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Subunit compositions of *Arabidopsis* RNA polymerases I and III reveal Pol I- and Pol III-specific forms of the AC40 subunit and alternative forms of the C53 subunit

Thomas S. Ream[1], Jeremy R. Haag[1,2], Frederic Pontvianne[2], Carrie D. Nicora[3], Angela D. Norbeck[3], Ljiljana Paša-Tolić[3] and Craig S. Pikaard[2,4,*]

[1]Division of Biology and Biomedical Sciences, Washington University, St. Louis, MO 63130, USA, [2]Department of Biology and Department of Molecular and Cellular Biochemistry, Indiana University, Bloomington, IN 47405, USA, [3]Environmental and Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99352, USA and [4]Howard Hughes Medical Institute, Indiana University, Bloomington, IN 47405, USA

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ABSTRACT

Using affinity purification and mass spectrometry, we identified the subunits of *Arabidopsis thaliana* multisubunit RNA polymerases I and III (abbreviated as Pol I and Pol III), the first analysis of their physical compositions in plants. In all eukaryotes examined to date, AC40 and AC19 subunits are common to Pol I (a.k.a. Pol A) and Pol III (a.k.a. Pol C) and are encoded by single genes. Surprisingly, *A. thaliana* and related species express two distinct AC40 paralogs, one of which assembles into Pol I and the other of which assembles into Pol III. Changes at eight amino acid positions correlate with the functional divergence of Pol I- and Pol III-specific AC40 paralogs. Two genes encode homologs of the yeast C53 subunit and either protein can assemble into Pol III. By contrast, only one of two potential C17 variants, and one of two potential C31 variants were detected in Pol III. We introduce a new nomenclature system for plant Pol I and Pol III subunits in which the 12 subunits that are structurally and functionally homologous among Pols I through V are assigned equivalent numbers.

INTRODUCTION

Eukaryotes have three essential nuclear multisubunit RNA polymerases, abbreviated as Pols I, II and III. Pol I functions in the nucleolus, transcribing repeated 45S ribosomal RNA genes whose precursor transcripts are then processed into the 18S, 5.8S and 25–28S RNAs of ribosomes (1–3). Pol II transcribes thousands of protein-coding genes and thousands of noncoding RNAs with diverse functions (4,5). Pol III transcribes tRNAs, 5S ribosomal RNA, a subset of retrotransposons, and multiple regulatory RNAs (6–8). In plants, two additional multisubunit RNA polymerases, Pol IV and Pol V, evolved as specialized forms of Pol II (9), generating noncoding RNAs that guide cytosine methylation and transcriptional silencing of transposons, repetitive elements and a subset of genes (10–12).

Pols I, II and III have been studied in greatest biochemical detail in yeast, which includes structural analyses using X-ray crystallography and/or cryo-electron microscopy (13–16). Yeast Pol I has 14 subunits, Pol II has 12 subunits and Pol III has 17 subunits. Available evidence indicates that Pol I and Pol III subunit compositions, as determined in yeast, are highly conserved in eukaryotes as diverse as mice, humans and trypanosomes (17–21), although complete subunit compositions have not been determined in most cases. Plant Pols IV and V have 12 subunits like Pol II, from which they evolved (9,22).

Within the five multisubunit RNA polymerases of eukaryotes, or within the sole RNA polymerase of archaea, are homologs of the ß′, ß, α and ω subunits that comprise bacterial RNA polymerase (23–25). The two largest subunits of Pols I through V are homologs of ß′ and ß; these interact to form the catalytic center for RNA synthesis (26). Eukaryotic homologs of the two bacterial α subunits are Rpb3 and Rpb11, in the case of Pols II, IV and V or AC40 and AC19, in the case of Pols I and III. AC40 and AC19 are named by virtue of their approximate masses in budding

*To whom correspondence should be addressed. Tel: +1 812 855 6621; Fax: +1 812 855 6082; Email: cpikaard@indiana.edu
Present addresses:
Thomas Ream, Monsanto Company, 700 West Chesterfield Parkway, Chesterfield, MO 63017, USA.
Jeremy Haag, Monsanto Company, 700 West Chesterfield Parkway, Chesterfield, MO 63017, USA.
Frederic Pontvianne, CNRS UMR5096, Laboratoire Génomes et Développement des Plantes, Université de Perpignan Via Domitia, Perpignan, France.

yeast (~40 and ~19 kDa, respectively) and their polymerase affiliations, using nomenclature introduced by Chambon *et al.* (27), with polymerases A, B and C being equivalent to Pols I, II and III (28). In humans, the *POLR1C* and *POLR1D* genes encode orthologs of yeast AC40 and AC19, respectively. Mutations in either of these genes can cause the craniofacial disorder, Treacher-Collins Syndrome (29). The eukaryotic homolog of the bacterial ω subunit is Rpb6, a subunit that is common to Pols I, II, III (30,31) as well as Pols IV and V in plants (9). Rpb6 is named according to a different nomenclature system in which budding yeast Pol II subunits are named in order of size, from largest to smallest; thus, Rpb6 is the sixth-largest subunit of Pol II (in yeast; not necessarily in other eukaryotes).

All five eukaryotic nuclear multisubunit RNA polymerases have a core set of 12 subunits that share homology with proteins in Archaea, indicative of a common ancestry (23). Among these are five subunits that are common to Pols I, II and III and are encoded by the same genes: Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12; by convention, the Pol II-specific Rpb(n) names are generally used for these common subunits (30,32). The common use of Rpb6, Rpb8, Rpb10 and Rpb12 by eukaryotic RNA polymerases extends to Pols IV and V in plants (9). However, this is not the case for Rpb5. In *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*), Pol IV makes use of the same Rpb5 protein as Pols I, II and III, but a different gene, NRPE5 encodes the subunit used primarily by Pol V (9,33,34). In plants, the prefix NRP denotes 'nuclear RNA polymerase', with E, as the fifth letter of the alphabet, denoting Pol V, by extension of Chambon and colleagues' Pol A, B, C naming system. In maize, the ortholog of *Arabidopsis NRPE5* is used by both Pols IV and V and is thus named *NRP(D/E)5*, whereas two distinct NRPB5 subunit variants (NRPB5a or NRPB5b) are used by Pol II (22).

Subunits that are homologous, but not identical, among eukaryotic nuclear RNA polymerases include the A12.2, Rpb9 and C11 subunits of yeast Pols I, II and III, respectively. A12.2 is named in yeast to reflect Pol I-specificity and mass in kilodaltons, Rpb9 is named according to Pol II-specificity and subunit order, and C11 is named according to Pol III-specificity and mass in kilodaltons. These subunits are important for enzyme accuracy and processivity, possessing RNA cleavage activities that allow for removal of misincorporated nucleotides or rescue from stalling via polymerase backtracking (35–38). In the case of Pol II, the RNA cleavage activity of Rpb9 is tuned by transcription factor TFIIS, which shares sequence and structural similarity with Rpb9 (38). In *Arabidopsis*, two genes encode Rpb9 orthologs, and either variant can assemble into Pols II, IV or V (9,39). However, despite being 92% identical in amino acid sequence, genetic evidence indicates that the alternative ninth subunits, or their genes, are not redundant in the context of Pols II or V (40). Interestingly, 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 (22).

In yeast Pols I, II or III, orthologous sub-complexes are formed by A14–A43, Rpb4–Rpb7 and C17–C25 heterodimers, respectively (13–16). These stalk-like subcomplexes protrude from the main bodies of the enzymes adjacent to the RNA exit channels (41,42). In *Arabidopsis* and maize, the Pol II NRPB4–NRPB7 sub-complex is paralogous to, but distinct from, the corresponding subcomplexes of Pols IV and V (9,22). This stems, in part, from the fact that the 4th subunit of Pols IV and V, NRP(D/E)4 differs from the NRPB4 subunit of Pol II, in both *Arabidopsis* and maize. Moreover, in *Arabidopsis*, gene duplication and sub-functionalization gave rise to distinct *NRPB7, NRPD7* and *NRPE7* genes used by Pols II, IV and Pol V, respectively (9,43). By contrast, in maize, the seventh subunits of Pols IV and V are encoded by the same gene, *NRP(D/E)7,* which is distinct from the Pol II *NRPB7* gene (22).

The preceding discussion illustrates the fact that RNA polymerase subunit gene duplications have given rise to unprecedented polymerase diversity in plants. This includes the emergence of Pol IV and Pol V, whose subunit compositions vary in different plant lineages and whose functional diversification is ongoing (9,22,40). Thus far, the subunit compositions of RNA polymerases I and III in plants have not been defined. To fill this gap in our understanding, we affinity-purified *Arabidopsis* Pol I and Pol III and identified their subunits using mass spectrometry. Homologs of all 17 yeast Pol III subunits, and of 12 of the 14 yeast Pol I subunits, were identified via a combination of homology searching and mass spectrometry. As with Pols II, IV and V, alternative versions of several subunits are utilized by Pol I or Pol III. Surprisingly, alternative AC40-like proteins are used by Pol I or Pol III, reflecting a functional diversification that occurred in a common ancestor of *Arabidopsis* and other species of the plant family, Brassicaceae. To simplify functional and structural comparisons between Pols I and III and other polymerases, we introduce a subunit nomenclature system, based on the yeast Pol II subunit numbering system, in which the 12 homologous subunits of all five nuclear nuclear multisubunit RNA polymerases bear the same numbers.

MATERIALS AND METHODS

Plant materials

Plants were grown in a greenhouse or in growth chambers under long day photoperiods (16 h light, 8 h dark). *A. thaliana nrpa3-1* corresponds to T-DNA insertion line SALK_088247; *nrpc3-1* is T-DNA line SALK_132788 (44) and *nrpa11-1* is T-DNA line WiscDsLox_419G02 (45). Primers used for genotyping are provided in Supplementary Table S1.

Affinity purification of Pol I and Pol III

150–250 g of fresh or frozen leaf tissue expressing FLAG-tagged *NRPA2* or *NRPC2* was ground in extraction buffer (300 mM NaCl, 20 mM Tris pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1:200 plant protease inhibitor cocktail (Sigma)) at 4°C, filtered through two layers of Miracloth (Calbiochem) and centrifuged twice at 7000–10 000 x g, 25 min, 4°C. Supernatants were incubated with anti-FLAG-M2 resin for 3 h and a 15 ml tube using 50 ul of resin per 14 ml of extract. Pooled resin was washed seven times in 14 ml of extraction buffer containing 0.4% Nonidet P-40

(Sigma). Wash buffer was removed from the resin before adding 1 volume of Ag/Ab Elution Buffer (Pierce) at 4°C. The sample was mixed thoroughly and incubated for 3 min on ice. The resin was pelleted, and the supernatant, containing eluted proteins, was recovered in 500 ul aliquots, concentrated using YM-10 Centricon columns (Millipore) at 4°C and desalted twice using a Pierce 500 ul desalting column. Final samples, eluted in 25 mM ammonium bicarbonate, were 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 then incubated 2 h at 60°C with shaking at 300 rpm. Proteins were reduced with 2 mM DTT, 37°C for 1 h, then diluted 5-fold with 50 mM ammonium bicarbonate. 1 mM $CaCl_2$ and sequencing-grade modified porcine trypsin (Promega) was added, at a 1:50 trypsin-to-protein mass ratio. After 3 h 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 using filtering criteria that provided a False Discovery Rate (FDR) <5%. Additional details are provided in the supplemental information.

Cloning, vectors and transgenic lines

NRPD1-FLAG, NRPE1-FLAG, NRPA2-FLAG, NRPB2-FLAG and NRPC2-FLAG transgenes were previously described (46,47). NRPE3a, NRPE3b, NRPE5, NRPB6a, NRPB6b, NRPB7, NRPB8a, NRPB8a, NRPB9a, NRPB10, NRPB11 transgenic lines have been described (9). NRPA13 (At3g13940), NRPC7 (At1g06790), NRPA9 (Ag3g25940), NRPB9b (At4g16265), NRPC9a (At4g07950), NRPC9b (At1g01210), NRPB7-like (At4g14520) and NRPB12-like (At1g53690) cDNAs were amplified by RT-PCR from oligo-T primed cDNA, or by PCR amplification of existing DNA clones obtained from the Arabidopsis Biological Resource Center (ABRC), and were then cloned into pENTR-D-TOPO or pENTR-TEV-TOPO. cDNAs were recombined into pEarleyGate 202 to generate FLAG tag fusions (48). Genomic NRPB12, NRPA11, NRPA3 and NRPC3 clones were amplified with Pfu Ultra (Stratagene) and cloned into pEarleyGate 302 to generate FLAG tag fusions. Primers used for PCR amplification of target gene sequences are provided in Supplementary Table S1.

Immunoprecipitation and immunoblotting

Two to four gram of leaves were ground in extraction buffer (see above), filtered through Miracloth and centrifuged at 10 000 x g for 15 min. Supernatants were incubated 3 h at 4°C with 30–50 μl of anti-FLAG-M2 resin (Sigma). Beads were washed three times in extraction buffer + 0.5% Nonidet P-40 (Sigma), eluted with two bed volumes of 2× sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and 5–20 μl were subjected to SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore). Blots were incubated with antibodies in TBST (Tris-buffered saline supplemented with Tween 20 detergent) + 5% (w/v) non-fat dried milk. Antibody dilutions were: 1:100 (NRPC7, NRPA3), 1:250 (NRPD2/NRPE2), 1:500 (anti-Pol I, II and/or III) and 1:2000–1:20 000 (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 4× 4 min in TBST and visualized by chemiluminescence (GE Healthcare). In some cases, blots were stripped for 35 min in 25 mM glycine pH 2.0, 1% SDS and re-equilibrated in TBST before probing with additional antibodies. Additional information is provided in the supplemental methods section.

Phylogenetic analyses

Unrooted neighbor-joining trees were generated from sequence alignments of AC40 or C53 protein family sequences using ClustalW. For C53 family proteins, it was necessary to use aligned sequences of the most conserved region, corresponding to amino acids 358–422 of budding yeast C53. Neighbor joining trees were created using Geneious software, with 1000 bootstrap iterations and a support threshold of 80%.

Protein structural modeling

Protein Data Bank file PDB:4C2M, corresponding to the yeast RNA Polymerase I crystal structure determined by Engel *et al.* (14), was visualized using using PyMol software in order to render space-filling models of AC40, and interacting subunits, shown in Figure 5.

Additional methods are described in the supplemental materials.

RESULTS

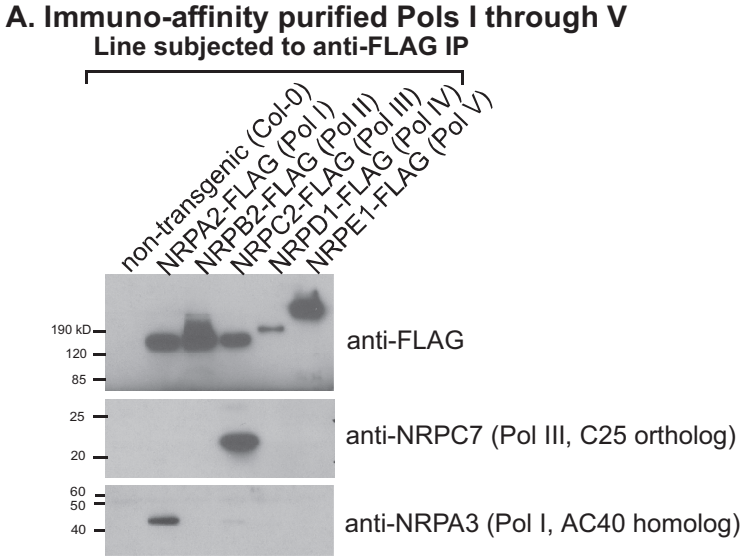
Similarity-based predictions of Pol I- and Pol III-specific subunits

Yeast Pol I or Pol III subunits were used as query sequences to search for potential *Arabidopsis* homologs using the BLASTp algorithm (Table 1, Supplementary Table S2). Putative homologs for 13 of the 14 yeast Pol I subunits were identified, the missing homolog being the A14 subunit (Table 1). Predicted *Arabidopsis* subunits were subsequently confirmed by mass spectrometry (see later section), and named using a nomenclature system in which the prefix NRP denotes Nuclear multisubunit RNA Polymerase and the letters A, B, C, D or E denote polymerases, I, II, III, IV or V, respectively. We previously established a Pol IV and Pol V subunit nomenclature based on homologies to the 12 yeast Pol II subunits, Rpb1 through Rpb12 (9). This nomenclature system has also been extended to Archaeal polymerases (23), greatly simplifying structural, functional and evolutionary comparisons. We have therefore chosen to extend this convention to plant RNA polymerases I and III, such that NRPA7, NRPB7 and NRPC7, for example, denote orthologous subunits of Pols I, II and III (Table 1). By comparison, in budding yeast these proteins are known

Table 1. Arabidopsis RNA Polymerase I or III subunits and their homologs

Subunit					Pol I (% coverage)		Pol III (% coverage)	
Bacteria	**Archaea**	**Yeast (S.c)**	**Arabidopsis**	**Arab. Gene locus ID**	**Total**	**Unique**	**Total**	**Unique**
B'	Rpo1 (RpoA)	Rpb1	NRPB1	At4g35800	0	0	0	0
			NRPD1	At1g63020	0	0	0	0
			NRPE1	At2g40030	0	0	0	0
		Rpa1	NRPA1	At3g57660	49	49	0	0
		Rpc1	NRPC1	At5g60040	0.6	0	22	22
B	Rpo2 (RpoB)		NRP(D/E)2	At3g23780	0	0	0	0
			NRPD2b	At3g18090	0	0	0	0
		Rpa2	NRPA2	At1g29940	51	51	0	0
		Rpb2	NRPB2	At4g21710	2	2	0	0
		Rpc2	NRPC2	At5g45140	0	0	39	39
α	Rpo3 (RpoD)	Rpb3	NRP(B/D/E)3a	At2g15430	4	4	0	0
			NRPE3b	At2g15400	4	4	0	0
		AC40	NRPA3	At1g60850	61	61	0	0
			NRPC3	At1g60620	7	3	57	57
	Rpo4 (RpoF)	A14	NRPA4	?	0	0	0	0
		C17	NRPC4	At5g62950	0	0	55	41
			NRPC4-like	At3g28956	0	0	14	0
		Rpb4	NRPB4	At5g09920	0	0	0	0
			NRP(D/E)4	At4g15950	0	0	0	0
	Rpo5 (RpoH)	Rpb5	NRP(A/B/C/D)5	At3g22320	59	59	44	44
			NRPB5-like	At5g57980	0	0	0	0
			NRPE5	At3g57080	0	0	0	0
			NRPE5-like	At2g41340	0	0	0	0
			NRPE5-like	At3g54490	0	0	0	0
ω	Rpo6 (RpoK)	Rpb6	NRP(A/B/C/D/E)6a	At5g51940	0*	0*	36	16
			NRP(A/B/C/D/E)6b	At2g04630	0*	0*	35	15
	Rpo7 (RpoE)	Rpb7	NRPE7	At4g14660	0	0	0	0
			NRPD7	At3g22900	0	0	0	0
			NRPB7-like	At4g14520	0	0	0	0
			NRPB7	At5g59180	0	0	0	0
		C25	NRPC7	At1g06790	0	0	48	48
		A43	NRPA7	At1g75670	5	5	0	0
	Rpo8 (RpoG)	Rpb8	NRP(A/B/C/D/E)8a	At1g54250	65	56	37	28
			NRP(A/B/C/D/E)8b	At3g59600	62	37	33	24
	TFS/RpoX	Rpb9	NRP(B/D/E)9a	At3g16980	0	0	0	0
			NRP(B/D/E)9b	At4g16265	0	0	0	0
		A12	NRPA9	At3g25940	60	60	0	0
		C11	NRPC9a	At4g07950	0	0	11	0
			NRPC9b	At1g01210	0	0	11	0
	Rpo10 (RpoN)	Rpb10	NRP(A/B/C/D/E)10	At1g11475	70	55	87	72
			NRPB10-like	At1g61700	15	0	15	0
α	Rpo11 (RpoL)	Rpb11	NRP(B/D/E)11	At3g52090	0	0	0	0
		AC19	NRP(A/C)11	At2g29540	29	29	50	50
	Rpo12 (RpoP)	Rpb12	NRP(A/B/C/D/E)12	At5g41010	0	0	16	16
			NRPB12-like	At1g53690	0	0	0	0
Pol I –specific subunits		A49	NRPA13	At3g13940	53	53	0	0
		A34.5	NRPA14	At5g64680	61	61	0	0
Pol III- specific subunits		C82	NRPC13	At3g49000	0	0	27	27
		C53	NRPC14a	At4g25180	0	0	57	57
			NRPC14b	At5g09380	0	0	32	32
		C37	NRPC15	At5g49530	0	0	38	38
		C34	NRPC16	At5g23710	0	0	25	25
		C31	NRPC17	At4g01590	0	0	54	33
			NRPC17-like	At4g35680	0	0	8	0

* confirmed by immunoblot analyses but not detected by LC-MS/MS



B.Transmission of *NRPA3, NRPC3 and NRP(A/C)11* mutant alleles

Parental genotype	T-DNA insertion site in mutant allele	Progeny			Total
		Homozygous wild-type	Heterozygous	Homozygous mutant	
NRPA3 / nrpa3-1	**exon**	**39**	**40**	**0**	**79**
NRPC3 / nrpc3-1	**intron**	**59**	**28**	**0**	**87**
NRP(A/C)11 / nrp(a/c)11-1	**intron**	**58**	**34**	**0**	**92**

Figure 1. Immunological and genetic tests of Pol I or Pol III–specific subunits. (**A**) Transgene-expressed FLAG-tagged catalytic subunits of Pols I, II, III, IV or V were used to immunoprecipitate the five polymerases from cell-free extracts using anti-FLAG resin. Immunoprecipitated proteins were then subjected to SDS-PAGE and immunoblotting. Duplicate immunoblots were probed with anti-FLAG, anti-NRPC7 or anti-NRPA3 antibodies. (**B**) Genetic tests of insertional mutations disrupting *NRPA3*, *NRPC3* or *NRP(A/C)11*. Plants heterozygous for T-DNA insertion alleles were allowed to self-fertilize and resulting progeny plants were genotyped using PCR primers that discriminate between wild-type and mutant alleles. The number of plants corresponding to each of the three possible genotypes is shown.

as A43, Rpb7 and C25; names that give no hint as to their relatedness.

In addition to the 12 core subunits that are homologous, or identical, among eukaryotic RNA polymerases, Pols I and III have unique subunits. In our nomenclature system, Pol I- or Pol III-specific subunits are numbered, beginning with the number 13, in decreasing order according to the mass of their orthologs in budding yeast (Table 1, Supplementary Figure S1). Thus, *Arabidopsis* NRPA13 and NRPA14 are the Pol I-specific subunits orthologous to yeast A49 and A34, respectively. Likewise, the *Arabidopsis* ortholog of yeast C82 is NRPC13; the ortholog of C53 is NRPC14; the C37 ortholog is NRPC15; the C34 ortholog is NRPC16 and the C31 ortholog is NRPC17 (Table 1, Supplementary Table S2).

Arabidopsis has unique genes homologous to the yeast A49, C34, C37 and C82 proteins (Table 1, Supplementary Figures S2–S5), but two putative homologs of C31 (gene loci At4g01590 and At4g35680; Supplementary Figure S6), two homologs of C53 (gene loci At4g25180 and At5g09380; Supplementary Figure S7) and two homologs of AC40 (see later section). An *Arabidopsis* ortholog of the yeast Pol I subunit, A43 and orthologs of the Pol III proteins C17 and C25 were also identified. In the case of NRPC4, which corresponds to yeast C17, there are two paralogs, encoded by loci At5g62950 and At3g28956 (Table 1, Supplementary Figure S8). Surprisingly, a clear *Arabidopsis* ortholog of yeast A14 was not identified, using BLASTp or hidden homology (HHpred) search algorithms.

Identification of *Arabidopsis* Pol III subunits by mass spectrometry

We affinity-purified Pol III by virtue of an epitope-tag (FLAG tag) fused to recombinant NRPC2. The *NRPC2-FLAG* transgene, expressed using the native *NRPC2* promoter, rescued a homozygous *nrpc2–1* null mutant, which is otherwise lethal due to maternal gametophyte lethality (46), indicating that the recombinant NRPC2-FLAG protein is functional. An initial immunoprecipitation test of NRPC2-FLAG resulted in the co-immunoprecipitation of the Pol III-specific subunit, NRPC7, but not subunits unique to other polymerases, such as the NRPA3 subunit of Pol I (Figure 1A).

Pol III immunoprecipitated from cell-free extracts of leaf tissue by virtue of NRPC2-FLAG was subjected to trypsin digestion and LC–MS/MS analysis. Peptides corresponding to the predicted *Arabidopsis* homologs of all 17 yeast Pol III subunits were identified (Supplementary Figure S9, Table 1). No peptides corresponding to Pol I, II, IV or V subunits were detected, as expected. Peptides unique to the Pol III largest subunit, NRPC1, represented 22% of the full-length protein (Table 1, Supplementary Figure S9). Likewise 39% unique peptide coverage was observed for NRPC2 (Table 1, Supplementary Figure S9).

Arabidopsis has two genes homologous to yeast AC40, encoded by loci At1g60620 and At1g60850. Peptides of the protein encoded by At1g60620 were detected in Pol III, collectively representing 57% of the protein sequence (Table 1, Supplementary Figure S9). This protein had previously been named AtRPAC43, because of its similarity to yeast AC40, and its predicted mass of 43 kDa (49). We have assigned an alternative name to the protein, NRPC3, because it is primarily used by Pol III and is paralogous to the Rpb3 subunit of Pol II. No peptides corresponding to the closely related protein encoded by gene locus At1g60850 (a.k.a. AtRPAC42; (49,50)) were detected in Pol III. Instead, the At1g60850 locus encodes the major NRPA3 subunit of Pol I (Supplementary Figures S10, S11, S12; Table 1).

Peptides of NRPC3 were detected at trace levels in Pol I (Supplementary Figures S10 and S11), suggesting that NRPC3 might function in the context of Pol I to some extent. However, the *NRPA3* and *NRPC3* genes are not functionally redundant, as indicated by genetic tests (Figure 1B). Upon selfing of heterozygous *NRPA3/nrpa3* or *NRPC3/nrpc3* plants, homozygous wild-type and heterozygous progeny were obtained in each case, but not homozygous *nrpa3* or *nprc3* progeny, indicating that *NRPA3* and *NRPC3* are both essential genes. The fact that heterozygous progeny do not outnumber homozygotes 2:1 likely reflects female gametophyte-specific lethality, and reduced transmission via male gametophytes, as is the case for other essential genes encoding RNA polymerase subunits (46).

Immunoblot assays using a polyclonal antibody raised against NRPA3 detected a faint signal of appropriate size in Pol III immunoprecipitated by virtue of FLAG-tagged NRPC2 (Figure 1A). However, Pol III immunoprecipitated by virtue of FLAG-tagged NRPC3 also yielded a faint signal upon immunoblotting using the anti-NRPA3 antibody (Figure 2, lane 4). Because NRPC3 and NRPA3 could not be present in the same enzyme at the same time, we conclude that the anti-NRPA3 polyclonal antisera cross-reacts with NRPC3 to some extent.

In yeast, AC40 and AC19 form a heterodimer in the context of both Pol I and Pol III. We obtained 50% unique peptide coverage for the predicted AC19 ortholog, AtRPAC19 (49,50) in affinity-purified Pol III and Pol I (Supplementary Figures S9, S11). In our nomenclature system, the protein is assigned the name NRP(A/C)11, reflecting use by Pol I and Pol III and homology to the Rpb11 subunit of Pol II. Like *NRPA3* and *NRPC3*, the *NRP(A/C)11* gene is essential for viability (Figure 1B).

Subunits shared by Pols I, II and III in yeast include Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12. A small multigene family encodes Rpb5-like proteins in *Arabidopsis*, one of which, encoded by gene locus At3g22320, was identified in Pol III with 44% unique peptide sequence coverage (Table 1, Supplementary Figure S9). This same protein has been shown to be used by Pols I, II, III and IV (9,51,52) and is thus assigned the full name of NRP(A/B/C/D)5 (see Table 2). Two genes encode Rpb6 orthologs and two genes encode Rpb8 orthologs in *Arabidopsis*. In purified Pol III, we identified peptides unique to NRPB6a, NRPB6b, NRPB8a and NRPB8b, showing that Pol III can make use of either variant form for these subunits (see Table 2) (9). Based on their detection in other polymerases, these subunits are assigned the full names of NRP(A/B/C/D/E)6a, NRP(B/C)6b, NRP(A/B/C/D/E)8a and NRP(A/B/C/D/E)8b. We obtained 72% coverage for the yeast Rpb10 ortholog (At1g11475), and 16% coverage for the yeast Rpb12 ortholog (At5g41010) in purified Pol III (Table 1, Supplementary Figure S9). These same proteins were previously identified in Pols II, IV and V (9) (Table 2). The yeast Rpb10 ortholog, encoded by locus At1g11475, was detected in Pol I (Supplementary Figure S10), as well as Pols II, II, IV and V (Table 2), thus the protein is assigned the full name NRP(A/B/C/D/E)10.

The yeast Pol III subunits, C25 and C17 are orthologous to the Rpb7 and Rpb4 subunits of Pol II, respectively. These subunit pairs form stalk-like sub-complexes that protrude from the main bodies of the enzymes near the RNA exit channel. We obtained 48% peptide coverage for the C25 homolog (Table 1, Supplementary Figure S9), which is NRPC7 in our nomenclature. Two genes encode potential Rpc17 homologs, but only peptides corresponding to one of these loci, At5g62950, were identified in Pol III; this protein is assigned the name NRPC4 (Table 1).

Two closely related genes encode potential NRPC9 subunits (the homologs of yeast C11): NRPC9a (At4g07950) and NRPC9b (At1g01210). A single NRPC9 peptide was identified in the mass spectrometry dataset, but both paralogs have this sequence, such that it is unclear if one or both proteins serve as Pol III subunits (Supplementary Figure S9, Table 1).

Arabidopsis contains a single gene (At3g49000) encoding the presumptive ortholog of yeast Rpc82 (NRPC13 in our nomenclature), and we detected the protein in Pol III with 27% unique peptide sequence coverage (Table 1, Supplementary Figure S9). Two Rpc53 (NRPC14) homologs are expressed in *Arabidopsis*, and both were detected in Pol III, with 57% unique peptide sequence coverage for NRPC14a (At4g25180) and 32% unique coverage for NRPC14b (At5g09380), indicating that either paralog can assemble into Pol III (Table 1, Supplementary Figure S9). 38% unique coverage was obtained for the Rpc37 homolog, NRPC15 (At5g49530), and 25% unique coverage was observed for the Rpc34 homolog, NRPC16 (At5g23710). The *Arabidopsis* genome encodes two potential Rpc31-like subunits, but peptides unique to only one of these proteins (encoded by locus At4g01590) were identified; this protein is assigned the name, NRPC17 (Table 1, Supplementary Figure S9).

Table 2. Peptide coverage for subunits of affinity-purified Pols I, II, III, IV or V

		Pol I coverage (%)		Pol III coverage (%)		Pol II coverage (%)		Pol IV coverage (%)		Pol V coverage (%)	
Subunit	Gene ID	Total	Unique	Total	unique	Total	Unique	Total	Unique	Total	Unique
NRPA1	At3g57660	49	49	0	0	0	0	0	0	0	0
NRPB1	At4g35800	0	0	0	0	59	59	0	0	0	0
NRPC1	At5g60040	0.6	0	22	22	0	0	3	3	4	4
NRPD1	At1g63020	0	0	0	0	0	0	58	58	0	0
NRPE1	At2g40030	0	0	0	0	0	0	0	0	63	63
NRPA2	At1g29940	51	51	0	0	0	0	0	0	0	0
NRPB2	At4g21710	2	2	0	0	63	63	0	0	3	3
NRPC2	At5g45140	0	0	39	39	0	0	0	0	0	0
NRP(D/E)2	At3g23780	0	0	0	0	0	0	18	4	37	13
NRPD2b	At3g18090	0	0	0	0	0	0	15	0	27	0
NRPA3	At1g60850	61	61	0	0	0	0	0	0	0	0
NRP(B/D/E)3a	At2g15430	4	4	0	0	72	57	32	28	58	45
NRPE3b	At2g15400	4	4	0	0	23	4	3	0	53	41
NRPC3	At1g60620	7	3	57	57	0	0	0	0	0	0
NRPA4	?	0	0	0	0	0	0	0	0	0	0
NRPB4	At5g09920	0	0	0	0	61	61	0	0	0	0
NRPC4	At5g62950	0	0	55	41	0	0	0	0	0	0
NRPC4-like	At3g28956	0	0	14	0	0	0	0	0	0	0
NRP(D/E)4	At4g15950	0	0	0	0	0	0	13	13	8	8
NRP(A/B/C/D)5	At3g22320	59	59	44	44	63	63	15	15	0	0
NRPE5	At3g57080	0	0	0	0	0	0	0	0	39	39
NRPB5-like	At5g57980	0	0	0	0	0	0	0	0	0	0
NRPE5-like	At2g41340	0	0	0	0	0	0	0	0	0	0
NRPE5-like	At3g54490	0	0	0	0	0	0	0	0	0	0
NRP(A/B/C/D/E)6a	At5g51940	0*	0*	36	16	40	15	33	15	33	15
NRP(A/B/C/D/E)6b	At2g04630	0*	0*	35	15	40	15	18	0*	18	0*
NRPA7	At1g75670	5	5	0	0	0	0	0	0	0	0
NRPB7	At5g59180	0	0	0	0	51	51	0	0	0	0
NRPB7-like	At4g14520	0	0	0	0	0	0	0	0	0	0
NRPC7	At1g06790	0	0	48	48	0	0	0	0	0	0
NRPD7	At3g22900	0	0	0	0	0	0	52	52	0	0
NRPE7	At4g14660	0	0	0	0	0	0	9	9	33	33
NRP(A/B/C/D/E)8a	At1g54250	65	56	37	28	66	30	0	0	9	0
NRP(A/B/C/D/E)8b	At3g59600	62	37	33	24	66	30	18	18	9	0
NRPA9	At3g25940	60	60	0	0	0	0	0	0	0	0
NRP(B/D/E)9a	At3g16980	0	0	0	0	30	22	0	0	22	22
NRP(B/D/E)9b	At4g16265	0	0	0	0	37	28	22	22	22	22
NRPC9a	At4g07950	0	0	11	0	0	0	0	0	0	0
NRPC9b	At1g01210	0	0	11	0	0	0	0	0	0	0
NRP(A/B/C/D/E)10	At1g11475	70	55	87	72	70	55	54	54	70	55
NRPB10-like	At1g61700	15	0	15	0	15	0	0	0	15	0
NRP(B/D/E)11	At3g52090	0	0	0	0	75	75	56	56	68	68
NRP(A/C)11	At2g29540	29	29	50	50	0	0	0	0	7	7
NRP(A/B/C/D/E)12	At5g41010	0	0	16	16	16	16	16	16	16	16
NRPB12-like	At1g53690	0	0	0	0	0	0	0	0	0	0
NRPA13	At3g13940	53	53	0	0	0	0	0	0	0	0
NRPA14	At5g64680	61	61	0	0	0	0	0	0	0	0
NRPC13	At3g49000	0	0	27	27	0	0	0	0	0	0
NRPC14a	At4g25180	0	0	57	57	0	0	0	0	0	0
NRPC14b	At5g09380	0	0	32	32	0	0	0	0	0	0
NRPC15	At5g49530	0	0	38	38	0	0	0	0	0	0
NRPC16	At5g23710	0	0	25	25	0	0	0	0	0	0
NRPC17	At4g01590	0	0	54	33	0	0	0	0	0	0
NRPC17-like	At4g35680	0	0	8	0	0	0	0	0	0	0

* confirmed by immunoblot analyses but not detected by LC-MS/MS

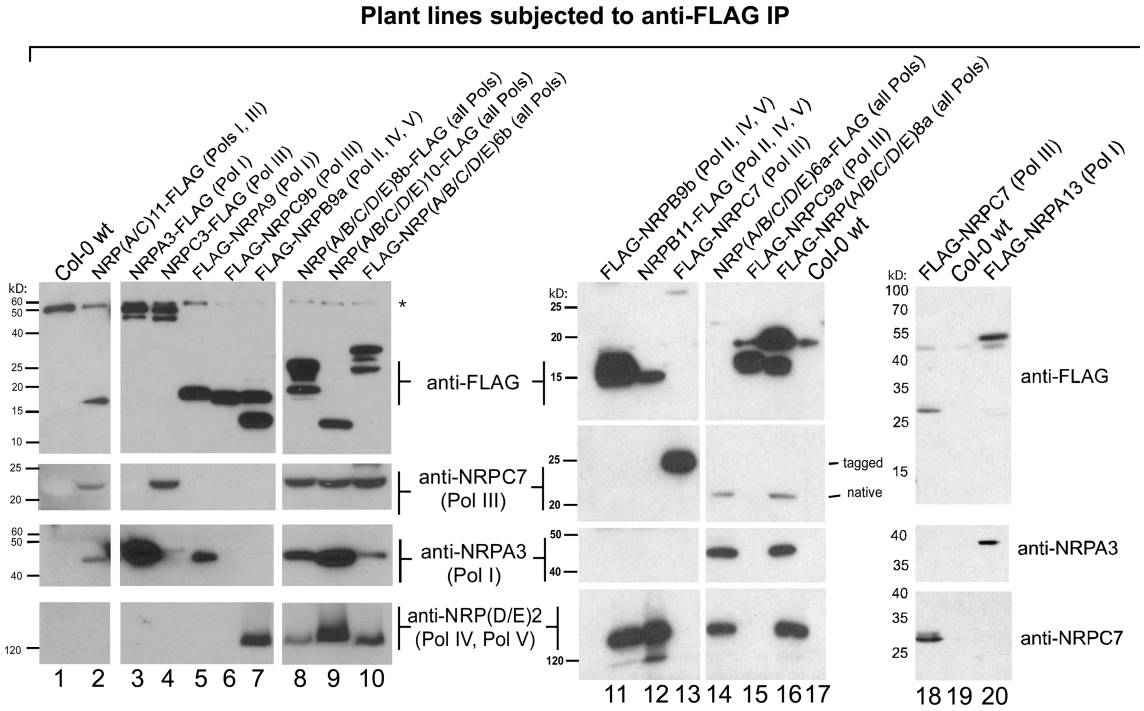


Figure 2. Immunological tests of Pol I and Pol III subunit associations. Cell-free extracts of wild-type plants (ecotype Col-0), or transgenic plant lines expressing specific FLAG epitope-tagged RNA polymerase subunits (indicated at the top of the lanes) were subjected to immunoprecipitation using anti-FLAG resin. Immunoprecipitated proteins were then subjected to SDS-PAGE and immunoblotting. For the experiment yielding the data of lanes 1–10, the experiment yielding the data in lanes 11–17, and the experiment yielding the data of lanes 18–20, duplicate immunoblots were probed with anti-FLAG, anti-NRPC7, anti-NRPA3 or anti-NRP(D/E)2 as indicated. For each of these experiments, immunoblot images using a given antibody are from the same exposure of the same blot. '*' represents a background protein band that cross-reacts with the anti-FLAG antibody in all samples.

Mass spectrometry analyses of *Arabidopsis* Pol I

Pol I was affinity-purified by virtue of a FLAG epitope-tag fused to the C-terminus of NRPA2. The resulting *NRPA2-FLAG* transgene was expressed using the native promoter and rescued the *nrpa2-1* null mutation, which is otherwise lethal when homozygous (46). Mass spectrometry analyses yielded 49% unique sequence coverage for NRPA1, the largest subunit of Pol I, and 51% coverage for NRPA2 (Supplementary Figures S10 and S11). As discussed previously, one of the two yeast AC40 homologs, encoded by locus At1g60850, was detected in Pol I with extensive sequence coverage and assigned the name NRPA3 (Supplementary Figures S10 and S11; see also Supplementary Figure S12 for alignment of NRPA3 with NRPC3, yeast AC40 and other orthologs). Single (but different) NRPC3 peptides were detected in Pol I samples in two independent experiments, and single peptides of the Pol II second subunit, NRPB2, the pol II third subunit, NRPB3 or the Pol V alternative third subunit, NRPE3b (Pol V) were also detected in one of the two Pol I mass spectrometry datasets (Supplementary Figure S10). The significance, if any, of these peptides of other polymerases in Pol I samples is unclear.

In the Pol I mass spectrometry datasets, we identified three of the five subunits common to Pol I, II, III, IV or V, namely homologs of yeast Rpb5, Rpb8 and Rpb10 (Table 1). NRP(A/B/C/D)5 was detected with 59% unique sequence coverage, in agreement with prior immunoblot studies that showed that *Arabidopsis* Pols I, II, III and IV make use of the same Rpb5-like subunit (9,51). Unique peptides for each of the two Rpb8 paralogs were detected in Pol I, a result confirmed by co-immunoprecipitation experiments (Figure 2, lanes 8 and 16), indicating that either variant can assemble into Pol I, as is also the case for Pol II, III, IV and V (9). We observed 55% peptide coverage for NRP(A/B/C/D/E)10 (encoded by locus At1g11475) in Pol I, showing that this subunit is common to all five polymerases. No peptides corresponding to the *NRPB10*-like gene, At1g61700, were detected (Table 1).

Unexpectedly, peptides corresponding to yeast Rpb6 or Rpb12, which are common to Pols I, II and III in yeast, and to Pols IV and V in plants, were not detected in the Pol I mass spectrometry dataset. However, immunoprecipitation of FLAG-tagged NRPA6a or NRPA6b co-immunoprecipitated subunits of Pols I and III, as well as subunits of Pols IV and V (Figure 2, lanes 10 and 14). The latter data suggest that 6th subunit peptides escaped detection by mass spectrometry, despite being present in Pol I.

Peptides of the predicted ortholog of yeast A12 were detected in our Pol I sample, with 60% unique sequence coverage (Table 1, Supplementary Figures S10, S11). This protein, encoded by locus At3g25940, is assigned the name NRPA9 in our nomenclature due to its homology to the Pol II subunit Rpb9.

Yeast A49 is a subunit unique to Pol I. We detected multiple peptides for the *Arabidopsis* ortholog of A49 (locus At3g13940), and assigned it the name NRPA13 (Table 1, Supplementary Figures S10, S11). Yeast A49 and A34.5 form a heterodimeric complex, as do their mammalian ho-

mologs, PAF49 and PAF53. A rice ortholog of A34.5 that interacts with the rice ortholog of A49 in yeast two-hybrid assays was recently identified (53), and the corresponding protein in *Arabidopsis*, encoded by locus At5g64680, was detected in our Pol I mass spectrometry dataset, with 61% unique sequence coverage. The protein encoded by locus At5g64680 was also shown to be enriched in the nucleolus in a previous study, as expected for a Pol I subunit (54). In our subunit nomenclature system, the A34.5 homolog is assigned the name NRPA14. Interestingly, plant NRPA14 proteins display little primary sequence conservation when compared to yeast A34.5 or human PAF53, but have C-terminal regions rich in lysines, serines, threonines and glutamates, similar to the lysine, glutamate, aspartate and serine rich C-terminal region of yeast Rpa34 (Supplementary Figure S13).

In yeast Pol I, the A14 and A43 subunits form a heterodimeric subcomplex that is homologous to the Rpb4–Rpb7 subcomplex of yeast Pol II, or the C17–C25 subcomplex of yeast Pol III. As noted previously, there is no obvious ortholog of A14 encoded by the *Arabidopsis* genome, making it unclear which protein (if any) fulfills its role (hence, the question mark in Table 1). Also surprisingly, only a single peptide corresponding to the predicted *Arabidopsis* homolog of yeast A43 (NRPA7 in our nomenclature) was detected, and only in one of the two mass spectrometry datasets (Supplementary Figure S11).

Confirmation of subunit associations using co-immunoprecipitation tests

To independently test for associations among Pol I and Pol III subunits identified by mass spectrometry, we immunoprecipitated a set of epitope-tagged subunits, constitutively expressed in transgenic plants, and performed immunoblotting using anti-NRPA3 and anti-NRPC7 antibodies specific for Pol I or Pol III (Figure 2). Recombinant proteins subjected to immunoprecipitation included subunits unique to Pol I (NRPA3, NRPA9, NRPA13), subunits unique to Pol III (NRPC3, NRPC7, NRPC9a, NRPC9b), subunits common to Pols II, IV or V (NRPB9a, NRPB9b, NRPB11), subunits common to all five nuclear polymerases (NRP(A/B/C/D/E)6a, NRP(A/B/C/D/E)6b, NRP(A/B/C/D/E)8a, NRP(A/B/C/D/E)8b, NRP(A/B/C/D/E)10), or the unique common subunit of Pols I and III, NRP(A/C)11. The anti-NRPC7 antibody cross-reacted with a protein of the expected size in samples resulting from immunoprecipitation of tagged NRP(A/C)11, NRPC3, NRPC7 (Figure 2, lanes 2, 4, 13, 18) or the common subunits, NRP(A/B/C/D/E)6a, NRP(A/B/C/D/E)6b, NRP(A/B/C/D/E)8a, NRP(A/B/C/D/E)8b, and NRP(A/B/C/D/E)10 (Figure 2, lanes 8–10, 14, 16), consistent with all of these subunits being present in Pol III. Interestingly, NRPC7 was not detected in immunoprecipitated FLAG-NRPC9a or FLAG-NRPC9b (At4g07950 or At1g01210, respectively) samples (Figure 2, lanes 6 and 15), despite LC-MS/MS evidence showing that NRPC7 and NRPC9 are present in purified native Pol III. A possibility is that fusion of the FLAG epitope to the amino terminus of NRPC9a or NRPC9b interferes with Pol III assembly.

Immunoblots revealed the presence of NRPA3 in samples immunoprecipitated using tagged NRP(A/C)11, NRPA3, NRPA9, NRP(A/B/C/D/E)8b, NRP(A/B/C/D/E)10, NRP(A/B/C/D/E)6b, NRP(A/B/C/D/E)6a, NRP(A/B/C/D/E)8a or NRPA13 (Figure 2, lanes 2, 3, 5, 8–10, 14, 16, 20) indicating that all of these proteins are present in Pol I, confirming the mass spectrometry results.

As a control, we probed immunoblots with an antibody against NRP(D/E)2, the second-largest subunit of both Pol IV and Pol V (9,55). NRPD2 is detected in NRPB6a, NRPB6b, NRPB8a, NRPB8b, NRPB9a, NRPB9b, NRPB10 and NRPB11 IP samples, confirming previous studies demonstrating that these subunits are common to Pols II, IV and V (Figure 2, lanes 7, 8–12, 14, 16) (9,34,39). In the case of subunits 6, 8, and 10, the current study shows that these proteins are also common to Pols I and III. Conversely, NRP(D/E)2 was not detected in immunoprecipitated NRPA3, NRPC3, NRPC7, NRPA9, NRPC9a, NRPC9b or NRP(A/C)11 samples, consistent with these being Pol I or Pol III-specific subunits not present in Pols IV or V.

Collectively, the co-immunoprecipitation and immunoblotting assays of Figure 2 support the mass spectrometry results of the current study, as well as our prior studies of Pol II, IV and V subunit compositions.

Predicted alternative RNA polymerase subunits

The *Arabidopsis* genome includes NRPC9b, NRPB7-like, NRPB10-like and NRPB12-like subunits whose peptides have not been detected in Pols I, II, III, IV or V. RT-PCR assays for *NRPB10*-like (encoded by locus At1g61700) and *NRPB12*-like (At1g53690) revealed that both genes are differentially expressed in different organs or at different times in development (Figure 3A). By contrast, *NRP(A/B/C/D/E)10* and *NRP(A/B/C/D/E)12* (abbreviated as *NRPB10* and *NRPB12* in Figure 3A), are constitutively expressed (Figure 3A). Thus, it is possible that different subtypes of one or more of the five polymerases may assemble in specific organs, specific cell types, or at different times in development, by using the variant forms of the tenth and twelfth subunits, despite not being detected in our studies.

We also asked whether the putative alternative subunits, NRPC9b (At1g01210), NRPB7-like (At4g14520) or NRPB12-like (At1g53690), associate with one or more of the five polymerases. FLAG epitope-tagged versions of the proteins were overexpressed using the Cauliflower Mosaic Virus 35S promoter, which is active in virtually all cell types. We then tested whether or not the tagged proteins co-immunoprecipitate with catalytic subunits of Pols I, II, III, IV or V (Figure 3B). The recombinant NRPC9b, NRPB7-like and NRPB12-like proteins were all detected following immunoprecipitation and immunoblotting with anti-FLAG antibody (Figure 3B, top panel), indicating that the recombinant proteins were expressed in the transgenic plants. However, unlike NRPE5, NRPA9 or NRP(B/D/E)9a controls, none of them co-immunoprecipitated the second-largest subunits of Pol I, II, III, IV or V (Figure 3B, middle and bottom panels), making their polymerase associations (if any) unclear.

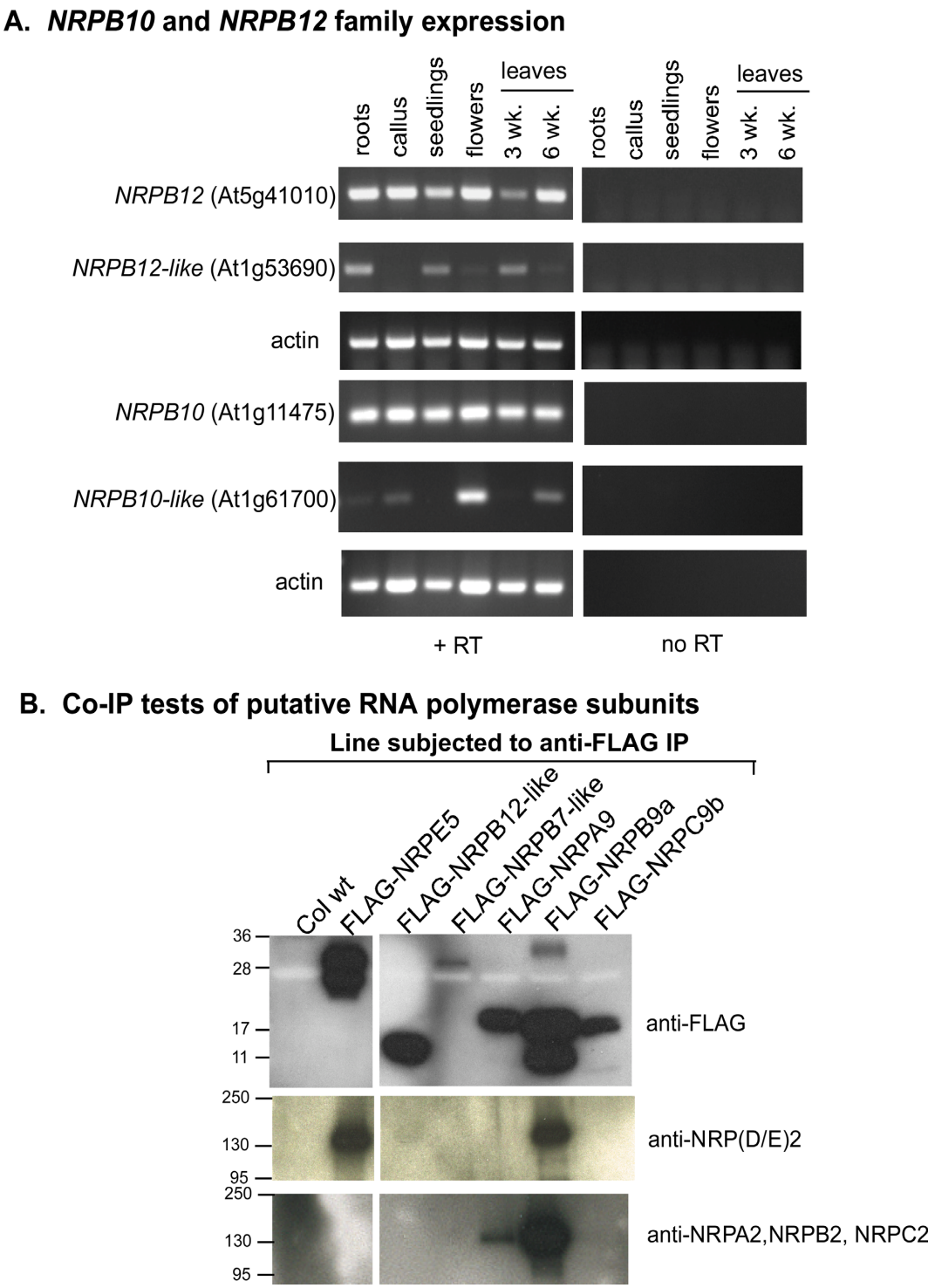


Figure 3. Tests of potential RNA polymerase subunits not detected by mass spectrometry. (**A**) Detection of RNA transcripts using reverse transcription and PCR amplification (RT-PCR). RNA isolated from roots, callus, whole seedlings, flowers, or leaves of 3-week or 6-week old plants was subjected to RT-PCR using primers specific for *NRPB12*, *NRPB12-like*, *NRP10*, *NRPB10-like* or an actin gene. Control reactions in which reverse transcriptase was omitted (-RT) are shown for each gene-specific primer pair. (**B**) Tests for affiliation of putative RNA polymerase subunits with Pols I through V. Cell-free extracts of wild-type (ecotype Col-0) plants, or transgenic plants expressing FLAG-tagged NRPE5, NRPB12-like (At1g53690), NRPB7-like (At4g14520), NRPA9, NRPC9b or NRPB9a were subjected to immunoprecipitation using anti-FLAG resin. Immunoprecipitated proteins were then subjected to SDS-PAGE and immunoblotting. Duplicate immunoblots were then probed with anti-FLAG, anti-NRP(D/E)2 or an anti-peptide antibody that specifically cross-reacts with the second-largest subunits of Pols I, II, and III but not with the NRP(D/E2)2 subunits of Pols IV or V (9).

Pol I and Pol III subunit diversity in other plants

To gain insight into how our findings in *Arabidopsis* might extend to other plants, we performed BLASTp searches using *Arabidopsis* Pol I-specific and Pol III-specific subunits to identify homologous genes in other plant species, including monocots (maize, rice, *Brachypodium*), dicots (grape, cottonwood, castor bean, and the crucifer (Brassicaceae) family) and the moss, *Physcomitrella*. At least one homolog of each subunit is present in each of these species or groups (Supplementary Table S3). Two yeast C34 paralogs are encoded by maize and *Brachypodium*, two yeast C82 paralogs are apparent in grape, two paralogs of yeast C31 are observed in poplar (as in *Arabidopsis*), and two paralogs of both yeast A34 and AC40 are encoded by the rice genome (Supplementary Table S3; for proteins alignments, see Supplementary Figures S2–S8, S12–13).

Phylogenetic analyses reveal that the NRPA3 and NRPC3 subunits of *Arabidopsis* each group with orthologous proteins of related species within the family Brassicaceae, of which *Arabidopsis* is a member, indicating that the functional divergence of AC40-like proteins for differential use by Pol I or Pol III occurred in a common ancestor (Figure 4A; see Supplementary Table S4 for gene ID information). This common ancestor apparently arose after the divergence of monocots and dicots, and after the divergence of different lineages within dicots. However, there are plant species outside of the Brassicaceae that have more than one AC40-like protein encoded by their genomes, including rice (*Oryza sativa*), soybean (*Glycine max*), and millet (*Setaria italica*). In each of these species, the paralogs are more similar within each species than between species, indicative of relatively recent duplication events independent to each lineage (Figure 4A).

Phylogenetic analyses of the NRPC14 family (yeast C53 homologs) reveals that multiple paralogs (as many as four) occur in diverse plant species, including dicots such as *Arabidopsis* (and related *Brassicaceae* species), grape (*Vitis vinifera*), poplar (*Populus trichocarpa*), beans *(Proteus vulgaris, G. max*) or tomato (*Solanum lycopersicum*) as well as monocots that include maize (*Zea mays*), rice (*O. sativa*), millet (*S. italica*) or Brachypodium (*Brachypodium distachyon*) (Figure 4B; see Supplementary Table S5 for gene ID information). In some cases, the C53 paralogs of a species are distinct, associated with unique branches of the neighbor-joining tree that are supported by strong bootstrap values; this is the case for each of the two NRPC14 paralogs in Brassicas (e.g. *A. thaliana*, *Arabidopsis lyrata*, *Brassica rapa*, *Capsella rubella*), a branch for one of the NRPC14 subtypes present in monocots (e.g. *Z. mays, O. sativa, B. distachyon, S. italica*), distinct branches for the NRPC14 paralogs in poplar and soybean, and a branch represented by mouse (*Mus musculus*) and human (*Homo sapiens*) C53 orthologs. Other plant paralogs are not clearly resolved from C53 of budding yeast, fission yeast or Drosophila (Figure 4B). In *Arabidopsis*, both NRPC14 subtypes are detected in Pol III (see Table 1), suggesting that multiple subtypes of NRPC14 may also incorporate into Pol III in other plant species.

DISCUSSION

Arabidopsis orthologs of all 17 yeast RNA polymerase III subunits and 12 of the 14 predicted Pol I subunits were identified in our study (see Table 1), extending to plants the conclusion that RNA polymerases I and III are similar in composition throughout eukaryotes. Subunits of Pol I we did not detect are those orthologous to yeast A14 (NRPA4 in our nomenclature) and Rpb12. In the case of Rpb12, its lack of detection by mass spectrometry is likely due to its small size and limited tryptic peptide profile given that Rpb12 is common to Pols I, II and III in yeast and other eukaryotes, as well as to Pols IV and V in plants. However, in the case of A14, no clear ortholog was detected in the *A. thaliana* genome. In fact, A14 orthologs differ considerably in sequence even among yeast species, such that sequence homology and hidden homology search algorithms, including BLAST, PSI-BLAST, Delta-BLAST or HHpred, do not identify high confidence homologs in more distantly related eukaryotes. Thus, the identity of the NRPA4 subunit in *Arabidopsis* (and other plants) is a question not answered by our study. The possibility that there is no NRPA4 subunit seems unlikely, given the structural conservation of the stalk-like sub-complexes formed by NRPA4–NRPC7 (a.k.a. A14–A43), NRPB4–NRPB7, NRPC4–NRPC7 (a.k.a. C17–C25) in Pols I, II and III, respectively of yeast, and the corresponding Rpo4–Rpo7 subcomplex of archaeal RNA polymerases. However, in yeast, Rpb4 is not absolutely essential (unlike Rpb7) (56,57), providing some basis for entertaining the possibility that plant RNA polymerase I might somehow function without an NRPA4 subunit.

Our current study, and our previous studies of Pols II, IV and V (9,22), shows that the use of alternative forms of numerous RNA polymerase subunits is commonplace in *Arabidopsis* (see Table 2), unlike yeast in which single genes encode each RNA polymerase subunit. However, plants are not unique in this regard. For instance, trypanosomes express more than one Rpb5, Rpb6 and Rpb10 variant (20), with Pol I utilizing variant forms of the fifth and sixth subunits distinct from those used by Pols II and III (17,20,58). In humans, several Rpb11 homologs are encoded by the genome, although only one is known to assemble into Pol II (59). Humans also have alternative RPC32 proteins that participate in functionally distinct forms of Pol III; one that is ubiquitous and essential and the other that is tissue-specific and important in undifferentiated embryonic stem cells and tumors (60). Likewise, alternative ninth subunits, and alternative second subunits, utilized by Pols II, IV and V in *Arabidopsis* and maize, give rise to functionally distinct RNA polymerase subtypes in plants (22,40). For *Arabidopsis* subunits NRP(A/B/C/D/E)6, 8, 10 and 12, commons to Pols I through V, and for Pol III-specific subunits NRPC9 and NRPC14, there are alternative subunit variants, some of which we have identified in affinity-purified polymerase samples by mass spectrometry. Whether the alternative forms of the subunits generate redundant, or functionally distinct, polymerase subtypes will require further investigation.

The most surprising finding of our study pertains to the orthologs of yeast AC40 and AC19, which are common to

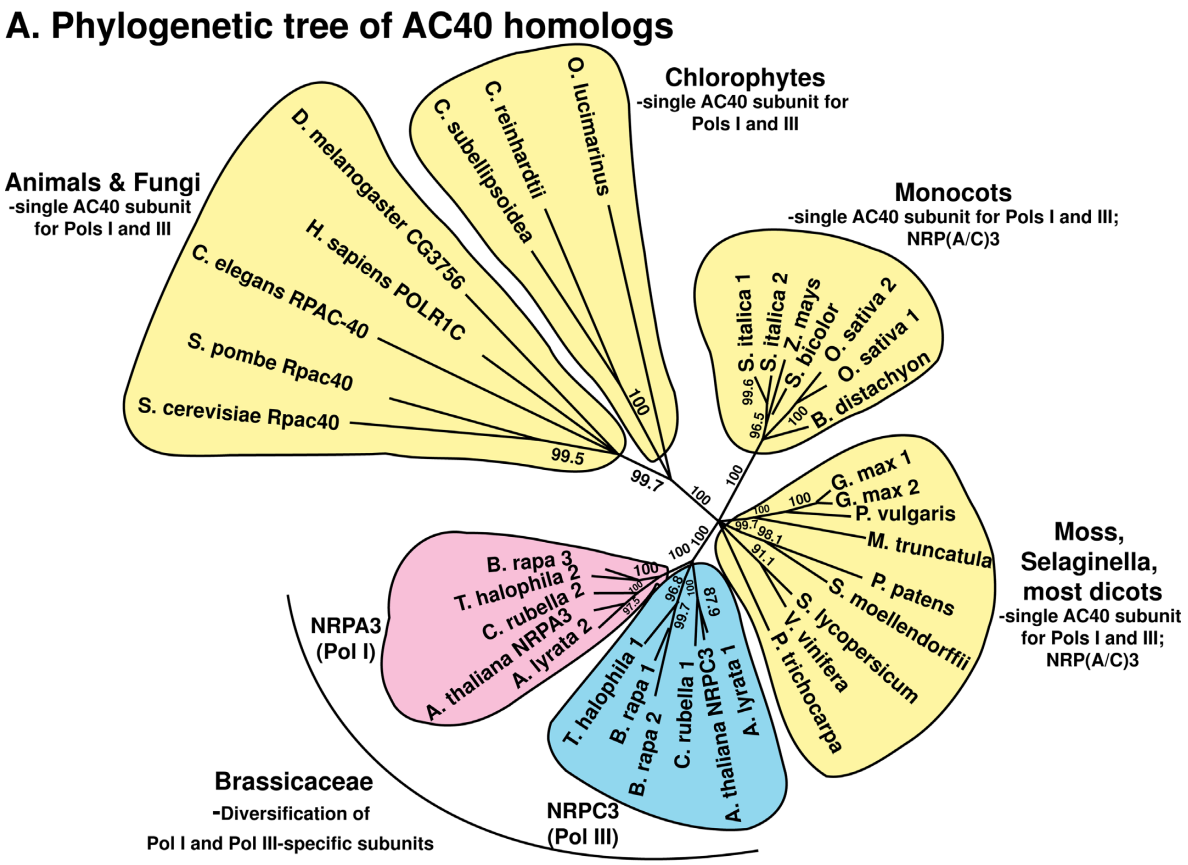
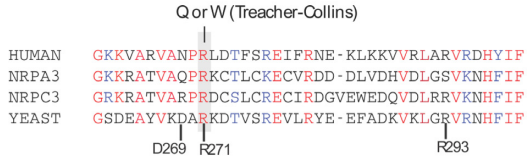


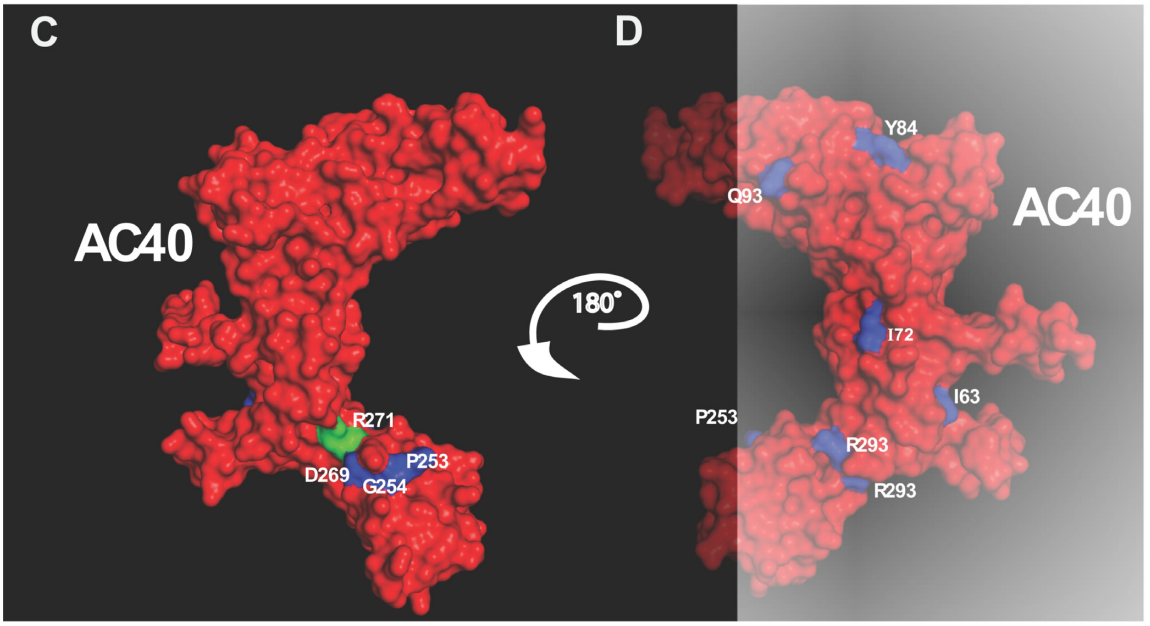
Figure 4. Phylogenetic trees of AC40 and C53 homologs. (**A**) Unrooted neighbor-joining tree of AC40 homologs of yeast, *Caenorhabditis elegans*, fruit fly, human and plants, revealing the diversification of NRPA3 and NRPC3 subunits specific for Pol I or Pol III, respectively, in species of the dicot family Brassicaceae. Numbers shown are bootstrap values. (**B**) Unrooted neighbor-joining tree of C53 homologs of yeast, flies, mouse, human and plants. Highlighted in red are non-plant C53 orthologs and homologs present in *Arabidopsis* (a dicot) and rice (a monocot). Numbers are bootstrap values.

A. Amino acids correlated with the NRPA3/NRPC3 split

Yeast (S.c) AC40	NRPC3 (Brassicaceae)	NRPA3 (Brassicaceae)
I63	I	F
I72	L	I
Y84	F/Y	L
Q93	Q	I/V
P253	K/V/E/E	Q
G254	K/K/K/N	N
D269	R	Q
R293	R	S

B. Alignment in vicinity of Treacher-Collins mutation





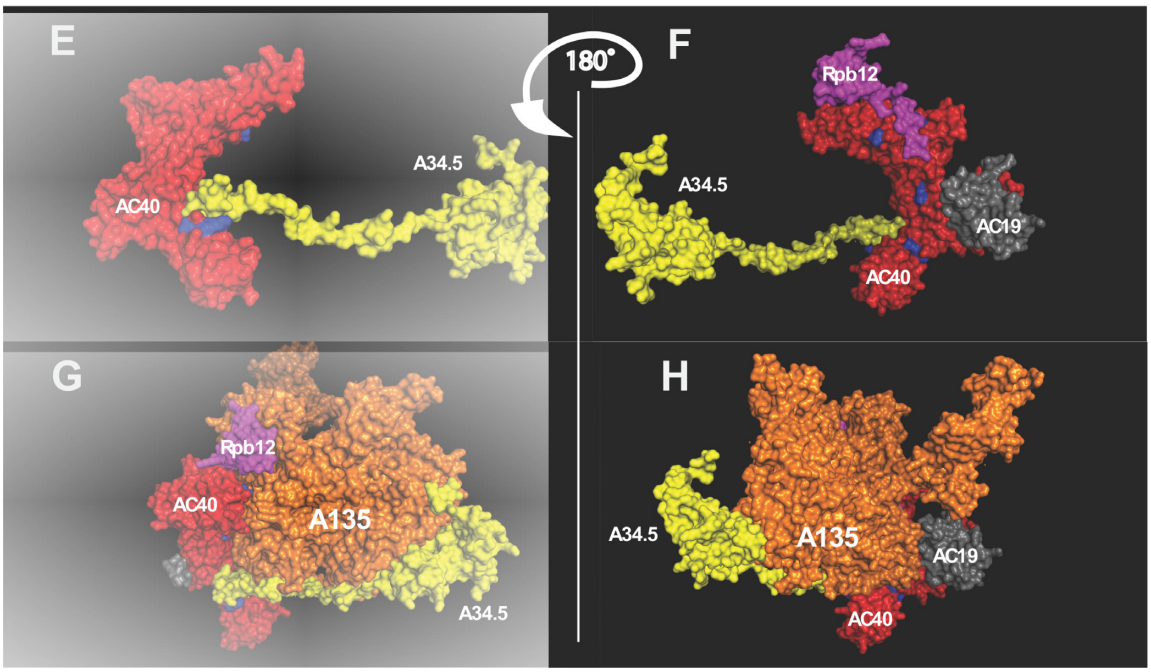


Figure 5. Amino acid positions implicated in functional divergence of NRPA3 and NRPC3 and Treacher–Collins syndrome. (**A**) Amino acid positions of yeast AC40 at which the corresponding amino acids of NRPA3 or NRPC3 proteins of *Arabidopsis*, and related species, are different (see also Supplementary Figure S14). (**B**) Alignment of the human AC40 ortholog, POLR1C, with *Arabidopsis* NRPA3, NRPC3 and yeast AC40 in the vicinity of the conserved arginine (R) that is mutated (to Q or W) in some individuals afflicted with Treacher-Collins Syndrome. Amino acids that are identical in the human and plant proteins are highlighted in red. Amino acids that are similar in the human and plant proteins are shown in blue. Amino acid numbering is based on the yeast AC40 protein. (**C** and **D**) Space filling model of yeast AC40, derived from the PDB:4C2M crystal structure of Pol I, showing the positions of R271 (green) and the eight amino acids, identified in panel (A), at which the corresponding amino acids of NRPA3 and NRPC3 are distinct (blue). (**E** and **F**) Space filling models (also derived from PDB:4C2M) showing the interaction sites of yeast subunits A34.5, AC19 and Rpb12 relative to the amino acid positions highlighted in panels (C) and (D). (**G and H**) Space filling models in which the A135 subunit has been added to the complexes shown in panels (E) and (F).

Pols I and III in yeast, humans and other eukaryotes examined to date. As expected, the AC19 ortholog, named NRP(A/C)11 in our nomenclature system, is shared by Pol I and III in *Arabidopsis*. However, two AC40 orthologs are expressed in *Arabidopsis*, which we have named NRPA3 and NRPC3 due to their differential incorporation into Pol I or Pol III, respectively. Mutations disrupting *NRPA3* or *NRPC3* are lethal when homozygous, indicating that both genes are essential and non-redundant (Figure 1B). At the amino acid level, *Arabidopsis* NRPA3 and NRPC3 are 71% identical, such that there are numerous amino acid changes that could potentially contribute to the divergence of Pol I- and Pol III-specific forms. However, alignment of *Arabidopsis* NRPA3 and NRPC3 with their orthologs in related species of the Brassicaceae family identified eight amino acid positions that distinguish NRPA3 from NRPC3 (Figure 5A; see also Supplementary Figure S14, in which these eight amino acid positions are enclosed in rectangles). Three correspond to amino acids (Proline 253, Glycine 254 and Aspartate 269) that are adjacent to one another in the yeast AC40 crystal structure (14,15) and are also adjacent to the conserved arginine (position R271 of yeast AC40) whose mutation in human AC40 (POLR1C) is associated with Treacher-Collins Syndrome (Figure 5B and C) (29,61,62). In the yeast Pol I crystal structure (Protein Data Bank structure 4C2M, (14)), this patch of amino acids in AC40 contacts the Pol I-specific A34.5 subunit (Figure 5E), suggesting that differences at these conserved positions in NRPA3 and NRPC3 of *Arabidopsis* (and related species) are good candidates for specifying Pol I- versus Pol III-specific interactions. By contrast, amino acid differences in NRPA3 versus NRPC3 at positions corresponding to yeast Y84 or I63 are less likely to be critical because these positions interact with Rpb12 and AC19 in yeast AC40 (Figure 5F), which are subunits common to Pols I and III in yeast and *Arabidopsis* (with the caveat that Rpb12 association with Pol I was not confirmed by mass spectrometry in our study). Amino acid differences in NRPA3 and NRPC3 at positions corresponding to yeast I72, Q93 or R293 are also potential candidates for mediating Pol I versus Pol III-specific interactions, as these positions make polymerase-specific contacts, such as with the A135 catalytic subunit of Pol I (Figure 5G and H).

Treacher-Collins Syndrome can be caused by mutations in the human orthologs of yeast AC40 or AC19, suggesting that impaired activity of Pol I, Pol III or both, is responsible for the impaired craniofacial development associated with the disorder. The fact that *Arabidopsis*, and related species, has evolved distinct AC40 orthologs for assembly into Pol I or Pol III, coupled with the fact that these proteins each have the conserved arginine whose mutation is linked to Treacher-Collins Syndrome (see Figure 5B), provides an intriguing opportunity. By conducting site-directed mutagenesis of the conserved arginine in the NRPA3 or NRPC3 proteins, to mimic the changes in human afflicted with Treacher-Collins Syndrome, Pol I- versus Pol III-specific phenotypes might be disentangled. If so, *Arabidopsis* may prove to be a useful model system for understanding AC40 subunit functions relevant to a devastating human genetic disorder.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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