Paternal easiRNAs regulate parental genome dosage in Arabidopsis

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

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

the biogenesis of 21/22-nt and 24-nt siRNAs for their effect on establishing the triploid block. We generated double mutants with the *omission of second division* (*osd1*) mutant that forms unreduced (2n) male gametes [5]. Thus, crossing *osd1* as pollen donor to wild-type (wt) plants results in triploid (3x) seed formation at almost 100% frequency [5], with the majority of 3x seeds being collapsed and non-viable [6] (Fig. 1a). Strikingly, using the double mutant *nrpd1a osd1* as pollen donor, viability of 3x seeds derived from those crosses was largely restored, with more than half of those seeds being non-collapsed and able to germinate (Fig. 1a,b). *NRPD1a* encodes for the largest subunit of RNA polymerase IV (Pol IV), a plant specific polymerase that transcribes heterochromatic regions of the Arabidopsis genome and leads to the production of 24 nt sRNAs that mediate DNA methylation [7,8]. The effect of *NRPD1a* on triploid seed rescue was exclusively paternal, there was no significant rescue of triploid seed viability when *nrpd1a* plants were used as maternal parents in crosses with *osd1* and *nrpd1a osd1* (Fig. 1c). Triploid seed rescue by *nrpd1a* pollen was associated with restored endosperm cellularization (Fig. 1d), similar as observed for other mutants able to rescue triploid seed abortion [6,9]. Together with *nrpd1a*, mutants in *DCL4*, *RDR6*, *AGO2*, and *AGO6* also significantly increased viable seed formation by a minimum of two-fold over background level, while no significant effect was observed for mutants in *DCL3*, *RDR2*, and *Pol V* (Fig. 1a). DCL2, DCL4, RDR6, AGO1, AGO2, and AGO6 are required for the production and function of 21/22nt easiRNAs targeting transcriptionally active TEs [10-14]. In pollen, easiRNAs are produced in the vegetative cell and loaded into sperm cells [14,15]; nevertheless, how they are produced and their downstream response remained to be identified. Given the strong effect of *nrpd1a* on triploid seed rescue, we addressed the question whether Pol IV and not Pol II could influence the production of 21/22-nt easiRNAs in pollen. We therefore sequenced sRNAs of pollen grains derived from 2x and 4x Col

and *nrpd1a* mutant plants (1n and 2n Col and *nrpd1a* pollen, respectively, Fig. S1, Table S1). Pollen derived from 2x and 4x *nrpd1a* plants had not only strongly reduced levels of TE-derived 24-nt siRNAs, but as well strongly reduced levels of 21-nt and 22-nt siRNAs, pinpointing an undiscovered new role of Pol IV in the biogenesis of these sRNA classes in the male germline in plants (Fig. 2a, b). Analysis of the bias between sRNAs derived from the plus or minus strand of TEs indicated that indeed Pol IV is the major producer of dsRNA precursors for TE-derived sRNA biogenesis in pollen (Fig 2c). This effect is moreover exclusive to TEs and not to other epigenetically-regulated regions, like rRNA genes (Fig. 2d). However, in contrast to the dependency of Pol IV RNA biogenesis on RDR2 in somatic plant tissues [16,17], our genetic data do not support an exclusive role of RDR2 in this process in pollen, but rather implicate a role for RDR6 and possible redundancies with other RDRs, similar as suggested for diRNA synthesis [18]. Consistently, production of 21/22-nt siRNA is reduced in *rdr6* and *rdr6 dcl4* double mutants (Fig. S2), supporting a role of DCL4 and RDR6 in the production of 21/22-nt siRNAs [19-21]. Thus, our data revealed a new Pol IV-dependent pathway leading to the production of 21/22-nt and 24-nt siRNAs from TEs in pollen that potentially avoids the risk of forming potentially harmful full-length TE transcripts.

We hypothesized that enlarged pollen grains of higher ploidy (Fig. S3) could potentially have an excess of 21/22-nt easiRNAs, building the triploid block. To test this, we purified RNA of 1n and 2n wt and *nrpd1a* mutant pollen samples normalized for an approximately equal number of pollen grains (Fig. 2e)[12] and ribosomal RNA in 2n pollen RNA samples of wt and *nrpd1a* mutant plants compared to RNA samples of 1n pollen (Fig. 2f), strongly suggesting that there is a general increase of RNA in 2n pollen compared to 1n pollen.

Interploidy hybridizations using *osd1* pollen donors cause strong reduction of CHH methylation (H corresponds to A, T, or C) [22]. Based on the previously proposed antagonistic relationship between post-transcriptional gene silencing (PTGS) mediated by 21/22-nt siRNAs and RNA-dependent DNA methylation (RdDM) [12], we raised the hypothesis that increased dosage of pollen-derived easiRNAs in 2n pollen negatively interferes with CHH methylation establishment in the endosperm. We tested this hypothesis by analyzing whether depletion of 21/22-nt easiRNAs from pollen restores CHH methylation in the endosperm. We analyzed DNA methylation in purified endosperm of 2x and 3x seeds derived from pollinations of L*er* maternal plants with wt Col or *nrpd1a* 1n or 2n pollen (referred to as 2x or 3x *nrpd1a* seeds, respectively, Fig. S4, Table S2). Consistent with previous work [22], CHH methylation at TEs was strongly reduced in the endosperm of 3x seeds and slightly increased in 3x *nrpd1a* seeds (Fig. 3a), indicating that loss of paternal PolIV function restores CHH methylation at defined TEs. About one quarter of TEs losing CHH methylation in 3x seeds restored CHH methylation in 3x *nrpd1a* seeds (Table S3, Fig. 3b, c). Strikingly, loci that experienced the strongest loss of CHH methylation in 3x seeds, had the highest gain of CHH methylation in 3x *nrpd1a* seeds (Fig. 3d), suggesting that easiRNAs negatively interfere with CHH methylation when present in increased dosage. Consistently, levels of 21/22-nt easiRNAs in pollen correlated with loss of CHH methylation in 3x seeds (Fig. 3e; Fig. S5a), suggesting that their increased dosage in 2n pollen causes the negative effect on CHH methylation establishment in 3x seeds. Loss of CHH methylation was PolIV dependent and correlated with loss of 21/22-nt easiRNAs in *nrpd1a* pollen (Fig. 3f, Fig. S5b) rather than with the presence of 24 nt siRNAs in 2n pollen (Fig 3h). Conversely, gain of CHH methylation in 3x *nrpd1a* seeds correlated with loss of 21/22-nt easiRNAs in pollen (Fig. 3g, Fig. S5c) and was not associated with reduced expression of *NRPD1a* in the endosperm (Fig.

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

We further challenged the hypothesis that 2n pollen contributes an increased dosage of 21/22-nt easiRNAs by testing whether increased levels of 21/22-nt siRNAs in 3x seeds correlate with loss of 21/22-nt siRNAs in 3x *nrpd1a* seeds (Fig. S7, Table S4). Loci with highest levels of 21/22-nt siRNAs in 3x seeds experienced the strongest loss of 21/22-nt siRNAs in 3x *nrpd1a* seeds (Fig. 3i), strongly supporting the idea that 2n pollen contributes an increased dosage of Pol IV-dependent easiRNAs that negatively interfere with CHH methylation in 3x seeds. Consistently, increased abundance of 21/22-nt siRNAs in 3x seeds correlated with increased loss of CHH methylation (Fig. 3j). Gain of 21/22-nt siRNAs in 3x seeds was associated with loss of 24-nt siRNAs (Fig. 3k), consistent with the loss of CHH methylation observed at loci targeted by 21/22-nt easiRNAs (Fig. 3j).

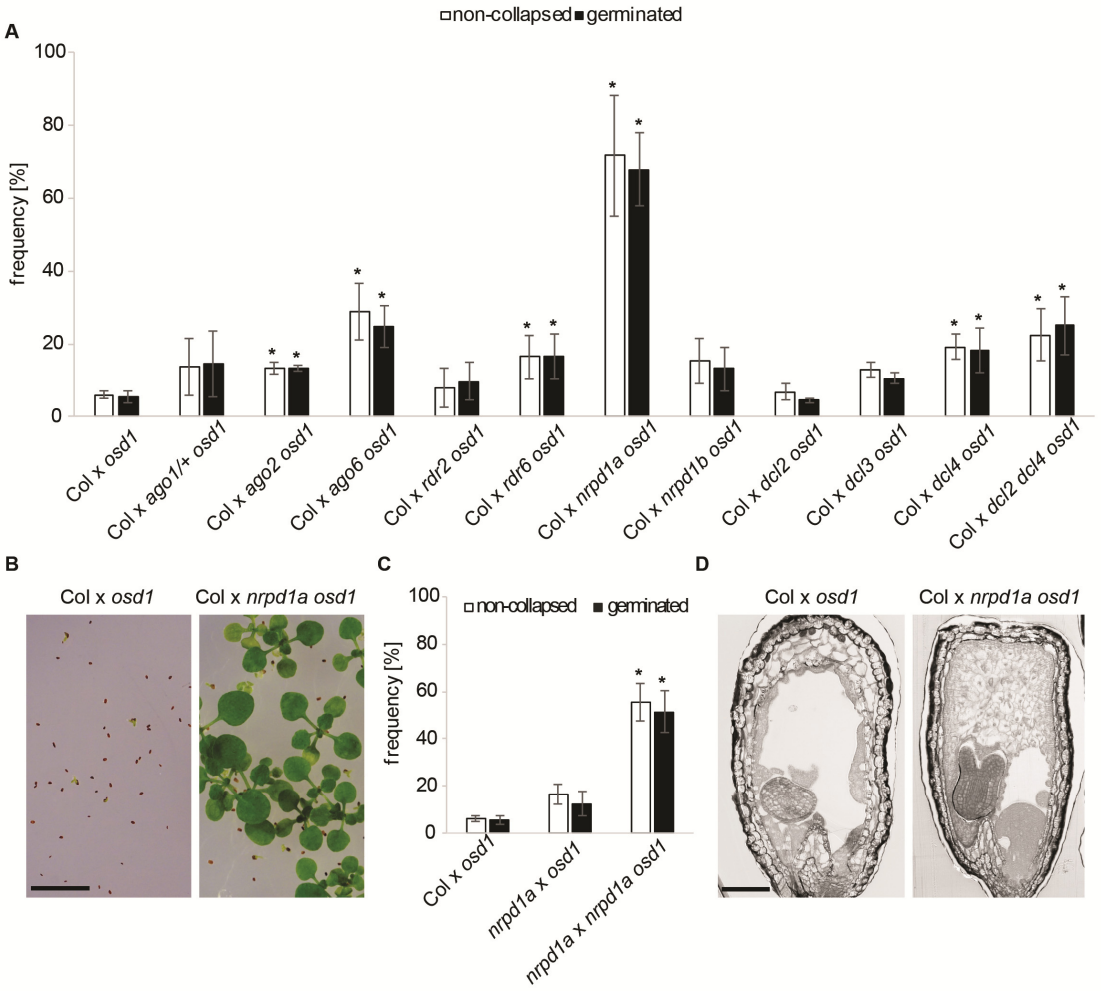
To address the question whether increased abundance of Pol IV-dependent easiRNAs in pollen associate with increased gene expression in the endosperm of 3x seeds, we generated transcriptome data of purified endosperm (Table S5). Increased abundance of Pol IV-dependent easiRNAs in pollen was associated with upregulated gene expression in the endosperm of 3x seeds (Fig. 4a), supporting the idea that increased abundance of easiRNAs negatively interferes with gene repression. Consistently, depletion of pollen-derived easiRNAs normalized gene expression in the endosperm of 3x seeds (Fig. 4b). Out of 57 PEGs with significantly increased expression in 3x seeds, 19 PEGs were at least 2-fold downregulated in 3x *nrpd1a* seeds (Fig. 4c[6,9]. Those 19 PEGs accumulated Pol IV-dependent easiRNAs at flanking regions (Fig. 4d), correlating with reduced CHH methylation in 3x seeds and partially restored CHH methylation in 3x *nrpd1a* seeds (Fig. 4e,f). Together we

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

Accordingly, we propose that Pol IV-dependent production of TE transcripts in gametic tissues is a genomic safeguard mechanism that sustains production of 21/22-nt easiRNAs of potentially harmful active TEs without Pol II transcription. Our data reveal a striking analogy of easiRNAs in establishing the triploid block with Piwi-interacting RNAs (piRNAs) in hybrid dysgenesis in flies [23]. In both models, TE-derived sRNAs transmit epigenetic information transgenerationally, pointing to a conserved role of TE-derived sRNAs in assessing gamete compatibility. Pollen-delivered 21/22-nt easiRNAs could possibly target transcriptionally active TEs for degradation in the hypomethylated endosperm and thus prevent transposition [24]. When different species hybridize, hybrid failure will result if pollen 21/22-nt easiRNAs do not recognize their maternal target TEs or, conversely, if paternal TEs are too sequence divergent to be recognized by maternal 24-nt siRNAs. Importantly, our data show that also inadequate dosage of parental siRNA populations can cause hybrid seed failure, revealing that the balanced dosage of maternal and paternal siRNA populations is essential for viable seed formation (Fig. 4e). One possible scenario is that increased dosage of 21/22-nt easiRNAs causes these siRNAs to target scaffolding transcripts produced by either Pol II or Pol V and thus negatively interfere with DNA methylation establishment, as previously proposed [12].

The triploid block has been a mystery to geneticists and breeders, formalized as the “endosperm balance number" hypothesis more than 35 years ago [1]. Our discovery that paternal easiRNAs form the genetic basis of dosage sensitivity and establish the triploid block provides the means for targeted strategies bypassing this hybridization 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 *nrpd1*a 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

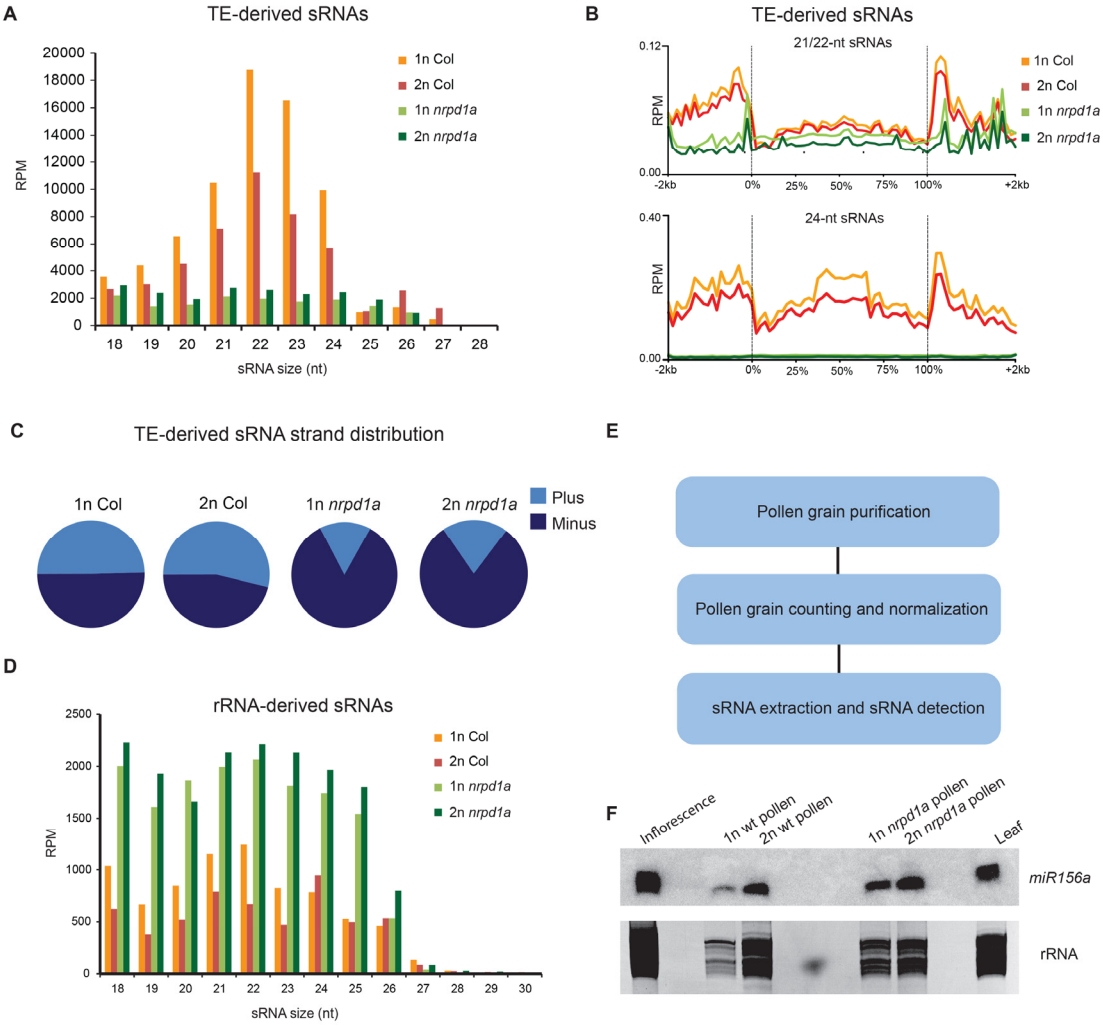


Fig. 2. Pol IV is responsible for the biogenesis of pollen TE-derived sRNA in the size range of 19-24 nt. (**A**) sRNA profiles of TE-derived sRNAs of 1n pollen from Col wild-type (wt) and *nrpd1a* mutants and 2n pollen from tetraploid wt and *nrpd1a* mutants. (**B**) Metaplots of siRNA distribution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

Figure 3

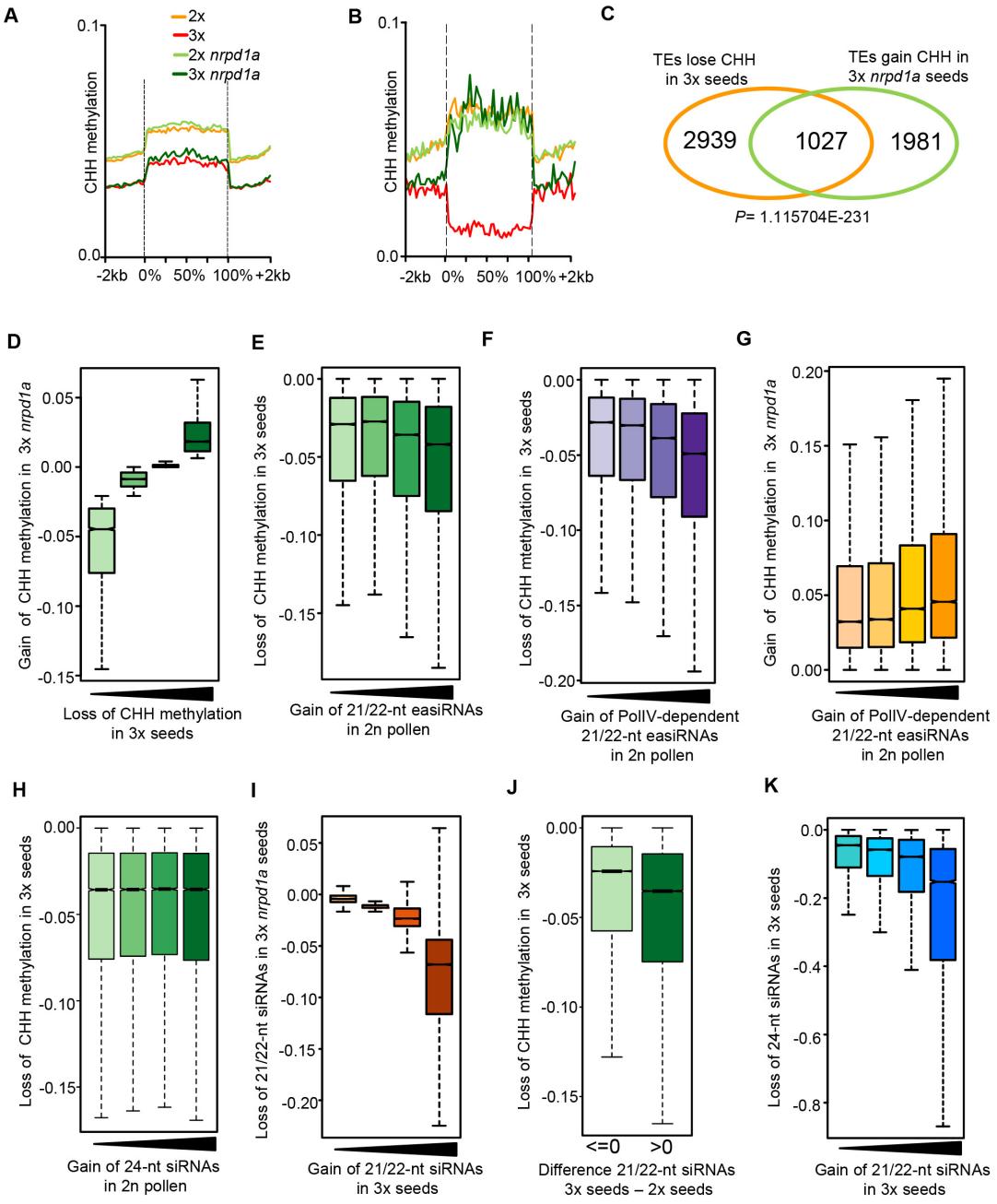


Fig. 3. Triploid seeds have lower CHH methylation as a consequence of increased pollen-derived 21/22-nt easiRNAs. (**A**) CHH methylation metaplots over all TEs in the endosperm derived from 2x, 3x, 2x *nrpd1a*, and 3x *nrpd1a* seeds. (**B**) CHH methylation metaplots over TEs that lose CHH methylation in the endosperm of 3x seeds and restore CHH methylation in 3x *nrpd1a osd1* seeds. Color code as specified in A. **(C)** Venn-diagram showing overlap of TEs specified in **B**. Hypergeometric testing was used to test for significance of overlap. (**D-F**) Loss of CHH methylation in the endosperm of 3x seeds associates with increasing levels of CHH methylation in 3x *nrpd1a* seeds (**D**), increasing levels of 21/22-nt easiRNAs in 2n pollen (**E**), increasing levels of POL IV-dependent

21/22-nt easiRNAs in 2n pollen (**F**). (**G**) Gain of CHH methylation in the endosperm of 3x *nrpd1a* seeds associates with increasing loss of 21/22-nt easiRNAs in 2n *nrpd1a* pollen. (**H**) Increasing levels of 24-nt easiRNAs in 2n pollen do not associate with CHH methylation differences in the endosperm of 3x seeds. (**I**) Increasing levels of 21/22-nt siRNAs in 3x seeds associate with increasing loss of 21/22-nt siRNAs in 3x *nrpd1a* seeds. (**J**) Increasing levels of 21/22-nt siRNAs in 3x seeds associate with loss of CHH methylation in 3x seeds. Plotted are differences of CHH methylation in the endosperm of 3x and 2x seeds at those 50 bps bins where differences in 21/22-nt siRNAs between 3x and 2x seeds are smaller or larger than zero. (**K**) Increasing levels of 21/22-nt siRNAs in 3x versus 2x seeds associate with increasing loss of 24-nt siRNAs in triploid seeds. (**D-I, K**) Plotted are CHH methylation or siRNA differences sorted by quantiles of 50-bp genome bins against differences of CHH methylation or siRNAs in the endosperm of indicated genotypes. Differences between first and last categories in **A-G** and **I-K** are significant ($P<0.00001$, Kolmogorov-Smirnov test).

Figure 4

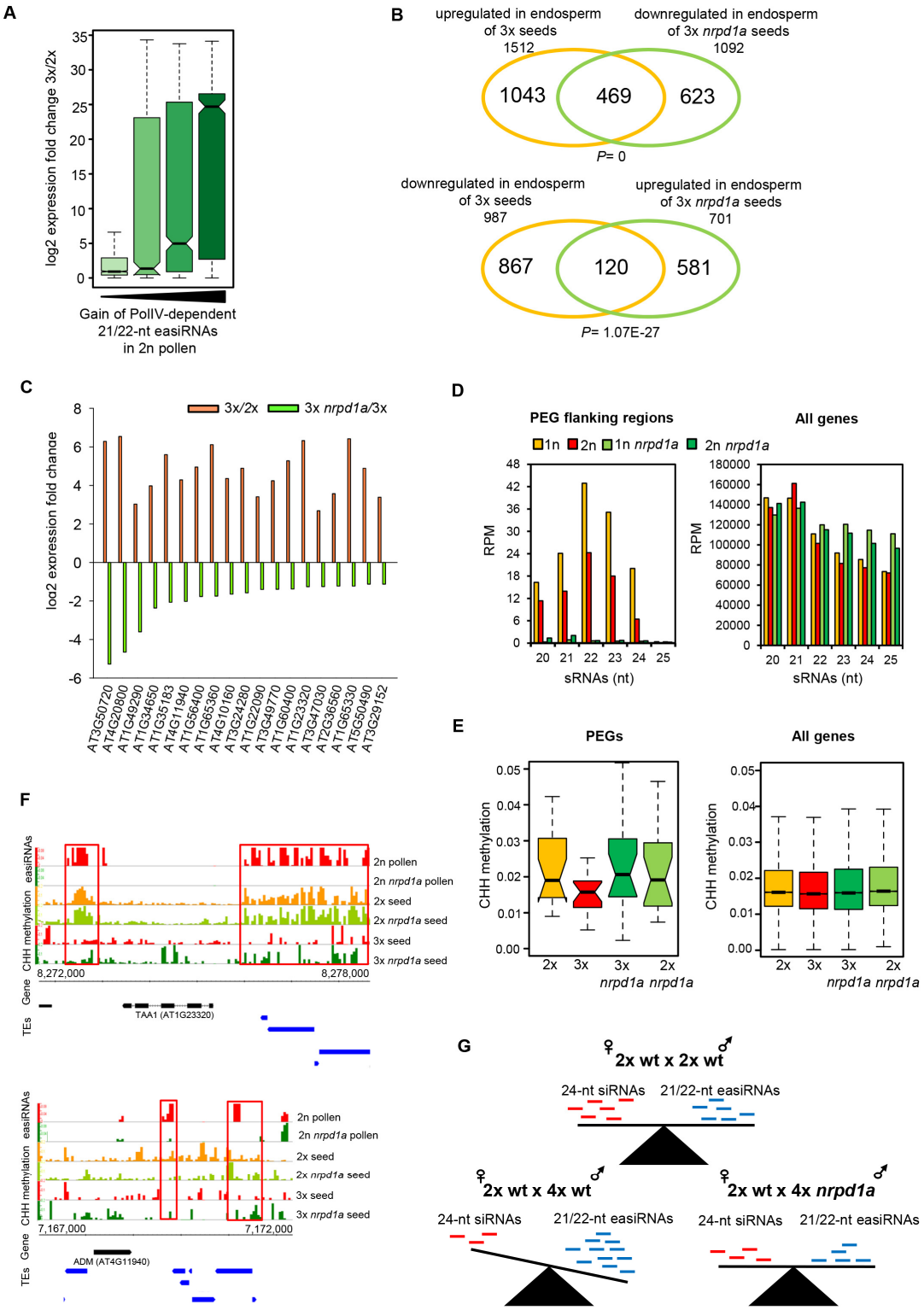


Fig. 4. Pollen-derived 21/22-nt easiRNAs associate with gene expression changes in the endosperm of triploid seeds. (**A**) Increasing levels of 21/22-nt easiRNAs in 2n versus 2n *nrpd1a*

pollen associate with upregulated gene expression in the endosperm of 3x seeds. Plotted are differences in 21/22-nt siRNAs in 2n and 2n *nrpd1a* pollen against log2-fold expression changes in the endosperm of 3x and 2x seeds. Differences between categories are significant (P<0.00001, Kolmogorov-Smirnov test). (**B**) Venn diagram showing overlap of deregulated genes in in the endosperm of 3x and 3x *nrpd1a* seeds compared to the corresponding 2x and 3x controls, respectively. Hypergeometric testing was used to test for significance of overlap. (**C**) Log2-fold change expression of PEGs in the endosperm of 3x and 3x *nrpd1a* compared to compared to the corresponding 2x and 3x controls, respectively. (**D**) sRNAs mapping to 2kb flanking regions of PEGs (specified in C) and flanking regions of all genes (left and right panels, respectively) in 1n and 2n wild-type and *nrpd1a* pollen. (**E**) Boxplots of CHH methylation in 2kb flanking regions of PEGs (specified in C) and all genes (left and right panels, respectively). (**F**) Representative PEGs accumulating 21/22-nt easiRNAs in pollen and experiencing changes in CHH methylation in the endosperm of 3x and 3x *nrpd1a* seeds. Red rectangles mark regions where easiRNA accumulation co-occurs with changes of CHH methylation. (**G**) Viable seed formation requires balanced populations of pollen-derived 21/22-nt easiRNAs and ovule-derived 24-nt siRNAs. Increased levels of 21/22-nt easiRNAs delivered from a higher ploidy pollen parent will reduce 24-nt siRNAs post-fertilization in the seed, resulting in unviable seed formation. *NRPD1a* deficient pollen restores viable seed formation by reducing pollen-derived easiRNAs.

Methods:

Plant material and growth conditions

The mutants used in this study were described previously as *ago1-36* (Salk_087076; [25], *ago2-1* (Salk_003380; [26], *ago6-2* (Salk_031553; [27], *rdr2-2* (Salk_059661; [20], *rdr6-15* (Sail_617H07; [28], *nrpd1a-3* (Salk_128428; [29], *nrpd1b-12* (Salk_033852; [30], *dcl2-1* (Salk_064627; [31], *dcl3-1* (Salk_005512; [31], *dcl4-2* [31]. The *osd1-1* mutant [5] was kindly provided by Raphael Mercier. Being originally identified in the Nossen background, the mutant was introgressed into Col by repeated backcrosses over five generations. Tetraploid *nrpd1a-3* plants were generated using colchicine treatment as previously described [22]. Plants were grown in a growth cabinet under long day photoperiods (16 hr light and 8 hr dark) at 22˚C. After 10 days, seedlings were transferred to soil and plants were grown in a growth chamber at 60% humidity and daily cycles of 16 hr light at 22˚C and 8 hr darkness at 18˚C. For all crosses, designated female plants were emasculated and the pistils were hand-pollinated 2 days after emasculation.

Germination analysis

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

Microscopy

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

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

Pollen normalization and Northern blotting

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

RNA sequencing

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

Small RNA sequencing

Pure mature pollen samples from inflorescences of approximately 500 plants were collected as previously described [33] with minor modifications. Flowers were harvested in a beaker, covered with 9% sucrose solution and shaken vigorously for 5 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 L*er* 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 L*er* x Col-0, L*er* x *osd1*, L*er* x *osd1 nrpd1a* and L*er* 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 L*er* SNPs (Table S2).

321 **mRNA sequencing data analysis**

For each replicate, 50 bp long reads were mapped to the Arabidopsis (TAIR10) genome, masked for rRNA genes, using TopHat v2.1.0 (Trapnell et al, 2009) (parameters adjusted as -g 1 -a 10 -i 40 -I 5000 -F 0 -r 130). Gene and TE expression was normalized to reads per kilobase per million mapped reads (RPKM) using GFOLD [37]. Expression level for each condition was calculating using the mean of the expression values in both replicates. Differentially regulated genes and transposable elements across the two replicates were detected using the rank product method as implemented in the Bioconductor RankProd Package [38]. The test was run with 100 permutations and gene selection was corrected for multiple comparison errors using a pfp (percentage of false prediction) < 0.05.

Small RNA sequencing data analysis

Adapters were removed from the 50 bp long single-end sRNA reads in each library. The resulting18-30 bp long reads were mapped to a Col genome (TAIR10) masked for L*er* SNPs using bowtie (-v 2 –best). All reads mapping to chloroplast and mitochondria and to structural noncoding RNAs (tRNAs, snRNAs, rRNAs, or snoRNAs) were removed. Mapped reads from both replicates were pooled together, sorted in two categories (21-22-nt and 24-nt long) and remapped to the same reference masked genome mentioned above using ShorStack (--mismatches 2 --mmap f) [39] in order to improve the localization of sRNAs mapping to multiple genomic locations. We normalized the alignments by converting coverage values to reads per million mapped reads (RPM) values. The sRNA mapping profiles were visualized with bedGraph files based in 50 bp bins. Metagene plots over TEs were constructed between -2 kb and + 2 kb by calculating mean levels of sRNA (RPM) in 100 bp bins in the flanks of the TEs and in 40 equally long bins between the transcriptional start and stop.

DNA methylation analysis

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

Reads were mapped to the TAIR10 Arabidopsis genome using the Bismark read mapper [40] allowing up to one mismatch per read. Duplicated reads (aligning to the same genomic position) were eliminated before calculating methylation levels. Methylation levels for each condition were calculated as the mean of the two replicates. Cytosine conversion efficiency was estimated as the percentage of CHH methylation in the chloroplast. Cytosine methylation was visualized separately for CG, CHG and CHH cytosine contexts with bedGraph files representing average methylation values in 50 bp bins across the genome. Differential analysis on the levels of CHH methylation in TEs between conditions considered the cytosine methylation reports from both replicates and was performed using linear modelling (p-value < 0.05) as implemented in the R package Limma [41].

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Extended data:

Tables 1 to 5

Figs. 1 to 7

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486 analyzed the data. GM, PW and CK wrote the manuscript.

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