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## Chemically-induced epimutagenesis allows bypassing reproductive barriers in hybrid seeds

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1 **Chemically-induced epimutagenesis allows bypassing**  
2 **reproductive barriers in hybrid seeds**

3

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14

15 **Short title:** Bypassing the triploid block with 5-Azacytidine

16

17 **One sentence summary:** Genome-wide loss of DNA methylation induced by 5-Azacytidine  
18 allows bypassing interploidy and interspecific hybridization barriers in Arabidopsis and  
19 Capsella.

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## 1 **Abstract**

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3         The "triploid block" prevents interploidy hybridizations in flowering plants, and is  
4 characterized by failure in endosperm development, arrest in embryogenesis, and seed  
5 collapse. Many genetic components of triploid seed lethality have been successfully identified  
6 in the model plant *Arabidopsis thaliana*, most notably the paternally expressed imprinted  
7 genes (PEGs) that are up-regulated in the tetraploid endosperm with paternal excess. Previous  
8 studies have shown that the paternal epigenome is a key determinant of the triploid block  
9 response, as the loss of DNA methylation in diploid pollen suppresses the triploid block  
10 almost completely. Here, we demonstrate that triploid seed collapse is bypassed in  
11 *Arabidopsis* plants treated with the DNA methyltransferase inhibitor 5-Azacytidine during  
12 seed germination and early growth. We have identified strong suppressor lines showing stable  
13 transgenerational inheritance of hypomethylation in CG context, as well as normalized  
14 expression of PEGs in triploid seeds. Importantly, differentially methylated loci segregate in  
15 the progeny of "epimutagenized" plants, which may allow the identification of epialleles  
16 involved in the triploid block response in future studies. Finally, we demonstrate that  
17 chemically-induced epimutagenesis allows bypassing hybridization barriers in crosses  
18 between different *Capsella* species, thus potentially emerging as a novel strategy for  
19 producing triploids and interspecific hybrids with high agronomical interest.

20

## 21 **Introduction**

22

23         Early studies in plants showed some of the first evidence of distinctive phenotypes  
24 dependent on the nature and dosage of parental chromosomes (Belling and Blakeslee, 1923;  
25 Blakeslee et al., 1920). This phenomenon, currently known as heterosis, is often observed in  
26 interploid and interspecific hybrids that display phenotypic values exceeding those in their  
27 parents, but its genetic and epigenetic basis remain poorly understood (Birchler et al., 2010;  
28 Hochholdinger and Baldauf, 2018). Plant breeders have been exploiting heterosis for  
29 thousands of years to create elite varieties of domesticated crops with enhanced growth and  
30 yield. However, more progress has been hindered by the existence of strong reproductive  
31 barriers that prevent heterosis, and a faster introgression of valuable alleles from wild species  
32 into domesticated cultivars (Kaneko and Bang, 2014).

33

1 In many angiosperms, interploidy crosses between diploid females and tetraploid males  
2 lead to abnormal endosperm development and seed collapse, which is known as the “triploid  
3 block” response (Köhler et al., 2021). The endosperm of most flowering plants is an essential  
4 triploid tissue that nourishes early embryo development in the seed, and is formed in the  
5 embryo sac by fertilization of the diploid central cell by an haploid sperm cell (Dresselhaus et  
6 al., 2016). Endosperm cellularization is a critical event for seed development, and relies on  
7 the correct balance between the dosage of maternal and paternal genomes (2m:1p), and  
8 distinct epigenetic features that are pre-established in the gametes to control genomic  
9 imprinting after fertilization (Gehring and Satyaki, 2017; Kawashima and Berger, 2014).  
10 Maternally expressed genes (MEGs) encode components of the Polycomb Repressive  
11 Complex 2 (PRC2), which is essential to silence the maternal allele of paternally expressed  
12 genes (PEGs) via deposition of tri-methylation at lysine 27 of histone 3 (H3K27me3)  
13 (Gehring and Satyaki, 2017; Köhler and Lafon-Placette, 2015). For a long time, this  
14 mechanism was sufficient to explain how imprinted gene expression is established in the  
15 endosperm. However, additional models emerged with the discovery of a paternally-inherited  
16 small RNA-directed DNA methylation (RdDM) pathway that is also involved in genomic  
17 imprinting (Vu et al., 2013; Calarco et al., 2012).

18  
19 The triploid block traces back to the Endosperm Balance Number (EBN) hypothesis,  
20 which was developed in the early 80’s in potato and then extended to many other crops  
21 (Johnston and Hanneman, 1982; Ehlenfeldt and Hanneman, 1984). The EBN, or “effective  
22 ploidy”, is the ratio between maternal and paternal chromosomes or genetic factors required  
23 for development of a normal seed (Carputo et al., 1999, 1997; Johnston and Hanneman, 1982;  
24 Ehlenfeldt and Hanneman, 1984). These studies are highly relevant for plant breeding, as the  
25 success of interspecific and intergeneric hybridizations may be predicted and manipulated  
26 based on the EBN of each parent (Tonosaki et al., 2018), although this system seems to be  
27 restricted to only certain genera (Carputo et al., 1999). Additional breeding strategies to  
28 overcome hybridization barriers require ovule/ovary cultures and embryo rescue techniques  
29 prior to seed collapse, which have limited efficiency depending on the species (Cisneros and  
30 Tel-Zur, 2010; Sauer and Friml, 2008; Eeckhaut et al., 2006). In the model plant *Arabidopsis*  
31 *thaliana*, loss-of-function mutations in PEGs are able to suppress the triploid block (Kradolfer  
32 et al., 2013; Batista et al., 2019; Wolff et al., 2015). This clearly indicates that endosperm  
33 failure during interploidy hybridization results from up-regulation of PEGs, which is also  
34 observed in crosses between different *Arabidopsis* species (Kirkbride et al., 2015; Josefsson

1 et al., 2006), and may have role in establishing interspecific hybridization barriers as well  
2 (Lafon-Placette et al., 2018). However, its potential application in plant breeding is limited, as  
3 imprinted genes are generally not well conserved (Kradolfer et al., 2013; Rodrigues and  
4 Zilberman, 2015), in a way that studies in Arabidopsis cannot be directly tested in crops.  
5 More recently, several studies have shown that the paternal epigenome triggers the triploid  
6 block response in Arabidopsis, which provided new ideas for plant breeding. Loss of DNA  
7 and histone methylation, as well as small-interfering RNAs (siRNAs) in diploid pollen  
8 restored viability of triploid seeds almost completely (Martinez et al., 2018; Borges et al.,  
9 2018; Erdmann et al., 2017; Schatlowski et al., 2014; Jiang et al., 2017; Satyaki and Gehring,  
10 2019), suggesting that the paternal epigenome mediates genomic imprinting and endosperm  
11 balance in the developing seed.

12

13 Here, we show that transient genome-wide epimutagenesis induced by the chemical  
14 inhibitor of DNA methylation 5-Azacytidine allows bypassing the triploid block in  
15 Arabidopsis. We identified and characterized strong epigenetic suppressors of the triploid  
16 block response, showing stable transgenerational loss of CG methylation, and down-  
17 regulation of imprinted genes that are well-known triggers of triploid seed collapse. Finally,  
18 we demonstrate that epimutagenesis induced by 5-Azacytidine allows bypassing hybridization  
19 barriers in crosses between Capsella species, thus potentially emerging as a new method to  
20 facilitate the production of triploid plants and interspecific hybrids with high socio-  
21 economical interest for agriculture and crop improvement.

22

## 23 **Results**

24

### 25 Exposure to 5-Azacytidine during seed germination and early growth allows bypassing the 26 triploid block

27

28 Chemical inhibition of DNA methyltransferases has been widely used to study the  
29 function of DNA methylation in several plant systems (Pecinka and Liu, 2014). For instance,  
30 cytosine analogs such as 5-Azacytidine and Zebularine are incorporated into newly replicated  
31 DNA, but do not become methylated (Pecinka and Liu, 2014), in a way that DNA  
32 methylation is passively erased during cell divisions (Jones and Taylor, 1980; Creusot et al.,  
33 1982; Santi et al., 1984). Recent whole-genome analysis of DNA methylation in Arabidopsis  
34 has shown that both chemicals lead to widespread loss of DNA methylation in all sequence

1 contexts, and in a dose-dependent manner (Griffin et al., 2016), which can be stably inherited  
2 into subsequent generations or fully restored before fertilization (Baubec et al., 2009;  
3 Akimoto et al., 2007).

4  
5 In order to test if epigenetic variation induced by 5-Azacytidine allows suppressing  
6 the triploid block response, we used the Arabidopsis mutant *jason* (Storme and Geelen, 2011;  
7 Erilova et al., 2009). In our growth conditions, plants homozygous for the *jas-3* allele in Col-0  
8 background produce 30-40% of diploid pollen (Supplemental Figure 1), while the female  
9 gametophyte is haploid. This way, the triploid block response may be quantified after self-  
10 fertilization. In untreated *jas-3* plants and DMSO-treated controls, triploid seed abortion  
11 varied between 30 and 40% (Figure 1A), thus reflecting the amount of diploid pollen in these  
12 plants (Supplemental Figure 1). Strikingly, plants treated with different concentrations of 5-  
13 Azacytidine showed a variable and dose-dependent effect in the triploid block response  
14 (Figure 1A), which was significantly reduced to ~20% in plants treated with 100µg/mL of the  
15 chemical (Figure 1A). In particular lines (e.g. Aza1), seed collapse was reduced to less than  
16 10% (Figure 1A), while the amount of diploid pollen remained unchanged (Supplemental  
17 Figure 1), thus suggesting strong suppression of the triploid block response. Close inspection  
18 of the seed set showed the presence of enlarged seeds (Figure 1B) that were routinely  
19 confirmed to be triploids by flow cytometric analysis of ploidy (Supplemental Figure 2).

20  
21 In Arabidopsis, known epigenetic suppressors of the triploid block show a suppressive  
22 effect only when paternally inherited (Schatlowski et al., 2014; Borges et al., 2018; Martinez  
23 et al., 2018; Satyaki and Gehring, 2019). We therefore asked if there is also a parental effect  
24 in triploid block suppression caused by 5-Azacytidine treatment, and tested this hypothesis by  
25 performing reciprocal crosses between DMSO control plants and the strong suppressor line  
26 Aza1. Indeed, we found that the suppressive effect caused by 5-Azacytidine is transmitted via  
27 the paternal genome (Supplemental Figure 3).

28  
29 We then selected diploid seeds from individual 5-Azacytidine-treated lines to inspect  
30 the stability of the suppressive effect in the next generation (Supplemental Figure 4), which  
31 will be hereafter designated as eM1 (epimutagenized population 1). Seeds from six individual  
32 plants of two suppressor lines (Aza1 and Aza14) and two non-suppressors (Aza5 and Aza26)  
33 were compared to the DMSO controls, to show that seed abortion and germination rates were  
34 similar to those observed in eM0 plants that had been directly exposed to the chemical (Figure

1 1C). Taken together, these results demonstrate that exposing *jas-3* plants to 5-Azacytidine  
2 during seed germination and early growth allows bypassing the triploid block response at  
3 variable levels, in a dose-dependent and transgenerational manner.

#### 4 5 Genome-wide CG hypomethylation observed in suppressor lines

6  
7 Whole-genome bisulfite sequencing (WGBS) was performed to test whether  
8 suppression of the triploid block in 5-Azacytidine-treated *jas-3* plants correlated with the loss  
9 of DNA methylation (Supplemental Data Set 1). Therefore, comparative methylome analyses  
10 were performed between the strong suppressor lines Aza1 (3,7% collapsed seeds), Aza14  
11 (11,3%), Aza18 (6,6%) and Aza25 (6,3%), and the non-suppressor lines Aza5 (27,8%),  
12 Aza10 (30,7%), Aza16 (26,9%) and Aza26 (28,7%) that showed levels of seed collapse closer  
13 to the controls *jas-3* (mean 33,7%) and DMSO (mean 33,4%). Indeed, in suppressor lines,  
14 strong loss of CG methylation was observed at protein-coding genes and transposable  
15 elements (TEs), as compared to untreated *jas-3* and DMSO controls (Figure 2A and  
16 Supplemental Figure 5). Analysis of differentially methylated regions (DMRs) allowed the  
17 identification of 6393 hypomethylated CG DMRs among all suppressor lines (Figure 2B),  
18 which occurred primarily at protein-coding genes (Figure 2C and Supplemental Data Set 2).  
19 Interestingly, this analysis also showed variable patterns of hypomethylation in the different  
20 suppressors (Figure 2B), as the overlap between the three strongest suppressors (Aza1, Aza18  
21 and Aza25) is limited to ~1/10 of the amount of DMRs detected in each individual line  
22 (Figure 2D). In contrast, CG methylation levels were mostly unchanged in all non-  
23 suppressors (Figure 2A and Supplemental Figure 5), confirming that genome-wide levels of  
24 CG methylation correlate with the triploid block response. Importantly, cytosine methylation  
25 in CHG and CHH contexts was mostly unchanged in both suppressor and non-suppressor  
26 lines (Supplemental Figure 5 and Supplemental Data Set 1). This indicates that non-CG  
27 methylation was rapidly restored in the first generation after 5-Azacytidine treatment, and is  
28 likely not responsible for the suppressive effect.

29  
30 In DNA methylation mutants, such as *met1* and *ddm1*, ectopic CHG methylation is  
31 often observed in gene bodies (Stroud et al., 2013), and may lead to transcriptional gene  
32 silencing. Similarly, ectopic CHG methylation was observed at paternally expressed genes  
33 down-regulated in the endosperm of viable triploid seeds after pollination with diploid *met1*  
34 pollen (Schatlowski et al., 2014). It was hypothesized that ectopic CHG methylation at the

1 paternal *met1* genome leads to reduced expression of PEGs during endosperm development,  
2 thus contributing to viability of seeds with paternal excess (Schatlowski et al., 2014).  
3 Although this effect was not previously reported in plants treated with 5-Azacytidine (Griffin  
4 et al., 2016), we detected 95 hypermethylated CHG DMRs in the progeny of the strongest  
5 suppressor line Aza1 (Supplemental Figure 6). This indicates that newly formed epialleles  
6 with ectopic CHG methylation are induced by 5-Azacytidine treatment, and are stably  
7 inherited to the next generation. However, this effect was not consistently observed among  
8 the four suppressor lines tested (Supplemental Figure 6), suggesting that ectopic CHG is also  
9 not responsible for the suppressive effect.

### 11 The triploid block response and DNA methylation levels were partially restored two 12 generations after 5-Azacytidine treatment

13  
14 Epigenetic variation induced by 5-Azacytidine is known to be a transient effect  
15 (Pecinka and Liu, 2014), and only a few epialleles have been detected in subsequent  
16 generations after treatment (Akimoto et al., 2007). However, we found approximately 3000  
17 hypomethylated CG DMRs in the eM1 generation of independent suppressor lines (Figure  
18 2C, Supplemental Data Set 2), which confirms that hypomethylation induced by 5-  
19 Azacytidine is stable for at least one generation after treatment. In order to investigate further  
20 the transgenerational stability of the suppressive effect, two diploid eM1 siblings from the  
21 strongest suppressor Aza1 were selected and selfed (Aza1-1 and Aza1-2), and six diploid eM2  
22 plants were analyzed individually for each line (Supplemental Figure 1). Seed abortion and  
23 germination analysis showed clear signs of recovery of the triploid block response in both  
24 lines, although only Aza1-2 was significantly higher compared to the levels detected in the  
25 eM1 generation (Figure 3A). We also performed WGBS analysis of bulked seedlings from  
26 each eM2 line, and confirmed that CG methylation was fully restored at many loci (Figure  
27 3B), while ectopic CHG methylation was no longer detected (Supplemental Figure 6). This  
28 strongly suggests that a progressive recovery of CG methylation contributes to a stronger  
29 triploid block response in eM2 plants. However, additional analyses will be required to  
30 determine if DNA methylation and triploid block response are fully restored to *jas-3* levels in  
31 subsequent generations.

32  
33 Interestingly, some DMRs segregated in the eM2 generation, including a large region  
34 in chromosome 1 that restored CG methylation almost completely in Aza1-2, but remained



1 hypomethylated in Aza1-1 (Figure 3C). Perhaps such differences explain the higher levels of  
2 triploid block in Aza1-2 (Figure 3A), although the majority of DMRs in all five chromosomes  
3 restored CG methylation to similar levels in both lines (Figure 3B and 3C), thus indicating  
4 that certain epigenetic states were already fixed. Further analyses with methylomes of  
5 additional lines and in different generations will be required for a robust evaluation of  
6 epiallele segregation. However, since the triploid block response remained relatively low in  
7 eM2 lines as compared to DMSO controls (Figure 3A), we can conclude that DMRs that fully  
8 restored CG methylation in eM2 are likely not major contributors for the suppressive effect  
9 observed in the eM1 generation.

### 11 Paternally expressed genes are transiently down-regulated in suppressor lines

13 Previous studies in *Arabidopsis* and crops have shown that the triploid block leads to  
14 striking changes in the gene expression program of the developing triploid seed (Schatlowski  
15 et al., 2014; Stoute et al., 2012). Most notably, expression of many PEGs is up-regulated in  
16 abortive triploid seeds, and restored to wild-type diploid levels when the triploid block is  
17 genetically or epigenetically suppressed (Schatlowski et al., 2014; Kradofer et al., 2013;  
18 Batista et al., 2019; Satyaki and Gehring, 2019; Martinez et al., 2018; Wolff et al., 2015).  
19 Therefore, we performed transcriptome analysis by mRNA sequencing of developing siliques  
20 collected 6 to 9 days post anthesis, as previously described (Mizzotti et al., 2018).  
21 Comparisons between wild-type Col-0 (WT) and *jas-3* mutant siliques allowed to detect 668  
22 down-regulated and 1804 up-regulated genes in *jas-3* (Figure 4A, Supplemental Data Set 3).  
23 Similar analysis between WT and DMSO control plants showed a strong overlap between  
24 genes up- and down-regulated in untreated *jas-3* and DMSO control plants (Supplemental  
25 Figure 7 and Supplemental Data Set 3). These observations, together with a strong correlation  
26 between *jas-3* and DMSO replicates (Supplemental Figure 8), show that DMSO treatment  
27 does not induce major changes in the transcriptome of developing siliques.

29 We then compared suppressor Aza1 plants from eM1 generation with DMSO controls,  
30 and found 470 genes down-regulated in eM1, while only 48 were up-regulated (fold change  
31  $\geq 2$ ,  $p < 0.01$ ) (Supplemental Data Set 1). As expected, the vast majority of genes down-  
32 regulated in Aza1 eM1 plants (93%) overlapped with genes up-regulated in *jas-3* (vs WT)  
33 (Supplemental Figure 7). We also performed transcriptome profiling in siliques of four  
34 different siblings in the eM2 generation, as CG methylation and the triploid block response

1 were partially restored in these plants (Figure 3). Indeed, we found 368 genes significantly  
2 up-regulated in eM2 siliques as compared to eM1, while only 28 genes were found down-  
3 regulated in eM2 (fold change  $\geq 2$ ,  $p < 0.01$ ). Strikingly, most of the 368 genes up-regulated  
4 in eM2 (90%) overlapped with the genes down-regulated in eM1 plants (vs DMSO)  
5 (Supplemental Figure 7), and are thus strong candidates to explain triploid block suppression  
6 in plants treated with 5-Azacytidine. Indeed, well-known PEGs involved in the triploid block  
7 response were found among the down-regulated genes in the eM1 generation, such as  
8 *PHE1/PHE2* and *ADM* (Figures 4B and 4C) (Batista et al., 2019; Kradofer et al., 2013), for  
9 which expression is partially restored in eM2 (Figures 4B and 4C).

### 11 Chemically-induced hypomethylation allows bypassing interploidy and interspecific 12 hybridization barriers

14 Endosperm-based hybridization barriers are frequently observed during interploidy  
15 and interspecific crosses in a variety of plant species (Lafon-Placette and Köhler, 2016), and  
16 we asked if genome-wide epimutagenesis induced by 5-Azacytidine could suppress  
17 hybridization barriers in crosses between plants of different ploidy or between different  
18 species. To test this hypothesis, we first treated tetraploid Arabidopsis plants in Col-0  
19 background with 100 $\mu$ M 5-Azacytidine during seed germination. In agreement with the  
20 results in *jas-3* mutants, we obtained 40% of viable triploid seeds when using pollen from 5-  
21 Azacytidine-treated tetraploid plants to pollinate non-treated diploid Col-0 plants (Figure 5A),  
22 while only 10% of triploid seeds were viable in the control cross using pollen from DMSO-  
23 treated plants. These results show that triploid block suppression after treatment with 5-  
24 Azacytidine is not dependent on the method used to produce diploid pollen.

26 We then performed interspecific hybridizations between *Capsella rubella* and  
27 *Capsella grandiflora*, as crosses between these species resemble the triploid block when *C.*  
28 *grandiflora* is used as the male parent (Rebernik et al., 2015). *C. grandiflora* plants were  
29 treated with 100 $\mu$ M 5-Azacytidine, and pollen from treated plants was used to pollinate non-  
30 treated *C. rubella* plants. Strikingly, approximately 30% of hybrid seeds were plump and  
31 appeared normal, and about 40% of seeds were less severely collapsed, as compared to the  
32 control DMSO crosses that resulted in 100% completely collapsed seeds (Figure 5B). When  
33 these hybrid seeds were tested in germination assays, we found that about 15-20% of hybrid  
34 seeds could germinate in the crosses where the male parent was treated with 5-Azacytidine

1 (Figure 5B), while none of the seeds germinated in the control cross. Collectively, our data  
2 shows that DNA hypomethylation induced by 5-Azacytidine treatment can suppress hybrid  
3 seed lethality in both interploidy and interspecific crosses.

## 4 5 **Discussion**

6  
7 The triploid block is a classic example of dosage regulation, and our recent studies  
8 have demonstrated how plants use epigenetic mechanisms to sense and control parental  
9 genome dosage in crosses between parents with differing ploidy (Martinez et al., 2018;  
10 Borges et al., 2018; Schatlowski et al., 2014; Jiang et al., 2017; Wang et al., 2018; Dziasek et  
11 al., 2021; Florez-Rueda et al., 2021; Satyaki and Gehring, 2019). Differential DNA  
12 methylation between parental genomes is essential for seed development and genomic  
13 imprinting (Adams et al., 2000; Xiao et al., 2006; Choi et al., 2002), and DNA methylation of  
14 the paternal genome is required to trigger the triploid block in Arabidopsis (Schatlowski et al.,  
15 2014; Wang et al., 2021; Satyaki and Gehring, 2019). However, and despite the recent  
16 advances, the mechanistic aspects of this complex process remain largely unclear.

17  
18 In this study, we demonstrate that the triploid block is bypassed in epimutagenized  
19 plants treated with the cytosine analog 5-Azacytidine, which induces DNA hypomethylation.  
20 We found that the suppression level was highly correlated with a genome-wide and  
21 transgenerational loss of DNA methylation that occurred mostly in the CG context (Figure 2  
22 and Supplemental Figure 4). Interestingly, independent suppressor lines showed strong loss of  
23 CG methylation at different loci, while non-suppressors had almost normal levels of CG  
24 methylation (Figure 4B). The reason for this variability among treated plants remains to be  
25 explored, but one possibility is that only a fraction of plants treated with 5-Azacytidine is able  
26 to transmit DNA hypomethylation to their germline, and from there to the next generation.

27  
28 We were able to validate previous results showing that DNA hypomethylation of the  
29 paternal genome is what allows viability of triploid seeds (Schatlowski et al., 2014; Wang et  
30 al., 2021; Satyaki and Gehring, 2019). In previous studies, ectopic CHG methylation at PEGs  
31 was observed in the endosperm carrying a hypomethylated paternal genome derived from  
32 diploid *met1* pollen, and was associated with triploid block suppression (Schatlowski et al.,  
33 2014). However, only one of the suppressor lines in our study showed a small number of loci  
34 with ectopic CHG methylation (Supplemental Figure 6), thus suggesting that this mechanism

1 is unlikely responsible for PEG suppression in 5-Azacytidine-treated plants. Nevertheless,  
2 DNA methylation analyses in the developing endosperm of different suppressor lines will be  
3 required to finally evaluate this hypothesis.

4  
5 Notably, we found a significant number of DMRs overlapping between the three  
6 strongest suppressors lines Aza1, Aza18 and Aza25 (Figure 2D). It is tempting to speculate  
7 that a particular epiallele (or epialleles) involved in the triploid block is within this list of 253  
8 DMRs (Supplemental Data Set 2), although there is no clear overlap with genes up-regulated  
9 in *jas-3*, or down-regulated in eM1 (Supplemental Data Set 3). Alternatively, triploid block  
10 suppression may simply require that a certain amount of the genome remains hypomethylated,  
11 independent of location. Comparisons between plants in eM1 and eM2 generations support  
12 this idea, as DNA methylation was restored at only a fraction of hypomethylated CG DMRs  
13 detected in eM1 (Figure 3B), but the majority of genes down-regulated in eM1 were  
14 significantly up-regulated in eM2 (Supplemental Figure 7). This way, genome-wide CG  
15 methylation levels of the paternal genome could somehow function as a "ploidy sensor" in the  
16 developing endosperm, by attracting or repulsing epigenetic modulators of genomic  
17 imprinting. The most obvious candidates for this interplay are the paternal DNA methylation  
18 and maternal PRC2 pathways that are often mutually exclusive (Deleris et al., 2012; Rougée  
19 et al., 2020), and have been independently implicated in the triploid block (Erilova et al.,  
20 2009; Martinez et al., 2018; Satyaki and Gehring, 2019; Wang et al., 2021).

21  
22 Our work furthermore shows that chemically-induced epimutagenesis allows  
23 bypassing interspecific hybridization barriers in crosses between the *Capsella* species *C.*  
24 *rubella* and *C. grandiflora* (Figure 5B). Interestingly, CHG and CHH methylation was shown  
25 to decrease in *Capsella* hybrid endosperm, while CG methylation increased (Dziasek et al.,  
26 2021). However, it remains to be explored whether the increase in CG methylation is what  
27 causes hybrid seed defects in interspecific crosses, rather than the decrease in CHG and CHH  
28 methylation.

29  
30 In conclusion, our study demonstrates that 5-Azacytidine can be successfully used as a  
31 tool to facilitate the generation of triploid seeds and interspecific F1 hybrids in different plant  
32 systems. We believe this method could be applicable to a wide range of species, including  
33 crops with high agronomical interest, thus providing a convenient and cheap strategy to  
34 facilitate modern plant breeding.

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**Methods**

Plant growth and chemically-induced epimutagenesis

The mutant allele *jas-3* (SAIL\_813\_H03, Col-0 background) was used in this study. Diploid seeds from *jas-3* mutants were surface sterilized with 50% bleach for five minutes, rinsed with sterile deionized water, and sowed on agar plates containing 0.5X Murashige and Skoog (MS) medium, 1% sucrose, pH = 5.7 and different concentrations (25, 50 and 100µg/mL) of 5-Azacytidine (Sigma), under long day conditions (16h photoperiod). DMSO solvent was used as control treatment. Seedlings were transferred to soil after two weeks, and maintained in greenhouse long-day conditions to complete the life cycle.

Triploid block quantification

The triploid block was quantified by counting the number of aborted seeds in 5 siliques after selfing. The same set of seeds was then surface-sterilized using 50% bleach and ethanol, rinsed once with ethanol 96%, and air-dried. Seeds were then sown on agar plates containing 0.5X MS medium, 1% sucrose, pH = 5.7, then stratified for 2 days at 4 °C, and transferred to growth chambers under long-days conditions (16h day, 8h dark). Germination rate was initially quantified on 4 to 5-day-old seedlings, then adjusted after 7 days if necessary to account for germination delays.

Pollen ploidy analysis by flow cytometry

Pollen ploidy in *jas-3* mutants was analyzed by collecting open flowers from individual plants into eppendorf tubes, vortexing in 2mL of 100mM sodium phosphate buffer (pH 7) for 3min, and filtering through a 50µm nylon mesh. Pollen population is characterized by an elevated high angle scatter (SSC) and autofluorescence, which allows discrimination of haploid (1n) and diploid (2n) pollen, as previously described (Storme and Geelen, 2011;

1 Erilova et al., 2009). These two populations were gated and quantified (Supplemental Figure  
2 2). For ploidy analysis of nuclei, leaf tissue was chopped in 2mL of Galbraith buffer (45 mM  
3 MgCl<sub>2</sub>, 20 mM MOPS, 30 mM sodium citrate, 1 % (v/v) Triton X-100. Adjust pH to 7.0)  
4 using a razor blade, filtered through a 50µm mesh, stained with SYBR Green dye (Lonza),  
5 and analyzed on a CyFlow Space cytometer (Sysmex).

#### 6 7 Whole-genome bisulfite sequencing and DNA methylation analysis

8  
9 Bulked seed from each Aza line, two *jas-3* and two DMSO plants were germinated on  
10 MS plates, genomic DNA was isolated from ten pooled seedlings using the Quick DNA  
11 purification kit (Zymo), and library preparation and sequencing was performed by BGI  
12 Genomics (Hong Kong). Briefly, genomic DNA was fragmented by sonication, end-repaired  
13 and ligated to methylated adaptors. After bisulfite treatment, bisulfite-treated fragments were  
14 PCR amplified and sequenced as paired-end 100bp reads (PE100) with DNBSEQ technology  
15 (BGI). Pre-processed and high-quality reads were mapped to the TAIR10 genome using  
16 bismark with default settings for paired-end libraries (Krueger and Andrews, 2011), and all  
17 figures and downstream analysis were performed using R. DMRs in CG context were defined  
18 as 100-bp bins containing at least 4 differentially methylated CGs, and with an absolute  
19 methylation difference of 0.4. DMRs localizing within 200 bp of each other were merged.  
20 The statistical significance of the observed overlaps between differentially methylated 100bp  
21 bins was calculated using the R package SuperExactTest (Wang et al., 2015). A summary of  
22 all bisulfite sequencing data generated in this study is presented in Supplemental Data Set 1,  
23 and is accessible through the NCBI's Gene Expression Omnibus (GSE179702).

#### 24 25 RNA sequencing and analysis

26  
27 Total RNA was extracted from three siliques 6-9 days after anthesis, as previously  
28 described (Mizzotti et al., 2018), and using the RNeasy Plant Mini Kit (Qiagen) following  
29 manufacturer's recommendations for seed tissues (RLC buffer). Sequencing of messenger  
30 RNA was performed by BGI Genomics (Hong Kong) using DNBSEQ technology. High  
31 quality raw reads were aligned to the TAIR10 genome using STAR (Dobin et al., 2013).  
32 Reads were counted and normalized using the R package DESeq2 (Love et al., 2014). We  
33 considered differentially expressed genes those displaying a log<sub>2</sub> fold-change  $\geq 2$ , and with an  
34 adjusted *p*-value  $< 0.01$ . Graphical outputs were produced using the R packages ggplot2,

1 heatmap and ComplexHeatmap. The statistical significance of the observed overlaps  
2 between differentially expressed genes was calculated using the R package SuperExactTest  
3 (Wang et al., 2015). A summary of all RNA sequencing data generated in this study is  
4 presented in Supplemental Data Set 1, and is accessible through the NCBI's Gene Expression  
5 Omnibus (GSE179697).

## 6 7 Interploidy and interspecific hybridizations

8  
9 Seeds of *Capsella rubella* (accession 48.21) and *Capsella grandiflora* (accession  
10 23.5), as well as diploid and tetraploid Col-0 seeds were surface sterilized with 30% bleach  
11 and 70% ethanol, rinsed with distilled water and sown on agar plates containing 0.5X  
12 Murashige and Skoog (MS) medium and 1% sucrose. Seeds of *C. grandiflora* and tetraploid  
13 Col-0 were also sown on agar plates with 0.5X MS medium, 1% sucrose and 100 $\mu$ M 5-  
14 Azacytidine (Sigma). All plates were put in a growth chamber with long-day photoperiod  
15 (16h and 22°C light, 8h and 19°C darkness) with a light intensity of 110  $\mu$ E. 7 days-old  
16 seedlings were transferred to pots filled with sterile soil and plants were grown in a growth  
17 chamber with 60% humidity and daily cycles of 16h light at 21 °C and 8 h darkness at 18°C  
18 with light intensity of 150  $\mu$ E. Flower buds were manually emasculated and pollinated after 2  
19 days. Dry seeds were stored for 30 days for "after-ripening". They were then surface sterilized  
20 and sown on agar plates containing 0.5X MS medium and 1% sucrose. Plates were stratified  
21 for 2 days at 4°C and then moved to the growing chamber. Germination rate was scored after  
22 7 days in the growing chamber. The experiment was done in 3 biological replicates (each  
23 replicate is offspring of different parental plants).

## 24 25 **Authors contributions and acknowledgments**

26  
27 F.B. designed the study, J.H., K.D., K.P. and F.B. performed experiments, J.H., T.W. and  
28 F.B. analyzed the data, C.K. provided materials and advised on experimental design, and F.B.  
29 wrote the manuscript with contributions from all authors.

30  
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3  
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6  
7 **Figure legends**

8  
9 **Figure 1.** The triploid block is suppressed in *jas-3* plants treated with 5-Azacytidine.

10 **(A)** The triploid block response was quantified by counting the number of aborted seeds in  
11 five siliques of untreated *jas-3* plants and DMSO controls, and plants exposed to three  
12 different concentrations of 5-Azacytidine (25, 50 and 100  $\mu\text{g}/\text{mL}$ ). Numbers on top of each  
13 box represent the number of individual plants used. Statistically significant difference in the  
14 percentage of collapsed seeds was calculated by ANOVA with a post hoc Dunnett test, using  
15 *jas-3* as the reference group (n.s. is not significant, \* is  $p \leq 0.05$ , and \*\*\* is  $p \leq 0.001$ ).

16 **(B)** Representative images of seeds from DMSO controls and the strong suppressor Aza1,  
17 showing a decrease in the level of seed abortion in the suppressor line.

18 **(C)** Seed abortion and germination assays were performed in the first generation after  
19 treatment with 5-Azacytidine (eM1) to evaluate the transgenerational stability of the  
20 suppressive effect. Numbers on top of each box indicate the number of siblings (top) and total  
21 number of seeds (bottom) counted. Statistically significant difference in the percentage of  
22 non-germinated seeds was calculated by ANOVA with a post hoc Dunnett test, using DMSO  
23 as the reference group (n.s. is not significant, \* is  $p \leq 0.05$ , and \*\*\* is  $p \leq 0.001$ ).

24  
25 **Figure 2.** Transgenerational inheritance of CG hypomethylation occurs specifically in  
26 suppressor lines.

27 **(A)** Metaplots show CG methylation levels at protein-coding genes annotated according to the  
28 TAIR10 reference genome, and aligned at the 5' and 3' ends (dashed lines). Average CG,  
29 methylation was calculated for 100-bp intervals and plotted for untreated *jas-3* and DMSO  
30 controls, suppressor and non-suppressor lines, showing that loss of CG methylation occurs  
31 specifically in the suppressor lines.

32 **(B)** Heatmap representation of CG methylation levels at hypomethylated CG DMRs detected  
33 in the suppressor lines (Aza1, Aza14, Aza18 and Aza25) as compared to the untreated control



1 *jas-3*. Average CG methylation mapping to these DMRs is presented as heatmap for untreated  
2 *jas-3* (2 replicates) and DMSO controls (2 replicates), suppressors and non-suppressors.

3 (C) Hypomethylated CG DMRs detected in each suppressor line were mapped to the genomic  
4 features annotated in the TAIR10 reference genome, showing that the majority of DMRs  
5 overlap with protein-coding genes and transposable elements (TEs).

6 (D) Venn diagram shows a significant overlap between differentially methylated 100bp bins  
7 detected in the three strongest suppressor lines (Aza1, Aza18 and Aza25). The statistical  
8 significance of the observed regions was calculated using the R package SuperExactTest  
9 (Wang et al., 2015).

10

11 **Figure 3.** The triploid block response and CG methylation are partially restored in the second  
12 generation after treatment with 5-Azacytidine.

13 (A) Seed abortion and germination assays were performed for two consecutive generations  
14 after treatment with 5-Azacytidine (eM1 and eM2), showing that the triploid block response  
15 is only partially restored in two independent lines in the second generation after  
16 epimutagenesis (eM2). Numbers on top of each box indicate the number of siblings (top) and  
17 total number of seeds (bottom) counted. Statistical significance was calculated by ANOVA  
18 with a post hoc Dunnett test, using Aza1 as the reference group (n.s. is not significant, and \*  
19 is  $p \leq 0.05$ ).

20 (B) Average CG methylation mapping to hypomethylated CG DMRs detected between the  
21 suppressor line Aza1 and the untreated control *jas-3* is presented as heatmap for untreated *jas-*  
22 *3* (2 replicates) and DMSO (2 replicates), Aza1 eM1, and two eM2 lines (Aza1-1 and Aza1-  
23 2), to show that CG methylation is also partially restored in eM2 lines.

24 (C) CG methylation of untreated *jas-3*, DMSO, eM1 and eM2 lines was mapped to 100kb  
25 bins across the Arabidopsis genome. CG methylation levels in DMSO, eM1 and eM2 datasets  
26 were then divided by the levels in *jas-3*, and plotted separately for all five chromosomes to  
27 show patterns of hypomethylation. This shows that for most of the genome, the distribution of  
28 CG hypomethylation is identical between the two eM2 lines, although there are particular loci  
29 where CG methylation is segregating.

30

31 **Figure 4.** Paternally expressed genes are down-regulated in suppressor lines.

32 (A) Differential expression analysis was performed between wild-type Col-0 (WT) and *jas-3*  
33 mutant siliques, and 1804 genes were found up-regulated in *jas-3* (fold change  $\geq 2$ , adjusted  
34  $p$ -value  $< 0.01$ ). Transformed raw counts for these genes are presented as an heatmap for WT,

1 *jas-3* and DMSO, Aza1 eM1 and eM2 biological replicates (see Methods), to show that a  
2 proportion of genes up-regulated in untreated *jas-3* and DMSO controls are transiently down-  
3 regulated in the first generation after treatment with 5-Azacytidine.

4 **(B)** Genome browser tracks display CG methylation (pooled seedlings) and mRNA levels  
5 (siliques) in *jas-3*, DMSO, Aza1 eM1 and Aza1 eM2 plants.

6 **(C)** Raw counts were normalized as fragments per kilobase per million (FPKM) and plotted  
7 as barplots with mean values and standard error.

8

9 **Figure 5.** Chemically-induced epimutagenesis allows bypassing interploidy and interspecific  
10 hybridization barriers.

11 **(A)** Pollen from tetraploid Arabidopsis plants in Col-0 background was used to pollinate  
12 diploid Col-0 plants. Triploid seeds resulting from this cross abort at high frequencies when  
13 the paternal parent is treated with DMSO, while 40% of viable triploid seeds were detected  
14 when pollen was derived from plants that have been treated with 5-Azacytidine.

15 **(B)** In interspecific crosses between diploid *Capsella rubella* (*Cr*) and *Capsella grandiflora*  
16 (*Cg*), hybrid seeds abort, resembling interploidy hybridizations with paternal excess. When *C.*  
17 *grandiflora* plants are treated with 5-Azacytidine, approximately 20% of hybrid seeds  
18 germinated.

19

## 20 **Supplemental Data**

21

22 **Supplemental Figure 1.** Quantification of diploid pollen in *jas-3* plants. (Supports Figure 1)

23 **(A)** The proportion of haploid (1n) and diploid (2n) pollen produced in *jas-3* mutants was  
24 quantified by flow cytometry, as pollen populations are characterized by an elevated high  
25 angle scatter (SSC) and autofluorescence (FL1).

26 **(B)** Boxplot was used to show the distribution of diploid (2n) pollen among individual DMSO  
27 and Aza1 plants. Number on top of the boxes represent the number of plants used. A  
28 Wilcoxon test was used to compare the mean values between the suppressor Aza1 and the  
29 DMSO control. n.s. is not significant ( $p=0.937$ ).

30

31 **Supplemental Figure 2.** Ploidy analysis by flow cytometry. (Supports Figure 1)

32 Leaf tissue was chopped in Galbraith buffer with a razor blade, stained with SYBR Green dye  
33 (Lonza), and analyzed on a CyFlow Space cytometer (Sysmex). Nuclei of young diploid

1 leaves in Arabidopsis is characterized by two prominent 2C and 4C peaks. Triploid  
2 individuals show a proportional increase in signal intensity.

3

4 **Supplemental Figure 3.** Suppression of the triploid block in plants treated with 5-  
5 Azacytidine is a paternal effect. (Supports Figure 1)

6 **(A)** A parental effect in triploid block suppression after epimutagenesis was investigated by  
7 performing reciprocal crosses between the strong suppressor Aza1 and DMSO control plants.  
8 Numbers on top of each bars represent the number of plants used (top) and total amount of  
9 seeds counted (bottom). Statistically significant difference in the percentage of collapsed  
10 seeds was calculated by ANOVA with a post hoc Dunnett test, using Aza1 as the reference  
11 group (n.s. is not significant, and \*\*\* is  $p \leq 0.001$ ).

12 **(B)** Representative seeds images are shown for the controls (selfed plants), and reciprocal  
13 crosses. Scale bars represent 1mm.

14

15 **Supplemental Figure 4.** Schematic depicting *jas-3* epimutagenesis and transgenerational  
16 analysis of the triploid block in suppressor lines. (Supports Figures 1, 2 and 4)

17 Diploid seeds from *jas-3* were treated with 100 $\mu$ g/mL of 5-Azacytidine during germination  
18 and early growth. Treated eM0 plants were then transferred to soil to recover, and allowed to  
19 self-fertilize. Selfed *jas-3* mutants produce diploid seeds that are viable, and triploid seeds  
20 that abort. Diploid plants were selected after each generation and allowed to self-fertilize, and  
21 the triploid block was quantified by counting the number of aborted seeds in individual  
22 siblings.

23

24 **Supplemental Figure 5.** CG, CHG and CHH methylation profiles at protein-coding genes  
25 and transposable elements. (Supports Figure 2)

26 Protein-coding genes (left panels) and transposable elements (TEs, right panels) annotated  
27 according to the TAIR10 reference genome were aligned at the 5' and 3' ends (dashed lines),  
28 and average CG, CHG and CHH methylation levels for 100-bp intervals were plotted for  
29 untreated *jas-3* and DMSO controls, suppressor and non-suppressor lines. Loss of DNA  
30 methylation at genes and TEs occurs mainly in the CG context and is observed only in  
31 suppressor lines.

32

33 **Supplemental Figure 6.** Ectopic CHG methylation in suppressor lines. (Supports Figure 2)

1 (A) Heatmap representation of CHG methylation levels at ectopic CHG DMRs detected in the  
2 suppressor line Aza1, as compared to the untreated control *jas-3*. Average CHG methylation  
3 mapping to these DMRs is presented as heatmap for two independent replicates of untreated  
4 *jas-3* and DMSO controls, suppressor and non-suppressor lines.

5 (B) Genome browser tracks show DNA methylation at CG and CHG context for selected loci  
6 showing ectopic CHG methylation.

7

8 **Supplemental Figure 7.** Differentially expressed genes in suppressor lines. (Supports Figure  
9 4)

10 (A) Venn diagram shows overlap between genes up-regulated in untreated *jas-3* (vs WT), and  
11 genes up-regulated in DMSO controls (vs WT).

12 (B) Venn diagram shows the overlap of genes up-regulated in *jas-3* (vs WT) with genes  
13 transiently down-regulated in eM1 (vs DMSO), and up-regulated in eM2 (vs eM1). The  
14 majority of genes down-regulated in eM1 are genes that are up-regulated in *jas-3* mutant  
15 siliques, and expression of these genes is restored in eM2.

16 The statistical significance of the observed overlaps between differentially expressed genes  
17 was calculated using the R package SuperExactTest (Wang et al., 2015).

18

19 **Supplemental Figure 8.** Clustering of RNA-seq datasets. (Supports Figure 4)

20 Transformed data was used for sample clustering to visualize sample-to-sample distances (see  
21 Methods). The heatmap of this distance matrix shows an overview over similarities and  
22 dissimilarities between all RNA-seq datasets produced in this study.

23

24 **Supplemental Data Set 1.** Summary whole-genome bisulfite and RNA-seq datasets.

25 Whole-genome bisulfite sequencing (WGBS). Mean genomic coverage and methylation  
26 percentages for CG, CHG and CHH context. Cytosines covered by 3 or more reads were used  
27 to calculate genome-wide methylation percentages.

28 RNA-seq. High quality raw reads were aligned to the TAIR10 genome using STAR (Dobin et  
29 al., 2013)

30

31 **Supplemental Data Set 2.** Lists of differentially methylated regions.

32 Differentially methylated regions (DMRs) in CG context were defined as 100-bp bins  
33 containing at least 4 differentially methylated CGs, and with an absolute methylation  
34 difference of 0.4. DMRs localizing within 200 bp of each other were merged.

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**Supplemental Data Set 3.** Lists of differentially expressed genes.

Reads counts were normalized using the R package DESeq2 (Love et al., 2014). We considered differentially expressed genes (DEGs) those displaying a log<sub>2</sub> fold-change  $\geq 2$ , and with an adjusted *p*-value  $< 0.01$ .

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