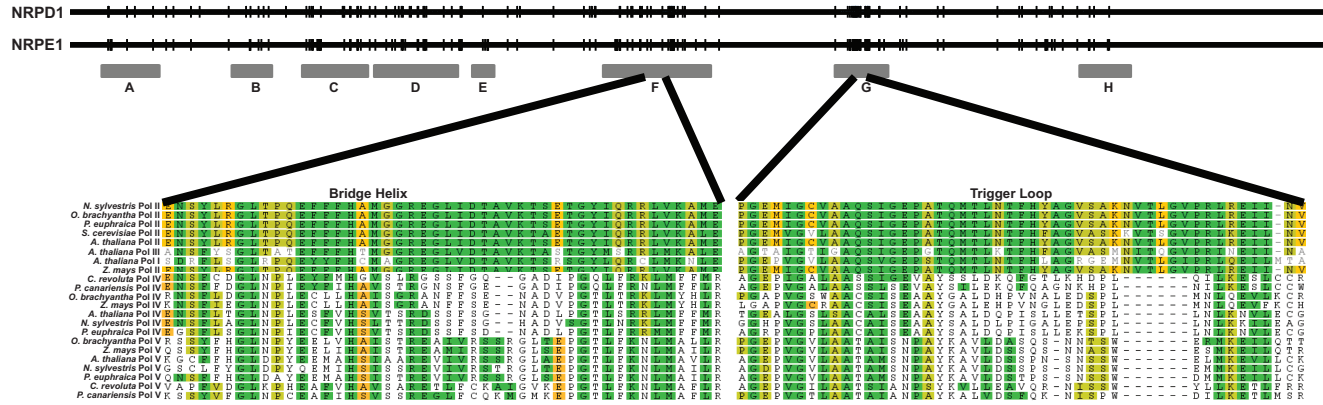


A. Positions of ultra-conserved amino acids that are different in Pol IV and V largest subunits, as detailed in Table S1.



B. Positions of ultra-conserved amino acids that are different in the Pol IV/V second-largest subunit, as detailed in Table S1.

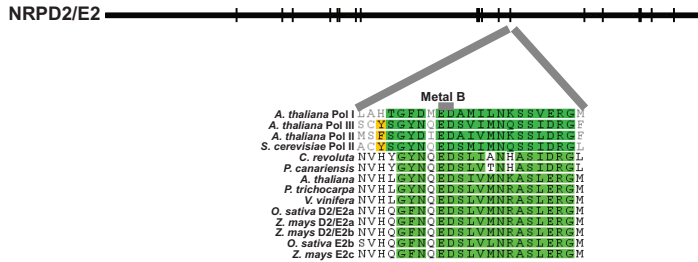
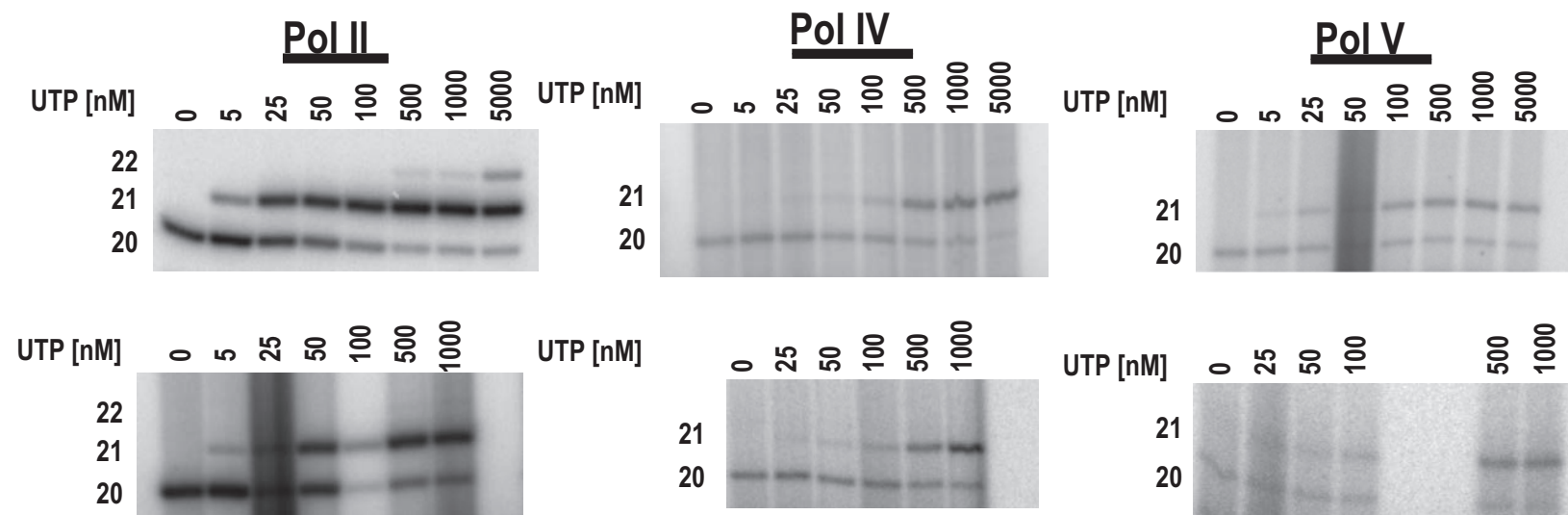


Figure S1. Pol IV and V have diverged at over 140 amino acid positions that are invariant in Pools I, II, and III and yeast Pol II (Table S1). (A) Divergent amino acids are depicted as vertical hash marks on a linear representation of the *S. cerevisiae* Rpb1 protein. Conserved RNA polymerase domains A through H are shown. Sequence alignments for bridge helix and trigger loop are highlighted. (B) Divergent amino acids of NRPD2/E2, common to Pol IV and Pol V, are mapped onto a linear depiction of the *S. cerevisiae* Rpb2 protein sequence. Location of the Metal B site of the catalytic center is shown.

A. Replicate UTP incorporation experiments of Fig. 2



B. Replicate ATP incorporation experiments of Fig. 2

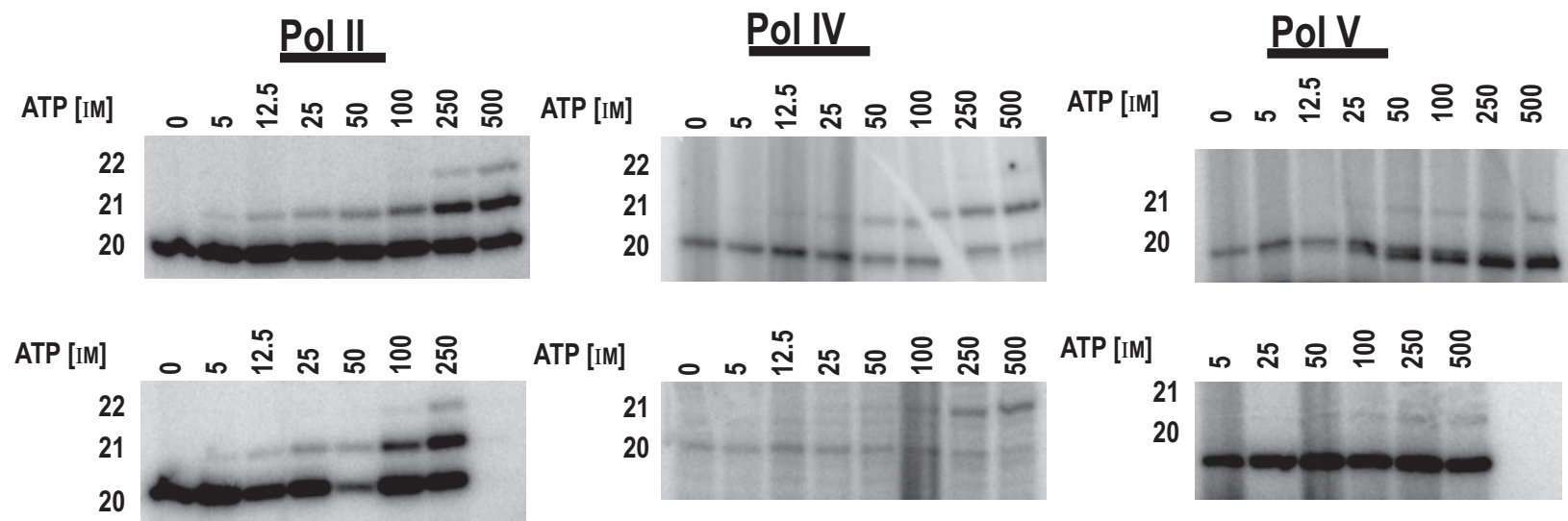


Figure S2. Data for complementary and non-complementary NTP kinetics calculations. (A) Replicate primer elongation experiments for calculating complementary (UTP) kinetics data for Pols II, IV, and V (Fig. 2). (B) Replicate primer elongation experiments for calculating non-complementary (ATP) kinetics data for Pols II, IV, and V (Fig. 2).

A. Assay design



B. dTTP incorporation replicate experiments

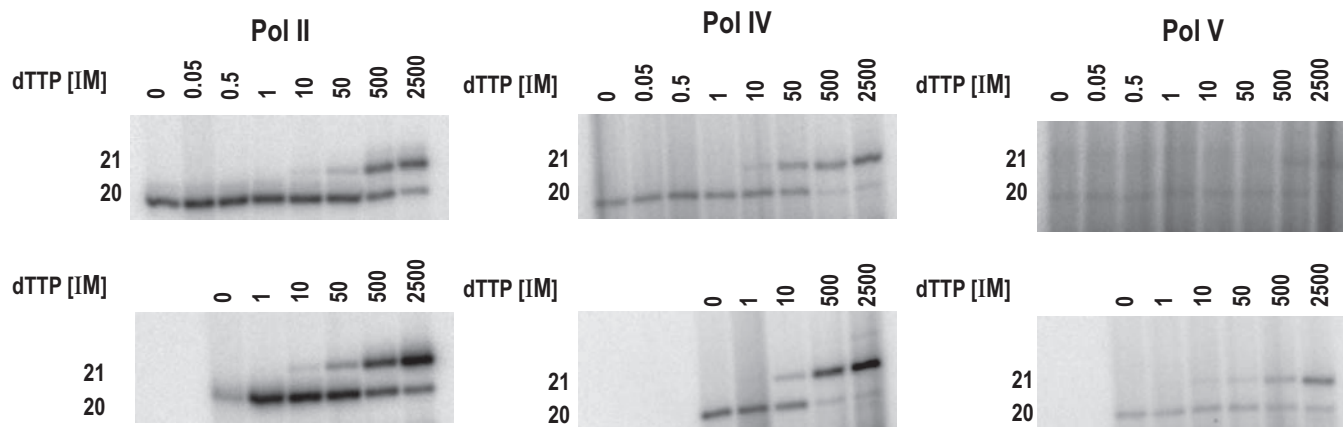


Figure S3. Differences in kinetics of dNTP incorporation contribute to differences in rNTP vs. dNTP discrimination of Pols IV and V relative to Pol II. (A) Primer elongation kinetics assays were used to determine the relative affinities of Pols II, IV, and V for dNTP versus rNTP. (B) Representative gels showing the incorporation of a complementary dNTP (dTTP) by Pols II, IV, and V across a range of dTTP concentrations. Incorporation of dTTP elongates the 20 nt RNA to 21 nts.

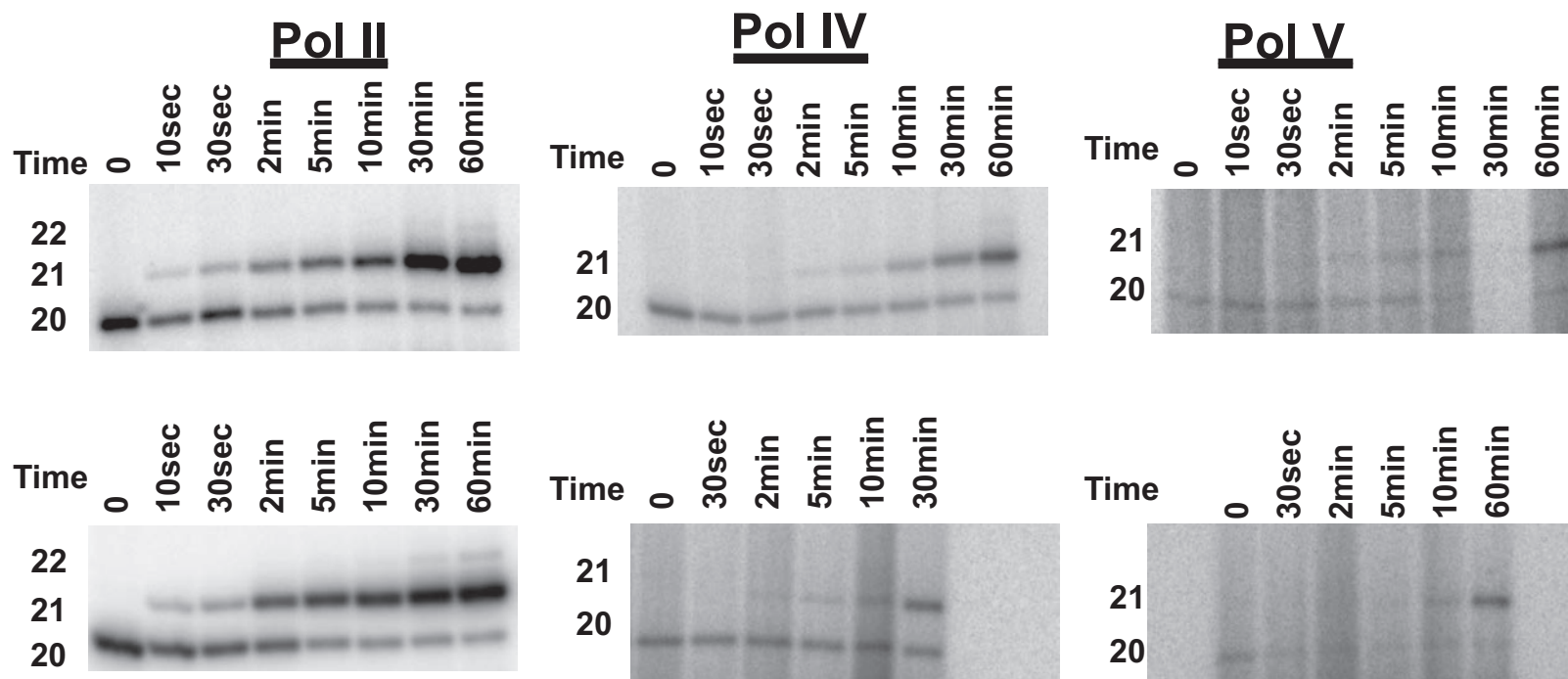


Figure S4. Replicate data for calculation of rate constants for Pols II, IV, and V shown in Figure 5 of the main figures.

Table S1. Invariant amino acids of Arabidopsis Pol I, II, III and *S. cerevisiae* Pol II catalytic subunits that are different in Pols IV and V

yeast Rpb1 aa	NRPD1 (Pol IV) aa	yeast Rpb1 aa	NRPE1 (Pol V) aa	yeast Rpb2 aa	NRPD2/E2 (Pols IV & V) aa
G52	Q41	G52	Q42	I269	K266
L53	V42	D55	N45	H363	G361
P78	E68	R57	F47	R384	C378
P89	S79	P78	E69	L461	F456
L114	R104	L114	K105	R476	V471
K132	deletion	K132	deletion	S480	V475
C142	deletion	C142	deletion	L514	P509
G166	deletion	G166	deletion	P524	F519
G178	S143	D218	S191	Q763	H711
D218	L207	R247	S219	P765	R713
P242	T226	P248	V220	N767	V715
P248	V232	E259	V232	M773	H722
E259	F246	T263	R236	R807	L756
L266	Y253	N273	V246	S831	L780
N273	E260	I325	R292	R983	M917
Q297	S267	L329	S296	M1021	Q955
I325	A291	K332	W307	R1096	V1041
L329	Q295	G334	E309	V1099	S1044
K332	deletion	L340	F315	G1121	D1066
G334	R307	G342	R317	G1167	K1112
V345	S318	R344	G319		
T375	Q348	V345	S320		
Y376	V349	P377	E352		
P377	S350	I385	R360		
I385	K358	D386	G361		
G395	T368	G395	K370		
P396	L369	P396	L371		
P400	K373	P400	Y375		
R412	deletion	R412	deletion		
R420	I386	R420	S388		
H435	S400	H435	R403		
Q447	P412	L443	F411		
K452	Q417	Q447	P415		
M456	I421	M456	Q424		
H458	M423	H458	L426		
T467	V433	K461	Y429		
R469	S435	Y478	L446		
N479	R445	N479	S447		
E486	C452	E486	C454		
N488	H454	N488	H456		
H490	Y456	H490	F458		
P514	R480	P514	S482		
D538	N505	Q525	S493		
T539	C506	T535	deletion		
P568	A532	R537	E504		
D602	L561	D538	R505		
G615	F574	T539	V506		
K619	S578	P568	S532		
G665	S623	L571	A538		
G707	deletion	G574	V541		
E715	S697	K575	F542		
V718	A700	D602	G557		
L722	F704	G615	F570		
N723	K705	G665	S620		
I756	G738	G707	deletion		
N757	K739	E715	R654		
Q767	L749	N723	E660		
G772	A753	G750	K687		
R774	S755	K752	N689		
L784	T765	G753	S690		
P785	C766	I756	T693		
F787	A768	N757	K694		
P794	Y790	Q767	L704		
F799	V795	R774	F712		
F815	V811	L784	A722		
G823	S819	P785	I723		
L824	S820	P794	S735		
I825	F821	F799	I740		
D826	S822	F815	A756		

T827	G823	G823	V764
A828	deletion	D826	R767
V829	deletion	T827	S768
K830	N824	A828	S769
T831	A825	V829	R770
Y836	T830	K830	G771
K843	F837	T831	L772
E846	R840	Y836	T777
D874	deletion	K843	A784
F893	deletion	E846	R787
F1053	deletion	G861	S802
Q1070	C883	D874	R816
G1073	S886	F893	deletion
P1075	A888	F1053	G817
T1077	Y890	Q1070	T834
Q1078	S891	G1073	S837
M1079	A892	E1074	N838
T1080	L893	T1077	Y841
L1081	D894	Q1078	K842
N1082	Q895	M1079	A843
T1083	P896	T1080	V844
H1085	S898	N1082	D846
A1087	L900	T1083	S847
G1088	E901	H1085	P849
V1094	deletion	A1087	deletion
T1095	deletion	G1088	S851
G1097	deletion	V1094	deletion
V1098	deletion	T1095	deletion
P1099	L906	G1097	deletion
R1100	N907	V1098	deletion
T1113	S926	P1099	E856
P1114	L927	R1100	L857
T1142	S955	T1113	I878
E1151	M964	P1114	L879
R1194	H983	L1120	H885
M1202	K994	T1142	S907
V1276	D1079	V1146	T911
L1306	deletion	E1151	L916
G1310	deletion	R1194	938
N1330	D1145	M1202	W949
L1348	F1163	V1276	D1040
E1351	N1166	L1306	deletion
R1366	E1181	G1310	deletion
R1386	A1201	N1330	Y1110
E1403	S1223	E1342	S1122
L1409	F1229	A1343	C1123
L1430	A1249	R1345	F1125
		E1351	R1131
		V1355	S1135
		R1366	E1146
		R1386	S1166
		F1402	L1187
		E1403	I1188
		L1409	F1194
		V1428	C1213
		L1430	S1214

Table S2. Oligonucleotides used in primer elongation assays

Oligonucleotide Name	Oligonucleotide Sequence (5'-->3')
RNA primer	UGUGUCACAGCGAGUCU
GTP incorp. fidelity DNA template	CATCGTAGACTGACCCAGACTCGCTCAACTCA
CTP incorp. fidelity DNA template	CATCGTAGACTTAGGGAGACTCGCTCAACTCA
UTP incorp. fidelity DNA template	CATCGTAGACTGGAAAAGACTCGCTCAACTCA
ATP incorp. fidelity DNA template	CATCGTAGACTGATTTAGACTCGCTCAACTCA
UTP incorp. fidelity, methylated template	CATCGTAGACTG ^{me} CAAAAGACTCGCTCAACTCA
ATP incorp. fidelity, methylated template	CATCGTAGACTG ^{me} CTTTAGACTCGCTCAACTCA
UTP incorp. fidelity, unmethylated template	CATCGTAGACTGCAAAAGACTCGCTCAACTCA
ATP incorp. fidelity, unmethylated template	CATCGTAGACTGCTTTAGACTCGCTCAACTCA
CTP incorp. fidelity, methylated template	CATCGTAGACTT ^{me} CGGGAGACTCGCTCAACTCA
CTP incorp. fidelity, unmethylated template	CATCGTAGACTTCGGGAGACTCGCTCAACTCA