Aberrant imprinting may underlie evolution of parthenogenesis

Olga Kirioukhova1,2, Jubin N. Shah1, Danaé S. Larsen3, Muhammad Tayyab1, Nora E. Mueller7, Geetha Govind1,2,10, Célia Baroux3, Michael Federer3, Jacqueline Gheysselinck7, Philippa J. Barrett3,11, Hong Ma1,4,5, Stefanie Sprunck2, Bruno Huettel7, Helen Wallace6, Ueli Grossniklaus4 & Amal J. Johnston1,2,3,9

Genomic imprinting refers to epigenetic gene regulation that leads to the parent-of-origin-specific expression of alleles, and it was proposed to differentially control offspring development1 reviewed in 1. Because genomic imprinting causes parental genomes to be non-equivalent, it prevents parthenogenetic embryo development by enforcing contribution of both parental genomes2. In flowering plants, most imprinted genes are mono-allelically expressed in the embryo-nourishing endosperm tissue, and a few are imprinted in the embryo3 reviewed in 1. Imprinting mechanisms may also serve as barriers to inter-specific or inter-ploidy hybridization in sexual plants, perhaps as a sensor that detects the correct parental gene dosage2 reviewed in 1. In contrast, apomicts can tolerate a skewed parental genome constitution, e.g. absence of the paternal genome in the embryo and sometimes an altered parental genome dosage in the endosperm4,6.

DNA and histone methylation play predominant roles in genomic imprinting. An imprinted and paternally expressed MADS-box gene encoding the transcription factor PHERES1 (PHE1) promotes embryo growth and is selectively repressed by a H3K27me3 histone methyltransferase MEDEA (MEA), which restricts growth in the sexual species Arabidopsis thaliana1,4,7. The contrasting imprinting effects between MEA and PHE1 lend support to the parental offspring theory4. Since parthenogenetic embryos lack a direct paternal contribution, we hypothesized that a relief of imprinting may have played a role in the evolution of parthenogenesis in plant species that reproduce asexually through seeds via apomixis. The Boechera genus is closely related to Arabidopsis, belonging to the same major clade within the Brassicaceae phylogeny6, and it consists of both sexual and apomictic (parthenogenetic) populations8. The genetic basis of parthenogenesis in Boechera is currently unknown. Here, we asked whether changes in the status of imprinting are involved in parthenogenesis in Boechera. For this, we analysed the spatio-temporal expression pattern and DNA methylation status of the Boechera homolog of PHE1, which is a paternally-expressed imprinted gene in Arabidopsis. We examined a diploid sexual B. stricta (Sex-1) and a triploid

1University of Heidelberg, Centre for Organismal Studies, Laboratory of Germline Genetics & Evo-Devo, Heidelberg, Germany. 2Jacobs University, Life Sciences & Chemistry, Laboratory of Germline Genetics & Evo-Devo, Bremen, Germany. 3University of Zurich, Department of Plant and Microbial Biology and Zurich-Basel Plant Science Center, Zurich, Switzerland. 4The Pennsylvania State University, the Huck Institute of Life Sciences, Department of Biology, the University Park, Pennsylvania, USA. 5Fudan University, State Key Laboratory of Genetic Engineering, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai, China. 6University of Regensburg, Cell Biology and Plant Biochemistry, Regensburg, Germany. 7Max-Planck-Institute for Plant Breeding, Cologne, Germany. 8University of the Sunshine Coast, Faculty of Science, Health, Education and Engineering, Genealogy Research Centre, Maroochydore, Australia. 9ETH Zurich, Department of Biology and Zurich-Basel Plant Science Center, Zurich, Switzerland. 10Present address: University of Agricultural Sciences, College of Agriculture Sciences, Department of crop physiology, Hassan, India. 11Present address: New Zealand Institute for Plant and Food Research, Christchurch, New Zealand. Jubin N. Shah and Danaé S. Larsen contributed equally to this work. Correspondence and requests for materials should be addressed to U.G. (email: grossnik@botinst.uzh.ch) or A.J.J. (email: a.johnston@jacobs-university.de)
Apomictic reproduction requires two major alterations of the sexual pathway: meiosis is avoided to generate unreduced gametes, followed by parthenogenesis enabling embryo development without a paternal contribution. In apomictic Boechera, both female and male meioses are equally circumvented (Supplementary Figs 1 and 2 and explanations therein) and the female and male gametogenesis produce unreduced egg and sperm cells, respectively (Supplementary Fig. 3). In Arabidopsis, EGG-CELL 1.1 (EC1.1) peptides accumulate in the egg cell prior to fertilization and prevent multiple sperm fusions likely through male-female signalling processes\(^5\). Abundant transcripts of EC1.1 were detected in Boechera by heterologous mRNA in situ hybridization with an Arabidopsis probe. The Boechera EC1.1 was expressed in the egg cell before fertilization in the sexual Sex-1 line as well as at the onset of parthenogenesis in the apomictic Apo-1 line (Fig. 1a,b). This likely reflects the requirement of egg-sperm signalling in the apomict similarly to the sexual Arabidopsis\(^1\). Preventing pollination in Apo-1 did not lead to parthenogenetic embryo development (Fig. 1b), further supporting the view that some aspects of fertilization are necessary for parthenogenesis in Boechera. In the sexual Sex-1 as well as the apomict Apo-1, the central cell exhibited weak but detectable signal of MEA transcripts (Fig. 1c,d) similar to Arabidopsis\(^12\), in which it has been shown to prevent autonomous divisions in the central cell\(^12\). In both the sexual Sex-1 and the apomicts, Apo-1 and Apo-2, the pollen tube enters the embryo sac, and the two sperm cells each target the egg and central cell, respectively (Fig. 11–p). Regardless of the reproductive mode, nuclear fusion occurs between one of the two sperm cells and the central cell, which is followed by primary endosperm divisions (Fig. 11–n). However, unlike egg-sperm karyogamy leading to zygote formation in the sexual (Fig. 11,l), the second sperm nucleus in the apomicts persisted in the vicinity of the nucleus of the egg but no fusion occurred even at a stage when mitotic divisions in the endosperm advanced (Fig. 1k.m–p). This observation suggests that presence of the sperm near the egg cell of the apomict might serve as activation source to induce pseudogamous parthenogenesis. The resulting parthenogenetic embryos were indistinguishable from the sexual ones in terms of morphology and expression of the cell-division marker gene CYCB1;1 (Fig. 1q,r). Furthermore, self-pollination in Boechera apomicts seems to be required for maintaining genome integrity in the progeny. The majority of the individuals arising from inter-specific pollination between apomicts displayed a range of genomic alterations and partial breakdown of genome integrity (Supplementary Fig. 4 and discussions therein). Some of the progeny that was of solely maternal genotype (i.e. of parthenogenetic origin) upon inter-specific pollination exhibited an array of vegetative and reproductive defects including self-incompatibility, which was not observed in the apomictic self-progeny. Global epigenetic changes in the maternal genome could possibly account for such morphological aberrations in clonal offspring. Collectively, our observations suggest that male cues from the self-parent are likely essential at fertilization for the initiation of pseudogamous parthenogenesis, and might be necessary for the maintenance of epigenetic states.

To understand the impact of apomictic mode of development on genomic imprinting we analysed the PHERES\(^1\) locus of Boechera. In Arabidopsis, the maternal allele is silenced by MEA, but the paternal allele is expressed during seed development\(^17\). Following incompatible hybridizations, the maternal PHE1 allele can be de-repressed\(^15\). We identified two Boechera homologs of PHE1: PHERES-LIKE 1 (PHEL1) and PHEL2. PHEL1 has up to 64% amino acid sequence identity with Arabidopsis PHE1 or PHE2 (Supplementary Fig. 5). PHEL2 represents a pseudo-gene without detectable expression (Supplementary Fig. 6a). In order to characterize the imprinting status of PHEL1 in Boechera sexuals and distinguishing the maternal and paternal alleles, we used another sexual diploid species, B. perennis (Sex-2) offering a sequence polymorphism in PHEL1. In reciprocal crosses between Sex-1 and Sex-2, we analyzed allele-specific expression of PHEL1 by RT-PCR experiments. Only the maternal allele of PHEL1 is expressed in the embryo and endosperm tissues of the Boechera sexuals (Fig. 2a, Supplementary Fig. 6b,c), unlike its Arabidopsis counterpart PHE1\(^14\). It is likely that expression of the maternal PHEL1 allele results from a switch in the state of imprinting within sexual Boechera species, which likely arose in response to hybridization-driven speciation and subsequent genome modifications as previously proposed\(^17\).

In sexual Boechera species, PHEL1 is expressed at very low levels both in the female (gynoecia) and male (anthers) reproductive organs, yet expression in the mature female tissues is nearly three-fold greater than in the male (Fig. 2b). PHEL1 expression could not be detected in situ in sexual ovules due to its low abundance, but the levels of the corresponding transcripts were quantified by qRT-PCR. PHEL1 transcripts were abundant in the gynoecia and siliques of Apo-1 and Apo-2; the asexual gynoecia had up to 40-fold higher PHEL1 expression levels compared to the sexual ones irrespective of the ploidy level of the apomict (Fig. 2b). In addition, faint but specific in situ expression of PHEL1 was detected in the apomictic embryo sac of Apo-1 (Fig. 1g, compare to Sex-1 in Fig. 1e and to the sense probe in Supplementary Fig. 6d). PHEL1 expression in the apomicts showed 250–400-fold higher transcript levels in female than male floral organs and in the embryos compared to the levels of expression in the sexual (Fig. 2b). We propose that these high levels of the maternal PHEL1 transcript prior and during embryo development may play a role in parthenogenetic contribution.

In Arabidopsis, METHYLTRANSFERASE 1 (MET1) is pivotal for maintenance of CG DNA methylation, while CHROMOMETHYLASE 3 (CMT3) maintains non-CG-methylation; and DOMAINS-REARRANGED METHYLTRANSFERASE1 and 2 (DRM1/2) control RNA-dependent DNA methylation (RdDM) de-novo in all contexts\(^18\). Their function is thought to be critical for epigenetic reprogramming and genomic imprinting during gametogenesis and seed development. When we examined genes expression levels of the corresponding Boechera homologs, we noticed a complex situation with respect to common and/or taxon-specific expression patterns of genes coding for DNA methyltransferases (Fig. 2d, Supplementary Fig. 7). In apomicts, MET1 was marginally lower in gynoecia, and significantly down-regulated in anthers, in comparison to the sexual lines (Fig. 2c). This situation persisted after fertilization in Apo-1. DRM2 was significantly upregulated in gynoecia and siliques of both apomicts (Fig. 2d). In Arabidopsis, DNA methylation at the PHE1 locus is regulated by cytosine methylation machinery involving MET1 and DRM2, and influences its parental expression levels\(^15\). Taken together, reduced
Figure 1. Pseudogamous parthenogenesis in *Boechera* is accompanied by sexual-like gene expression patterns and deregulation of a MADS-box gene. (a,b) Heterologous mRNA *in situ* signals of EC1.1 in sexual versus parthenogenetic egg cells. Arrow-heads: red – egg cell, green – synergids, white – central cell nuclei. (c,d) MEA signals in central cells. (e–g) Heterologous signals of PHELI in apomictic versus sexual embryo sacs. (h) An apomictic egg cell of Apo-1 (red arrow-head) at 3 days after emasculation. (i–n) Fertilized ovules (dark-blue arrow-heads – pollen tube entry, black arrow-heads – endosperm). (m,n) An unfused sperm nucleus (light-blue arrow-head) is visible proximal to the parthenogenetic egg cell (red arrow-heads). (o,p) Confocal micrographs of Apo-1 ovaries at fertilization. (o) Two sperm cells (light-blue arrow-heads) discharged into an apomictic embryo sac. (p) Sperm cell arrival coincides with polar nuclei fusion (white arrow-head). (q,r) CYCB1;1 mRNA *in situ* signals at one-celled embryo stage (red arrow-heads). Scale bars in (a–r) 20 µm.
MET1 and increased DRM2 levels in female floral tissues might provide an explanation for high levels of maternal PHEL1 in Boechera lines. Furthermore, in seedlings of the Boechera apomicts, increased PHEL1 expression also correlated with upregulation of DRM2 and down-regulation of MET1 (Supplementary Fig. 7d–f). Although cell-type-specific comparative gene expression and DNA methylation profiling remains to be elucidated (and poses a significant challenge in non-model systems like Boechera) we propose that MET1/DRM2-mediated DNA methylation changes might be responsible for the elevated expression of PHEL1 in Boechera apomicts.

We tested by bisulfite sequencing whether the active maternal PHEL1 allele in apomictic Boechera exhibits DNA methylation footprints distinct from those in the sexual. We found that the most distal 3′ region ca. 2 kb downstream of the PHEL1 gene showed strong DNA methylation in a non-CG context in both sexual and apomictic gynoecia (3′-#3, Fig. 3b). Intriguingly, we found that a 0.6 kb DNA fragment distal to the PHEL1 gene (3′-#2) is present only in the Sex-1 line, and is heavily methylated primarily in CG but also in non-CG contexts (Fig. 3a,b). This methylated region (3′-#2, or 3′MR) consisted of several repeats (Supplementary Fig. 8). The 3′MR was absent in an Apo-1 PHEL1 allele; a similar deletion was also found in Apo-2. In brief, a heavily methylated distal DNA fragment was absent in two apomict-specific PHEL1 alleles but present in a sexual, and this deletion positively correlated with elevated PHEL1 expression in the apomicts.

Apomixis is reported in only about 0.5% of the Brassicaceae genera, which mostly occur in extreme environmental conditions (discussed in 10). In particular, the North-American Boechera species are likely to have arisen from millions of years of evolutionary bottlenecks and reticulate evolution. Hybridization between sexual Boechera genotypes may have paved the way for genomic imprinting to become relieved from tight control, based on a genomic landscape with two contrasting genomes17,19. Ultimately, some Boechera hybrids may have had an epigenetic environment conducive for the evolution of novel apomictic traits, such as parthenogenesis. Existence of multiple independently evolved apomictic Boechera population allow us to propose that some convergent (epi)genetic mechanisms may play a prominent role here. Our findings suggest that parent-of-origin expression of PHEL1 or PHEL15 across genera is highly correlated with DNA methylation pattern of the corresponding loci; however, this regulation is modified in Boechera in terms of a) reversion of the imprinting status resulting in expression of the maternal PHEL1 allele; and b) deletion of the heavily methylated 3′MR in the alleles specific to apomicts, with a concomitant increase in expression. Figure 3c proposes a model where distinct epigenetic regulation of PHEL1/PHEL1 based on DNA methylation may have enabled parthenogenesis to evolve in Boechera. Our findings in Boechera show similarity to an artificially induced case of parthenogenesis in mice20, where loss of distal DNA methylation causing maternal activation of the paternally expressed Insulin-like growth factor 2
The *Igf2* gene was sufficient to induce parthenogenesis. We thus propose that alterations in the control of genomic imprinting enable the adjustment of parental gene dosage necessary for parthenogenesis to evolve.

**Materials and Methods**

**Plant material and growth conditions.** Diploid sexual and/or triploid asexual *Boechera* seeds were kind donations from various sources. Both triploid *Apo-1* and diploid *Apo-2* were first analyzed for ploidy by bulked seed flow-cytometry, which revealed the presence of occasional 6 C (hexaploid) and 4 C (tetraploid) embryo peaks, respectively. The offspring seedlings were ploidy-analyzed using a FacsCantoII cytometer (BD Biosciences, USA), and rare hexaploid *Apo-1* plants were eliminated from further analyses. Single seed flow-cytometry gave an over-estimate of apomeiosis per plant as only the fertile seeds were taken for analyses; therefore, expressivity of apomeiosis and parthenogenesis was determined by individual seed ploidy analyses by flow-cytometry, and subsequently by ovule clearing and seed counts. Plants were grown under long-day conditions at 18–21°C.

**RNA extraction, cDNA synthesis, real-time qRT-PCR.** RNA isolation and cDNA synthesis were performed as described. Locus-specific fragments across all *Boechera* strains were PCR-amplified based on conserved *Arabidopsis* and *Boechera* sequences available from public repositories (NCBI, Phytozome) and sequenced. We cloned the entire *PHEL1* and *PHEL2* loci in all *Boechera* species analyzed here based on a *Sex-1*-specific template. Allele-specific *PHEL1* transcript fragments were genotyped upon *BstUI* digestion (New England Biolabs, USA). qRT-PCR SYBR Green assays were performed in a StepOnePlus Real-Time-PCR System (Applied Biosystems, USA) with three biological replicates and normalized as described using *RPS18* gene as a reference. Primer sequences are given in Supplementary Table 1.

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**Figure 3.** DNA methylation analysis of the *PHEL1* loci in a sexual and an apomictic *Boechera* line, and a proposed epigenetic model of *PHEL1* regulation in sexual (sex) versus apomictic (apo) lines. (a,b) Scheme of *PHEL1* loci and corresponding percent methylation identified by bisulfite-sequencing. (c) An illustration of the proposed epigenetic regulation of *Arabidopsis PHE1* versus *Boechera PHEL1* by DNA/histone methylation. PRC2, Polycomb Repressive Complex 2 containing the MEA histone methyltransferase.
**DNA methylation analysis by bisulfite sequencing.** DNA methylation conversion was performed using Epifect Bisulfite kit (Qiagen, Germany). For each library, 8–10 egg cell-containing gynoecia at the stage just prior to fertilization were processed according to the manufacturer instructions yielding two BS-seq libraries (Sex-1 120 ng and Apo-1 200 ng). Library was sequenced using standard Illumina 2500 pipeline at the MPIZ Genome Centre, Cologne, Germany. In brief, library quality check was performed with FastQC method. BS-seq was set to 6 Gb for Sex-1 and 18 Gb for Apo-1 aiming 30× genome coverage. Conversion efficiency was evaluated using Bismark alignment and methylation caller21. Detected genome-wide methylation levels of cytosines in the CG, and non-CG contexts were 19.6% and 8.1%, respectively, which was similar to Arabidopsis22 and indicated a very good level of bisulfite conversion; the high conversion efficiency was further confirmed by detecting long stretches (ca. 0.5–1 kb) of fully bisulfite converted DNA. Sequence reads were mapped to the corresponding PHEL1 sequences from Sex-1 and Apo-1 using Bismark platform.

**Heterologous in situ mRNA hybridization.** Arabidopsis-specific heterologous in situ probes were prepared from corresponding cDNA clones; and mRNA in situ hybridization23 was modified to include an additional RNase A treatment step (20 μg/ml for 30 min incubation at 37 °C) to remove unspecific background signals. The mRNA in situ mRNA hybridization worked efficiently, particularly when the transcripts were abundant. For MEA and PHE1 RNA probes, it was necessary to add hybridization solution on slides during each day for up to three days to enhance its very weak signal; the specificity of probe binding was ensured by RNase A treatment (see above). In the case of probing against PHERES-like genes in Boechera, although the in situ probe used cannot distinguish gene-specific transcripts due to a high degree (~80%) of nucleotide identity between PHEL1 and PHE2, we are confident that we indeed detected PHEL1-specific transcripts in situ because PHE2 signals were barely detectable even in qRT-PCR assays. Ovule and seed clearing, DIC and confocal microscopy upon propidium-iodide staining, and image analyses using Imaris (Bitplane, Switzerland) were performed as described24,25.

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Author Contributions
A.J.J. and U.G. conceived the idea, and A.J.J. designed the study. Sequencing of genomic loci, cytology, in situ mRNA hybridization (I.S.H.), DNA methylation and real-time qRT-PCR analyses across several Boechera strains were performed by O.K., J.N.S., D.S.L., M.T., N.E.M., L.L., G.G., B.H., H.W., and A.J.J., J.G. and M.F. contributed in ISH (for PHEL1) and flow cytometry experiments, respectively. H.M., C.B., P.B. and O.K. performed the cytological analyses of meiosis and fertilization events. S.S. provided an unpublished gene probe of EC1. O.K. and A.J.J. wrote the manuscript, which was further text-edited by H.W., H.M., C.B. and U.G.

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