZM2G347402			4	AGO121

Numerical values are the percentage of the full-length protein represented by unique peptides, excluding peptides that could match related paralogs. For additional details, see Tables S2–S4.

[a]Peptides matching the protein were detected but also match one or more paralogs.

[b]Proteins are identical and indistinguishable.

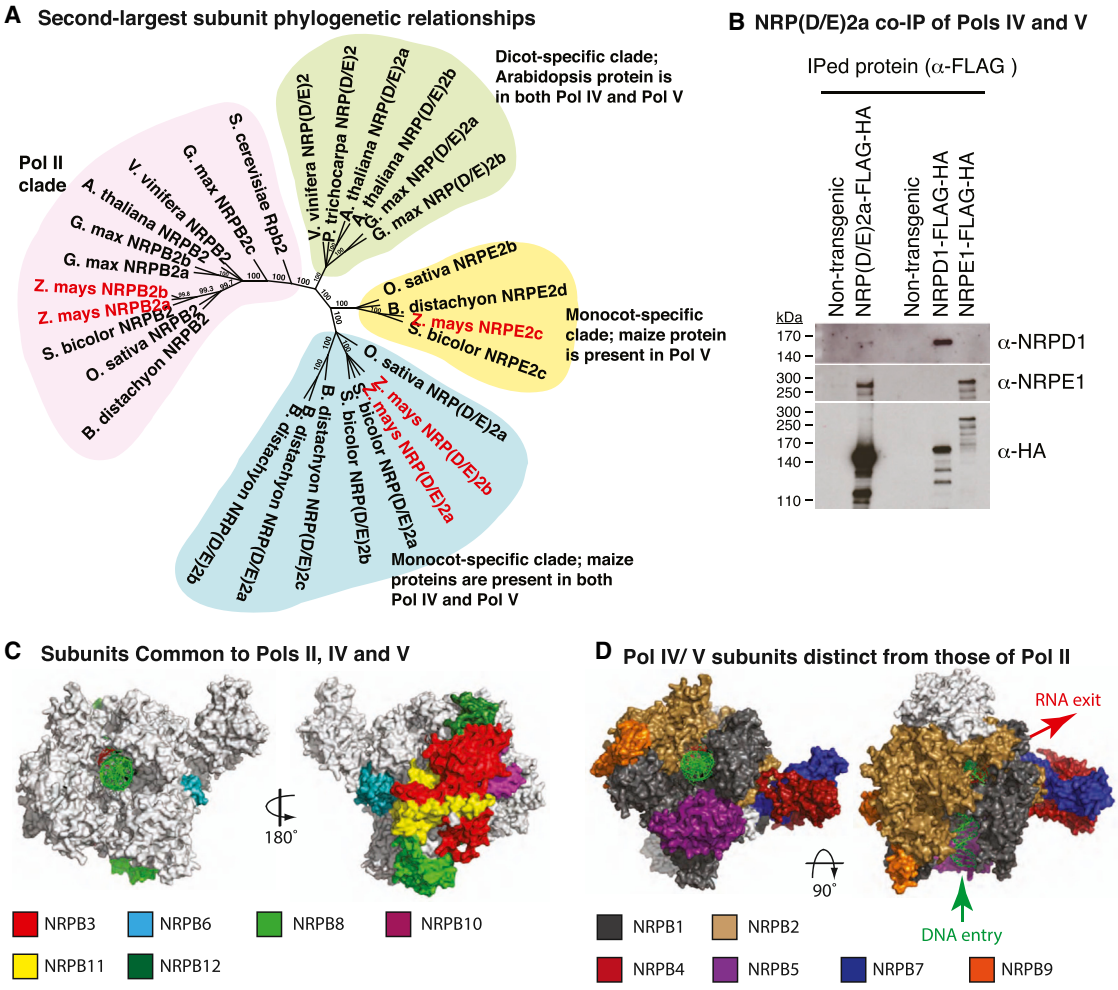

Figure 2. Second-Subunit Diversity and Positions of Pol II, IV, and V Common versus Distinct Subunits

(A) Neighbor-joining tree, with bootstrap values, generated from MUSCLE alignment of full-length RNAP second subunits. Maize subunits are highlighted in red.

(B) NRPD1 and NRPE1 coimmunoprecipitate with epitope-tagged NRP(D/E)2a-FLAG-HA. Proteins immunoprecipitated from the indicated lines using anti-FLAG resin were subjected to immunoblotting using antibodies recognizing native NRPD1, native NRPE1, or the HA epitope.

(C) Based on yeast Pol II (Protein Data Bank no. 2VUM), positions of subunits common to Pols II, IV, and V (in color) are displayed in views of the leading face (left) or backside (right) of the enzyme. The DNA helix (green) and nascent RNA (red) are visible at the center of the leading face image.

(D) Positions of subunits that differ in Pols IV and V compared to Pol II are displayed in views of the leading face (left) or from above, looking down onto the template DNA within the enzyme. Pol-IV/Pol-V-specific subunits occupy the leading face of the enzyme; common subunits cluster on the trailing face.

See also Figures S1B–S1L and Table S1.

the seventh subunits of Pols IV and V in maize (Tables 1, S3, and S4; Figures 3B and S1G). Whereas the same two ninth-subunit variants are used alternatively by Pols II, IV, and V in *Arabidopsis*, maize Pols IV and V make use of a unique ninth subunit, NRP(D/E)9, that is distinct from two NRPB9 variants used by Pol II (Table 1; Figure S1I).

Pol IV/Pol V-Associated Proteins Include Chromatin Binding, Chromatin Remodeling, Paramutation, and Argonaute Proteins

A number of proteins implicated in paramutation or RdDM were detected in association with Pols IV and/or V (Table 1). These include two proteins similar to *Arabidopsis* SHH2 (Law et al., 2011) associated with both Pol IV and Pol V (Table 1; Figures 3C and S1Q). The related *Arabidopsis* protein, SHH1 (also known as DTF1), helps target Pol IV to heterochromatin by binding to histone H3 dimethylated on lysine 9 and unmethylated on lysine 4 (Law et al., 2013; Zhang et al., 2013). Association of maize SHH2 with Pol IV and Pol V suggests similar modes of chromatin recruitment for both enzymes.

A known paramutation protein detected in association with Pol IV is MOP1, the maize ortholog of *Arabidopsis* RDR2 (Alleman et al., 2006). Pol IV-MOP1 association was confirmed in reciprocal coimmunoprecipitation experiments, in which epitope-tagged versions of the proteins were isolated from cell extracts using antibodies specific for the epitope tags and then detected on immunoblots using antibodies recognizing native NRPD1 or MOP1 (Figures 4A and 4B).

Table 2. Proteins Copurifying with Maize NRP(D/E)2a Epitope-Tagged at the Amino or Carboxyl Terminus

		Recombinant Form of NRP(D/E)2a Analyzed		
Yeast Protein	Maize Homologs	N-Terminal FLAG Tag	C-Terminal FLAG–HA Tag	Names/Synonyms
DNA-Dependent RNA Polymerase Subunits				
Rpb1	GRMZM2G044306			NRPB1
	GRMZM2G007681		2	NRPD1/RMR6
	GRMZM2G153797	16	9	NRPE1
Rpb2	GRMZM2G084891			NRPB2a
	GRMZM2G113928			NRPB2b
	GRMZM2G054225	14	18	NRP(D/E)2a/MOP2/RMR7
	GRMZM2G427031	[a]	[a]	NRP(D/E)2b
	GRMZM2G133512	[a]	[a]	NRPE2c
Rpb3	GRMZM2G402295	47	54	NRP(B/D/E)3
Rpb4	GRMZM2G119393			NRPB4
	GRMZM2G453424	30	8	NRP(D/E)4
Rpb5	GRMZM2G476009			NRPB5a
	GRMZM2G099183			NRPB5b
	GRMZM2G469969	36	23	NRP(D/E)5
Rpb6	GRMZM2G013600	[a]		NRP(B/D/E)6a
	GRMZM2G086904	[a]		NRP(B/D/E)6b
Rpb7	GRMZM2G179346			NRPB7
	GRMZM2G040702	7		NRP(D/E)7
Rpb8	GRMZM2G034326	52	26	NRP(B/D/E)8
	GRMZM2G347789			NRPB8-like
Rpb9	GRMZM2G046061			NRPB9a
	GRMZM2G023028			NRPB9b
	GRMZM5G898768		12	NRP(D/E)9
Rpb10	NP_001152395	[a]	42	NRP(B/D/E)10a
	GRMZM5G803992	35[b]	[a]	NRP(B/D/E)10b
	GRMZM5G834335	35[b]	[a]	NRP(B/D/E)10c
Rpb11	GRMZM2G130207	33	41	NRP(B/D/E)11
	GRMZM2G043461	[a]	[a]	NRPB11-like
Rpb12	GRMZM2G146331			
	GRMZM2G322661			
	GRMZM2G540834			
Polymerase-Associated Proteins				
RNA-dep RNAP	GRMZM2G042443		3	MOP1/RDR101
	GRMZM2G145201			RDR102
Swi2/Snf2	GRMZM2G154946			RMR1
	GRMZM2G178435			CHR167/RMR1-like
	GRMZM5G574858			CHR127/Zm_DRD1a
	GRMZM2G393742			CHR156/Zm_DRD1b
-	GRMZM2G309152			Zm_DMS3
-	GRMZM2G111204	10	5	Zm_SHH2a
	GRMZM2G126170	15	5	Zm_SHH2b
Iwr1	GRMZM2G098603			IWR1/Zm_DMS4/Zm_RDM4
Ago1	GRMZM2G589579			AGO105
	GRMZM2G089743			AGO119
	GRMZM2G347402			AGO121

Annotation is the same as for Table 1. For additional details, see Tables S5 and S6.
[a]Peptides matching the protein were detected but also match one or more paralogs.
[b]Proteins are identical and indistinguishable.

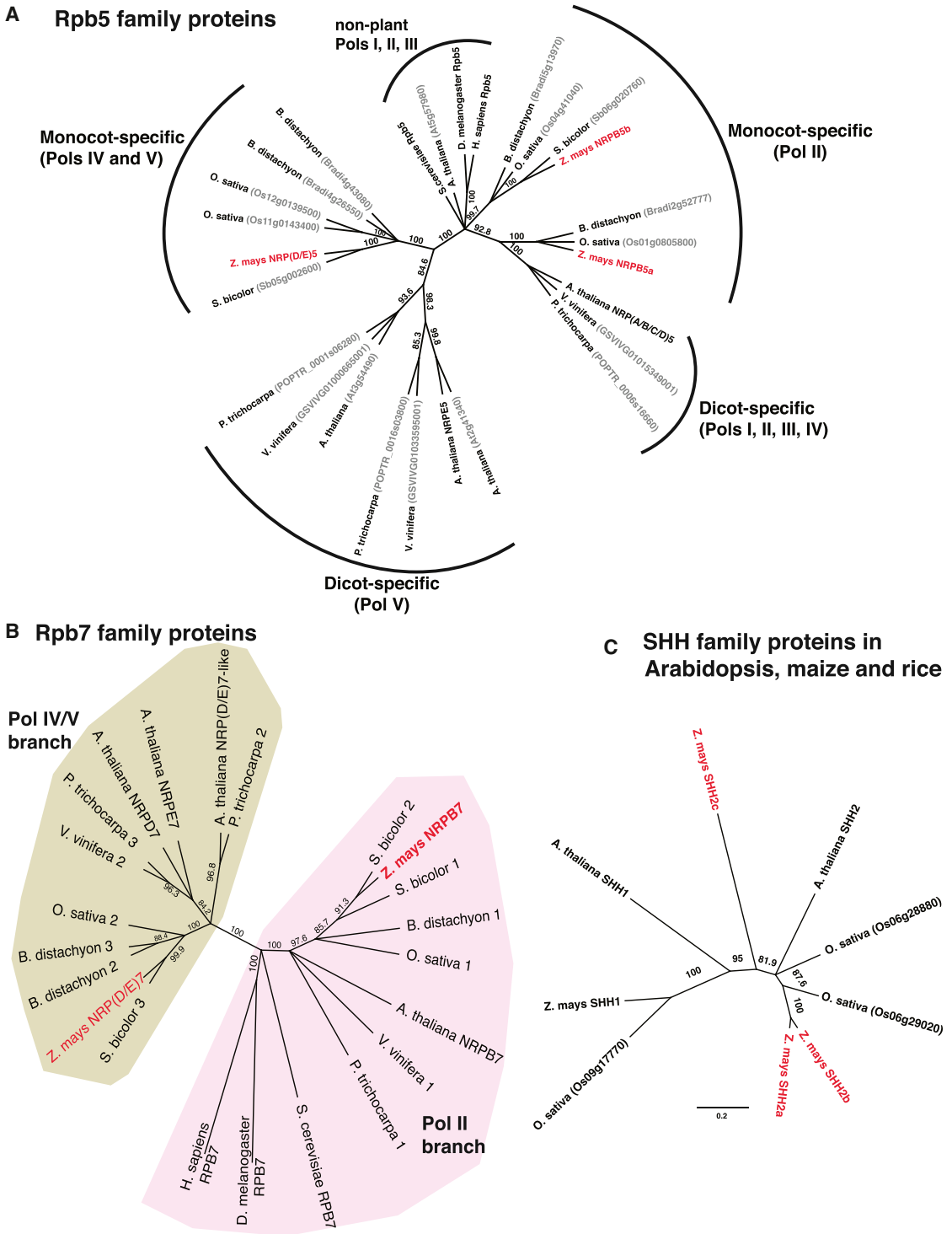

Figure 3. Phylogenetic Analyses of Proteins Homologous to Yeast Rpb5, Yeast Rpb7, or *Arabidopsis* SHH1

(A) Neighbor-joining tree, with bootstrap values, for Rpb5 homologs; maize NRP(D/E)5, NRPB5a, and NRPB5b proteins are highlighted in red.

(B) Neighbor-joining tree for Rpb7 homologs, with NRP(D/E)7 and NRPB7 proteins highlighted in red.

(C) Neighbor-joining tree for SHH1 homologs in *Arabidopsis*, rice (*O. sativa*), and maize.

See also Figures S1E, S1G, and S1Q and Table S1.

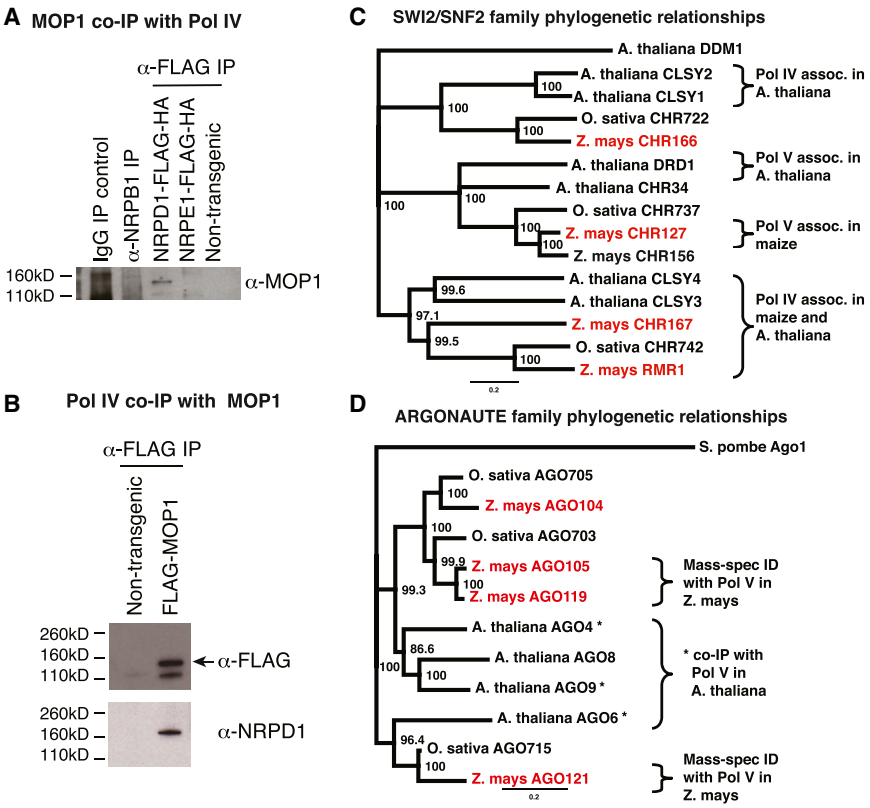

Figure 4. Proteins Associated with Pol IV and/or Pol V in Maize

(A) MOP1 associates with NRPD1 (Pol IV). Immunoprecipitated Pols II, IV, or V, and controls, were subjected to immunoblot analysis using anti-MOP1 antibody.

(B) NRPD1 detection in association with MOP1. FLAG-MOP1 was immunoprecipitated and subjected to immunoblot analysis using anti-FLAG (to verify IP) or anti-NRPD1 antibodies.

(C) Neighbor-joining tree, with bootstrap values, generated from MUSCLE alignment of *Arabidopsis*, rice, and maize proteins most similar to maize RMR1, CHR167, CHR127, and CHR166 (in red). DDM1 serves as an outgroup.

(D) Neighbor-joining tree, with bootstrap values, generated from MUSCLE alignment of full-length *Arabidopsis*, rice, and maize Argonaute proteins most similar to *Arabidopsis* AGO4. Fission yeast (*S. pombe*) Ago1 serves as an outgroup.

See also Figures S1O and S1P and Table S1.

Pol IV-MOP1 association was also confirmed by LC-MS/MS analyses of affinity-purified FLAG-MOP1 complexes (Table 3; see also Table S7). MOP1-associated proteins include the Pol IV largest subunit, NRPD1, and the Pol IV-associated protein, RMR1 (see later section), but not the Pol V largest subunit, NRPE1, or Pol V-associated proteins. Pol IV associated with MOP1 included the NRP(D/E)10b or NRP(D/E)10c variant forms of the tenth subunit, whose alternative use was unclear upon analysis of NRPD1-associated proteins (compare Tables 1 and 3). Moreover, MOP1 appears to preferentially associate with the Pol IV subtype containing NRP(D/E)2a; no peptides for NRP(D/E)2b were detected.

RMR1 is a known paramutation protein (Hale et al., 2009) that we found in association with Pol IV (NRPD1), along with its paralog, CHR167 (Table 1; Figure 4C). These are SWI2/SNF2-family proteins and thus putative ATP-dependent DNA translocases. Both proteins are related to *Arabidopsis* CLSY proteins (Figures 4C and S1P), which associate with Pol IV and are involved in Pol IV and RDR2-dependent siRNA biogenesis (Law et al., 2011; Smith et al., 2007), and to DRD1 (Figure 4C), which facilitates Pol V transcription in *Arabidopsis*. However, a more closely related DRD1-like protein, CHR127, was detected in association with maize Pol V (Table 1). A maize ortholog of *Arabidopsis* DMS3 (Kanno et al., 2008), a protein that associates with DRD1 and is required for Pol V transcription (Law et al., 2010; Wierzbicki et al., 2008, 2009), was also identified in association with Pol V (Table 1; Figure S1M).

AGO121, a maize protein similar to *Arabidopsis* AGO6, associates with maize Pol V (Table 1; Figures 4D and S1O). AGO4 and AGO6 are the principal 24 nt siRNA-binding Argonaute proteins involved in RNA-directed DNA methylation in vegetative tissues of *Arabidopsis*, suggesting that AGO121 plays an analogous role in 24 nt siRNA-mediated silencing in maize.

The maize ortholog of the yeast Iwr1 protein was detected in association with Pols II, IV, and V (Table 1; Figure S1N). This is consistent with the involvement of yeast Iwr1 in the nuclear import of RNA polymerases assembled in the cytoplasm and with the previous identification of IWR1 as a protein involved in RdDM that can associate with *Arabidopsis* Pol V (Czeko et al., 2011; He et al., 2009; Kanno et al., 2010; Law et al., 2011). The presence of IWR1 in affinity-purified MOP1 complexes (Table S7) suggests that MOP1 and Pol IV associate prior to IWR1 dissociation from Pol IV.

DISCUSSION

Functionally Distinct Polymerase Subtypes Use Alternative Catalytic Subunits

Catalytic centers of multisubunit RNA polymerases are formed by the largest and second-largest subunits (Cramer et al., 2008) such that mutants defective for either subunit are expected to have the same phenotypes. The fact that maize *nrpd1* (*rmr6*) and *nrp*(*d*/*e*)*2*$_a$ (*mop2*/*rmr7*) loss-of-function mutants are both impaired for paramutation fits this expectation. However, developmental abnormalities observed in *nrpd1* mutants are not typical of *nrp*(*d*/*e*)*2*$_a$ mutants (Sidorenko et al., 2009; Stonaker et al., 2009), an observation made even more surprising by our finding that *NRP*(*D*/*E*)*2*$_a$ is the preferred second subunit for both Pol IV and Pol V (based on peptide abundance), indicating that *nrp*(*d*/*e*)*2*$_a$ mutants should be impaired for both activities. The fact that Pol IV can also assemble using *NRP*(*D*/*E*)*2*$_b$ and Pol V can make alternative use of *NRP*(*D*/*E*)*2*$_b$ or *NRP*(*D*/*E*)*2*$_c$ provides a solution to this apparent paradox, allowing the deduction that Pol IV and/or Pol V subtypes assembled

Table 3. Proteins Copurifying with FLAG-Tagged MOP1

Yeast Protein	Maize Homologs	Coverage by Peptides Unique to Indicated Protein (%)	Protein Names and Synonyms
DNA-Dependent RNA Polymerase Subunits			
Rpb1	GRMZM2G044306		NRPB1
	GRMZM2G007681	18	NRPD1/RMR6
	GRMZM2G153797		NRPE1
Rpb2	GRMZM2G084891		NRPB2a
	GRMZM2G113928		NRPB2b
	GRMZM2G054225	14	NRP(D/E)2a/ MOP2/RMR7
	GRMZM2G427031	[a]	NRP(D/E)2b
	GRMZM2G133512	[a]	NRPE2c
Rpb3	GRMZM2G402295	43	NRP(B/D/E)3
Rpb4	GRMZM2G119393		NRPB4
	GRMZM2G453424	44	NRP(D/E)4
Rpb5	GRMZM2G476009		NRPB5a
	GRMZM2G099183		NRPB5b
	GRMZM2G469969	32	NRP(D/E)5
Rpb6	GRMZM2G013600	[a]	NRP(B/D/E)6a
	GRMZM2G086904	[a]	NRP(B/D/E)6b
Rpb7	GRMZM2G179346		NRPB7
	GRMZM2G040702	16	NRP(D/E)7
Rpb8	GRMZM2G034326	47	NRP(B/D/E)8
	GRMZM2G347789		NRPB8-like
Rpb9	GRMZM2G046061		NRPB9a
	GRMZM2G023028		NRPB9b
	GRMZM5G898768	12	NRP(D/E)9
Rpb10	NP_001152395	[a]	NRP(B/D/E)10a
	GRMZM5G803992	35[b]	NRP(D/E)10b
	GRMZM5G834335	35[b]	NRP(D/E)10c
Rpb11	GRMZM2G130207	33	NRP(B/D/E)11
	GRMZM2G043461	[a]	NRPB11-like
Rpb12	GRMZM2G146331		NRPB12a
	GRMZM2G322661		NRPB12b
	GRMZM2G540834		NRPB12c
Polymerase-Associated Proteins			
RNA-dep RNAP	GRMZM2G042443	55	MOP1/RDR101
	GRMZM2G145201	[a]	RDR102
Swi2/Snf2	GRMZM2G154946		RMR1
	GRMZM2G178435		CHR167/RMR1-like
	GRMZM5G574858		CHR127/Zm_DRD1a
	GRMZM2G393742		CHR156/Zm_DRD1b
-	GRMZM2G309152		Zm_DMS3
-	GRMZM2G111204	3	Zm_SHH2a
	GRMZM2G126170	13	Zm_SHH2b
Iwr1	GRMZM2G098603	4	IWR1/Zm_DMS4/ Zm_RDM4
Ago1	GRMZM2G589579		AGO105
	GRMZM2G089743		AGO119
	GRMZM2G347402		AGO121

Annotation is the same as for Table 1. For additional details, see Table S7.
[a]Peptides matching the protein were detected but also match one or more paralogs.
[b]Proteins are identical and indistinguishable.

using alternative second subunits are functionally distinct. The evidence suggests that Pol $IV_{NRP(D/E)2a}$ and Pol $IV_{NRP(D/E)2b}$ subtypes are redundant with respect to functions important for development, such that a *nrp*(*d*/*e*)2_a *nrp*(*d*/*e*)2_b double mutant would be necessary to recapitulate *nrpd1* developmental phenotypes. By contrast, the Pol $IV_{NRP(D/E)2b}$ subtype must not be redundant with Pol $IV_{NRP(D/E)2a}$ with respect to paramutation, given that *nrp*(*d*/*e*)2_a single mutants are impaired for paramutation, like *nrpd1* mutants.

One plausible explanation for polymerase subtype nonredundancy might be different interactions with partner proteins. In this regard, it is noteworthy that Pol IV associated with MOP1 contains the NRP(D/E)2a subunit, but no NRP(D/E)2b peptides were detected. Different Pol IV and Pol V subtypes might also target different subsets of loci or may have different enzymatic properties. Alternatively, the different polymerase subtypes may be enriched in different cell types, tissues, or organs, consistent with observation that *NRPE2c* is highly expressed only in tassels and pollen (Sidorenko et al., 2009), perhaps explaining why the protein was detected only in trace amounts in Pol V isolated from callus cells in our study. Variation in noncatalytic subunits, such as the sixth and tenth subunits, may contribute additional functional diversity to maize Pols IV and V subtypes, as well as to Pol II subtypes.

Insights into the Pol II-Pol IV/Pol V Evolutionary Split and Pol IV/Pol V Diversification

The fundamental difference between Pols IV and V in maize is their use of different largest subunits. This is surprising in light of our prior results in *Arabidopsis*, in which we showed that Pols IV and V differ in three subunits: their largest, fifth-largest, and seventh-largest subunits (Ream et al., 2009). *Arabidopsis* Pol V makes use of a fifth subunit encoded by a gene (*NRPE5*) that is distinct from the single-copy gene that encodes the corresponding subunits of Pols I, II, III, and IV. Importantly, the maize ortholog of *Arabidopsis NRPE5* (Figure 3A) encodes the fifth subunit of both Pols IV and V (see Table 1). This argues against the *Arabidopsis*-centric hypothesis that emergence of a Pol-V-specific fifth subunit was a critical event in the functional diversification of Pols IV and V.

Maize Pols IV and V also make use of seventh subunits encoded by the same gene, unlike *Arabidopsis*. This is less

surprising given that phylogenetic analyses indicate that the gene duplication giving rise to the *NRPD7* and *NRPE7* genes in *Arabidopsis* is not deeply rooted, even among dicots (Figure 3B; see also Tucker et al., 2010). The seventh subunit, in partnership with the fourth subunit, is expected to form a stalk-like subcomplex located adjacent to the RNA exit channel, as in archaeal RNA polymerases or eukaryotic Pols I, II, and III (Cramer et al., 2001, 2008; Werner and Grohmann, 2011). In the context of Pol II, the subunit 4/7 subcomplex is important for multiple aspects of RNA processing and, in yeast, can even dissociate from the core polymerase and traffic with RNA within the cell (Harel-Sharvit et al., 2010; Mitsuzawa et al., 2003; Sampath and Sadhale, 2005). Importantly, the fourth and seventh subunits of maize Pols IV and V are the same yet distinct from the corresponding subunits of Pols I, II, and III. This suggests that the 4/7 subcomplex is likely important for functions common to both Pols IV and V yet different from those of other polymerases.

Another maize-*Arabidopsis* difference concerns the ninth subunits of Pols II, IV, and V. In the context of Pol II, the ninth subunit is important for RNA cleavage activity, stimulated by TFIIS, for the correction of misincorporated nucleotides, backtracking to overcome polymerase stalling, or transcription termination (Hemming et al., 2000; Koyama et al., 2007; Nesser et al., 2006; Walmacq et al., 2009). *Arabidopsis* expresses two alternative ninth subunits that are 92% identical and are detected in similar abundance in Pols II, IV, or V (Law et al., 2011; Ream et al., 2009). Genetic experiments in *Arabidopsis* showed that these alternative subunits are redundant for Pol II functions required for viability, but not for RNA-directed DNA methylation (Tan et al., 2012). Instead, only the NRP(B/D/E)9b variant is required for RdDM; the 9a variant is dispensable (Tan et al., 2012). In light of these studies, it is intriguing that maize Pols IV and V make use of a ninth subunit distinct from the two alternative ninth subunits used by Pol II (see Table 1), suggesting important Pol-IV/Pol-V-specific ninth-subunit functions that await definition.

Maize Pols IV and V differ only in their largest subunits, and the most obvious difference between their NRPD1 and NRPE1 proteins is the presence of a longer C-terminal domain in NRPE1. Pol V interaction with AGO proteins is attributable to the CTD of NRPE1 in *Arabidopsis* (El-Shami et al., 2007), suggesting that maize Pol V interaction with AGO121 may similarly involve the WG- and GW-rich region of the CTD. Pol-V-specific interactions with CHR127/Zm-DRD1 and ZmDMS3 and Pol-IV-specific interactions with MOP1 and RMR1 must also be attributable, directly or indirectly, to the different largest subunits of Pols IV and V. By contrast, interactions with SHH2a and SHH2b, by both Pols IV and V, are presumably mediated by subunits common to Pols IV and V. Guided by these insights, identification of Pol IV or Pol V sequences that interact with partner proteins to coordinate RNA-directed DNA methylation and/or paramutation is a priority for future studies.

EXPERIMENTAL PROCEDURES

Identification and Cloning of RNA Polymerase Subunits

Gene models for maize *NRPD1*, *NRPE1*, and *NRP(D/E)2a* were deduced based on sequence similarity to *Arabidopsis* and rice orthologs. Sequences corresponding to GRMZM2G007681, GRMZM2G153797, and GRMZM2G054225 were targeted for cloning and overexpression. Full-length cDNAs were obtained by RT-PCR amplification of isolated total RNA.

C-terminal FLAG-HA tagged *NRPD1*, *NRPE1*, and *NRP(D/E)2a* were generated by cDNA amplification using KOD DNA polymerase (Novagen) and cloning into pCR-Blunt II-TOPO (Invitrogen). Plant-expression vectors pMON124573 (NRPD1-C tag), pMON124574 (NRPDE2a-C tag), and pMON124576 (NRPE1-C tag) were used for Agrobacterium-mediated plant transformation (Frame et al., 2002). Transgenic (R0) plants were selected on medium containing glyphosate and verified by PCR and Southern blot hybridization.

To generate N-terminal FLAG-tagged *NRP(D/E)2a* (National Center for Biotechnology Information [NCBI] no. GQ453405), cDNA from 5-day-old seedlings (Brzeska et al., 2010) was amplified with primers vc2817F and vc2817R. *MOP1* (NCBI no. JQ248126) was amplified using primers KM510F and KM511R and RNA from B73 embryos dissected from midmaturation kernels using the Agilent Plant RNA Isolation Mini Kit. Primers carried *attB* sites for GATEWAY LR cloning (Invitrogen) into the plant expression vector, pEarleyGate402 (http://sites.bio.indiana.edu/~pikaardlab/Vectors%20homepage.html). Resulting constructs were transformed into *Hill* embryos using *A. tumefaciens* at the Iowa State Plant Transformation Facility.

Transgenic Plant Production, Growth, and Genotyping

FLAG::MOP1 plants, grown in a greenhouse with 16 hr of light and 8 hr of dark, were screened for transgene expression by RT-PCR using FLAG tag primer KM666F and *MOP1* coding region primer, KM668R. Tissues were flash frozen in liquid nitrogen and stored at −80°C.

FLAG::NRP(D/E)2a callus was screened for transgene expression by RT-PCR using a FLAG primer (vc4856F) and *NRP(D/E)2a*-specific primer, vc4856R3. The transgene was detected by PCR using either the *Ubiquitin1* promoter primer vc6139F and *NRP(D/E)2a* coding region primer vc6139R or the *NRP(D/E)2a* coding region primer vc2987F and OCS3′R1 (NCBI no. MCG524A) primer vc2556AR.

Type II corn callus AT824 was initiated (Gordon-Kamm et al., 1990) and cryopreserved and reinitiated as described in Gordon-Kamm et al. (1991). AT824 callus suspension was maintained on N6 media at 28°C as described (Gordon-Kamm et al., 1990, 1991). NRPD1-C, NRP(D/E)2a-C, and NRPE1-C transgenic corn cell lines were created by microprojectile bombardment (Biolistic particle delivery system PDS-1000/He) at 1,350 psi. Postbombardment, cells were cultured for 7 days and then spread on selection plates containing 25 mg/l paramomycin. Resistant colonies were identified 5 weeks postselection, confirmed by PCR and immunoblot analyses, and maintained as individual cell lines on medium containing 25 mg/l paramomycin.

Sample Preparation for Mass Spectrometry

Frozen cells (70 g) from callus suspension cultures (nontransgenic, NRPD1-FLAG-HA, NRPE1-FLAG-HA, or NRP(D/E)2a-FLAG-HA genotypes) or from 100 g combined husk and ear tissues harvested 3–5 days post-silk emergence (nontransgenic, FLAG-NRP(D/E)2a, and FLAG-MOP1) were homogenized at 4°C in a blender with extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 10% glycerol, 0.1% NP-40, 5 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride, and 1:300 plant protease inhibitor cocktail [Sigma]), filtered through two layers of Miracloth (Calbiochem), and centrifuged twice at 10,000 × *g* for 20 min at 4°C. Supernatants were incubated with anti-FLAG-M2 resin (Sigma) for 3 hr in 15 ml conical tubes using 25 μl of resin per 14 ml of extract. Resin was pelleted at 200 × *g* for 2 min, was washed five times with 10 ml extraction buffer, and resuspended in an equal volume Gentle Ag/Ab Elution Buffer (Pierce). After 2 min, resin was pelleted and proteins in the supernatant were concentrated in an Ultracel-10k centrifugal filter (Millipore), with three serial buffer exchanges of 25 mM ammonium bicarbonate, to a final volume of ~100 μl.

NRPB1 was purified from 34 g of nontransgenic husk and ear tissues as above. The cell-free extract was precleared with Protein G agarose (Pierce) for 30 min at 4°C before the supernatant was split for control (mouse immunoglobulin G [IgG] serum [Calbiochem]) or anti-NRPB1 (clone 8WG16 [Millipore]) immune-affinity purifications. Samples were incubated with antibodies 2 hr; 50 μl Protein G agarose (Pierce) was added and incubated an additional 2 hr; and resin was washed, eluted, and concentrated, as above.

Mass Spectrometry of Affinity-Purified Protein Complexes

Samples (100 μl) were mixed with 400 μl of 0°C methanol, 100 μl of 0°C chloroform, and 300 μl of ice-cold water; incubated 2 min on ice; and subjected to centrifugation at 12,000 × *g* for 2 min at 0°C. The supernatant was removed, mixed by vortexing with 300 μl methanol, and subjected to centrifugation at 12,000 × *g* for 5 min. The supernatant was removed and the protein pellet dried briefly at room temperature prior to addition of 50 μl of 50 mM ammonium bicarbonate (pH 8.5). Protein concentration was estimated using a Coomassie dye-binding assay (Pierce). 2,2,2-trifluoroethanol (Sigma) was added to a final concentration of 50% (v/v). The sample was sonicated in an ice-water bath for 1 min and incubated at 60°C for 2 hr with shaking at 300 rpm. Samples were reduced with 2 mM DTT (Sigma) for 1 hr at 37°C with shaking at 300 rpm and then diluted 5-fold with 50 mM ammonium bicarbonate. One millimolar CaCl2 and sequencing-grade modified porcine trypsin (Promega) were added at a 1:50 (w/w) trypsin-to-protein ratio and incubated 3 hr at 37°C. Samples were concentrated in a Speed Vac to a volume of ~30 μl, centrifuged at 12,000 × *g*, and supernatants subjected to LC-MS analysis using LTQ-Orbitrap and LTQ-Orbitrap Velos mass spectrometry (Thermo Scientific) as previously described (Ream et al., 2009). Acquired tandem mass spectra were searched against the annotated *Z. mays* genome (http://ensembl.gramene.org/Zea_mays/Info/Index; release 5b.60) supplemented with additional RNA polymerase subunit and chromatin modifier sequences identified in The Chromatin Database (http://www.chromdb.org/), Qian et al. (2011), and NCBI GenBank by tblastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using SEQUEST (Eng et al., 1994), allowing for partial tryptic cleavage and methionine oxidation. Nonmodified peptides or those only containing an oxidized methionine were filtered to a 2% false discovery rate, based on MS generating function scores (http://proteomics.ucsd.edu/software-tools/ms-gf/).

Antibody Production

Antibodies against unique NRPD1 and NRPE1 sequences were raised in rabbits by immunization with multiple peptide antigens (KLH linked). NRPD1 antibodies were raised against peptides ELHREPPEAILNAIKFDC and KVRNFEKNHLDTRRQSTE. NRPE1 sera were raised against peptides CDGTGLLGKAPQADWGPRFDAD and SQRNNPGRPPRRPDER.

Anti-MOP1 antibodies were raised against a peptide comprising amino acids 1,028–1,127 and affinity purified from crude sera using bacterially expressed 6xHis-MOP1(amino acids [aas] 1,028–1,127) immobilized on polyvinylidene fluoride membrane, as previously described (Haag et al., 2012).

Immunoprecipitation and Immunoblotting

Frozen cell pellets of maize callus (1 to 2 g), or husk and ear tissues (4 g), were ground in liquid nitrogen and suspended in 14 ml extraction buffer (see above), filtered through Miracloth, and subjected to centrifugation at 16,000 × *g* for 15 min at 4°C. Supernatants were incubated 2 to 3 hr at 4°C with 50 μl anti-FLAG-M2 slurry or precleared with mouse IgG serum for 30 min at 4°C prior to incubation with mouse IgG serum or anti-NRPB1 (clone 8WG16) for 2 hr followed by incubation with 50 μl Protein G agarose slurry for 1 to 2 hr at 4°C. Resin was washed three times in extraction buffer and eluted in two bed volumes of 2× SDS sample buffer by boiling. Proteins were subjected to SDS-PAGE on 7.5% Tris-glycine gels and transferred to nitrocellulose membranes. Blots were incubated with antibodies in Tris-buffered saline supplemented with Tween-20 + 5% (w/v) nonfat dried milk. Antibody dilutions were 1:3,000 anti-FLAG-horseradish peroxidase (HRP) (Sigma); 1:3,000 anti-HA, clone 9E10 (Sigma); 1:500 anti-NRPD1; 1:500 anti-NRPE1; 1:250 anti-MOP1; 1:2,000 anti-NRPB1, clone 8WG16 (Millipore); and 1:5,000–1:10,000 donkey anti-mouse-HRP (Santa Cruz Biotechnology). Enhanced chemiluminescence (ECL) and ECL Plus reagents (GE Healthcare) were used for chemiluminescent detection on film.

Phylogenetic Analyses

Aligned proteins were identified by tblastn searches using *A. thaliana* and *S. cerevisiae* protein sequences as queries against NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), maize sequence (http://ensembl.gramene.org/Zea_mays/Info/Index), The Chromatin Database (http://www.chromdb.org/), Joint Genome Institute (http://www.phytozome.net/index.php), and Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml) databases. Sequences were aligned using ClustalW2 or MUSCLE and conserved sequences highlighted using BOXSHADE v3.31. Phylogenetic analysis was by the neighbor-joining method, with 1,000 bootstrap replications, using Geneious software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one figure, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.067.

AUTHOR CONTRIBUTIONS

J.R.H. and C.S.P. designed the study and wrote the paper. FLAG-NRP(D/E)2a and FLAG-MOP1 plants were generated by L.S. and A.I., respectively. J.B. generated the MOP1 antibody. LC-MS/MS was performed by C.D.N., A.D.N., and L.P.-T. Maize polymerase subunit genes were identified and annotated by J.R.H., L.S., and B.B.-T. All other experiments and analyses were performed by J.R.H.

ACKNOWLEDGMENTS

Monsanto generated transgenic callus lines and NRPD1 and NRPE1 antibodies. We thank John Lemon (IU) and Benjamin Echalier (UA) and their staffs for helping with plant care; Hong Liu and Jiyan Ma for technical assistance; and Jason Osborne (Monsanto Immunoassay) and Todd Ziegler (Monsanto Custom Expression) for reagents. Portions of this research were supported by the NIH National Center for Research Resources (RR18522) and the W.R. Wiley Environmental Molecular Science Laboratory, a national scientific user facility sponsored by the US Department of Energy, located at PNNL and operated by Battelle Memorial Institute under DOE contract DE-AC05-76RL01830. C.S.P. laboratory studies were supported by NIH grant GM077590 and C.S.P.'s support as an Investigator of the Howard Hughes Medical Institute and the Gordon & Betty Moore Foundation. Opinions are those of the authors and do not necessarily reflect the views of our sponsors.

Received: June 12, 2014
Revised: July 9, 2014
Accepted: August 26, 2014
Published: October 2, 2014

REFERENCES

Alleman, M., Sidorenko, L., McGinnis, K., Seshadri, V., Dorweiler, J.E., White, J., Sikkink, K., and Chandler, V.L. (2006). An RNA-dependent RNA polymerase is required for paramutation in maize. Nature *442*, 295–298.

Frame, B.R., Shou, H., Chikwamba, R.K., Zhang, Z., Xiang, C., Fonger, T.M., Pegg, S.E., Li, B., Nettleton, D.S., Pei, D., and Wang, K. (2002). Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol. *129*, 13–22.

Arteaga-Vazquez, M.A., and Chandler, V.L. (2010). Paramutation in maize: RNA mediated trans-generational gene silencing. Curr. Opin. Genet. Dev. *20*, 156–163.

Gordon-Kamm, W.J., Spencer, T.M., O'Brien, J.V., Start, W.G., Daines, R.J., Adams, T.R., Mangano, M.L., Chambers, S.A., Zachwieja, S.J., Willetts, N.G., et al. (1991). Transformation of maize using microprojectile bombardment: an update and perspective. In Vitro Cell. Dev. Biol. *27P*, 21–27.

Bellaoui, M., and Gruissem, W. (2004). Altered expression of the Arabidopsis ortholog of DCL affects normal plant development. Planta *219*, 819–826.

Brzeska, K., Brzeski, J., Smith, J., and Chandler, V.L. (2010). Transgenic expression of CBBP, a CXC domain protein, establishes paramutation in maize. Proc. Natl. Acad. Sci. USA *107*, 5516–5521.

Cramer, P., Bushnell, D.A., and Kornberg, R.D. (2001). Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. Science *292*, 1863–1876.

Cramer, P., Armache, K.J., Baumli, S., Benkert, S., Brueckner, F., Buchen, C., Damsma, G.E., Dengl, S., Geiger, S.R., Jasiak, A.J., et al. (2008). Structure of eukaryotic RNA polymerases. Annu. Rev. Biophys. *37*, 337–352.

Czeko, E., Seizl, M., Augsberger, C., Mielke, T., and Cramer, P. (2011). Iwr1 directs RNA polymerase II nuclear import. Mol. Cell *42*, 261–266.

Ebright, R.H., and Busby, S. (1995). The Escherichia coli RNA polymerase alpha subunit: structure and function. Curr. Opin. Genet. Dev. *5*, 197–203.

Egloff, S., and Murphy, S. (2008). Cracking the RNA polymerase II CTD code. Trends Genet. *24*, 280–288.

El-Shami, M., Pontier, D., Lahmy, S., Braun, L., Picart, C., Vega, D., Hakimi, M.A., Jacobsen, S.E., Cooke, R., and Lagrange, T. (2007). Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. Genes Dev. *21*, 2539–2544.

Eng, J.K., McCormack, A.L., and Yates, J.R., III. (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. *5*, 976–989.

Erhard, K.F., Jr., and Hollick, J.B. (2011). Paramutation: a process for acquiring trans-generational regulatory states. Curr. Opin. Plant Biol. *14*, 210–216.

Erhard, K.F., Jr., Stonaker, J.L., Parkinson, S.E., Lim, J.P., Hale, C.J., and Hollick, J.B. (2009). RNA polymerase IV functions in paramutation in Zea mays. Science *323*, 1201–1205.

Gao, Z., Liu, H.L., Daxinger, L., Pontes, O., He, X., Qian, W., Lin, H., Xie, M., Lorkovic, Z.J., Zhang, S., et al. (2010). An RNA polymerase II- and AGO4-associated protein acts in RNA-directed DNA methylation. Nature *465*, 106–109.

Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien, J.V., Chambers, S.A., Adams, W.R., Jr., Willetts, N.G., et al. (1990). Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants. Plant Cell *2*, 603–618.

Haag, J.R., and Pikaard, C.S. (2011). Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing. Nat. Rev. Mol. Cell Biol. *12*, 483–492.

Haag, J.R., Ream, T.S., Marasco, M., Nicora, C.D., Norbeck, A.D., Pasa-Tolic, L., and Pikaard, C.S. (2012). In vitro transcription activities of Pol IV, Pol V, and RDR2 reveal coupling of Pol IV and RDR2 for dsRNA synthesis in plant RNA silencing. Mol. Cell *48*, 811–818.

Hale, C.J., Erhard, K.F., Jr., Lisch, D., and Hollick, J.B. (2009). Production and processing of siRNA precursor transcripts from the highly repetitive maize genome. PLoS Genet. *5*, e1000598.

Harel-Sharvit, L., Eldad, N., Haimovich, G., Barkai, O., Duek, L., and Choder, M. (2010). RNA polymerase II subunits link transcription and mRNA decay to translation. Cell *143*, 552–563.

Havecker, E.R., Wallbridge, L.M., Hardcastle, T.J., Bush, M.S., Kelly, K.A., Dunn, R.M., Schwach, F., Doonan, J.H., and Baulcombe, D.C. (2010). The Arabidopsis RNA-directed DNA methylation argonautes functionally diverge based on their expression and interaction with target loci. Plant Cell *22*, 321–334.

He, X.J., Hsu, Y.F., Zhu, S., Liu, H.L., Pontes, O., Zhu, J., Cui, X., Wang, C.S., and Zhu, J.K. (2009). A conserved transcriptional regulator is required for RNA-directed DNA methylation and plant development. Genes Dev. *23*, 2717–2722.

Hemming, S.A., Jansma, D.B., Macgregor, P.F., Goryachev, A., Friesen, J.D., and Edwards, A.M. (2000). RNA polymerase II subunit Rpb9 regulates transcription elongation in vivo. J. Biol. Chem. *275*, 35506–35511.

Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C. (2005). RNA polymerase IV directs silencing of endogenous DNA. Science *308*, 118–120.

Hsin, J.P., and Manley, J.L. (2012). The RNA polymerase II CTD coordinates transcription and RNA processing. Genes Dev. *26*, 2119–2137.

Huang, L., Jones, A.M., Searle, I., Patel, K., Vogler, H., Hubner, N.C., and Baulcombe, D.C. (2009). An atypical RNA polymerase involved in RNA silencing shares small subunits with RNA polymerase II. Nat. Struct. Mol. Biol. *16*, 91–93.

Kanno, T., Aufsatz, W., Jaligot, E., Mette, M.F., Matzke, M., and Matzke, A.J. (2005a). A SNF2-like protein facilitates dynamic control of DNA methylation. EMBO Rep. *6*, 649–655.

Kanno, T., Huettel, B., Mette, M.F., Aufsatz, W., Jaligot, E., Daxinger, L., Kreil, D.P., Matzke, M., and Matzke, A.J. (2005b). Atypical RNA polymerase subunits required for RNA-directed DNA methylation. Nat. Genet. *37*, 761–765.

Kanno, T., Bucher, E., Daxinger, L., Huettel, B., Böhmdorfer, G., Gregor, W., Kreil, D.P., Matzke, M., and Matzke, A.J. (2008). A structural-maintenance-of-chromosomes hinge domain-containing protein is required for RNA-directed DNA methylation. Nat. Genet. *40*, 670–675.

Kanno, T., Bucher, E., Daxinger, L., Huettel, B., Kreil, D.P., Breinig, F., Lind, M., Schmitt, M.J., Simon, S.A., Gurazada, S.G., et al. (2010). RNA-directed DNA methylation and plant development require an IWR1-type transcription factor. EMBO Rep. *11*, 65–71.

Koyama, H., Ito, T., Nakanishi, T., and Sekimizu, K. (2007). Stimulation of RNA polymerase II transcript cleavage activity contributes to maintain transcriptional fidelity in yeast. Genes Cells *12*, 547–559.

Lahmy, S., Pontier, D., Cavel, E., Vega, D., El-Shami, M., Kanno, T., and Lagrange, T. (2009). PolV(PolIVb) function in RNA-directed DNA methylation requires the conserved active site and an additional plant-specific subunit. Proc. Natl. Acad. Sci. USA *106*, 941–946.

Law, J.A., and Jacobsen, S.E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat. Rev. Genet. *11*, 204–220.

Law, J.A., Ausin, I., Johnson, L.M., Vashisht, A.A., Zhu, J.K., Wohlschlegel, J.A., and Jacobsen, S.E. (2010). A protein complex required for polymerase V transcripts and RNA- directed DNA methylation in Arabidopsis. Curr. Biol. *20*, 951–956.

Law, J.A., Vashisht, A.A., Wohlschlegel, J.A., and Jacobsen, S.E. (2011). SHH1, a homeodomain protein required for DNA methylation, as well as RDR2, RDM4, and chromatin remodeling factors, associate with RNA polymerase IV. PLoS Genet. *7*, e1002195.

Law, J.A., Du, J., Hale, C.J., Feng, S., Krajewski, K., Palanca, A.M.S., Strahl, B.D., Patel, D.J., and Jacobsen, S.E. (2013). Polymerase IV occupancy at RNA-directed DNA methylation sites requires SHH1. Nature *498*, 385–389.

Luo, J., and Hall, B.D. (2007). A multistep process gave rise to RNA polymerase IV of land plants. J. Mol. Evol. *64*, 101–112.

Mallory, A., and Vaucheret, H. (2010). Form, function, and regulation of ARGONAUTE proteins. Plant Cell *22*, 3879–3889.

Matzke, M., Kanno, T., Daxinger, L., Huettel, B., and Matzke, A.J. (2009). RNA-mediated chromatin-based silencing in plants. Curr. Opin. Cell Biol. *21*, 367–376.

Mitsuzawa, H., Kanda, E., and Ishihama, A. (2003). Rpb7 subunit of RNA polymerase II interacts with an RNA-binding protein involved in processing of transcripts. Nucleic Acids Res. *31*, 4696–4701.

Nesser, N.K., Peterson, D.O., and Hawley, D.K. (2006). RNA polymerase II subunit Rpb9 is important for transcriptional fidelity in vivo. Proc. Natl. Acad. Sci. USA *103*, 3268–3273.

Onodera, Y., Haag, J.R., Ream, T., Costa Nunes, P., Pontes, O., and Pikaard, C.S. (2005). Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. Cell *120*, 613–622.

Pikaard, C.S., Haag, J.R., Pontes, O.M., Blevins, T., and Cocklin, R. (2012). A transcription fork model for Pol IV and Pol V-dependent RNA-directed DNA methylation. Cold Spring Harb. Symp. Quant. Biol. *77*, 205–212.

Pontier, D., Yahubyan, G., Vega, D., Bulski, A., Saez-Vasquez, J., Hakimi, M.A., Lerbs-Mache, S., Colot, V., and Lagrange, T. (2005). Reinforcement of silencing at transposons and highly repeated sequences requires the concerted action of two distinct RNA polymerases IV in Arabidopsis. Genes Dev. *19*, 2030–2040.

Qian, Y., Cheng, Y., Cheng, X., Jiang, H., Zhu, S., and Cheng, B. (2011). Identification and characterization of Dicer-like, Argonaute and RNA-dependent RNA polymerase gene families in maize. Plant Cell Rep. *30*, 1347–1363.

Ream, T.S., Haag, J.R., Wierzbicki, A.T., Nicora, C.D., Norbeck, A.D., Zhu, J.K., Hagen, G., Guilfoyle, T.J., Pasa-Tolić, L., and Pikaard, C.S. (2009).

Subunit compositions of the RNA-silencing enzymes Pol IV and Pol V reveal their origins as specialized forms of RNA polymerase II. Mol. Cell *33*, 192–203.

Ream, T., Haag, J., and Pikaard, C.S. (2014). Plant multisubunit RNA polymerases IV and V. In Nucleic Acid Polymerases, K. Murakami and M. Trakselis, eds. (Berlin, Heidelberg: Springer-Verlag), pp. 289–308.

Sampath, V., and Sadhale, P. (2005). Rpb4 and Rpb7: a sub-complex integral to multi-subunit RNA polymerases performs a multitude of functions. IUBMB Life *57*, 93–102.

Sidorenko, L., Dorweiler, J.E., Cigan, A.M., Arteaga-Vazquez, M., Vyas, M., Kermicle, J., Jurcin, D., Brzeski, J., Cai, Y., and Chandler, V.L. (2009). A dominant mutation in mediator of paramutation2, one of three second-largest subunits of a plant-specific RNA polymerase, disrupts multiple siRNA silencing processes. PLoS Genet. *5*, e1000725.

Smith, L.M., and Baulcombe, D.C. (2007). Dissection of silencing signal movement in Arabidopsis. Plant Signal. Behav. *2*, 501–502.

Smith, L.M., Pontes, O., Searle, I., Yelina, N., Yousafzai, F.K., Herr, A.J., Pikaard, C.S., and Baulcombe, D.C. (2007). An SNF2 protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in Arabidopsis. Plant Cell *19*, 1507–1521.

Stonaker, J.L., Lim, J.P., Erhard, K.F., Jr., and Hollick, J.B. (2009). Diversity of Pol IV function is defined by mutations at the maize rmr7 locus. PLoS Genet. *5*, e1000706.

Tan, E.H., Blevins, T., Ream, T.S., and Pikaard, C.S. (2012). Functional consequences of subunit diversity in RNA polymerases II and V. Cell Reports *1*, 208–214.

Tucker, S.L., Reece, J., Ream, T.S., and Pikaard, C.S. (2010). Evolutionary history of plant multisubunit RNA polymerases IV and V: subunit origins via genome-wide and segmental gene duplications, retrotransposition, and lineage-specific subfunctionalization. Cold Spring Harb. Symp. Quant. Biol. *75*, 285–297.

Ulmasov, T., Larkin, R.M., and Guilfoyle, T.J. (1996). Association between 36- and 13.6-kDa alpha-like subunits of Arabidopsis thaliana RNA polymerase II. J. Biol. Chem. *271*, 5085–5094.

Walmacq, C., Kireeva, M.L., Irvin, J., Nedialkov, Y., Lubkowska, L., Malagon, F., Strathern, J.N., and Kashlev, M. (2009). Rpb9 subunit controls transcription fidelity by delaying NTP sequestration in RNA polymerase II. J. Biol. Chem. *284*, 19601–19612.

Werner, F., and Grohmann, D. (2011). Evolution of multisubunit RNA polymerases in the three domains of life. Nat. Rev. Microbiol. *9*, 85–98.

Wierzbicki, A.T. (2012). The role of long non-coding RNA in transcriptional gene silencing. Curr. Opin. Plant Biol. *15*, 517–522.

Wierzbicki, A.T., Haag, J.R., and Pikaard, C.S. (2008). Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. Cell *135*, 635–648.

Wierzbicki, A.T., Ream, T.S., Haag, J.R., and Pikaard, C.S. (2009). RNA polymerase V transcription guides ARGONAUTE4 to chromatin. Nat. Genet. *41*, 630–634.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., and Carrington, J.C. (2004). Genetic and functional diversification of small RNA pathways in plants. PLoS Biol. *2*, E104.

Zhang, H., and Zhu, J.K. (2011). RNA-directed DNA methylation. Curr. Opin. Plant Biol. *14*, 142–147.

Zhang, H., Ma, Z.Y., Zeng, L., Tanaka, K., Zhang, C.J., Ma, J., Bai, G., Wang, P., Zhang, S.W., Liu, Z.W., et al. (2013). DTF1 is a core component of RNA-directed DNA methylation and may assist in the recruitment of Pol IV. Proc. Natl. Acad. Sci. USA *110*, 8290–8295.