

1 **Evidence for a unique DNA-dependent RNA polymerase in cereal crops**

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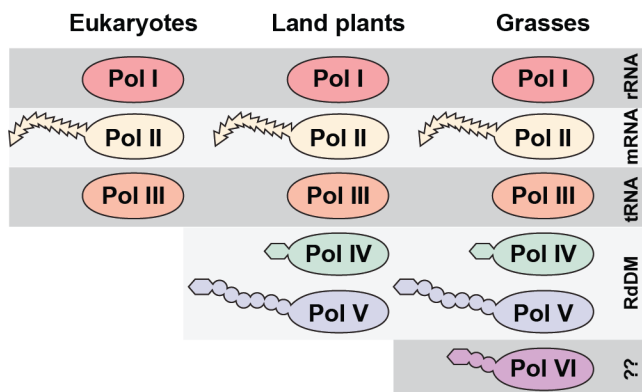
19 duplication, Poaceae

20 Abstract

21 Gene duplication is an important driver for the evolution of new genes and protein
22 functions. Duplication of DNA-dependent RNA polymerase (Pol) II subunits within plants
23 led to the emergence of RNA Pol IV and V complexes, each of which possess unique
24 functions necessary for RNA-directed DNA Methylation. Comprehensive identification of
25 Pol V subunit orthologs across the monocot radiation revealed a duplication of the
26 largest two subunits within the grasses (Poaceae), including critical cereal crops. These
27 paralogous Pol subunits display sequence conservation within catalytic domains, but
28 their carboxy terminal domains differ in length and character of the Ago-binding
29 platform, suggesting unique functional interactions. Phylogenetic analysis of the
30 catalytic region indicates positive selection on one paralog following duplication,
31 consistent with retention via neofunctionalization. Positive selection on residue pairs
32 that are predicted to interact between subunits suggests that paralogous subunits have
33 evolved specific assembly partners. Additional Pol subunits as well as Pol-interacting
34 proteins also possess grass-specific paralogs, supporting the hypothesis that a novel
35 Pol complex with distinct function has evolved in the grass family, Poaceae.

36

37 Graphical Abstract



38

39 **Significance statement**

40 The grass family is critically important for humans, as this group contains cereal grains
41 such as rice, wheat, and corn that form the bulk of the human diet. Here we provide
42 evidence that grasses have evolved a unique polymerase complex of unknown function,
43 suggesting a novel mechanism of gene regulation in the grass lineage. In addition to
44 implications for the biology of grasses, this system offers an opportunity to understand
45 how evolution shapes multi-subunit complexes through duplication of individual
46 components.

47

48 **Introduction**

49 Eukaryotic organisms possess three multi-subunit DNA-dependent RNA polymerase
50 complexes (Pol I-III), which are each responsible for transcription of a subset of cellular
51 RNA. Plants encode two additional DNA-dependent RNA polymerases (Pol IV and V),
52 which are specialized for RNA-directed DNA methylation (Haag and Pikaard 2011).
53 RNA Pol IV produces 24-nucleotide small RNAs that are bound by Argonaute4 (AGO4)
54 proteins. These siRNAs then guide AGO4 to sites of Pol V transcription and recruit *de*
55 *novo* methylation machinery to the locus (Wierzbicki et al. 2009). The carboxy terminal
56 domain (CTD) of Pol V helps to recruit AGO4 through an AGO-binding platform (El-
57 Shami et al. 2007; Lahmy et al. 2016).

58 DNA-dependent RNA polymerases are composed of multiple subunits, which are
59 named NRP_{xn}, where x=A-E for Pols I-V, respectively, and n=1-12 for the largest to
60 smallest subunit, respectively (Zhou and Law 2015). Pol II, IV, and V share many of
61 their 12 subunits, but also possess unique subunits that confer their distinct functions

62 (Huang et al. 2009; Lahmy et al. 2009; Ream et al. 2009; Haag et al. 2014). The largest
63 subunit of Pol IV (NRPD1) and Pol V (NRPE1) are non-redundant paralogs that
64 together are sister to the largest Pol II subunit (NRPB1) (Luo and Hall 2007). The
65 second, fourth, fifth, and seventh subunits have also duplicated and specialized for Pol
66 IV and V at different times during land plant evolution (Tucker et al. 2010; Huang et al.
67 2015). In addition, the Argonaute-binding platform in the CTD of NRPE1 is evolving
68 more rapidly than other regions of the protein (Trujillo et al. 2016).

69 Here, we identify retained duplicates of multiple polymerase subunits and
70 polymerase-associated proteins in Poaceae, the family that contains cereal grasses.
71 Phylogenetic analysis of the two largest subunits indicates positive selection for one
72 paralog following the duplication, consistent with retention via neofunctionalization.
73 Analysis of selection at sites of subunit interaction raises the possibility that evolution
74 favored specific polymerase assemblies. The CTDs of paralogous subunits are also
75 characteristically distinct. Together these results suggest that a sixth distinct RNA
76 polymerase complex exists in this critical plant family.

77

78 **Materials and Methods**

79 **Ortholog identification**

80 Published *NRPE1* ortholog sequences (Trujillo et al. 2016) were retrieved from
81 Phytozome versions 11 and 12 (Goodstein et al. 2012). Additional sequences, including
82 homologs of *NRPE1*, *NRPD1*, *NRPF1*, *NRPB2*, *NRPD/E2*, *NRPB/D5*, *NRPE5*, *NRPB9*,
83 *AGO4*, *SPT5/SPT5L*, and *DRD1/CLSY1*-like, were obtained through BLAST or
84 TBLASTX queries against whole genome sequences in Phytozome, CoGE (Lyons and

85 Freeling 2008), and Ensembl Plants (Kersey et al. 2017) using *Oryza sativa* nucleotide
86 sequences. *Streptochoeta angustifolia* and *Zea mays ssp. parviglumis* are available at
87 <http://gif-server.biotech.iastate.edu/arnstrm/mhufford/streptochoeta.html> and [http://gif-](http://gif-server.biotech.iastate.edu/arnstrm/mhufford/parviglumis.html)
88 [server.biotech.iastate.edu/arnstrm/mhufford/parviglumis.html](http://gif-server.biotech.iastate.edu/arnstrm/mhufford/parviglumis.html).

89 In unannotated genomes, or when gene model predictions were incomplete,
90 coding sequences were predicted using FGENESH+ (Softberry Inc. New York, NY,
91 USA) with *O. sativa* protein sequence as the homology template, followed by manual
92 curation. Orthology was confirmed by reciprocal BLAST searches against the *O. sativa*
93 genome and with phylogenetic analysis. Where species-specific duplications were
94 detected (often due to polyploidization), only one full-length coding sequence was used
95 for downstream analysis. All genes included in this study are listed in Supplemental
96 Table 1.

97

98 **Phylogenetic analysis**

99 Nucleotide sequences were aligned by translation using MUSCLE in Geneious version
100 6.1.8 (Kearse et al. 2012). Where necessary, manual curation was performed to correct
101 alignments. Maximum likelihood phylogenetic trees were inferred with RAxML version
102 7.2.8 (Stamatakis et al. 2008) using full-length coding sequences for most genes. For
103 the largest subunits (NRPE1 and NRPF1), only the catalytic region from domains B-H
104 were aligned. A General Time Reversible (GTR) model with gamma distributed rate of
105 heterogeneity was implemented, and support values were based on 100 bootstrap
106 replicates.

107 The branch-sites test for positive selection was performed using PAML version 4.9c

108 codeml (Yang 2007). Branches under positive selection were determined by likelihood
109 ratio test (χ^2). Parameter space was explored with various starting ω values (0.2, 0.4,
110 0.6, 0.8, and 1.0) to determine effect on likelihood calculation under M2 (branch-sites)
111 model. Robustness of likelihood values was evaluated by three replications of each
112 analysis under each parameter set.

113

114 **Expression analysis**

115 Total nucleic acids were isolated from *O. sativa* inflorescence as previously described
116 (Grover et al. 2017), followed by Turbo DNase-free treatment (Ambion). First-strand
117 cDNA was synthesized using SuperScript III (Invitrogen) with either polyT or random
118 hexamer primers. Ortholog expression was determined through PCR using primers in
119 Supplemental Table 2.

120

121 **Structural modeling**

122 Protein homology models of *O. sativa* NRPE1, NRPF1, NRPD/E2, and NRPF2 were
123 generated by Phyre2 intensive modelling (<http://www.sbg.bio.ic.ac.uk/~phyre2>) (Kelly et
124 al. 2015) to the *S. pombe* (PDB:3H0G) or *Bos taurus* (PDB:5FLM) PolII holoenzyme
125 structures for the largest and second largest subunits, respectively. Modelled subunits
126 were then aligned based on interaction of the cognate *S. pombe* subunits and
127 interaction between the subunits was analyzed. Sites under positive selection were
128 visualized in PyMol Molecular Graphics System (Schrödinger, LLC). Residues of *O.*
129 *sativa* NRPE1 and NRPF1 were compared to homologous residues of NRPE1 in *S.*
130 *pombe* and NRPE1 in *A. thaliana* with known functional importance. Experimentally

131 derived information regarding subunit interaction regions and specific interacting
132 residues was retrieved through UniProt database (<http://www.uniprot.org/>)(Bateman et
133 al. 2017).

134

135 **Results**

136 **Poaceae members encode paralogous Pol V subunits**

137 Previous studies investigating Pol V evolution revealed the presence of two *NRPE1*
138 paralogs in some monocot genomes (Trujillo et al. 2016). However, this observation
139 was restricted to species within Poaceae as most of the sequenced monocot genomes
140 are members of this agriculturally-important family. To understand the timing of this
141 duplication, we identified putative *NRPE1* homologs across the monocot lineage.
142 Phylogenetic analysis of these sequences reveals a single *NRPE1* ortholog in non-
143 Poaceae monocots and two well-supported clades of *NRPE1*-like sequences within
144 Poaceae (Figure 1A). *Ananus comosus* (pineapple) in Bromeliaceae, a sister lineage to
145 Poaceae in the order Poales, contains a single *NRPE1* ortholog, indicating that
146 duplication of *NRPE1* occurred after the divergence of the two families. Conversely,
147 *Streptochaeta angustifolia*, an early-diverging member of the Poaceae, has two *NRPE1*-
148 like sequences, indicating the duplication occurred in an early ancestor of extant
149 grasses. We have designated the paralog along the longer branch as *NRPF1*.

150 *NRPE1* and *NRPF1* copies were recovered from every Poaceae genome we
151 assessed, with the exception of *Z. mays*, which lacks a full-length *NRPF1* (Figure 1A). A
152 *NRPF1* homolog is present in the genome, but it appears to be a pseudogene in all *Z.*
153 *mays* varieties we analyzed (B73, PH207, CML247, and B104), possibly due to

154 insertion of a transposon within the coding sequence (Supplemental Figure 1). Analysis
155 of teosinte (*Zea mays ssp parviglumis*), the wild progenitor of cultivated *Z. mays*,
156 revealed that this pseudogenization occurred prior to domestication. It is not clear why
157 *Z. mays* has lost NRPF1, since retention of this gene in every other grass genome we
158 assessed indicates that these paralogs are not redundant.

159 In addition to NRPE1, Pol V is distinguished from Pol II by the smaller subunits
160 NRPD2/E2, NRPD4/E4, NRPE5, and NRPD7/E7. We therefore determined if these
161 subunits are also duplicated within Poaceae. We found no evidence of duplication for
162 *NRPD4/E4*, *NRPE5*, or *NRPD7/E7*, however the second largest subunit, *NRPD2/E2*,
163 which is shared by RNA Pol IV and V, has also duplicated within monocots (Figure 1B).

164 A single copy of *NRPD2/E2* is present in *A. comosus* but two well-supported clades
165 of Poaceae specific orthologs exist. As with the largest subunit, the clade with the
166 longer branch has been designated as *NRPF2*. It is clear that the *NRPD2/E2* duplication
167 occurred early in Poaceae diversification, however whether this duplication was
168 simultaneous or subsequent to the *NRPE1* duplication is not clear. One *NRPD2/E2*
169 paralog was identified in *S. angustifolia*, and this is sister to all other grass *NRPD2/E2*
170 sequences, suggesting that *NRPF2* might have been lost in *S. angustifolia*. However, it
171 is also possible that *S. angustifolia* diverged from other grasses prior to duplication
172 giving rise to the *NRPD2/E2* and *NRPF2* paralogs since support for the placement of
173 the single *S. angustifolia* *NRPD2/E2* homolog is weak.

174 Triplication of *NRPD2/E2* was reported in *Z. mays* (Sidorenko et al. 2009; Stonaker
175 et al. 2009; Haag et al. 2014). With the addition of other Poaceae homologs we show
176 that these three copies arose through a subsequent duplication of *NRPD2/E2* in the

177 maize (or possibly maize + sorghum) lineage, yielding *NRPD2a/E2a*, *NRPD2b/E2b*, and
178 *NRPF2* (previously called *NRPD2c/E2c*).

179 We confirmed expression of *NRPE1*, *NRPF1*, *NRPD/E2*, and *NRPF2* paralogs in *O.*
180 *sativa* floral tissue (Figure 1C). Publicly available expression data also support the
181 expression of both paralogs of each subunit (Supplemental Figure 2), confirming that
182 none of the paralogs are pseudogenes. Public expression data also indicate that the
183 paralogs differ in expression level and pattern, supporting our hypothesis that grasses
184 contain novel Pol subunits with non-redundant functions.

185

186 **NRPF1 has distinct CTD characteristics**

187 The largest subunits of Pols II, IV, and V have unique C-terminal domains (CTD).
188 *NRPB1* has a large region composed exclusively of heptad repeats; *NRPD1* and
189 *NRPE1* both contain a domain of unknown function with similarity to Defective
190 Chloroplast and Leaves (DeCL) genes at the extreme C-terminus, but only *NRPE1*
191 contains an Ago-binding platform between the catalytic and DeCL domains (Pontier et
192 al. 2005; El-Shami et al. 2007; Huang et al. 2015). The Ago-binding platform is
193 repetitive and disordered, and contains numerous Ago-hook motifs for association with
194 AGO4 (Trujillo et al. 2016). Because the *NRPE1* Ago-binding platform is evolutionarily
195 labile and only closely related sequences can be aligned, CTDs were not included in the
196 phylogenetic analysis that identified *NRPE1* and *NRPF1* clades. However, the same
197 two clades are identified when only characteristics of the CTDs are considered.

198 *NRPE1* CTDs possess an Ago-binding platform similar to that found in *NRPE1* from
199 non-Poaceae species, namely a long region with numerous Ago hook motifs. In *NRPF1*

200 proteins this region is reduced in length and in number of Ago hook motifs (Figure 2,
201 Supplemental Table 3). However, NRPF1 CTDs are not as short as NRPD1 CTDs, and
202 all but one NRPF1 ortholog contain at least one Ago hook in its CTD, as well as two
203 Ago hook motifs in the catalytic region. This change in CTD character maps to the
204 branch leading to the *NRPF1* clade on the NRPE1/NRPF1 gene tree, suggesting it
205 occurred immediately following duplication. The Ago-binding platform is required for Pol
206 V activity (Wendte et al. 2017), and the change in this domain following duplication
207 further indicates that *NRPF1* is a non-redundant paralog of *NRPE1*.

208

209 **Pol VI paralogs experienced positive selection following duplication**

210 Gene duplication allows evolutionary changes that can result in a paralog with a
211 novel function (neofunctionalization) or paralogs that partition the original function
212 (subfunctionalization), hypotheses that can be distinguished based on the pattern of
213 selection following duplication. The branch leading to the *NRPF1* clade is longer than
214 that of the Poaceae *NRPE1* clade, suggesting that *NRPF1* and *NRPE1* experienced
215 different selective pressure following duplication. A longer branch length could result
216 from relaxed selection permitting the accumulation of substitutions; alternatively,
217 positive selection on specific substitutions might have driven the progression towards a
218 novel function. We distinguished between these possibilities with a branch-sites model
219 (Zhang et al. 2005), which indicated that positive selection occurred along the branch
220 leading to the *NRPF1* and *NRPF2* clades while *NRPE1* and *NRPD2/E2* subunits
221 retained purifying selection.

222 The branch-sites test identified 12 codons with a high likelihood of being under

223 positive selection on the branch leading to *NRPF1* and numerous additional sites when
224 all branches of the clade are evaluated. (Figure 3A, Supplemental Table 4). Similarly,
225 the *NRPF2* branch has 9 sites predicted to be under positive selection immediately
226 following the duplication, and 59 sites across the whole clade (Figure 3B, Supplemental
227 Table 5). Indels also exist between *NRPE1* and *NRPF1* orthologs, suggesting that
228 structural differences were also selected following duplication (Supplemental Figure 3).
229 Only two sites were identified as under positive selection for *NRPE1* and no sites for
230 *NRPD2/E2* (Supplemental Tables 4-5). Positive selection along the *NRPF1* and *NRPF2*
231 branches and predominantly purifying selection for *NRPE1* and *NRPD2/E2* supports the
232 hypothesis of paralog retention due to neofunctionalization, and suggests that in
233 Poaceae, *NRPE1* and *NRPD2/E2* maintain the ancestral functions, while *NRPF1* and
234 *NRPF2* may have evolved novel functions.

235

236 **Sites under positive selection are exposed and predict additional subunit**
237 **duplications**

238 We mapped the predicted sites under positive selection on a homology-based model
239 of *O. sativa* paralogs to evaluate the structural significance of particular substitutions
240 (Figure 3C). *NRPE1* and *NRPF1* were aligned and modelled to the largest subunit of *S.*
241 *pombe* RNA pol II holoenzyme (PDB:3H0G chain A) with 100% confidence and
242 sequence identity of 23%. The second largest subunit paralogs were analyzed in the
243 same manner with *O. sativa* *NRPD2/E2* and *NRPF2* mapping with 100% confidence
244 and 36-37% sequence identity to a bovine RNA Pol II structure (PDB:5FLM chain B).

245 Most sites predicted to be under positive selection were located on the surface of

246 subunits or at interaction faces with other polymerase subunits, suggesting that the
247 substitutions do not impact the overall structure of the subunits, but might impact
248 assembly of the holoenzyme (Supplemental Tables 4-5). In one case, residues under
249 selection in NRPF1 and NRPF2 are predicted to interact, suggesting that they might be
250 compensatory, and selection may have acted to restrict the number of possible
251 holoenzyme assemblies (Figure 3D). Specific assembly of Pol subunits would indicate
252 that not only are the paralogous subunits functionally non-redundant, they assemble
253 into a unique polymerase complex, a Pol VI.

254 Based on the position of selected sites on the structure model, we hypothesized
255 additional duplications of smaller subunits. Six selected sites are in the region that
256 interacts with the fifth subunit (Figure 3E) and four sites near the interaction region for
257 the ninth subunit (Supplemental Tables 4-5). Although there is no evidence for
258 duplication of the Pol V-specific *NRPE5*, we identified multiple copies of *NRPB5/D5* in
259 Poaceae (Supplemental Figure 4). There is also evidence for a duplication of
260 *NRPB9/D9/E9* within the monocots (Supplemental Figure 5), although this duplication is
261 difficult to resolve given the limited sequence information in this small subunit.
262 Duplication of *NRPB5/D5* suggests that a Poaceae-specific Pol VI might have
263 assembled in a modular fashion, using paralogous modules/subunits from both Pol IV
264 and Pol V.

265

266 **Pol V-associated proteins are also duplicated in Poaceae**

267 Canonical RdDM involves RNA Pol V interacting with numerous other proteins to
268 accomplish DNA methylation. If Pol VI has a novel function that is diverged from Pol V,

269 we might expect duplication and neofunctionalization of Pol V interacting proteins. We
270 therefore investigated the evolution of known Pol V-interacting proteins, including the
271 small RNA-binding protein Argonaute 4 (AGO4) (El-Shami et al. 2007), the
272 transcriptional elongation factor SPT5-like (Huang et al. 2009), and the SWI/SNF-
273 related helicase DRD1 (Law et al. 2010; Zhong et al. 2012).

274 AGO4 associates with the NRPE1 CTD, enabling base-pairing of AGO4-bound small
275 RNAs with Pol V transcripts (Wierzbicki et al. 2009), or with the Pol V transcription
276 bubble (Lahmy et al. 2016). In *A. thaliana*, AGO4 is a part of a group of Argonautes
277 including the deeply-conserved AGO6, and Brassicaceae-specific AGO8 and AGO9
278 (Zhang et al. 2015; Rodríguez-Leal et al. 2016). In addition to an AGO6 group, we
279 detected three well-supported clades of AGO4 orthologs within grasses arising from
280 nested duplications at the base of Poaceae (Figure 4). *AGO4a* and *AGO15* are sister
281 groups that share high sequence similarity and are located within a few kilobases of one
282 another, suggesting that a tandem duplication of one paralog occurred following whole
283 genome duplication. Most predicted *AGO15* sequences consist of partial, fragmented
284 coding sequences, and there is no evidence for *AGO15* protein accumulation in rice
285 (Wu et al. 2010), suggesting that *OsAGO15* might be an expressed pseudogene. Public
286 expression data indicate that rice and maize AGO4 orthologs are broadly expressed
287 (Supplemental Figure 6), where they bind to different groups of small RNAs (Wu et al.
288 2010). Genetic data in maize also indicates that despite the fact that *ZmAGO4a*
289 (*ZmAGO119*) and *ZmAGO4b* (*ZmAGO104*) have broad and overlapping expression
290 patterns, these paralogs are not redundant (Singh et al. 2011).

291 SPT5L, a duplicate of the Pol II transcription elongation factor SPT5, interacts with

292 Pol V and contains its own Ago-binding platform in its carboxy terminus (Bies-Etheve et
293 al. 2009; Lahmy et al. 2016). Although SPT5L is the paralog that interacts with Pol V,
294 we do not detect a duplication of this gene. Rather, *SPT5*, which interacts with Pol II
295 and Pol IV, has undergone a duplication at the base of Poaceae (Supplemental Figure
296 7). This observation is additional evidence that only specific components of Pol V were
297 duplicated in grasses, and further supports the hypothesis that a sixth polymerase
298 complex formed through duplication of both Pol IV and Pol V machinery.

299 RNA Pol IV and V transcription is assisted through interaction with helicase proteins
300 of the DRD1-like family (Kanno et al. 2004; Smith et al. 2007; Law et al. 2011). We
301 discovered Poaceae-specific duplications within the *DRD1* and *CLSY3/4* clades, giving
302 rise to paralogs we have named *DRD1-like* and *CLSY5*, respectively (Supplemental
303 Figure 8). We discovered *DRD1L* and *CLSY5* copies only in Poaceae species, though
304 *DRD1* and *CLSY* trees suggest the duplication predates the evolution of the grasses.
305 We take the current placement as a preliminary assessment in need of more data from
306 additional species to more fully resolve these gene tree topologies. Whether one or both
307 helicases are required for transcription by a grass-specific Pol VI remains to be
308 determined.

309 *DRD1* interacts with *RDM1* and *DMS3* to form the DDR complex, which is required
310 for RdDM (Law et al. 2010). We identified only a single copy of *RDM1* and *DMS3* in
311 Poaceae, further demonstrating that many components of Pol V machinery remain in
312 single copy, while specific components of Pol VI and Pol V duplicated in grasses. The
313 duplication of Pol IV and Pol V interacting protein further supports our hypothesis that
314 grasses contain a distinct sixth polymerase with unique activity.

315

316 **Discussion**

317 Our evolutionary analysis of DNA-dependent RNA polymerases within the monocot
318 lineage of land plants identified duplications of multiple subunits and polymerase-
319 associated proteins. These duplications are coincident with the *rho* whole genome
320 duplication at the base of grasses (McKain et al. 2016). Most genes return to single
321 copy following whole genome duplication, therefore retention of duplicated genes is
322 evidence for the formation of non-redundant protein function (Hahn 2009). Verified
323 expression of *NRPE1*, *NRPF1*, *NRPD2/E2*, and *NRPF2* (Figure 1C); unique CTD
324 sequences for *NRPE1* and *NRPF1* (Figure 2); and phylogenetic evidence of positive
325 selection on *NRPF1* and *NRPF2* (Figure 3AB) indicate that these paralogous subunits
326 are not merely redundant, but rather are likely to encode unique functions.

327 Many of the sites with evidence for positive selection are at interfaces where Pol
328 subunits interact (Figure 3DE), suggesting that in addition to selection for unique
329 activity, there might have been selection for faithful assembly of subunits into unique
330 complexes (Beilstein et al. 2015). This idea is supported by biochemical evidence from
331 *Z. mays*, in which *NRPD/E2* and *NRPF2* display differential association with *NRPD1*
332 (Haag et al. 2014). However, pseudogenization of *NRPF1* in *Z. mays* makes this
333 species a poor representative for other grasses and further validation of Pol subunit
334 associations is required in a different grass species. The signature of selection at
335 predicted interacting sites, as well as the coordinated duplication of multiple subunits,
336 leads us to hypothesize that not only do *NRPF1* and *NRPF2* encode novel functions,
337 but that they assemble into a unique polymerase complex, Pol VI.

338 We identified duplications of many Pol V subunits and interacting proteins, but we
339 also found duplications of proteins that are not specific to Pol V. For example, we
340 detected duplication of *NRPB/D5*, but not *NRPE5* (Figure S3). Similarly, the Pol V-
341 specific transcription elongation factor *SPT5L* is not duplicated, but the paralogous
342 *SPT5*, which interacts with Pol II and Pol IV, occurs in multiple copies (Figure S6).
343 Duplication of Pol II/IV proteins suggest that Pol VI formed through neofunctionalization
344 of both Pol IV and Pol V subunits (Figure 5). Pol IV and Pol V both generate non-coding
345 transcripts from otherwise silent DNA, but they differ in their speed, accuracy, and
346 processivity (Wierzbicki et al. 2008; Zhai, Bischof, et al. 2015; Marasco et al. 2017).
347 Conservation of key enzymatic residues, including the metal binding sites, indicates that
348 Pol VI is capable of transcription, although such activity and how it differs from Pol IV
349 and Pol V remain to be studied.

350 One key difference between Pol IV and Pol V is association with AGO4. The NRPE1
351 CTD contains numerous Ago-hooks for interaction with AGO4. Likewise, it's binding
352 partner *SPT5L* also contains numerous Ago hooks and associates with AGO4 (Bies-
353 Etheve et al. 2009; Trujillo et al. 2016). In contrast, neither *NRPD1* nor *SPT5* contain
354 Ago-hook motifs for AGO4 interaction. *NRPF1* orthologs possess only a few Ago hooks,
355 suggesting that Pol VI might interact with AGO proteins, but in a manner distinct from
356 the Pol V-AGO4 interaction. Duplication of *SPT5* rather than *SPT5L* also hints that
357 numerous Ago hooks are not necessary for Pol VI function.

358 The biological role of Pol VI remains to be determined, but its similarity to Pol IV and
359 Pol V along with the presence of Ago hooks suggests a role in small RNA biology while
360 expression data indicate that Pol VI might accumulate during reproductive development

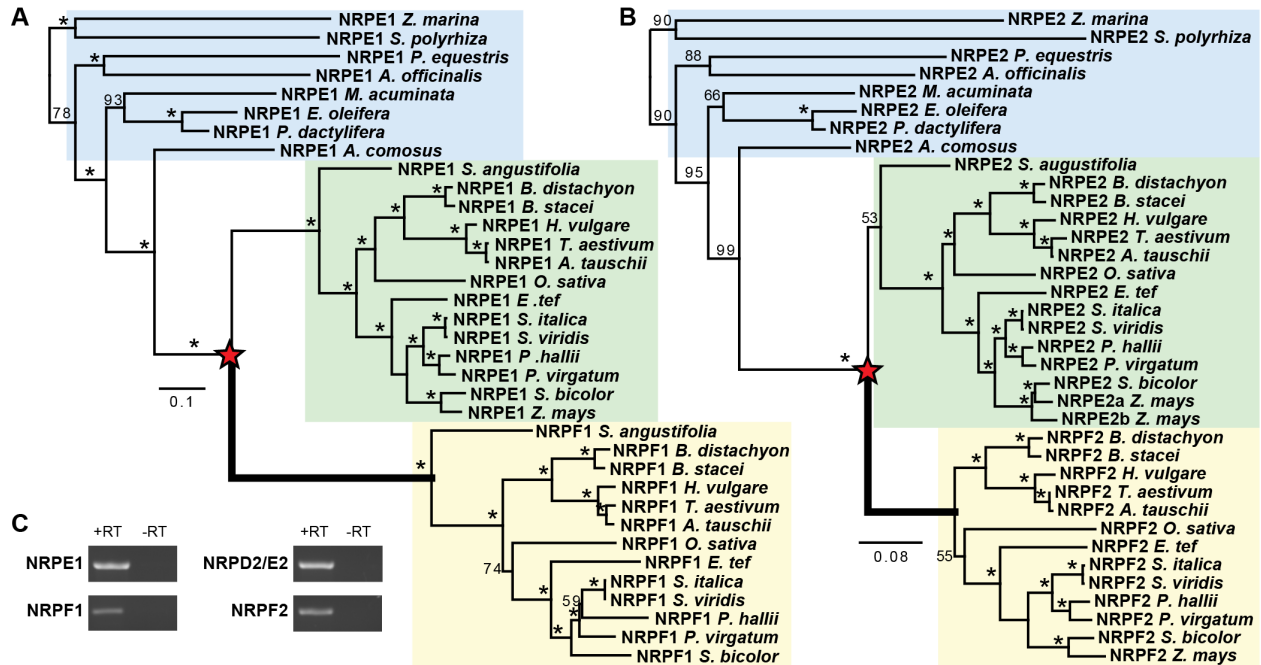
361 (Supplemental Figure 2). Pol VI might be required for the biosynthesis or function of a
362 number of novel small RNA classes, including highly expressed endosperm-specific
363 siRNA loci in rice (Rodrigues et al. 2013), 24-nt phased siRNAs required for microspore
364 development (Zhai, Zhang, et al. 2015; Fei et al. 2016), or rice “long” miRNAs (Wu et al.
365 2010). The potential role of Pol VI in reproductive development could make it an
366 important target of agricultural and biotechnology manipulation.

367 Grasses are one of the most successful radiations of land plants, covering vast
368 areas of natural habitat and agricultural land and forming the bulk of the human diet. We
369 are acutely dependent on grasses, both for food and environmental stability, and it is
370 therefore critical to understand the unique gene regulatory mechanisms of this family.
371 Our discovery of a novel sixth polymerase in Poaceae uncovers a previously unknown
372 aspect of grasses and offers an opportunity to learn more about this important plant
373 family.

374

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382

383

384 **Figure 1. Duplications of Pol V subunits are coincident with the emergence of the**

385 **grass family Poaceae.** The evolutionary relationships of *NRPE1* (A) and *NRPD2/E2*

386 (B) within the monocot lineage demonstrate that monocots outside of the Poaceae

387 family have a single gene copy (blue box), while most members of Poaceae have

388 paralogous genes (green and yellow boxes). Phylogenetic trees were inferred by

389 maximum likelihood analysis of mRNA sequence in the catalytic domain (regions B to

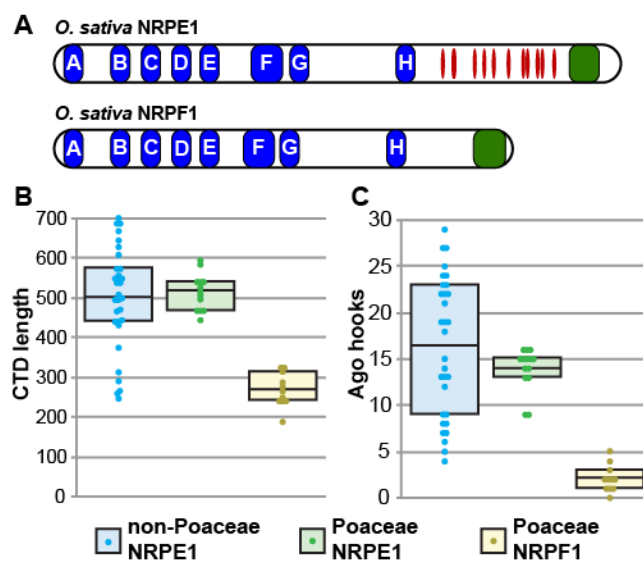
390 H). Bootstrap support values < 100 are shown and red stars mark the inferred

391 duplications. Thick branches indicate positive selection ($p < 0.05$). Full species names

392 and gene accession numbers are listed in Supplementary Table 1. (C) RT-PCR

393 demonstrates that all *O. sativa* paralogs are expressed.

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397 **Figure 2. Structural divergence of the CTDs between NRPE1 and NRPF1 paralogs.**

398 (A) Diagram of NRPE1 and NRPF1 from *O. sativa*. OsNRPE1 retains a canonical Ago-

399 binding platform between the catalytic A-H domains (blue ovals) and the DeCL domain

400 (green oval). The Ago-binding platform contains many Ago hooks (red ovals). In

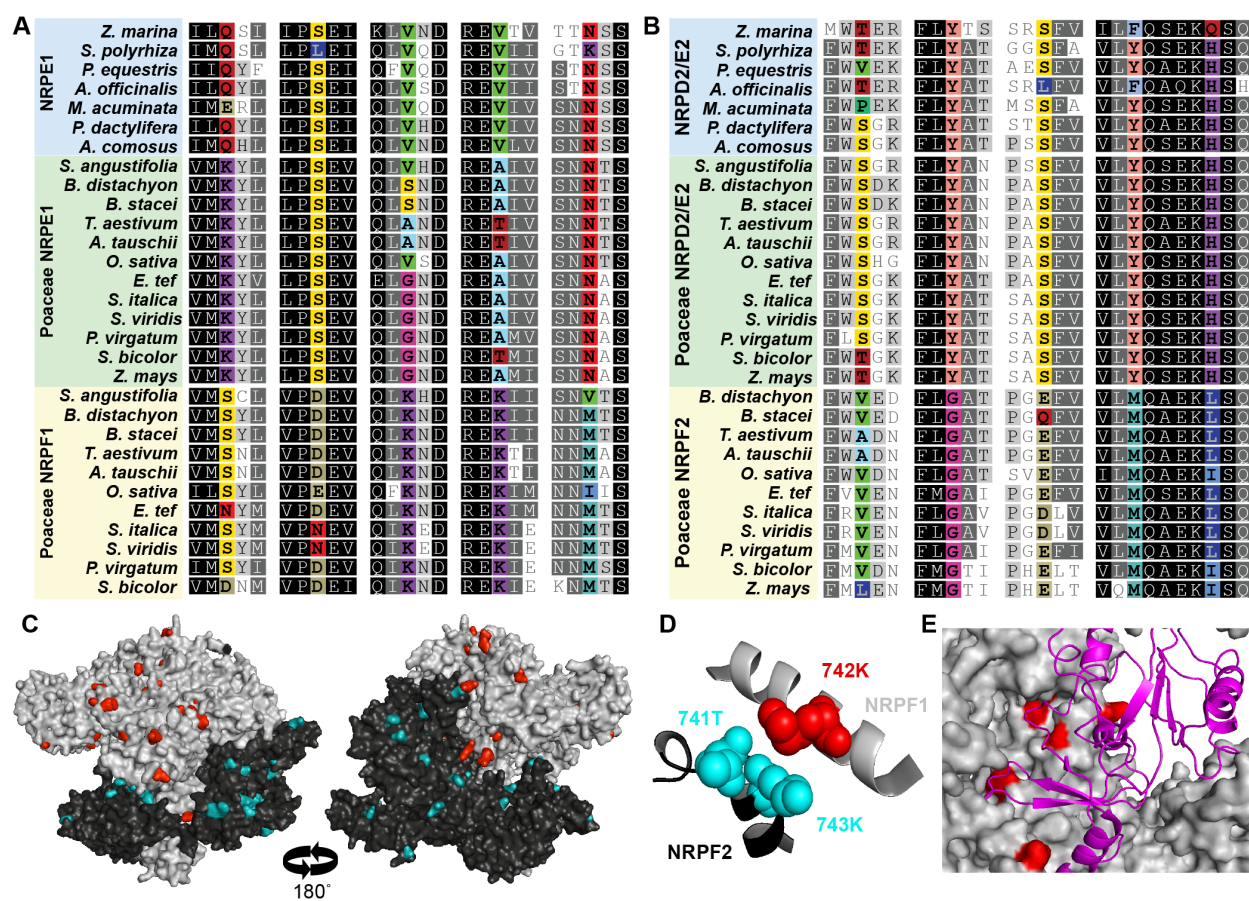
401 contrast, OsNRPF1 has a shorter CTD that lacks Ago hook motifs. (B, C) Poaceae

402 NRPE1 and non-Poaceae NRPE1 CTDs are similar in length and number of Ago hooks,

403 while NRPF1 CTDs are shorter and contain fewer Ago hooks. Data points for 31 non-

404 Poaceae, 14 Poaceae NRPE1, and 11 Poaceae NRPF1 are shown as colored circles;

405 boxes represent the interquartile range and the mean is shown by a black bar.



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408 **Figure 3. Sites under positive selection cluster on the surface of Pol VI subunits.**

409 Alignment of monocot largest (A) and second largest (B) subunits illustrates residues

410 that are under positive selection following gene duplication (colored) (A). Remaining

411 residues are colored based on sequence conservation. (C) Residues under positive

412 selection (colored) are found on the surface of homology-based structures of NRPF1

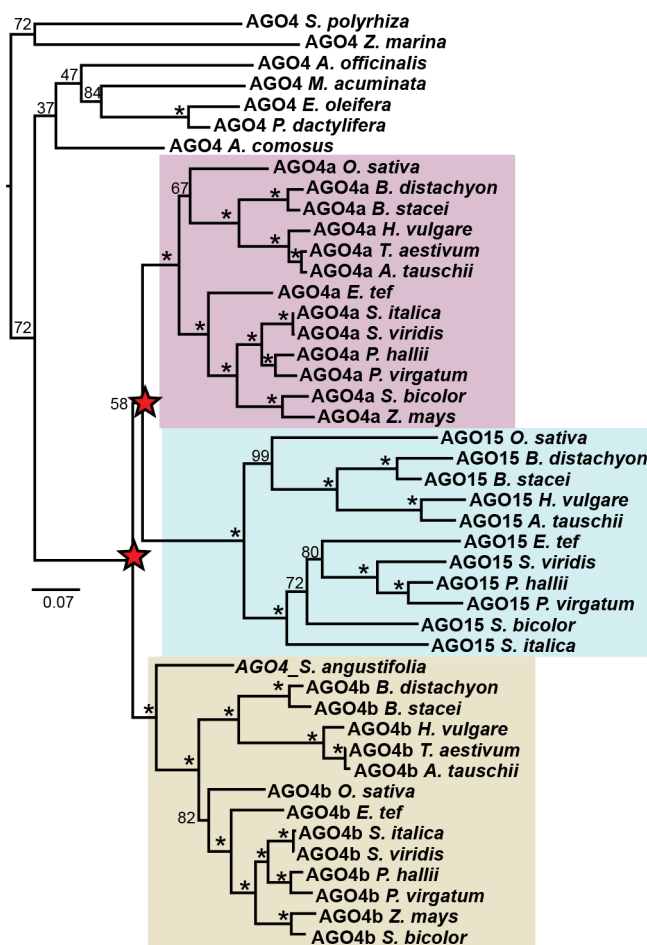
413 (gray) and NRPF2 (black). (D) Residues under positive selection also occur at the

414 interface between subunits, as demonstrated by 742K in NRPF1, which is directly

415 opposite 741T and 743K in NRPF2. (E) Several residues under positive selection are

416 found where the largest subunit (gray) interacts with the fifth subunit (magenta ribbon).

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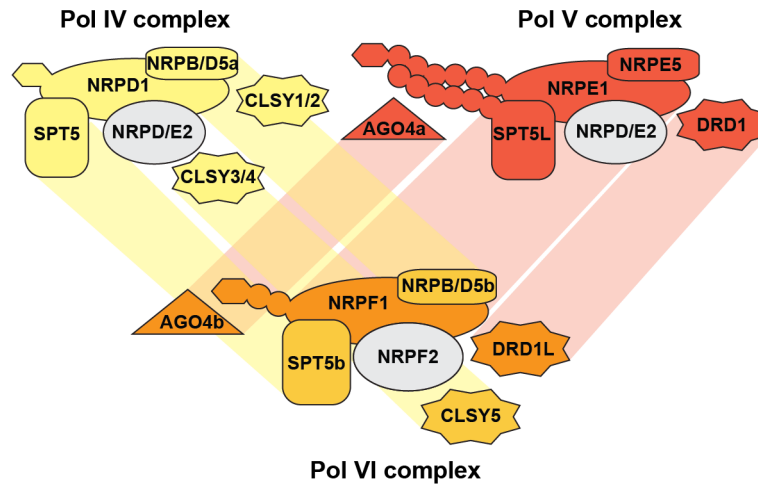


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420 **Figure 4. Nested duplications of AGO4 locus result in three paralogs in most**
421 **grasses.** Comparison of AGO4-orthologous sequences in monocots demonstrates that
422 grasses contain multiple AGO4 paralogs and that these duplications were coincident
423 with the emergence of the Poaceae family. Maximum likelihood phylogenetic tree of
424 AGO4 related nucleotide sequences in monocots. Support values for branches with
425 <100 bootstrap support are marked. Red stars mark the inferred duplication events.

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429 **Figure 5. Proposed Pol assembly in grasses.** We propose that NRPF1 and NRPF2
430 subunits assemble into Pol VI with a paralog of NRPB/D5. The Pol VI complex might
431 function with specific paralogs of SPT5, DRD1, CLSY3/4, and AGO4, highlighting the
432 use of paralogous proteins from both Pol V and Pol IV complexes. Paralogous subunits
433 are connected by shaded boxes.

434

435 **References**

- 436 Bateman A, Martin MJ, O'Donovan C, Magrane M, Alpi E, Antunes R, Bely B, Bingley
437 M, Bonilla C, Britto R, et al. 2017. UniProt: The universal protein knowledgebase.
438 Nucleic Acids Res. 45:D158–D169.
- 439 Beilstein MA, Renfrew KB, Song X, Shakirov E V., Zanis MJ, Shippen DE. 2015.
440 Evolution of the telomere-associated protein POT1a in arabidopsis thaliana is
441 characterized by positive selection to reinforce protein-protein interaction. Mol. Biol.
442 Evol. 32:1329–1341.
- 443 Bies-Etheve N, Pontier D, Lahmy S, Picart C, Vega D, Cooke R, Lagrange T. 2009.
444 RNA-directed DNA methylation requires an AGO4-interacting member of the SPT5
445 elongation factor family. EMBO Rep. [Internet] 10:649–654. Available from:
446 [http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2711833&tool=pmcentre](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2711833&tool=pmcentrez&rendertype=abstract)
447 [z&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2711833&tool=pmcentrez&rendertype=abstract)
- 448 El-Shami M, Pontier D, Lahmy S, Braun L, Picart C, Vega D, Hakimi M-A, Jacobsen SE,
449 Cooke R, Lagrange T. 2007. Reiterated WG/GW motifs form functionally and
450 evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related
451 components. Genes Dev. [Internet] 21:2539–2544. Available from:
452 [http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2000319&tool=pmcentre](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2000319&tool=pmcentrez&rendertype=abstract)
453 [z&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2000319&tool=pmcentrez&rendertype=abstract)
- 454 Fei Q, Yang L, Liang W, Zhang D, Meyers BC. 2016. Dynamic changes of small RNAs
455 in rice spikelet development reveal specialized reproductive phasiRNA pathways. J.
456 Exp. Bot. 67:6037–6049.
- 457 Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W,

- 458 Hellsten U, Putnam N, et al. 2012. Phytozome: A comparative platform for green
459 plant genomics. *Nucleic Acids Res.* 40:1178–1186.
- 460 Grover J, Kendall T, Baten A, King GJ, Mosher RA. 2017. Maternal RNA-directed DNA
461 methylation is required for seed development in *Brassica rapa*. *bioRxiv [Internet]:*1–
462 18. Available from: <http://www.pfaf.org/user/Plant.aspx?LatinName=Brassica+rapa>
- 463 Haag JR, Brower-Toland B, Krieger EK, Sidorenko L, Nicora CD, Norbeck AD, Irsigler
464 A, LaRue H, Brzeski J, McGinnis K, et al. 2014. Functional diversification of maize
465 RNA polymerase IV and V subtypes via alternative catalytic subunits. *Cell Rep.*
466 [Internet] 9:378–390. Available from: <http://dx.doi.org/10.1016/j.celrep.2014.08.067>
- 467 Haag JR, Pikaard CS. 2011. Multisubunit RNA polymerases IV and V: purveyors of non-
468 coding RNA for plant gene silencing. *Nat. Rev. Mol. Cell Biol.* [Internet] 12:483–
469 492. Available from: <http://dx.doi.org/10.1038/nrm3152>
- 470 Hahn MW. 2009. Distinguishing among evolutionary models for the maintenance of
471 gene duplicates. *J. Hered.* 100:605–617.
- 472 Huang L, Jones AMEE, Searle I, Patel K, Vogler H, Hubner NC, Baulcombe DC. 2009.
473 An atypical RNA polymerase involved in RNA silencing shares small subunits with
474 RNA polymerase II. *Nat. Struct. Mol. Biol.* [Internet] 16:91–93. Available from:
475 <http://www.nature.com/doi/10.1038/nsmb.1539>
- 476 Huang Y, Kendall T, Forsythe ES, Dorantes-Acosta A, Li S, Caballero-Pérez J, Chen X,
477 Arteaga-Vázquez M, Beilstein MA, Mosher RA. 2015. Ancient Origin and Recent
478 Innovations of RNA Polymerase IV and V. *Mol. Biol. Evol.* [Internet] 32:1788–1799.
479 Available from:
480 <http://mbe.oxfordjournals.org/content/early/2015/04/07/molbev.msv060.abstract>

- 481 Kanno T, Mette MF, Kreil DP, Aufsatz W, Matzke M, Matzke AJ. 2004. Involvement of
482 Putative SNF2 Chromatin Remodeling Protein DRD1 in RNA-Directed DNA
483 Methylation. *Curr. Biol.* [Internet] 14:801–805. Available from:
484 <http://linkinghub.elsevier.com/retrieve/pii/S0960982204002672>
- 485 Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper
486 A, Markowitz S, Duran C, et al. 2012. Geneious Basic: An integrated and
487 extendable desktop software platform for the organization and analysis of
488 sequence data. *Bioinformatics* 28:1647–1649.
- 489 Kelly LA, Mezulis S, Yates C, Wass M, Sternberg M. 2015. The Phyre2 web portal for
490 protein modelling, prediction, and analysis. *Nat. Protoc.* [Internet] 10:845–858.
491 Available from: <http://dx.doi.org/10.1038/nprot.2015-053>
- 492 Kersey PJ, Allen JE, Allot A, Barba M, Boddu S, Bolt BJ, Carvalho-Silva D, Christensen
493 M, Davis P, Grabmueller C, et al. 2017. Ensembl Genomes 2018: an integrated
494 omics infrastructure for non-vertebrate species. *Nucleic Acids Res.* [Internet]
495 46:802–808. Available from:
496 <http://academic.oup.com/nar/article/doi/10.1093/nar/gkx1011/4577569>
- 497 Lahmy S, Pontier D, Bies-Etheve N, Laudié M, Feng S, Jobet E, Hale CJ, Cooke R,
498 Hakimi M, Angelov D, et al. 2016. Evidence for ARGONAUTE4-DNA interactions in
499 RNA-directed DNA methylation in plants. *Genes Dev.* [Internet] 30:2565–2570.
500 Available from: <http://genesdev.cshlp.org/lookup/doi/10.1101/gad.289553.116>
- 501 Lahmy S, Pontier D, Cavel E, Vega D, El-Shami M, Kanno T, Lagrange T. 2009.
502 PolV(PollVb) function in RNA-directed DNA methylation requires the conserved
503 active site and an additional plant-specific subunit. *Proc. Natl. Acad. Sci. U. S. A.*

504 106:941–946.

505 Law JA, Ausin I, Johnson LM, Vashisht AA, Zhu JK, Wohlschlegel JA, Jacobsen SE.
506 2010. A Protein Complex Required for Polymerase V Transcripts and RNA-
507 Directed DNA Methylation in Arabidopsis. *Curr. Biol.* [Internet] 20:951–956.
508 Available from: <http://linkinghub.elsevier.com/retrieve/pii/S096098221000388X>

509 Law JA, Vashisht AA, Wohlschlegel JA, Jacobsen SE. 2011. SHH1, a Homeodomain
510 protein required for DNA Methylation, as well as RDR2, RDM4, and Chromatin
511 remodeling factors, associate with RNA Polymerase IV. *PLoS Genet.* 7.

512 Luo J, Hall BD. 2007. A multistep process gave rise to RNA polymerase IV of land
513 plants. *J. Mol. Evol.* 64:101–112.

514 Lyons E, Freeling M. 2008. How to usefully compare homologous plant genes and
515 chromosomes as DNA sequences. *Plant J.* 53:661–673.

516 Marasco M, Li W, Lynch M, Pikaard CS. 2017. Catalytic properties of RNA polymerases
517 IV and V : accuracy , nucleotide incorporation and rNTP / dNTP discrimination. :1–
518 12.

519 McKain MR, Tang H, McNeal JR, Ayyampalayam S, Davis JI, dePamphilis CW, Givnish
520 TJ, Pires JC, Stevenson DW, Leebens-Mack JH. 2016. A Phylogenomic
521 Assessment of Ancient Polyploidy and Genome Evolution across the Poales.
522 *Genome Biol. Evol.* 8:1150–1164.

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

527 [http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1199573&tool=pmcentre](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1199573&tool=pmcentrez&rendertype=abstract)
528 [z&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1199573&tool=pmcentrez&rendertype=abstract)

529 Ream TS, Haag JR, Wierzbicki AT, Nicora CD, Norbeck AD, Zhu J-K, Hagen G,
530 Guilfoyle TJ, Paša-Tolić L, Pikaard CS. 2009. Subunit Compositions of the RNA-
531 Silencing Enzymes Pol IV and Pol V Reveal Their Origins as Specialized Forms of
532 RNA Polymerase II. *Mol. Cell* [Internet] 33:192–203. Available from:
533 <http://linkinghub.elsevier.com/retrieve/pii/S1097276508008587>

534 Rodrigues JA, Ruan R, Nishimura T, Sharma MK, Sharma R, Ronald PC, Fischer RL,
535 Zilberman D. 2013. Imprinted expression of genes and small RNA is associated
536 with localized hypomethylation of the maternal genome in rice endosperm. *Proc.*
537 *Natl. Acad. Sci.* [Internet] 110:7934–7939. Available from:
538 <http://www.pnas.org/cgi/doi/10.1073/pnas.1306164110>

539 Rodríguez-Leal D, Castillo-Cobián A, Rodríguez-Arévalo I, Vielle-Calzada J-P. 2016. A
540 Primary Sequence Analysis of the ARGONAUTE Protein Family in Plants. *Front.*
541 *Plant Sci.* [Internet] 7:1–12. Available from:
542 <http://journal.frontiersin.org/Article/10.3389/fpls.2016.01347/abstract>

543 Sidorenko L, Dorweiler JE, Cigan AM, Arteaga-Vazquez M, Vyas M, Kermicle J, Jurcin
544 D, Brzeski J, Cai Y, Chandler VL. 2009. A dominant mutation in mediator of
545 paramutation2, one of three second-largest subunits of a plant-specific RNA
546 polymerase, disrupts multiple siRNA silencing processes. *PLoS Genet.* 5.

547 Singh M, Goel S, Meeley RB, Dantec C, Parrinello H, Michaud C, Leblanc O, Grimanelli
548 D. 2011. Production of viable gametes without meiosis in maize deficient for an
549 ARGONAUTE protein. *Plant Cell* [Internet] 23:443–458. Available from:

550 <http://www.ncbi.nlm.nih.gov/pubmed/21325139>
551 <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3077773/pdf/443.pdf>

552 Smith LM, Pontes O, Searle I, Yelina N, Yousafzai FK, Herr AJ, Pikaard CS, Baulcombe
553 DC. 2007. An SNF2 Protein Associated with Nuclear RNA Silencing and the
554 Spread of a Silencing Signal between Cells in Arabidopsis. *Plant Cell* [Internet]
555 19:1507–1521. Available from:
556 <http://www.plantcell.org/cgi/doi/10.1105/tpc.107.051540>

557 Stamatakis A, Hoover P, Rougemont J. 2008. A rapid bootstrap algorithm for the
558 RAxML web servers. *Syst. Biol.* 57:758–771.

559 Stonaker JL, Lim JP, Erhard KF, Hollick JB. 2009. Diversity of Pol IV function is defined
560 by mutations at the maize *rmr7* locus. *PLoS Genet.* 5.

561 Trujillo JT, Beilstein MA, Mosher RA. 2016. The Argonaute-binding platform of NRPE1
562 evolves through modulation of intrinsically disordered repeats. *New Phytol.*
563 [Internet]. Available from: <http://doi.wiley.com/10.1111/nph.14089>

564 Tucker SL, Reece J, Ream TS, Pikaard CS. 2010. Evolutionary History of Plant
565 Multisubunit RNA Polymerases IV and V: Subunit Origins via Genome-Wide and
566 Segmental Gene Duplications, Retrotransposition, and Lineage-Specific
567 Subfunctionalization. *Cold Spring Harb. Symp. Quant. Biol.* [Internet] 75:285–297.
568 Available from: <http://symposium.cshlp.org/cgi/doi/10.1101/sqb.2010.75.037>

569 Wendte JM, Haag JR, Singh J, McKinlay A, Pontes OM, Pikaard CS. 2017. Functional
570 Dissection of the Pol V Largest Subunit CTD in RNA-Directed DNA Methylation.
571 *Cell Rep.* [Internet] 19:2796–2808. Available from:
572 <http://dx.doi.org/10.1016/j.celrep.2017.05.091>

- 573 Wierzbicki AT, Haag JR, Pikaard CS. 2008. Noncoding transcription by RNA
574 polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and
575 adjacent genes. *Cell* [Internet] 135:635–648. Available from:
576 [http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2602798&tool=pmcentre](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2602798&tool=pmcentrez&rendertype=abstract)
577 [z&rendertype=abstract](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2602798&tool=pmcentrez&rendertype=abstract)
- 578 Wierzbicki AT, Ream TS, Haag JR, Pikaard CS. 2009. RNA polymerase V transcription
579 guides ARGONAUTE4 to chromatin. *Nat. Genet.* [Internet] 41:630–634. Available
580 from:
581 <http://www.ncbi.nlm.nih.gov/pubmed/19377477>
582 <http://www.ncbi.nlm.nih.gov/pubmed/19377477>
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2674513>
- 583 Wu L, Zhou H, Zhang Q, Zhang J, Ni F, Liu C, Qi Y. 2010. DNA Methylation Mediated
584 by a MicroRNA Pathway. *Mol. Cell* [Internet] 38:465–475. Available from:
585 <http://dx.doi.org/10.1016/j.molcel.2010.03.008>
- 586 Yang Z. 2007. PAML 4: Phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.*
587 24:1586–1591.
- 588 Zhai J, Bischof S, Wang H, Feng S, Lee T, Teng C, Chen X, Park SY, Liu L, Gallego-
589 Bartolome J, et al. 2015. A One Precursor One siRNA Model for Pol IV-Dependent
590 siRNA Biogenesis. *Cell* [Internet] 163:445–455. Available from:
591 <http://www.sciencedirect.com/science/article/pii/S0092867415011940>
- 592 Zhai J, Zhang H, Arikiti S, Huang K, Nan G-L, Walbot V, Meyers BC. 2015.
593 Spatiotemporally dynamic, cell-type-dependent premeiotic and meiotic phasiRNAs
594 in maize anthers. *Proc. Natl. Acad. Sci.* [Internet] 112:3146–3151. Available from:
595 <http://www.pnas.org/lookup/doi/10.1073/pnas.1418918112>

- 596 Zhang H, Xia R, Meyers BC, Walbot V. 2015. Evolution, functions, and mysteries of
597 plant ARGONAUTE proteins. *Curr. Opin. Plant Biol.* [Internet] 27:84–90. Available
598 from: <http://dx.doi.org/10.1016/j.pbi.2015.06.011>
- 599 Zhang J, Nielsen R, Yang Z. 2005. Evaluation of an improved branch-site likelihood
600 method for detecting positive selection at the molecular level. *Mol. Biol. Evol.*
601 22:2472–2479.
- 602 Zhong X, Hale CJ, Law JA, Johnson LM, Feng S, Tu A, Jacobsen SE. 2012. DDR
603 complex facilitates global association of RNA polymerase V to promoters and
604 evolutionarily young transposons. *Nat. Struct. Mol. Biol.* [Internet] 19:870–875.
605 Available from: <http://www.nature.com/articles/nsmb.2354>
- 606 Zhou M, Law JA. 2015. RNA Pol IV and V in gene silencing: Rebel polymerases
607 evolving away from Pol II's rules. *Curr. Opin. Plant Biol.* [Internet] 27:154–164.
608 Available from: <http://dx.doi.org/10.1016/j.pbi.2015.07.005>
609