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Parent-of-origin control of transgenerational retrotransposon proliferation in *Arabidopsis*

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Retrotransposons are ubiquitous mobile genetic elements constituting a major part of eukaryotic genomes. Yet, monitoring retrotransposition and subsequent copy number increases in multicellular eukaryotes is intrinsically difficult. By following the transgenerational accumulation of a newly activated retrotransposon EVADE (EVD) in Arabidopsis, we noticed fast expansion of activated elements transmitted through the paternal germ line but suppression when EVD-active copies are maternally inherited. This parent-of-origin effect on EVD proliferation was still observed when gametophytes carried mutations for key epigenetic regulators previously shown to restrict EVD mobility. Therefore, the main mechanism preventing active EVD proliferation seems to act through epigenetic control in sporophytic tissues in the mother plant. In consequence, once activated, this retrotransposon proliferates in plant populations owing to suppressed epigenetic control during paternal transmission. This parental gateway might contribute to the occasional bursts of retrotransposon mobilization deduced from the genome sequences of many plant species.
Keywords: epigenetics; transposable elements; retrotransposition; parent-of-origin effect

INTRODUCTION

A significant proportion of chromosomal DNA can be assigned to various families of transposable elements (TEs), with retrotransposons being the most abundant [1,2]. Uncontrolled retrotransposon activation might threaten the genome owing to the accumulation of mutations or to negative regulatory effects on host genes residing in the vicinity of their new insertions [3]. Homologous recombination between ectopic genomic regions through related transposon sequences might also lead to further genome rearrangements [4]. Thus, mechanisms limiting increased copies of self-replicating DNA elements in host genomes are advantageous. However, it was hypothesized that some TEs could also be associated with certain benefits, for example by creating novel, favourable regulatory networks or beneficial genome rearrangements [5,6], thus providing a selective advantage at the population level [3,7–9]. Just how the cost/benefit balance of TEs is regulated remains largely unclear.

Recently, much emphasis has been placed on elucidating epigenetic mechanisms restricting TE activities by transcriptional and post-transcriptional gene silencing [3,8,10–12]. These mechanisms generally involve small RNAs [13]. For example, Piwi-interacting RNA prevent transposon proliferation in *Drosophila melanogaster* and mammalian germ lines [14,15]. In plants, 21- and 24-nucleotide small interfering RNAs (siRNAs) influence transposons through post-transcriptional and transcriptional silencing mechanisms, respectively [3,16]. While such studies address important aspects of host genome regulation, they have not defined host responses to actively mobilized retrotransposons, how active retrotransposons are transmitted or how their copy numbers increase over generations. One underlying problem has been the paucity of mobile retrotransposons to study in 'real time' [9]. Moreover, precise analyses of genome-wide TE copy numbers and the parental transmission of specific elements is difficult for mobile TEs belonging to families composed of hundreds of copies [17,18].

Recently, we and others reported transpositional activation of the *Arabidopsis ATCOPIA93* retrotransposon EVADE (EVD) [19,20]. In wild-type (WT) plants, EVD transcription is suppressed by DNA methylation determined by the maintenance DNA METHYLTRANSFERASE 1 (MET1) in cooperation with the chromatin remodelling protein DECREASED DNA METHYLATION 1 (DDM1). Full-length EVD transcripts are immediately produced in *met1* and *ddm1* mutant plants but EVD transposition was observed only after prolonged inbreeding. In contrast, drastic EVD transposition was observed when the *met1*

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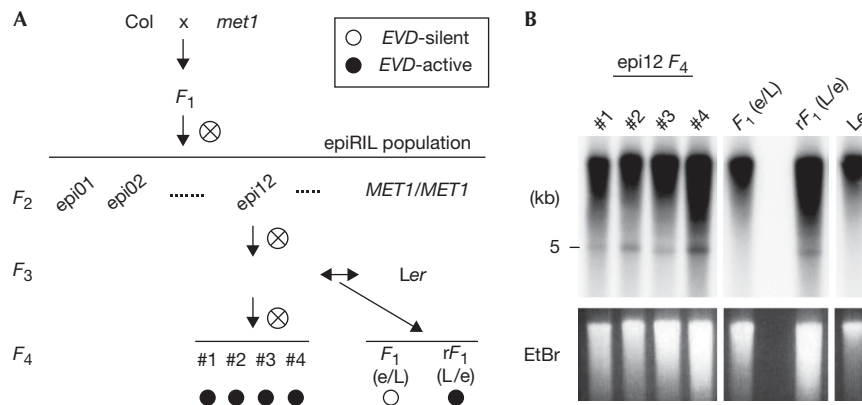E-mail: marie.mirouze@ird.fr

Fig 1 | Parent-of-origin effects on the transmission of the active *EVD* within inter-accession crosses. (**A**) Crossing scheme to obtain epi12 and its F3 cross to the L*er* accession. One plant from line 12 (epi12) of the epiRIL population at the F_3 generation was crossed reciprocally (double arrow) with L*er* to generate F_1 and reciprocal F_1 (rF_1) hybrids. The use of L*er* (*L*) or the epiRIL (*e*) parent is indicated (maternal/paternal) for each hybrid. The inferred *EVD* activity levels (*EVD*-silent or *EVD*-active) are indicated by white and black circles, respectively. (**B**) Southern blot detection of *EVD* ecDNA reveals pollen transmission of *EVD*. Undigested genomic DNA was probed for *EVD*. The presence of the ∼5-kb *EVD* ecDNA is indicated (black arrow). Individual epiRIL siblings were investigated, while the two F_1 samples and the L*er* parental control each comprised a bulked lot of eight individual plants. ecDNA, extrachromosomal DNA; L*er*, Landsberg *erecta*.

mutation was combined with mutations impairing the biogenesis of siRNAs or the di-methylation of histone H3 in lysine 9 [19]. Increased *EVD* copy number was also reported in plants defective in both DDM1 function and siRNA production [21]. Importantly, although dynamic increases in *EVD* copies were observed after inbreeding in these strains, potential differences in parental contributions to this accumulation have not been determined.

Here, we report on parent-of-origin control of *EVD* proliferation. Using reciprocal crosses, we have shown that *EVD* proliferation occurs predominantly by pollen transmission, whereas maternal transmission largely depresses the rate of *EVD* accumulation. Moreover, our genetic analyses demonstrated that this maternal suppression is maintained in maternal gametes deficient in the production of siRNAs (mutation in RNA polymerases IV/V), deficient in histone H3 in lysine 9 (mutation of histone methyltransferase KYP), or lacking Agronaute 5 (AGO5). Thus, our results suggest that maternal suppression of active *EVD* copies might not occur in the gametes but that epigenetic mechanisms involving siRNAs and/or methylation of histone H3 on lysine 9 might act in maternal sporophytic tissues of the flower to prevent transgenerational proliferation of active *EVDs*.

RESULTS
Parent-of-origin effects on *EVD* accumulation

We demonstrated previously that *EVD* was mobilized and increased in copies during inbreeding in ∼50% of WT epigenetic recombinant inbred lines [19], here referred to as epiRILs [22], derived from a cross between the WT Columbia-0 accession (Col) and the *met1-3* mutant with a complete loss of MET1 activity in the Col accession [23]. This increase was first observed in the F_4 generation of epiRILs, notably in the epi12 line, and was associated with the presence of *EVD* extrachromosomal DNA (ecDNA) that made up 10% of the total copies of *EVDs* (supplementary Fig S1 online) in all studied lines [19].

We aimed to examine possible *EVD* activation in reciprocal F_1 hybrids, as reflected by the appearance of its ecDNA. Hence, a randomly selected single F_3 epi12 plant [19] with a propensity to transmit actively replicating *EVD* (hereafter named *EVD*-active) was crossed reciprocally to the Landsberg *erecta* (L*er*) accession (*EVD*-silent) (Fig 1A). By Southern blot, we observed a clear parent-of-origin effect on the presence of *EVD* ecDNA in F_1 progeny of the reciprocal crosses (Fig 1B). We detected ecDNA in the bulked progeny when epi12 pollen was used to fertilize L*er* flowers, indicating completion of the replication cycle through reverse transcription. Conversely, ecDNA was absent when L*er* pollen fertilized epi12 flowers. This intriguing observation prompted further investigation of a possible parent-of-origin effect on transgenerational propagation of this retrotransposon.

In the F_3, individual epi12 plants had various levels of *EVD* activity. To detect *EVD*-active plants, we developed a long terminal repeat polymerase chain reaction (LTR-PCR) assay on the basis of the semi-quantitative detection of circular ecDNA forms (supplementary Fig S2A online). We then screened further F_3 epi12 siblings and selected three *EVD*-active epi12 plants differing in amounts of ecDNA (A–C) for further crosses, including one *EVD*-silent epi12 sibling (D) as a control (supplementary Fig S2B online). These plants were crossed reciprocally to L*er* to create four F_1 populations A–D (Fig 2A). The relative abundance of *EVD* DNA in the F_1 progeny was assayed using quantitative polymerase chain reaction (supplementary Fig S2C online), that reports on linear ecDNA, circular ecDNA and chromosomal *EVD* insertions, thus providing a general readout for progressive *EVD* accumulation. The *EVD* DNA was also determined in progeny from the self-fertilized parents (epi12 and L*er*) to provide controls for relative changes in *EVD* copies. The highest increases in *EVD* copies were observed on self-fertilization of *EVD*-active epi12 siblings (F_4, Fig 2B). There were no significant increases in *EVD* copies when the *EVD*-active epiRIL lines were fertilized with *EVD*-silent L*er* pollen (maternal suppression phenotype). Conversely, when *EVD*-silent L*er* plants were fertilized with
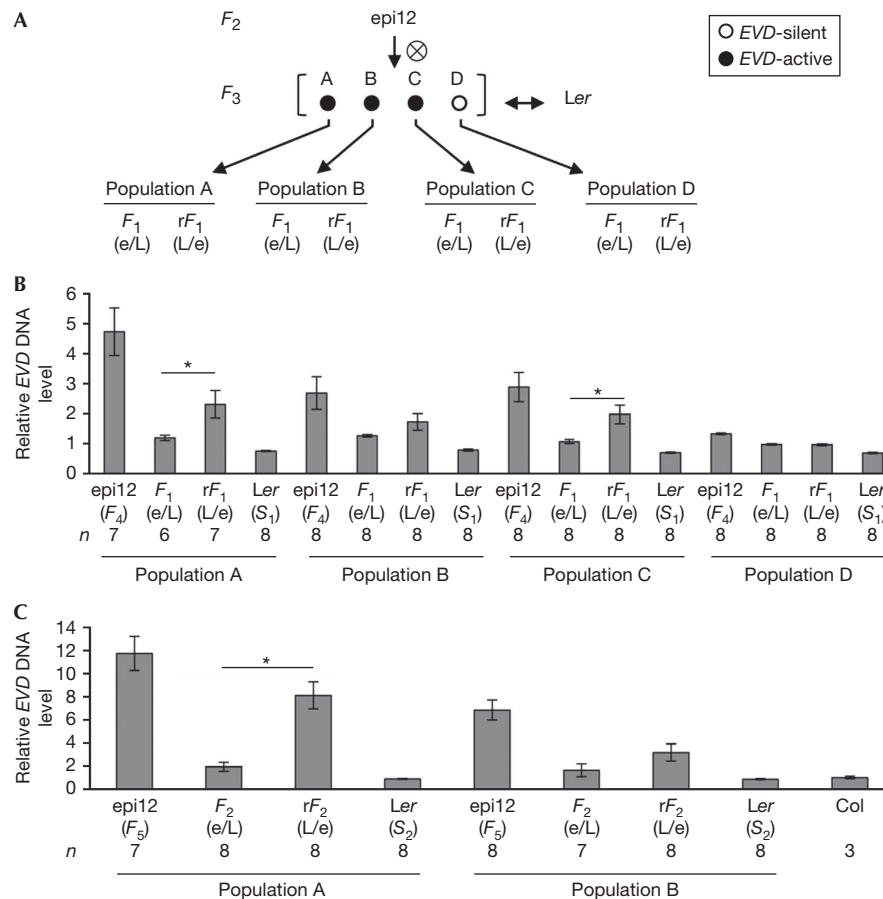
Fig 2 | Acceleration of *EVD* activity via pollen transmission within inter-accession crosses. (**A**) Crossing scheme between four epi12 F_3 individuals (marked as A, B, C, D) crossed reciprocally (double arrow) to L*er* parents to create populations A–D (see supplementary Fig S2B online for details). (**B**) Relative *EVD* DNA content observed in populations A–D at the F_1 with the corresponding self-fertilized parental controls. The maternal and paternal contributions (as described in Fig 1) of each cross and sample size (n) are listed along the X axis below the graphs. The average relative *EVD* content levels ($\pm$ s.e.m.) for each cross relative to the mid-parent level (set to 1 as midpoint of Col and L*er*) are shown along the Y axis. The significance ($P < 0.05$) of Student t-test comparisons of reciprocal crosses (F_1 and rF_1) for each population (horizontal bars) is indicated by asterisks. (**C**) Analyses of relative *EVD* DNA content in the epi12 x L*er* F_2 progenies of populations A and B. The maternal and paternal contribution and relative *EVD* DNA content for each population is shown as described in **A**. Significant differences between reciprocal crosses are shown as described in **B**. L*er*, Landsberg *erecta*.

EVD-active pollen increases in *EVD* copies were observed in all three *EVD*-active F_1 populations derived from epi12 siblings carrying active *EVD* (populations A–C, Fig 2B), with two out of three (populations A and C) being significantly different at $P < 0.05$ (Student's t-test).

We next examined how the relative abundance of *EVD* DNA changed in the F_2 progeny of self-fertilized reciprocal F_1 hybrids of *EVD*-active populations A and B. After self-fertilization, relative abundance of *EVD* DNA increased to nearly twofold levels above the WT (Fig 2C) in the F_2 progeny derived from F_1 plants exhibiting near parental levels (see Fig 2B). In contrast, *EVD* DNA levels in reciprocal F_1 siblings from populations A and B, differing in the initial degree of the paternal transmission (see Fig 2B), increased up to eight- and threefold levels above the WT, respectively (Fig 2C). Thus, the relative difference in *EVD* accumulation originating from the initial parent-of-origin effect

was perpetuated in the subsequent generation, with a significant difference between the F_2 and reciprocal F_2 entries of population A (Student's t-test, $P < 0.05$; Fig 2C).

Sporophytic parental control of *EVD*

We repeated the reciprocal crosses using the isogenic Col background to test whether the L*er* genome itself influences *EVD* mobility (Fig 3A). Moreover, we examined whether the maternal suppression phenotype is affected by mutations in particular epigenetic regulators by using mutants previously shown to promote *EVD* transposition [19]. Specifically, mutants lacking KYP or the common subunit of polymerase IV/V (NRPD2A) when combined with *met1-3* permitted massive *EVD* transposition [19]. Given *EVD* pollen transmission and the reported activity of TE-derived small RNA produced in the vegetative nucleus in reinforcing silence in male gametes [24], we
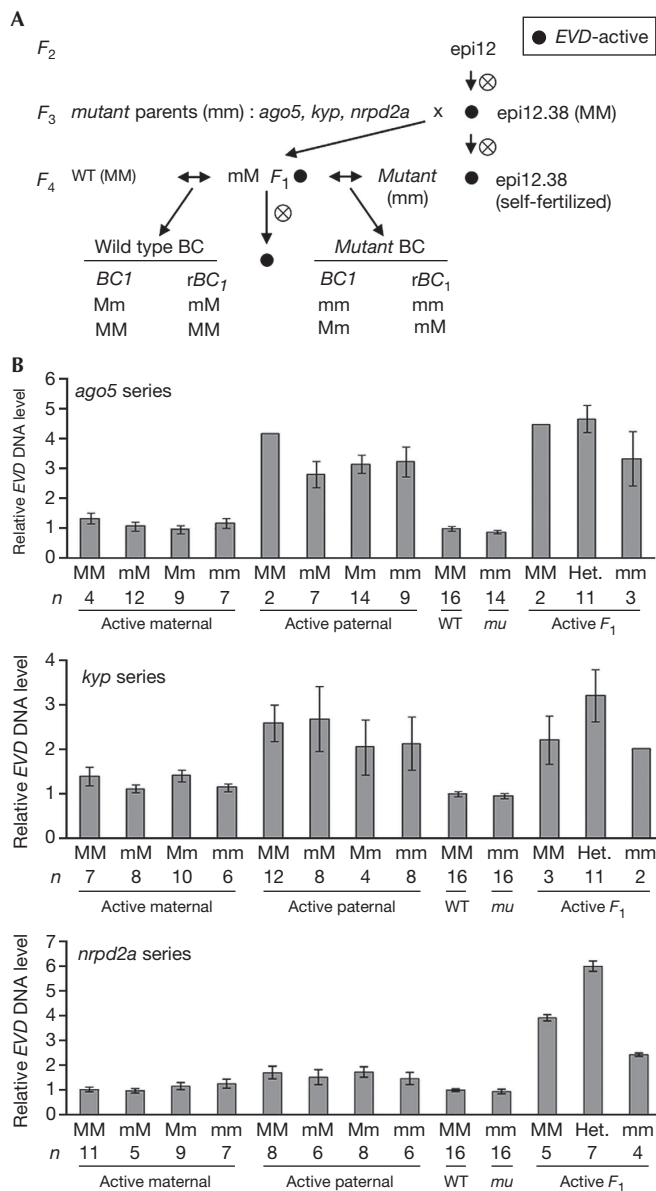
Fig 3 | EVD transmission rates in EVD-active first-generation reciprocal backcross (BC$_1$) populations. (**A**) Crossing scheme between one F_3 generation epi12 individual (epi12.38, paternal parent) and *ago5*, *kyp* and *nrpd2a* mutants (as maternal parents) to create *EVD*-active backcross populations. Resultant F_1 individuals selected as either *EVD*-active or *EVD*-silent (see supplementary Fig S3 online) was backcrossed reciprocally to the WT Col parent and the selected mutant parents (*ago5*, *kyp* and *nrpd2a*) to create the respective backcross populations. Shown here are the *EVD*-active populations; see supplementary Fig S4 online for *EVD*-silent population results. (**B**) Relative *EVD* DNA content levels (*Y* axis) shown for each entry (*X* axis) for the mutant populations: *ago5* (upper panel), *kyp* (middle panel), and *nrpd2a* (lower panel). The parent-of-origin effect (active maternal, active paternal or active F_1), sample size (*n*), and allelic origin at each respective epigenetic regulator for WT (M) and mutant (m) alleles are indicated with respect to parental contributions (maternal/paternal). Heterozygous *F2* generation progeny (*Het.*) comprise both mM and Mm progeny. WT, wild type.

also sought to determine whether perturbing a pollen-specific function would result in higher relative increases in *EVD* DNA during male or female transgenerational transmission. For this purpose, we selected a mutant allele of AGO5, an Argonaute family member known to be highly expressed in sperm cell nuclei [25].

In these crosses (Fig 3A), we used a single epi12 F_3 individual, epi12.38, with moderate *EVD* activity (supplementary Fig S3A online) as the pollen donor to fertilize each selected mutant (supplementary Figs S3B,C online). We selected F_1 individuals with *EVD*-silent or *EVD*-active copies according to quantitative polymerase chain reaction results in the *ago5*, *kyp* and *nrpd2a* populations (supplementary Fig S3C online). These selected F_1 progeny were backcrossed reciprocally to both the WT and respective mutant parent (Fig 3A and supplementary Fig S4A online) to examine the effect of these epigenetic regulators on *EVD* inheritance. In agreement with the previous results, self-fertilization of *EVD*-active F_1 individuals produced two- to over fourfold increases in *EVD* copies relative to the WT level, while copies of *EVD*-silent classes did not differ from the WT level (Fig 3B and supplementary Fig S4 online). By genotyping the BC_1 at the *AGO5*, *KYP* or *NRPD2A* loci pertaining to each population, we partitioned seedlings into WT or mutant classes. Given that reciprocal crosses were performed, this also permitted us to address parent-of-origin effects in all genotyped and partitioned populations. Abundance of *EVD* DNA in the *EVD*-silent class stayed at the WT level, regardless of crossing direction or gametophytic genotype (supplementary Fig S4B online). The BC_1 progeny of *EVD*-active populations exhibited parent-of-origin effects that reflected maternal suppression or paternal transmission (Fig 3B). This effect was less pronounced in the *nrpd2a* population, illustrating that rates for which *EVD* transmission occurs can vary (see also Fig 2), as reported for DNA transposons [26]. The presence of *EVD* neo-insertions was confirmed by transposon display analysis (supplementary Fig S5 online) in a subset of lines, but we acknowledge our method will only display neo-insertions near *Dra*I recognition motifs. As mutant or WT gametes did not differ in *EVD* levels within BC_1 derived populations, we conclude that the loss of AGO5, KYP or NRPD2A activity alone in the male and female gametophytes is not sufficient to explain the parent-of-origin effects on transgenerational propagation of *EVD*.

To further examine if parent-of-origin effects depend on parental sporophytic tissues rather than Pol IV/V activities within the gametophytes, we examined *EVD* DNA levels across several generations of plants segregating for *MET1* and/or *Pol IV/V* mutations (Fig 4A). Southern blot analyses showed WT *EVD* patterns in both *met1* and *nrpd2a* parents (Fig 4B). In the first generation when the two mutations were combined, there was no increase in *EVD* copy number (Fig 4B), which is consistent with the above results showing no gametophytic role for Pol IV/V activities (Fig 3). In contrast, a *met1 nrpd2a* double mutant selected in the second generation (from a *met1* heterozygous *nrpd2a* homozygous parent, thus devoid of Pol IV/V activities during sporophytic growth) showed a massive increase in *EVD* copy number (Fig 4B), consistent with our previous report [19]. This observation together with the parent-of-origin effects described above points towards a suppressive effect of Pol IV/V activities within the maternal sporophytic tissues in
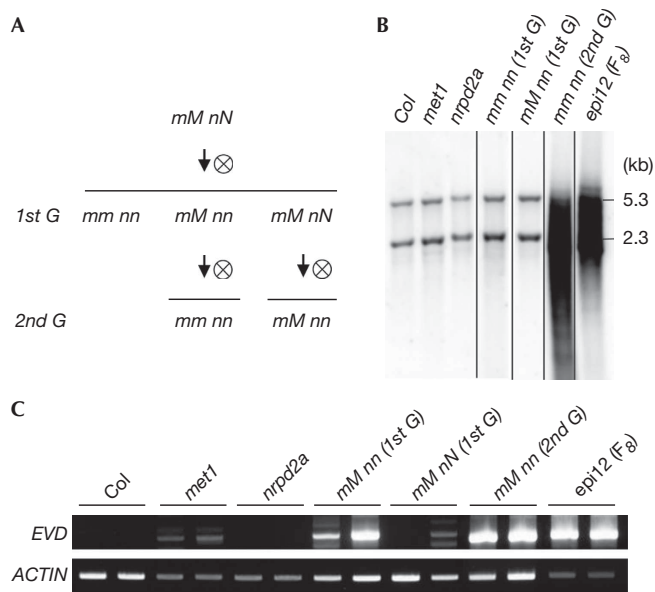
Fig 4 | Initiation of *EVD* proliferation is controlled by parental sporophyte. (**A**) F_1 plants *met1* (±) *nrpd2a* (±) (mM nN) originating from a cross between *met1* and *nrpd2a* parents were self-fertilized to produce segregating 1st and 2nd generation plants (1st and 2nd G, respectively). (**B**) Southern blot analysis of individual plants with *Ssp*I-digested DNA hybridized to an *EVD*-specific probe as previously reported [19]. (**C**) LTR-PCR showing *EVD* extrachromosomal forms diagnostic for *EVD* life cycle initiation in individual plants. Actin was a loading control and epi12 (F_8) a positive control for *EVD* activity. LTR-PCR, long terminal repeat polymerase chain reaction; met1, methyltransferase 1.

transgenerational *EVD* proliferation. To further examine *EVD* activation events in the maternal sporophyte, we compared *EVD* activities in heterozygous *met1* plants that acquired homozygosity of the *nrpd2a* mutation at different generations in respect to the F_1 hybrid. For this, we used the long terminal repeat polymerase chain reaction assay to reveal *EVD* activity through detection of its ecDNA copies, which cannot be clearly revealed by Southern blot (Fig 4B). Consistent with our previous observation, we detected initiation of *EVD* life cycle only in plants homozygous for the *nrpd2a* mutation (Fig 4C). As we repeatedly observed that progenies of such *EVD*-active plants will accumulate new chromosomal insertions ([19] and supplementary Fig S5 online), we conclude that sporophytic epigenetic control contributes to the early dynamics of *EVD* inheritance.

DISCUSSION

Most retrotransposons are transcriptionally silenced by DNA methylation with the developmental control of epigenome reprogramming likely contributing to retrotransposon 'immunity'. For example, active DNA demethylation in plant companion cells during gametogenesis and the movement of 21-nucleotide siRNAs from the vegetative cell to the germ cell is believed to reinforce continued transposon silencing in gametes [24,27–30]. Yet, host responses to actively replicating retrotransposons remain less clear. A notable example however is the maize DNA transposon *MuDR* where increased transmission rates in progeny inheriting

MuDR-1 from the male parent relative to the female parent was reported [31–33].

Our analysis of reciprocal *EVD* transmission during sexual reproduction in *A. thaliana* has likewise shown the rate of increase in *EDV* copy levels is affected by a parent-of-origin effect. The initial results with F_1 inter-accession hybrids (Fig 1) could have been due to structural variation between the Col and L*er* genomes leading to limited homology-dependent recognition of active elements. This possibility is not likely given the parental effect persisted in intra-accession Col × Col crosses (Fig 3B).

Our results are consistent with a mechanism for the maternal suppression of *EVD* activity, possibly dependent on the RNA dependent RNA Polymerase 6 (RDR6)–RdDM pathway functioning to re-silence transcriptionally activate TEs [34] and/or diminished canonical RdDM activity in microspores and sperm cells [27]. Given our previous results demonstrating a crucial role of the common subunit of Pol IV/V (NRPD2A) and KYP in preventing *EVD* mobilization in self-fertilized mutant backgrounds [19], we were surprised the maternal suppression persisted in gametophytes with NRPD2A and KYP mutant genotypes. Therefore, NRPD2A and KYP most likely contribute to sporophytic maternal suppression of *EVD* replication before gametophyte development. If small RNAs are produced within maternal sporophytic tissue of *EVD*-active individuals, AGO9-mediated transposon silencing acting in the somatically derived megaspore mother cell could be activated [35]. We speculate such pre-meiotic silencing would facilitate the maternal *EVD* suppression observed here, independent of the subsequent genotype of the maternal gametophyte. Indeed, NRPD2A activity is involved in restricting the movement and transgenerational accumulation of the heat-activated *ONSEN* retrotransposon within sporophytic tissues before gametogenesis [36]. In this case, however, as retrotransposition took place before separation of male and female organs in the flower, the parental effect was not expected and thus was not analysed.

Because the observed maternal restriction of *EVD* level occurs only within the maternal germ line but does not affect paternally transmitted *EVD* activity, we conclude that maternally-produced small RNAs do not protect the embryo against *EVD* proliferation after fertilization. This conclusion is supported by data showing that *de novo* DNA methyltransferases are expressed during female gametogenesis [37] but are barely detectable in microspores and sperm cells [27], despite that RdDM activity becomes highly active during embryogenesis resulting in DNA methylation levels equivalent to the adult sporophytic phase [27,37]. This example of retrotransposon control is in contrast to the transposon immunity provided by maternal-derived Piwi-interacting RNA that repress paternally active TEs in *Drosophila* [38,39].

In summary, we report that after loss of transcriptional silencing and subsequent activation of *EVD* retrotransposition, transgenerational proliferation depends on the source of the parental gametes. In contrast to the suppressive effect conferred by epigenetic control within the maternal sporophytic tissue, the rapid expansion observed through paternal germ line transmission illustrates how retrotransposons can amplify and proliferate within the plant populations through pollen dispersal. This proliferation in turn supports the accumulation of *de novo* variation, and the cost/benefit balance of TEs continues to influence genome evolution as natural selection proceeds.

METHODS

Plant Material. The epiRILs [22] and mutant lines (*ago5-1*, *kyp-7 nrpd2a-2*) are all in the Columbia background. See supplementary information online for *EVD*-active and *EVD*-silent lines selection.

EVD DNA Content Analyses. For Southern blot analysis, DNA was extracted from leaf tissue with the DNeasy MaxiPrep kit (Qiagen) and treated as previously described [19] using 5 µg of DNA. For DNA content analysis DNA was extracted from individual mature leaf tissue as previously described [22] and quantified by quantitative polymerase chain reaction. Assays are described in supplementary information online.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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