

1 **Paternal easiRNAs regulate parental genome dosage in Arabidopsis**

2

3 German Martinez^{1‡}, Philip Wolff^{1‡‡}, Zhenxing Wang¹, Jordi Moreno-Romero¹, Juan
4 Santos-González¹, Lei Liu Conze¹, Christopher DeFraia², Keith Slotkin², Claudia
5 Köhler^{1*}

6 ¹Department of Plant Biology, Uppsala BioCenter, Swedish University of Agricultural
7 Sciences and Linnean Center for Plant Biology, Uppsala, Sweden

8 †Present address: John Innes Centre, Colney Lane, Norwich, NR4 7UH, UK

9 ²Department of Molecular Genetics and Center for RNA Biology, The Ohio State
10 University, 500 Aronoff Laboratory, 318 West 12th Avenue, Columbus, Ohio 43210,
11 USA

12 ‡These authors contributed equally to this work

13 * For correspondence: claudia.kohler@slu.se

14

15

16 **The regulation of parental genome dosage is of fundamental importance in**
17 **animals and plants, exemplified by X chromosome inactivation and dosage**
18 **compensation. The "triploid block" is a classical example of dosage regulation**
19 **in plants that establishes a reproductive barrier between species differing in**
20 **chromosome number ^{1,2}. This barrier acts in the endosperm, an ephemeral**
21 **tissue that nurtures the developing embryo and induces the abortion of hybrid**
22 **seeds through a yet unknown mechanism. Interploidy hybridizations involving**
23 **diploid (2x) maternal parents and tetraploid (4x) pollen donors cause failure in**
24 **endosperm cellularization, leading to embryo arrest ³. Here we show that**
25 **paternal epigenetically activated small interfering RNAs (easiRNAs) are**
26 **responsible for the establishment of the triploid block-associated seed**
27 **abortion in *Arabidopsis thaliana*. Paternal loss of the plant-specific RNA**
28 **polymerase IV suppressed easiRNA formation and rescued triploid seeds by**
29 **restoring small RNA-directed DNA methylation at transposable elements (TEs),**
30 **correlating with reduced expression of paternally expressed imprinted genes**
31 **(PEGs). We propose that excess of paternally derived easiRNAs in diploid**
32 **pollen prevents establishment of DNA methylation, leading to triploid seed**
33 **abortion. Our data further suggest that easiRNAs form a quantitative signal for**
34 **chromosome number and their balanced dosage is required for post-**
35 **fertilization genome stability and seed viability.**

36

37 Previous work indicated that 24-nt small interfering RNAs (siRNAs) are sensitive to
38 genome dosage and that their level is strongly reduced in response to interploidy
39 hybridizations of 2x maternal parents and 4x pollen donors ⁴. To understand the
40 genetic basis of this phenomenon, we tested the effect of several mutants impaired in

41 the biogenesis of 21/22-nt and 24-nt siRNAs for their effect on establishing the
42 triploid block. We generated double mutants with the *omission of second division*
43 (*osd1*) mutant that forms unreduced (2n) male gametes⁵. Thus, crossing *osd1* as
44 pollen donor to wild-type (wt) plants results in triploid (3x) seed formation at almost
45 100% frequency⁵, with the majority of 3x seeds being collapsed and non-viable⁶
46 (Fig. 1a). Strikingly, using the double mutant *nrpd1a osd1* as pollen donor, viability of
47 3x seeds derived from those crosses was largely restored, with more than half of
48 those seeds being non-collapsed and able to germinate (Fig. 1a,b). *NRPD1a*
49 encodes for the largest subunit of RNA polymerase IV (Pol IV), a plant specific
50 polymerase that transcribes heterochromatic regions of the Arabidopsis genome and
51 leads to the production of 24 nt sRNAs that mediate DNA methylation^{7,8}. The effect
52 of *NRPD1a* on triploid seed rescue was exclusively paternal, there was no significant
53 rescue of triploid seed viability when *nrpd1a* plants were used as maternal parents in
54 crosses with *osd1* and *nrpd1a osd1* (Fig. 1c). Triploid seed rescue by *nrpd1a* pollen
55 was associated with restored endosperm cellularization (Fig. 1d), similar as observed
56 for other mutants able to rescue triploid seed abortion^{6,9}. Together with *nrpd1a*,
57 mutants in *DCL4*, *RDR6*, *AGO2*, and *AGO6* also significantly increased viable seed
58 formation by a minimum of two-fold over background level, while no significant effect
59 was observed for mutants in *DCL3*, *RDR2*, and *Pol V* (Fig. 1a). *DCL2*, *DCL4*, *RDR6*,
60 *AGO1*, *AGO2*, and *AGO6* are required for the production and function of 21/22nt
61 easiRNAs targeting transcriptionally active TEs¹⁰⁻¹⁴. In pollen, easiRNAs are
62 produced in the vegetative cell and loaded into sperm cells^{14,15}; nevertheless, how
63 they are produced and their downstream response remained to be identified. Given
64 the strong effect of *nrpd1a* on triploid seed rescue, we addressed the question
65 whether Pol IV and not Pol II could influence the production of 21/22-nt easiRNAs in
66 pollen. We therefore sequenced sRNAs of pollen grains derived from 2x and 4x Col

67 and *nripd1a* mutant plants (1n and 2n Col and *nripd1a* pollen, respectively, Fig. S1,
68 Table S1). Pollen derived from 2x and 4x *nripd1a* plants had not only strongly
69 reduced levels of TE-derived 24-nt siRNAs, but as well strongly reduced levels of 21-
70 nt and 22-nt siRNAs, pinpointing an undiscovered new role of Pol IV in the
71 biogenesis of these sRNA classes in the male germline in plants (Fig. 2a, b). Analysis
72 of the bias between sRNAs derived from the plus or minus strand of TEs indicated
73 that indeed Pol IV is the major producer of dsRNA precursors for TE-derived sRNA
74 biogenesis in pollen (Fig 2c). This effect is moreover exclusive to TEs and not to
75 other epigenetically-regulated regions, like rRNA genes (Fig. 2d). However, in
76 contrast to the dependency of Pol IV RNA biogenesis on RDR2 in somatic plant
77 tissues^{16,17}, our genetic data do not support an exclusive role of RDR2 in this
78 process in pollen, but rather implicate a role for RDR6 and possible redundancies
79 with other RDRs, similar as suggested for diRNA synthesis¹⁸. Consistently,
80 production of 21/22-nt siRNA is reduced in *rdr6* and *rdr6 dcl4* double mutants (Fig.
81 S2), supporting a role of DCL4 and RDR6 in the production of 21/22-nt siRNAs¹⁹⁻²¹.
82 Thus, our data revealed a new Pol IV-dependent pathway leading to the production
83 of 21/22-nt and 24-nt siRNAs from TEs in pollen that potentially avoids the risk of
84 forming potentially harmful full-length TE transcripts.

85 We hypothesized that enlarged pollen grains of higher ploidy (Fig. S3) could
86 potentially have an excess of 21/22-nt easiRNAs, building the triploid block. To test
87 this, we purified RNA of 1n and 2n wt and *nripd1a* mutant pollen samples normalized
88 for an approximately equal number of pollen grains (Fig. 2e)¹² and ribosomal RNA in
89 2n pollen RNA samples of wt and *nripd1a* mutant plants compared to RNA samples of
90 1n pollen (Fig. 2f), strongly suggesting that there is a general increase of RNA in 2n
91 pollen compared to 1n pollen.

92 Interploidy hybridizations using *osd1* pollen donors cause strong reduction of CHH
93 methylation (H corresponds to A, T, or C)²². Based on the previously proposed
94 antagonistic relationship between post-transcriptional gene silencing (PTGS)
95 mediated by 21/22-nt siRNAs and RNA-dependent DNA methylation (RdDM)¹², we
96 raised the hypothesis that increased dosage of pollen-derived easiRNAs in 2n pollen
97 negatively interferes with CHH methylation establishment in the endosperm. We
98 tested this hypothesis by analyzing whether depletion of 21/22-nt easiRNAs from
99 pollen restores CHH methylation in the endosperm. We analyzed DNA methylation in
100 purified endosperm of 2x and 3x seeds derived from pollinations of *Ler* maternal
101 plants with wt *Col* or *nripd1a* 1n or 2n pollen (referred to as 2x or 3x *nripd1a* seeds,
102 respectively, Fig. S4, Table S2). Consistent with previous work²², CHH methylation
103 at TEs was strongly reduced in the endosperm of 3x seeds and slightly increased in
104 3x *nripd1a* seeds (Fig. 3a), indicating that loss of paternal PolIV function restores
105 CHH methylation at defined TEs. About one quarter of TEs losing CHH methylation in
106 3x seeds restored CHH methylation in 3x *nripd1a* seeds (Table S3, Fig. 3b, c).
107 Strikingly, loci that experienced the strongest loss of CHH methylation in 3x seeds,
108 had the highest gain of CHH methylation in 3x *nripd1a* seeds (Fig. 3d), suggesting
109 that easiRNAs negatively interfere with CHH methylation when present in increased
110 dosage. Consistently, levels of 21/22-nt easiRNAs in pollen correlated with loss of
111 CHH methylation in 3x seeds (Fig. 3e; Fig. S5a), suggesting that their increased
112 dosage in 2n pollen causes the negative effect on CHH methylation establishment in
113 3x seeds. Loss of CHH methylation was PolIV dependent and correlated with loss of
114 21/22-nt easiRNAs in *nripd1a* pollen (Fig. 3f, Fig. S5b) rather than with the presence
115 of 24 nt siRNAs in 2n pollen (Fig 3h). Conversely, gain of CHH methylation in 3x
116 *nripd1a* seeds correlated with loss of 21/22-nt easiRNAs in pollen (Fig. 3g, Fig. S5c)
117 and was not associated with reduced expression of *NRPD1a* in the endosperm (Fig.

118 S6) Together, our data strongly suggest that pollen-derived 21/22-nt easiRNAs cause
119 loss of CHH methylation in 3x seeds after fertilization.

120 We further challenged the hypothesis that 2n pollen contributes an increased dosage
121 of 21/22-nt easiRNAs by testing whether increased levels of 21/22-nt siRNAs in 3x
122 seeds correlate with loss of 21/22-nt siRNAs in 3x *nripd1a* seeds (Fig. S7, Table S4).
123 Loci with highest levels of 21/22-nt siRNAs in 3x seeds experienced the strongest
124 loss of 21/22-nt siRNAs in 3x *nripd1a* seeds (Fig. 3i), strongly supporting the idea that
125 2n pollen contributes an increased dosage of Pol IV-dependent easiRNAs that
126 negatively interfere with CHH methylation in 3x seeds. Consistently, increased
127 abundance of 21/22-nt siRNAs in 3x seeds correlated with increased loss of CHH
128 methylation (Fig. 3j). Gain of 21/22-nt siRNAs in 3x seeds was associated with loss of
129 24-nt siRNAs (Fig. 3k), consistent with the loss of CHH methylation observed at loci
130 targeted by 21/22-nt easiRNAs (Fig. 3j).

131 To address the question whether increased abundance of Pol IV-dependent
132 easiRNAs in pollen associate with increased gene expression in the endosperm of 3x
133 seeds, we generated transcriptome data of purified endosperm (Table S5). Increased
134 abundance of Pol IV-dependent easiRNAs in pollen was associated with upregulated
135 gene expression in the endosperm of 3x seeds (Fig. 4a), supporting the idea that
136 increased abundance of easiRNAs negatively interferes with gene repression.
137 Consistently, depletion of pollen-derived easiRNAs normalized gene expression in
138 the endosperm of 3x seeds (Fig. 4b). Out of 57 PEGs with significantly increased
139 expression in 3x seeds, 19 PEGs were at least 2-fold downregulated in 3x *nripd1a*
140 seeds (Fig. 4c^{6,9}). Those 19 PEGs accumulated Pol IV-dependent easiRNAs at
141 flanking regions (Fig. 4d), correlating with reduced CHH methylation in 3x seeds and
142 partially restored CHH methylation in 3x *nripd1a* seeds (Fig. 4e,f). Together we

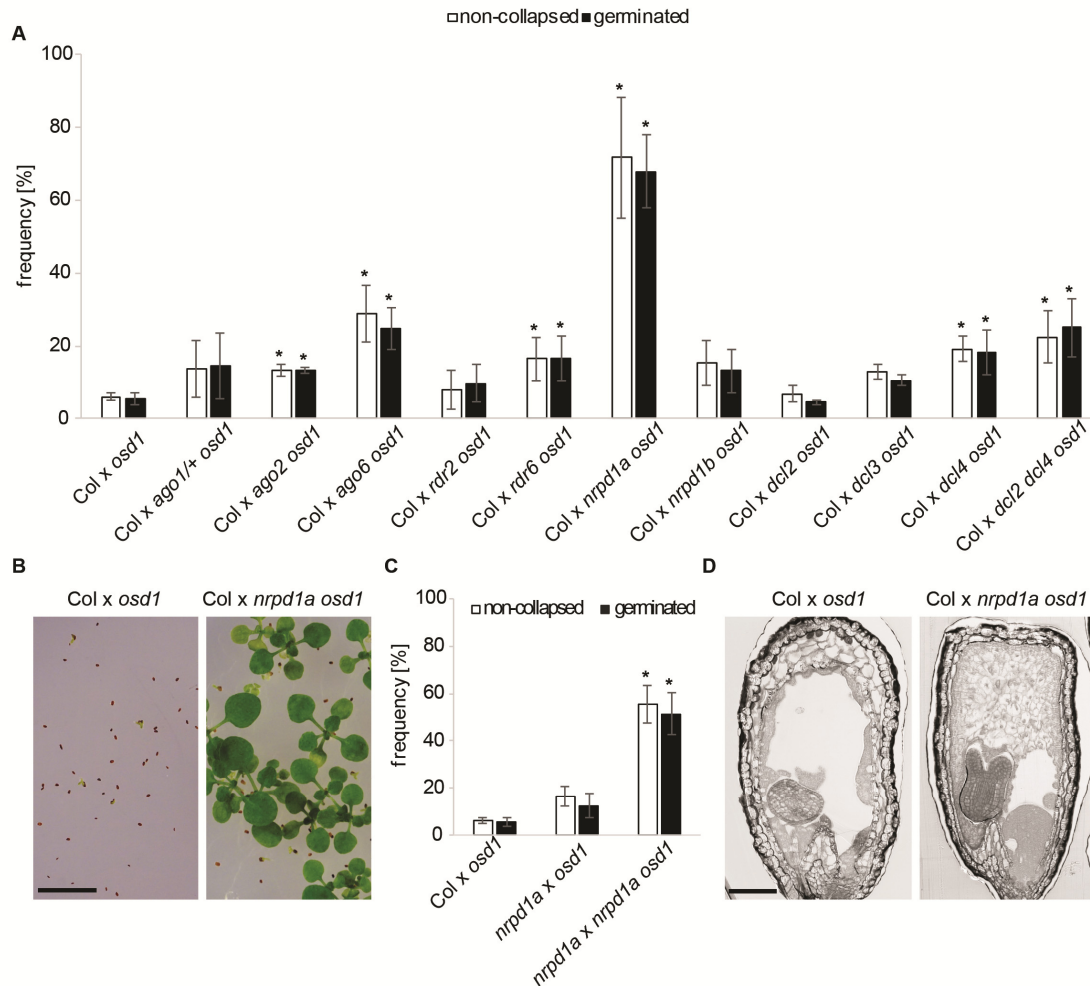
143 conclude that epigenetic changes at TEs are connected to changes of PEG
144 expression.

145 Accordingly, we propose that Pol IV-dependent production of TE transcripts in
146 gametic tissues is a genomic safeguard mechanism that sustains production of
147 21/22-nt easiRNAs of potentially harmful active TEs without Pol II transcription. Our
148 data reveal a striking analogy of easiRNAs in establishing the triploid block with Piwi-
149 interacting RNAs (piRNAs) in hybrid dysgenesis in flies ²³. In both models, TE-
150 derived sRNAs transmit epigenetic information transgenerationally, pointing to a
151 conserved role of TE-derived sRNAs in assessing gamete compatibility. Pollen-
152 delivered 21/22-nt easiRNAs could possibly target transcriptionally active TEs for
153 degradation in the hypomethylated endosperm and thus prevent transposition ²⁴.

154 When different species hybridize, hybrid failure will result if pollen 21/22-nt easiRNAs
155 do not recognize their maternal target TEs or, conversely, if paternal TEs are too
156 sequence divergent to be recognized by maternal 24-nt siRNAs. Importantly, our data
157 show that also inadequate dosage of parental siRNA populations can cause hybrid
158 seed failure, revealing that the balanced dosage of maternal and paternal siRNA
159 populations is essential for viable seed formation (Fig. 4e). One possible scenario is
160 that increased dosage of 21/22-nt easiRNAs causes these siRNAs to target
161 scaffolding transcripts produced by either Pol II or Pol V and thus negatively interfere
162 with DNA methylation establishment, as previously proposed ¹².

163 The triploid block has been a mystery to geneticists and breeders, formalized as the
164 "endosperm balance number" hypothesis more than 35 years ago ¹. Our discovery
165 that paternal easiRNAs form the genetic basis of dosage sensitivity and establish the
166 triploid block provides the means for targeted strategies bypassing this hybridization
167 barrier.

Figure 1



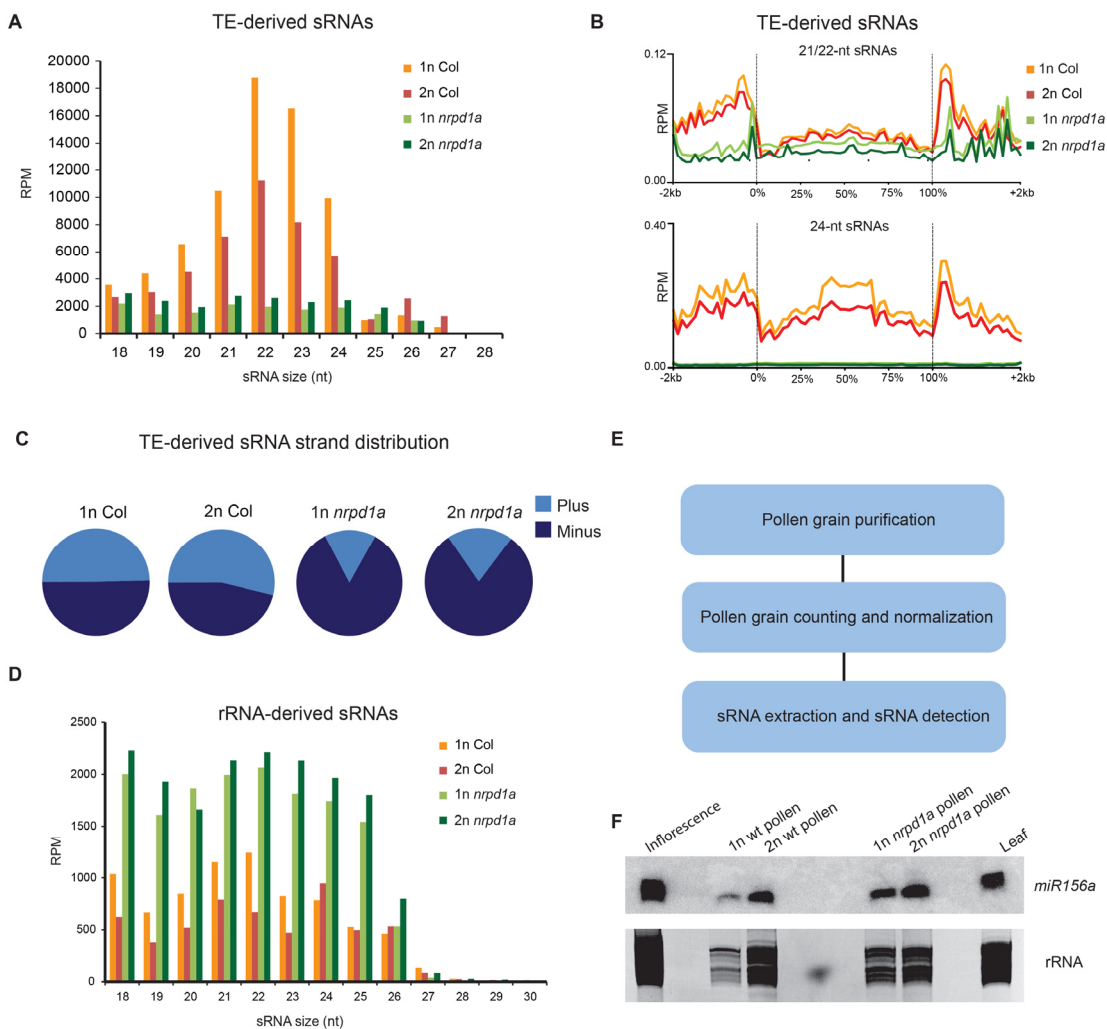
168

169 **Fig. 1. Pol IV mutant pollen is able to rescue seed abortion in triploid seeds.** (A) Frequency of
 170 non-collapsed and germinated seeds derived from crosses of wild-type (Col) maternal parents with
 171 *osd1* and *osd1* double mutants of indicated genotypes. Asterisks mark significant differences ($P < 0.05$)
 172 to the cross Col x *osd1* (Chi square test with Bonferroni correction). By convention, the female parent
 173 is always indicated first. (B) Pictures of non-germinating seeds (left panel) and seedlings (right panel)
 174 derived from the crosses Col x *osd1* and Col x *nrpd1a osd1*, respectively. Scale bar, 1 cm. (C)
 175 Analysis of the parent-of-origin effect of *nrpd1a* on triploid seed rescue. Asterisks mark significant
 176 differences ($P < 0.05$) to the cross Col x *osd1* (Chi square test with Bonferroni correction). (D) Sections
 177 of 6 DAP seeds derived from Col x *osd1* and Col x *nrpd1a osd1* crosses. Scale bar, 0.1 mm.

178

179

Figure 2



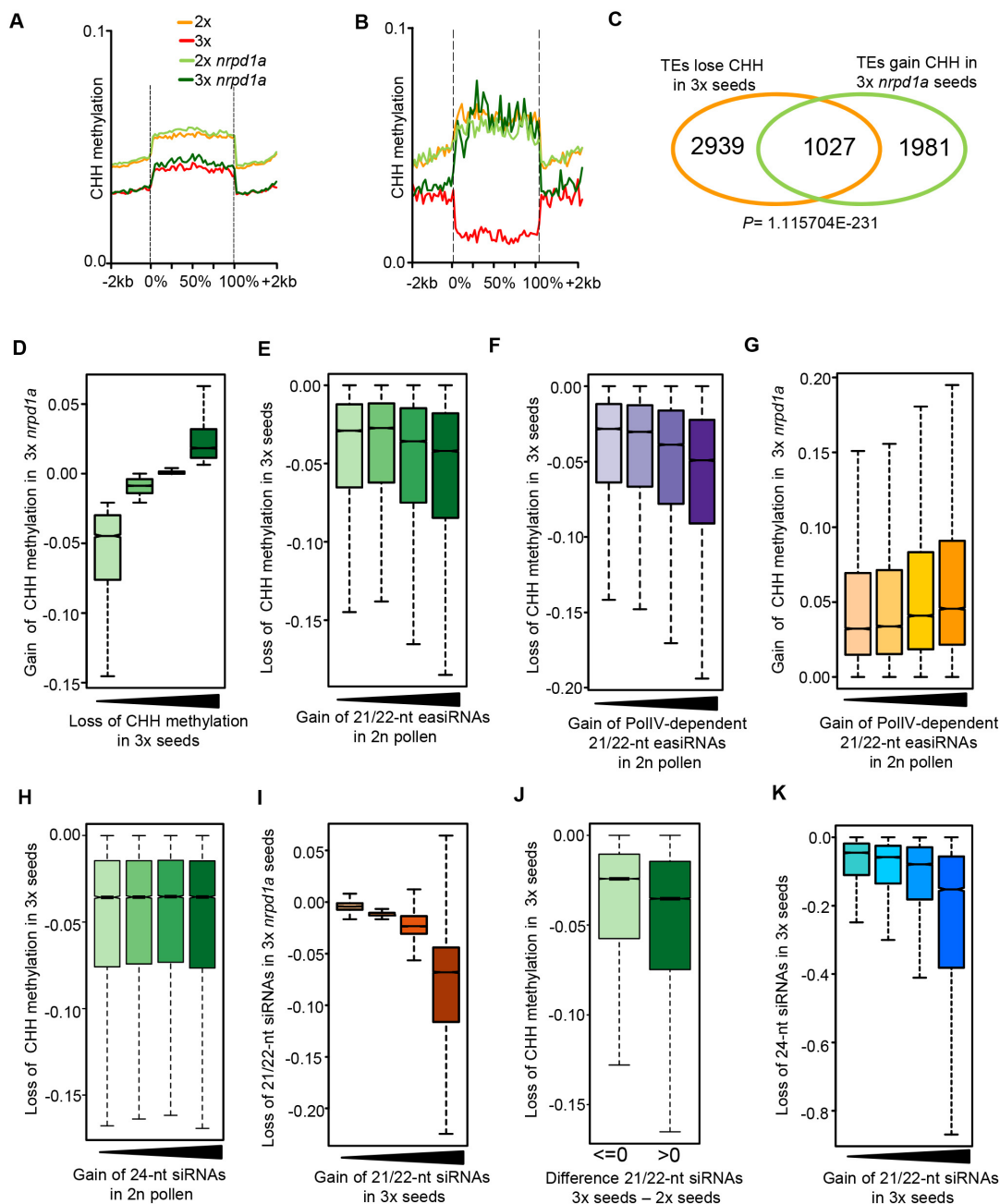
180

181 **Fig. 2. Pol IV is responsible for the biogenesis of pollen TE-derived sRNA in the size range of**
 182 **19-24 nt.** (A) sRNA profiles of TE-derived sRNAs of 1n pollen from Col wild-type (wt) and *nrpd1a*
 183 mutants and 2n pollen from tetraploid wt and *nrpd1a* mutants. (B) Metaplots of siRNA distribution over
 184 TE gene sequences in 1n and 2n pollen of Col wt and *nrpd1a* mutants. (C) sRNA strand origin
 185 percentage for TE-derived sRNAs in 1n and 2n pollen from Col wt and *nrpd1a* mutants. (D) sRNA
 186 profiles of ribosomal RNA (rRNA)-derived sRNAs of 1n and 2n pollen from Col wt and *nrpd1a* mutants.
 187 (E) Flowchart of steps for sRNA isolation used in the Northern blot shown in panel F. (F) Northern blot
 188 detection of *miR156a* in 1n and 2n pollen from Col wt and *nrpd1a* mutants. RNA samples were
 189 normalized to total amount of pollen grains. rRNA loading control from EtBr stained gel is shown in the
 190 lower panel.

191

192

Figure 3



193

194 **Fig. 3. Triploid seeds have lower CHH methylation as a consequence of increased pollen-**
 195 **derived 21/22-nt easiRNAs.** (A) CHH methylation metaplots over all TEs in the endosperm derived
 196 from 2x, 3x, 2x *nrpd1a*, and 3x *nrpd1a* seeds. (B) CHH methylation metaplots over TEs that lose CHH
 197 methylation in the endosperm of 3x seeds and restore CHH methylation in 3x *nrpd1a* seeds.
 198 Color code as specified in A. (C) Venn-diagram showing overlap of TEs specified in B.
 199 Hypergeometric testing was used to test for significance of overlap. (D-F) Loss of CHH methylation
 200 in the endosperm of 3x seeds associates with increasing levels of CHH methylation in 3x *nrpd1a* seeds
 201 (D), increasing levels of 21/22-nt easiRNAs in 2n pollen (E), increasing levels of POL IV-dependent

202 21/22-nt easiRNAs in 2n pollen. **(F)**. **(G)** Gain of CHH methylation in the endosperm of 3x *nripd1a*
203 seeds associates with increasing loss of 21/22-nt easiRNAs in 2n *nripd1a* pollen. **(H)** Increasing levels
204 of 24-nt easiRNAs in 2n pollen do not associate with CHH methylation differences in the endosperm of
205 3x seeds. **(I)** Increasing levels of 21/22-nt siRNAs in 3x seeds associate with increasing loss of 21/22-
206 nt siRNAs in 3x *nripd1a* seeds. **(J)** Increasing levels of 21/22-nt siRNAs in 3x seeds associate with loss
207 of CHH methylation in 3x seeds. Plotted are differences of CHH methylation in the endosperm of 3x
208 and 2x seeds at those 50 bps bins where differences in 21/22-nt siRNAs between 3x and 2x seeds are
209 smaller or larger than zero. **(K)** Increasing levels of 21/22-nt siRNAs in 3x versus 2x seeds associate
210 with increasing loss of 24-nt siRNAs in triploid seeds. **(D-I, K)** Plotted are CHH methylation or siRNA
211 differences sorted by quantiles of 50-bp genome bins against differences of CHH methylation or
212 siRNAs in the endosperm of indicated genotypes. Differences between first and last categories in **A-G**
213 and **I-K** are significant ($P < 0.00001$, Kolmogorov-Smirnov test).

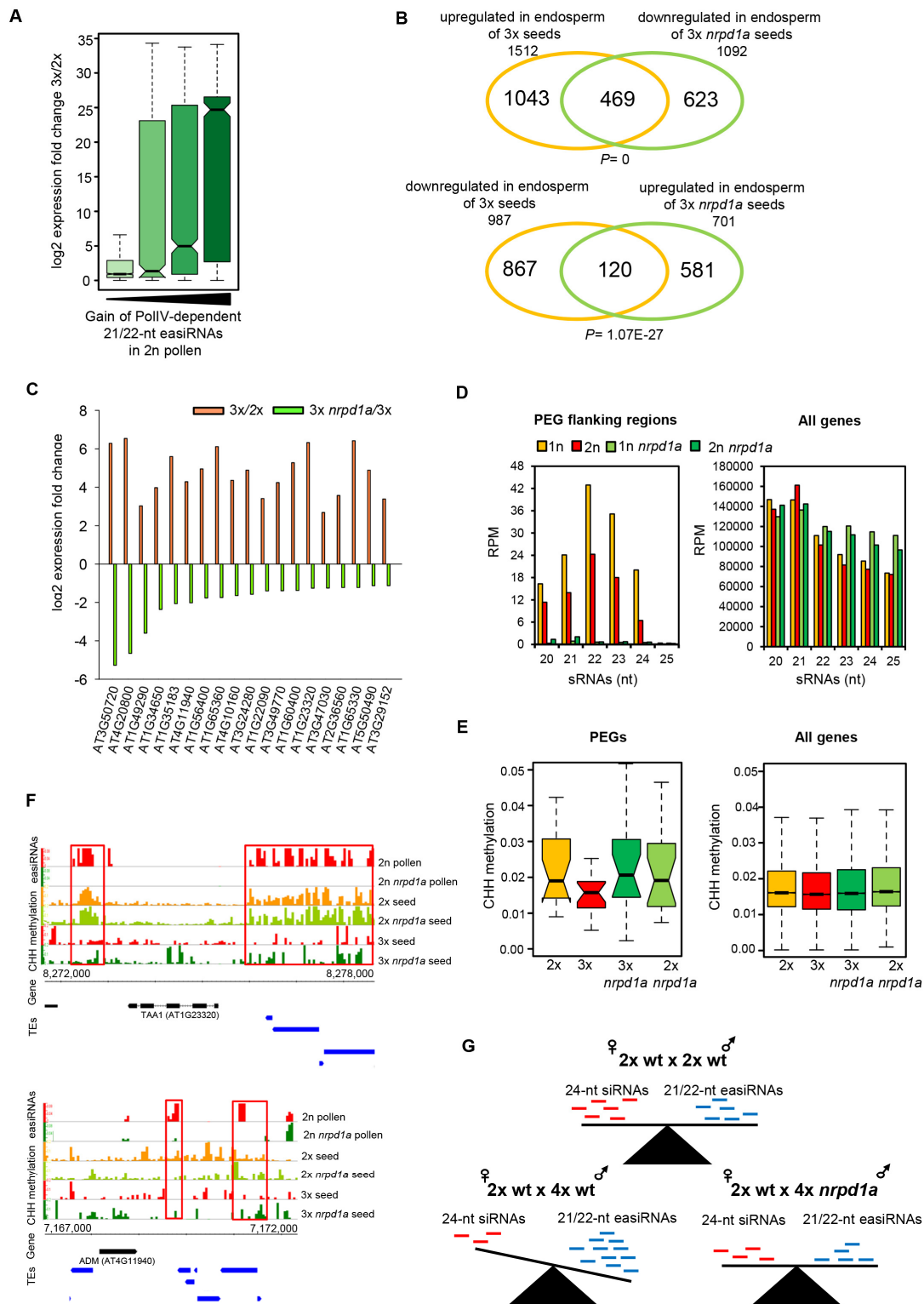
214

215

216

217

Figure 4



218

219 **Fig. 4. Pollen-derived 21/22-nt easiRNAs associate with gene expression changes in the**
 220 **endosperm of triploid seeds. (A) Increasing levels of 21/22-nt easiRNAs in 2n versus 2n *npd1a***

221 pollen associate with upregulated gene expression in the endosperm of 3x seeds. Plotted are
222 differences in 21/22-nt siRNAs in 2n and 2n *nripd1a* pollen against log₂-fold expression changes in the
223 endosperm of 3x and 2x seeds. Differences between categories are significant ($P < 0.00001$,
224 Kolmogorov-Smirnov test). **(B)** Venn diagram showing overlap of deregulated genes in in the
225 endosperm of 3x and 3x *nripd1a* seeds compared to the corresponding 2x and 3x controls,
226 respectively. Hypergeometric testing was used to test for significance of overlap. **(C)** Log₂-fold change
227 expression of PEGs in the endosperm of 3x and 3x *nripd1a* compared to compared to the
228 corresponding 2x and 3x controls, respectively. **(D)** sRNAs mapping to 2kb flanking regions of PEGs
229 (specified in C) and flanking regions of all genes (left and right panels, respectively) in 1n and 2n wild-
230 type and *nripd1a* pollen. **(E)** Boxplots of CHH methylation in 2kb flanking regions of PEGs (specified in
231 C) and all genes (left and right panels, respectively). **(F)** Representative PEGs accumulating 21/22-nt
232 easiRNAs in pollen and experiencing changes in CHH methylation in the endosperm of 3x and 3x
233 *nripd1a* seeds. Red rectangles mark regions where easiRNA accumulation co-occurs with changes of
234 CHH methylation. **(G)** Viable seed formation requires balanced populations of pollen-derived 21/22-nt
235 easiRNAs and ovule-derived 24-nt siRNAs. Increased levels of 21/22-nt easiRNAs delivered from a
236 higher ploidy pollen parent will reduce 24-nt siRNAs post-fertilization in the seed, resulting in unviable
237 seed formation. *NRPD1a* deficient pollen restores viable seed formation by reducing pollen-derived
238 easiRNAs.

239

240

241

242

243

244

245

246

247

248

249

250 **Methods:**

251 **Plant material and growth conditions**

252 The mutants used in this study were described previously as *ago1-36* (Salk_087076;
253 ²⁵, *ago2-1* (Salk_003380; ²⁶, *ago6-2* (Salk_031553; ²⁷, *rdr2-2* (Salk_059661; ²⁰, *rdr6-*
254 *15* (Salk_617H07; ²⁸, *nprpd1a-3* (Salk_128428; ²⁹, *nprpd1b-12* (Salk_033852; ³⁰, *dcl2-1*
255 (Salk_064627; ³¹, *dcl3-1* (Salk_005512; ³¹, *dcl4-2* ³¹. The *osd1-1* mutant ⁵ was kindly
256 provided by Raphael Mercier. Being originally identified in the Nossen background,
257 the mutant was introgressed into Col by repeated backcrosses over five generations.
258 Tetraploid *nprpd1a-3* plants were generated using colchicine treatment as previously
259 described ²². Plants were grown in a growth cabinet under long day photoperiods (16
260 hr light and 8 hr dark) at 22°C. After 10 days, seedlings were transferred to soil and
261 plants were grown in a growth chamber at 60% humidity and daily cycles of 16 hr
262 light at 22°C and 8 hr darkness at 18°C. For all crosses, designated female plants
263 were emasculated and the pistils were hand-pollinated 2 days after emasculation.

264 **Germination analysis**

265 Seeds were surface sterilized in a container using chlorine gas (10 ml hydrochloric
266 acid plus 50 ml sodium hypochlorite) and incubated for up to 3 hr. To determine
267 germination frequency, seeds were plated on ½ MS media containing 1% sucrose,
268 stratified at 4°C for 2 days in the dark and grown in a growth cabinet under long day
269 photoperiods (16 hr light and 8 hr dark) at 22°C for 10 days.

270 **Microscopy**

271 Seeds were fixed and embedded with Technovit 7100 (Heraeus, Germany) as
272 described ³². Five-micrometer sections were prepared with an HM 355 S microtome
273 (Microm, Germany) using glass knives. Sections were stained for 1 min with 0.1%

274 toluidine blue and washed three times with distilled water. Microscopy was
275 performed using a DMI 4000B microscope with DIC optics (Leica, Germany). Images
276 were captured using a DFC360 FX camera (Leica) and processed using Photoshop
277 CS5 (Adobe, San Jose, California).

278 **Pollen normalization and Northern blotting**

279 10 ul of total pollen extracts of different genotypes was counted under a microscope.
280 Based on the determined pollen density different volumes of the extracts were used
281 for subsequent downstream RNA extraction. Northern blotting was done as
282 previously described ¹⁰.

283 **RNA sequencing**

284 For RNA sequencing, endosperm from seeds derived from crosses *Ler* x *Col-0*, *Ler* x
285 *osd1*, *Ler* x *osd1 nrpd1a* and *Ler* x *nrpd1a* were dissected in biological duplicates
286 following previously described procedures ²². RNA was extracted following a modified
287 protocol for the RNAqueous kit (Ambion, Life Technologies). RNA was purified by
288 Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) after residual DNA was
289 removed by adding 2 uL DNaseI (Thermo-Scientific, Waltham, USA). Libraries were
290 prepared using the TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, USA) and
291 sequenced at the SciLife Laboratory (Uppsala, Sweden) on an Illumina HiSeq2000 in
292 50-bp single-end mode.

293 **Small RNA sequencing**

294 Pure mature pollen samples from inflorescences of approximately 500 plants were
295 collected as previously described ³³ with minor modifications. Flowers were
296 harvested in a beaker, covered with 9% sucrose solution and shaken vigorously for 5
297 min to release pollen grains into the solution. The subsequent centrifugation- and

298 filtering-steps were carried out in 9% sucrose solution. The obtained pollen pellet was
299 frozen in liquid nitrogen and stored at -70 °C. Total RNA was extracted using Trizol
300 reagent (Ambion/ Life Technologies, USA) and glass beads (1.25-1.55 mm; Carl
301 Roth) and 10 µg of total RNA were run on a 14% TBE UREA Polyacrylamide gel for
302 size selection. Gel slices containing RNA in the range of 17- to 25-nt were purified
303 following the Illumina TrueSeq small RNA protocol for gel extraction. Libraries were
304 constructed using the Illumina TruSeq Small RNA library preparation kit (RS-200-
305 0012) according to the manufacturer's instructions.

306 To generate sRNA libraries from seeds, we crossed male sterile *pistillata* maternal
307 plants (in *Ler* background ³⁴) with pollen from Col-0, 4x Col-0, *nrpd1a*, and 4x *nrpd1a*.
308 For each genotype 1000 seeds at 6-7 DAP were collected in duplicates in RNA later
309 (Sigma–Aldrich) and homogenized (Silamat S5) using glass beads. Total RNAs of
310 seeds were extracted using mirVana miRNA isolation kit (Ambion/Life Technologies)
311 and sRNAs were isolated by FDF-PAGE ³⁵. sRNA libraries were generated using the
312 NEBNext® Multiplex Small RNA Library Prep Set for Illumina. Libraries were
313 sequenced at the SciLife Laboratory (Uppsala, Sweden) on an Illumina HiSeq2000 in
314 50-bp single-end fashion.

315 **Bisulfite sequencing**

316 To generate bisulfite libraries we dissected endosperm from seeds derived from
317 crosses *Ler* x Col-0, *Ler* x *osd1*, *Ler* x *osd1 nrpd1a* and *Ler* x *nrpd1a* in biological
318 duplicates following previously described procedures ²². DNA purification and library
319 preparation were done as described in ³⁶. Quality of isolated endosperm was
320 calculated based on Col and *Ler* SNPs (Table S2).

321 **mRNA sequencing data analysis**

322 For each replicate, 50 bp long reads were mapped to the Arabidopsis (TAIR10)
323 genome, masked for rRNA genes, using TopHat v2.1.0 (Trapnell et al, 2009)
324 (parameters adjusted as -g 1 -a 10 -i 40 -l 5000 -F 0 -r 130). Gene and TE
325 expression was normalized to reads per kilobase per million mapped reads (RPKM)
326 using GFOLD³⁷. Expression level for each condition was calculating using the mean
327 of the expression values in both replicates. Differentially regulated genes and
328 transposable elements across the two replicates were detected using the rank
329 product method as implemented in the Bioconductor RankProd Package³⁸. The test
330 was run with 100 permutations and gene selection was corrected for multiple
331 comparison errors using a pfp (percentage of false prediction) < 0.05.

332 **Small RNA sequencing data analysis**

333 Adapters were removed from the 50 bp long single-end sRNA reads in each library.
334 The resulting 18-30 bp long reads were mapped to a Col genome (TAIR10) masked
335 for Ler SNPs using bowtie (-v 2 -best). All reads mapping to chloroplast and
336 mitochondria and to structural noncoding RNAs (tRNAs, snRNAs, rRNAs, or
337 snoRNAs) were removed. Mapped reads from both replicates were pooled together,
338 sorted in two categories (21-22-nt and 24-nt long) and remapped to the same
339 reference masked genome mentioned above using ShorStack (--mismatches 2 --
340 mmap f)³⁹ in order to improve the localization of sRNAs mapping to multiple genomic
341 locations. We normalized the alignments by converting coverage values to reads per
342 million mapped reads (RPM) values. The sRNA mapping profiles were visualized with
343 bedGraph files based in 50 bp bins. Metagene plots over TEs were constructed
344 between -2 kb and + 2 kb by calculating mean levels of sRNA (RPM) in 100 bp bins
345 in the flanks of the TEs and in 40 equally long bins between the transcriptional start
346 and stop.

347 **DNA methylation analysis**

348 For each mutant, the 125 bp reads from the Illumina BS sequence libraries from the
349 two biological replicates were merged and trimmed to 100 bp by cutting 5 bp from the
350 start and 20 bp from the end of each read. After this first trimming, each read was
351 split in two 50 bp long reads in order to improve mapping efficiency.

352 Reads were mapped to the TAIR10 Arabidopsis genome using the Bismark read
353 mapper⁴⁰ allowing up to one mismatch per read. Duplicated reads (aligning to the
354 same genomic position) were eliminated before calculating methylation levels.
355 Methylation levels for each condition were calculated as the mean of the two
356 replicates. Cytosine conversion efficiency was estimated as the percentage of CHH
357 methylation in the chloroplast. Cytosine methylation was visualized separately for
358 CG, CHG and CHH cytosine contexts with bedGraph files representing average
359 methylation values in 50 bp bins across the genome. Differential analysis on the
360 levels of CHH methylation in TEs between conditions considered the cytosine
361 methylation reports from both replicates and was performed using linear modelling
362 (p -value < 0.05) as implemented in the R package Limma⁴¹.

363 **References:**

- 364 1. Johnston, S., Nijs, T., Peloquin, S. & Hanneman, R. The significance of genic
365 balance to endosperm development in interspecific crosses. *Theor. Appl.*
366 *Genetics* **57**, 5-9 (1980).
- 367 2. Schatlowski, N. & Köhler, C. Tearing down barriers: understanding the
368 molecular mechanisms of interploidy hybridizations. *J Exp Bot* **63**, 6059-67
369 (2012).
- 370 3. Scott, R.J., Spielman, M., Bailey, J. & Dickinson, H.G. Parent-of-origin effects
371 on seed development in *Arabidopsis thaliana*. *Development* **125**, 3329-3341
372 (1998).
- 373 4. Lu, J., Zhang, C., Baulcombe, D.C. & Chen, Z.J. Maternal siRNAs as
374 regulators of parental genome imbalance and gene expression in endosperm
375 of *Arabidopsis* seeds. *Proc Natl Acad Sci U S A* **109**, 5529-5534 (2012).

- 376 5. d'Erfurth, I. et al. Turning meiosis into mitosis. *PLoS Biol* **7**, e1000124 (2009).
- 377 6. Kradofer, D., Wolff, P., Jiang, H., Siretskiy, A. & Köhler, C. An imprinted gene
378 underlies postzygotic reproductive isolation in *Arabidopsis thaliana*. *Dev Cell*
379 **26**, 525-535 (2013).
- 380 7. Law, J.A. & Jacobsen, S.E. Establishing, maintaining and modifying DNA
381 methylation patterns in plants and animals. *Nat Rev Genet* **11**, 204-220
382 (2010).
- 383 8. Havecker, E.R. et al. The *Arabidopsis* RNA-directed DNA methylation
384 argonautes functionally diverge based on their expression and interaction with
385 target loci. *Plant Cell* **22**, 321-334 (2010).
- 386 9. Wolff, P., Jiang, H., Wang, G., Santos-Gonzalez, J. & Kohler, C. Paternally
387 expressed imprinted genes establish postzygotic hybridization barriers in
388 *Arabidopsis thaliana*. *Elife* **4**, doi: 10.7554/eLife.1 (2015).
- 389 10. McCue, A.D., Nuthikattu, S., Reeder, S.H. & Slotkin, R.K. Gene expression
390 and stress response mediated by the epigenetic regulation of a transposable
391 element small RNA. *PLoS Genet* **8**, e1002474 (2012).
- 392 11. Nuthikattu, S. et al. The initiation of epigenetic silencing of active transposable
393 elements is triggered by RDR6 and 21-22 nucleotide small interfering RNAs.
394 *Plant Physiol* **162**, 116-131 (2013).
- 395 12. Creasey, K.M. et al. miRNAs trigger widespread epigenetically activated
396 siRNAs from transposons in *Arabidopsis*. *Nature* **508**, 411-415 (2014).
- 397 13. McCue, A.D. et al. ARGONAUTE 6 bridges transposable element mRNA-
398 derived siRNAs to the establishment of DNA methylation. *EMBO J* **34**, 20-35
399 (2015).
- 400 14. Martinez, G., Panda, K., Köhler, C. & Slotkin, R.K. Silencing in sperm cells is
401 directed by RNA movement from the surrounding nurse cell. *Nature Plants* **2**,
402 16030 (2016).
- 403 15. Slotkin, R.K. et al. Epigenetic reprogramming and small RNA silencing of
404 transposable elements in pollen. *Cell* **136**, 461-472 (2009).
- 405 16. Li, S. et al. Detection of Pol IV/RDR2-dependent transcripts at the genomic
406 scale in *Arabidopsis* reveals features and regulation of siRNA biogenesis.
407 *Genome Res* **25**, 235-245 (2015).
- 408 17. Zhai, J. et al. A One Precursor One siRNA Model for Pol IV-Dependent siRNA
409 Biogenesis. *Cell* **163**, 445-455 (2015).
- 410 18. Wei, W. et al. A role for small RNAs in DNA double-strand break repair. *Cell*
411 **149**, 101-112 (2012).
- 412 19. Gascioli, V., Mallory, A.C., Bartel, D.P. & Vaucheret, H. Partially redundant
413 functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in
414 producing trans-acting siRNAs. *Curr Biol* **15**, 1494-1500 (2005).

- 415 20. Vazquez, F. et al. Endogenous trans-acting siRNAs regulate the accumulation
416 of Arabidopsis mRNAs. *Mol Cell* **16**, 69-79 (2004).
- 417 21. Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H.L. & Poethig, R.S. SGS3
418 and SGS2/SDE1/RDR6 are required for juvenile development and the
419 production of trans-acting siRNAs in Arabidopsis. *Genes Dev* **18**, 2368-2379
420 (2004).
- 421 22. Schatlowski, N. et al. Hypomethylated pollen bypasses the interploidy
422 hybridization barrier in Arabidopsis. *Plant Cell* **26**, 3556-3568 (2014).
- 423 23. Brennecke, J. et al. An epigenetic role for maternally inherited piRNAs in
424 transposon silencing. *Science* **322**, 1387-1392 (2008).
- 425 24. Martienssen, R.A. Heterochromatin, small RNA and post-fertilization
426 dysgenesis in allopolyploid and interploid hybrids of Arabidopsis. *New Phytol*
427 **186**, 46-53 (2010).
- 428 25. Baumberger, N. & Baulcombe, D.C. Arabidopsis ARGONAUTE1 is an RNA
429 Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc*
430 *Natl Acad Sci U S A* **102**, 11928-11933 (2005).
- 431 26. Lobbes, D., Rallapalli, G., Schmidt, D.D., Martin, C. & Clarke, J. SERRATE: a
432 new player on the plant microRNA scene. *EMBO Rep* **7**, 1052-1058 (2006).
- 433 27. Zheng, X., Zhu, J., Kapoor, A. & Zhu, J.K. Role of Arabidopsis AGO6 in siRNA
434 accumulation, DNA methylation and transcriptional gene silencing. *EMBO J*
435 **26**, 1691-1701 (2007).
- 436 28. Allen, E. et al. Evolution of microRNA genes by inverted duplication of target
437 gene sequences in Arabidopsis thaliana. *Nat Genet* **36**, 1282-1290 (2004).
- 438 29. Herr, A.J., Jensen, M.B., Dalmay, T. & Baulcombe, D.C. RNA polymerase IV
439 directs silencing of endogenous DNA. *Science* **308**, 118-120 (2005).
- 440 30. Pontier, D. et al. Reinforcement of silencing at transposons and highly
441 repeated sequences requires the concerted action of two distinct RNA
442 polymerases IV in Arabidopsis. *Genes Dev* **19**, 2030-2040 (2005).
- 443 31. Xie, Z. et al. Genetic and functional diversification of small RNA pathways in
444 plants. *PLoS Biol* **2** (2004).
- 445 32. Hehenberger, E., Kradolfer, D. & Köhler, C. Endosperm cellularization defines
446 an important developmental transition for embryo development. *Development*
447 **139**, 2031-2039 (2012).
- 448 33. Schoft, V.K. et al. SYBR Green-activated sorting of Arabidopsis pollen nuclei
449 based on different DNA/RNA content. *Plant Reprod* **28**, 61-72 (2015).
- 450 34. Goto, K. & Meyerowitz, E.M. Function and regulation of the Arabidopsis floral
451 homeotic gene *pistillata*. *Genes Dev* **8**, 1548-1560 (1994).

- 452 35. Harris, C.J., Molnar, A., Muller, S.Y. & Baulcombe, D.C. FDF-PAGE: a
453 powerful technique revealing previously undetected small RNAs sequestered
454 by complementary transcripts. *Nucleic Acids Res* **43**, 7590-7599 (2015).
- 455 36. Moreno-Romero, J., Jiang, H., Santos-Gonzalez, J. & Kohler, C. Parental
456 epigenetic asymmetry of PRC2-mediated histone modifications in the
457 *Arabidopsis* endosperm. *EMBO J* **35**, 1298-1311 (2016).
- 458 37. Feng, J. et al. . GFOLD: a generalized fold change for ranking differentially
459 expressed genes from RNA-seq data. *Bioinformatics* **28**, 2782-2788 (2012).
- 460 38. Hong, F. et al. RankProd: a bioconductor package for detecting differentially
461 expressed genes in meta-analysis. *Bioinformatics* **22**, 2825-2827 (2006).
- 462 39. Johnson, N.R., Yeoh, J.M., Coruh, C. & Axtell, M.J. Improved Placement of
463 Multi-mapping Small RNAs. *G3 (Bethesda)* **6**, 2103-2111 (2016).
- 464 40. Krueger, F. & Andrews, S.R. Bismark: a flexible aligner and methylation caller
465 for Bisulfite-Seq applications. *Bioinformatics* **27**, 1571-1572 (2011).
- 466 41. Ritchie, M.E. et al. Limma powers differential expression analyses for RNA-
467 sequencing and microarray studies. *Nucleic Acids Res* **43**, e47 (2015).

468

469 **Extended data:**

470 Tables 1 to 5

471 Figs. 1 to 7

472 References (25–41)

473

474 **Acknowledgements:** We thank Rob Martienssen and Filipe Borges for critical
475 comments on the manuscript. This research was supported by a European Research
476 Council Starting Independent Researcher grant (to C.K.), a grant from the Swedish
477 Science Foundation (to C.K.) and a grant from the Knut and Alice Wallenberg
478 Foundation (to C.K.). G.M. was supported by a Marie Curie IOF Postdoctoral
479 Fellowship (PIOF-GA-2012-330069). Sequencing was performed by the SNP&SEQ
480 Technology Platform, Science for Life Laboratory at Uppsala University, a national

481 infrastructure supported by the Swedish Research Council (VRRFI) and the Knut and
482 Alice Wallenberg Foundation.

483 **Author Contributions:** GM, PW, RKS and CK designed the experiments. GM, PW,
484 ZW, J M-R, CDeF performed the experiments and generated the data. GM, JS-G and
485 LLC carried out the bioinformatic analysis. GM, PW, JS-G, LLC, RKS and CK
486 analyzed the data. GM, PW and CK wrote the manuscript.

487