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**Experimental evidence for short term directional  
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1  
2 **Title**

- 3 • Experimental evidence for short term directional selection of epigenetic trait variation  
4 • Epigenetic response to selection

5  
6 **Authors**

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16  
17 **Abstract**

18 Evolution by natural selection can occur when organisms harbor genetically inherited  
19 phenotypic variation, and phenotypic variants have differential fitness. Stable  
20 transgenerational epigenetic variation also exists for fitness-related traits and theory  
21 predicts that selection can act on this variation alone without a contribution of genetic  
22 variation. Here, we artificially selected for divergent rosette size and plant height in  
23 experimental *Arabidopsis thaliana* populations harboring DNA methylation  
24 polymorphism in an identical genetic background. We found significant epi-allele  
25 frequency changes in response to selection. Our results show how selection changed  
26 population trait values and their epigenetic basis in real time, over one generation of  
27 selection. Our results imply the role of the transgenerational epigenetic variation of  
28 populations as an additional source of short-term adaptive potential.

29  
30 **Teaser**

31 Trait based selection experiment in *Arabidopsis* recombinant inbred lines drives  
32 epigenetic changes in response to selection.

33  
34 **MAIN TEXT**

35  
36 **The manuscript should be a maximum of 15,000 words.**

37  
38 **Introduction**

39 Evolution by natural selection occurs when organisms express heritable variation for  
40 fitness. As a result, fitness-related traits change in response to selection (Walsh and Lynch  
41 2018). The heritable trait variation upon which selection can act is typically equated to  
42 variation in the DNA sequence of bases in most studies of adaptive evolution. However,  
43 transgenerational epigenetic variation exists for fitness-related traits (Kronholm et al.  
44 2017, Muyle et al. 2021, Stajic et al. 2019). Under the assumption that epigenetic variants  
45 are stable across generations, theory predicts that selection can act on this heritable  
46 variation alone, without any contribution of genetic variation (Bonduriansky and Day  
47 2009, Danchin et al. 2011, Jablonka et al. 2014).

48 Epigenome-wide association studies (EWAS) have been successfully used in the past  
49 (Ong-Abdullah et al. 2015) to identify population divergence based on heritable DNA

50 methylation patterns linked to phenotypic traits. The presence of epigenetic divergence  
51 between populations that have a variable genomic background is difficult to interpret as  
52 strong evidence that selection can shape standing epigenetic variation independently from  
53 DNA sequence variation but sheds light on this possible scenario.

54 The stakes are high for evaluating the potential role of epigenetic variation as a source of  
55 variation upon which selection can act. This is because the epigenetic response to  
56 selection may participate to adaptation of wild populations confronted to environmental  
57 constraints. Selection of standing epigenetic variation could also participate to breeding  
58 strategies (Kakoulidou et al. 2021). Epigenetic breeding might be rather straight forward  
59 in vegetatively propagated crops (Latutrie et al. 2019), and in seed crops if the response to  
60 selection can be predicted from the epigenetic heritability and the selection differential, as  
61 usually done on the basis of the standing genetic variation of traits (Walsh and Lynch  
62 2018). Another stake lies in the debate around the evolutionary significance of epigenetic  
63 variation (Charlesworth et al. 20017, Quadrana and Colot 2016, Richards et al. 2017,  
64 Verhoeven et al. 2016). This debate roots in the confounded genetic and epigenetic  
65 variations of traits that are technically and statistically difficult to separate (Thomson et al.  
66 2018). We therefore tested for an epigenetic signature of selection by using a selection  
67 experiment in artificial plant populations displaying DNA methylation polymorphism in  
68 the same genetic background.

69 Here, we tested the hypothesis that directional selection can act on heritable epigenetic  
70 trait variation in an identical genetic background by conducting artificial selection  
71 experiments in *Arabidopsis thaliana*. There is growing evidence for epigenetic variation,  
72 notably DNA methylation, at quantitative traits in animals and plants (Cortijo et al. 2014,  
73 Noshay et al. 2021, Vogt 2021). This is notably the case of size traits and flowering time  
74 in *A. thaliana* (Cortijo et al. 2014). Size traits and flowering time strongly influence the  
75 fitness of most plants in nature (Halbritter et al. 2018). There is evidence for the heritable  
76 epigenetic variation of these traits associated with differentially methylated regions  
77 (DMRs) that are stably inherited across generations in epiRILs (Johannes et al. 2009,  
78 Reinders et al. 2009, Roux et al. 2011, Zhang et al. 2018). EpiRILs are a set of 123 fixed  
79 homozygous lines, descending from an F2 population, that were obtained by an initial  
80 cross of isogenic parents with different DNA methylation profiles (Col-0 x *Col-ddm1-2*)  
81 (Johannes et al. 2009). The mutation in the DDM1 gene (*ddm1*) – a chromatin remodeler  
82 involved in DNA methylation – induced hypomethylation of 126 clusters of cytosines  
83 (DMRs). We therefore expect that a selection differential imposed to parental plants on  
84 the basis of their size and flowering time which variation would be caused by DMRs, e.g.,  
85 by allowing to breed only bigger or late flowering epiRILs, would produce the expected  
86 directional trait changes in the progeny.

87 Novel phenotypes can be selected in *A. thaliana* populations that only differ with respect  
88 to epigenetic variation in a fixed genomic background (Schmid et al. 2018). These  
89 changes were stably inherited over at least two generations in the absence of selection and  
90 correlated with changes in DNA methylation and transcription. Potential genetic  
91 differences that could have occurred over the 7-8 generations of this experiment were  
92 excluded by resequencing the complete genome of several individuals. The epigenetic  
93 divergence found between lab populations exposed to fragmented and disturbed landscape  
94 environments and natural plant populations could therefore be attributed to selection  
95 (Schmid et al. 2018). Today, the time is ripe to reach a better understanding of the  
96 epigenetic response to selection. Whether directional selection shapes the standing

97 epigenetic variation of traits as it would do with the standing genetic variation of traits  
98 remains to be clarified. Confirming this hypothesis would confirm that the genetical  
99 theory of natural selection also applies to epigenetic variation and unlock new potential for  
100 breeding strategies.

101 First, building upon the available knowledge, we described DNA methylation  
102 polymorphism acknowledged to be stable across generations (Cortijo et al. 2014, Johannes  
103 et al. 2009) and phenotypic variation in two different artificial *A. thaliana* epiRIL  
104 populations. We grew these two populations made of two totally different sets of *A.*  
105 *thaliana* epiRILs in similar conditions and at different times, in order to repeat the  
106 experiment in two different populations. Second, independently in each population and for  
107 each trait, we artificially generated a fitness differential between plants by selecting them  
108 based on their morphological and reproductive trait values. We selected in two different  
109 directions - for upper and lower trait values – and applied two different selection  
110 intensities - weak and strong - to explore the potential properties and limits of epigenetic  
111 selection. Third, we estimated the phenotypic response to selection in the next generation  
112 of plants and assessed whether changes in epigenetic allele frequencies can be directly  
113 attributed to the response to selection. To this aim, we developed a new approach that  
114 consists in growing all the progeny as if every parental plant had been selected to be a  
115 breeder and removing the progeny of counter selected parents *in silico*, which allowed us  
116 to build strong confidence intervals and conduct independent selection trials on different  
117 traits.

## 118 **Materials and Methods**

### 119 **Plant material**

120  
121  
122 Epigenetic Recombinant Inbred Lines (epiRILs) of *Arabidopsis thaliana* plants represent  
123 one of the few study systems available to date to investigate epigenetic variation  
124 associated with phenotypic trait variation, clearly identified as epiQTLs located on  
125 chromosomes, in an almost fixed genomic background (13, 18, 20, 25). We obtained 123  
126 epiRILs from the Institute Jean-Pierre Bourgin (IJPB - UMR1318 INRAE-AgroParisTech,  
127 Versailles, France). These epiRILs were originally created by crossing two parents from  
128 the same accession (*Columbia*, *Col*) that differed only at the *Decreased DNA Methylation*  
129 *1* gene (*DDM1*), with one parent homozygous for the wild type *DDM1* allele (*Col-wt*),  
130 and the other for the *ddm1-2* mutant allele (*Col-ddm1*) (Johannes et al. 2009). The latter is  
131 characterized by a widely different DNA methylation profile background. These epiRILs  
132 were obtained after six generations of single seed descent and harbor differentially  
133 methylated regions (DMR) across the entire genome. These DMRs are stably inherited  
134 over multiple generations and associated with substantial heritable variation in flowering  
135 time and plant height (Cortijo et al. 2014, Johannes et al. 2009, Roux et al. 2011).  
136 Genome-wide DNA methylation data are available for these 123 epiRILs as well as the  
137 derived recombination map for 126 meiotically stable DMRs covering 81.9% of the  
138 genome (Colomé-Tatché et al. 2012).  
139

140 We built two independent samples of 60 different epiRILs by randomly choosing lines  
141 without replacement out of the 123 lines available in order to create two independent  
142 experimental populations of *Col-wt* epiRILs and replicate our selection experiment in  
143 these two populations at different times. Population 1 included the following epiRILs  
144 (Colomé-Tatché et al. 2012): 14, 20, 36, 52, 54, 55, 62, 64, 70, 92, 99, 108, 112, 144, 164,

145 166, 169, 183, 193, 195, 215, 216, 238, 257, 260, 275, 277, 297, 305, 326, 333, 340, 344,  
146 350, 356, 361, 362, 368, 371, 393, 394, 400, 408, 432, 434, 437, 458, 466, 467, 473, 477,  
147 480, 488, 492, 493, 497, 503, 539, 561 and 567. Population 2 included the following  
148 epiRILs: 8, 11, 18, 24, 46, 53, 60, 69, 71, 73, 94, 95, 101, 114, 118, 137, 147, 148, 150,  
149 159, 170, 172, 202, 208, 218, 222, 225, 229, 232, 244, 252, 258, 262, 276, 315, 323, 363,  
150 366, 375, 391, 410, 425, 438, 439, 454, 471, 494, 495, 500, 506, 508, 523, 538, 556, 558,  
151 559, 570, 572, 573 and 579.

## 152 Plant cultivation

153  
154  
155 The selection experiment required growing two successive generations of plants (G0 and  
156 G1) for each of the two independent experimental populations of Col-wt epiRILs. G0 of  
157 population 1 was grown in June 2018 and G1 in January 2019. G0 of population 2 was  
158 grown January 2019 and G1 in August 2019. At G0, three plant replicates per epiRIL were  
159 grown (n = 180 plants for each population). Ninety-eight percent of G0 plants survived  
160 until flowering (n = 177 plants in population 1 and n = 177 plants in population 2). At G1,  
161 three descendants of every G0 plant were grown (n = 531 plants per population). Because  
162 of a final success rate of 96 %, 524 plants were available for population 1, and 521 plants  
163 for population 2. All the plants were grown in a growth chamber under controlled long-  
164 day conditions (day: 16 h – 20/22°C, night: 8 h – 16°C/18°C) with artificial light (120  
165 mmol/m<sup>2</sup>s<sup>-1</sup>), in 7x7x6.4 cm pots (Soparco, France) on a substrate composed of 80% BP  
166 Substrate K716 (Klassman, France) and 20 % of vermiculite (Souflet Vigne, France).  
167 About 5 to 10 seeds, equivalent in terms of lineage and parental plant, were sown in each  
168 pot and seedlings were thinned out to retain a single plant per pot. Plant location in the  
169 growth chamber and planting occurring over a few hours was fully randomized.  
170 Comparisons were carried out under identical environmental conditions and the design  
171 was fully randomized. G0 and G1 were grown 6 months apart in different seasons, but in  
172 an underground growth chamber under fully controlled environmental conditions.

## 173 Plant phenotypic measurements

174  
175  
176 Four growth- and flowering-related phenotypic traits were recorded: Flowering time (in  
177 number of days since sowing) and rosette diameter were recorded when the first flower  
178 opened. Height at first silique and above-ground biomass were measured after plants  
179 ceased flowering. Above-ground biomass was measured on an analytical high precision  
180 lab balance (Sartorius, Germany) after plants were dried for 24h at 40°C in a laboratory  
181 oven (France Etuves, France). These traits, and in particular growth traits, are not expected  
182 to be fully independent. They were chosen because they present transversal and  
183 comparative interest in ecological and evolutionary plant studies as they are usually  
184 recorded in *A. thaliana* and other plant studies (Halbritter et al. 2018, Roux et al. 2011,  
185 Herrera and Bazaga 2010).

## 186 Selection experiment

187  
188  
189 G0 populations formed the base generation upon which selection was conducted. In each  
190 population, we ranked plants based on their trait values. We randomly selected plants from  
191 this list without replacement to establish a control line. We then selected the top-ranked  
192 and bottom-ranked of the remaining plants to establish high and low selection lines,  
193 respectively. The high and low selection lines (HSL and LSL) respectively aim at  
194 increasing and decreasing trait value in response to selection at the next generation. In

195 order to test the limits of the selection protocol, the selection experiment was conducted at  
196 two selection intensities: weak selection, whereby each selection line (HSL and LSL)  
197 consisted of 33% of the plants (60 plants out of 180) and strong selection, whereby ca.  
198 11% of the plants were randomly sampled to build a control group and then the upper and  
199 lower ca. 11% (20 plants out of 180) of the remaining plants were selected to build each  
200 selection line (HSL and LSL). The sample size of control groups matched the size of  
201 selection lines. G1 control group phenotypes, their similarity to G0, and their difference  
202 with G1 selection lines, allows to control for confounding effects between the response to  
203 selection and the effect of temporal changes in the controlled environment of the growth  
204 chambers, which in itself is highly unlikely.

205  
206 Selection experiments conducted on outcrossing plants require cross-pollinating different  
207 sets of plants for the different traits under selection and the different selection lines, and  
208 sometimes require isolating the plants forming control and selection lines before pollen  
209 might be dispersed between groups. This was not necessary in our selection experiment  
210 because Col-wt epiRILs reproduce almost exclusively by self-fertilization and were grown  
211 in tubes that isolate flowering stems from each other. Here, we grew the progeny of every  
212 G0 plant as if it had been chosen and multiplied (three descendants per parent) to be part  
213 of the control, the HSL and LSL. This original protocol is challenging on the logistical  
214 front because the number of plants cultivated in the growth chamber, and the number of  
215 phenotypic records, grew exponentially between G0 and G1, but it has certain advantages.  
216 It allowed us to establish HSL, LSL and control lines and estimate the response to  
217 selection *in silico* based on the four different phenotypic traits after the two generations of  
218 plants had been grown and measured *in vivo*.

## 219 220 Statistical analysis

221  
222 The classical *in vivo* estimation of the response to selection can be biased because a  
223 unique event of random sampling without replacement of a fixed set of individuals from  
224 the base population is used to establish the control line. This protocol can potentially  
225 produce a sample of values that is upward or downward biased by chance alone. The  
226 remaining individuals available to establish the high and low selection lines are then  
227 biased towards average values. Our original protocol allowed us to overcome this potential  
228 bias although we applied the same logical random sampling of individuals without  
229 replacement to build the control line. This is because we replicated 1000 times this  
230 random sampling *in silico*. We built the corresponding distribution and estimated unbiased  
231 estimates of the trait mean, Standard Error, and 95% Confidence Interval, for each line  
232 (control group, HSL, LSL) and each selection intensity. We used the lack of overlap  
233 between the 95% confidence intervals of phenotypic estimates to test for a significant  
234 difference between high and low selection lines. This is a conservative approach that  
235 provides information on the statistical effects (range, direction, strength and reliability)  
236 that is not provided by p values 40. Scripts in R are publicly available on the ZENODO  
237 repository: <https://doi.org/10.5281/zenodo.7603324>.

## 238 239 Phenotypic selection analysis

240  
241 On the basis of the unbiased control and selection line values obtained on the basis of  
242 1000 replicates, we estimated the response to selection (R). In our selection experiment,  
243 the response to selection evaluates the change in trait mean obtained after one generation  
244 of selection. R was typically estimated by the difference of mean trait value between the

245 selection line and control line in G1, which allows controlling for potential between-year  
246 environmental variation of the mean phenotype (Walsh and Lynch 2018). We also  
247 estimated the different parameters of the breeder's equation,  $R = h^2S$ , where S is the  
248 selection differential and  $h^2$  is the realized narrow-sense heritability (Lush 1937). The  
249 selection differential evaluates the selection pressure imposed on the base population by  
250 measuring the trait mean difference between the population and the selection line. The  
251 selection differential (S) was typically estimated by the difference of mean trait value  
252 between the selection and control line in G0 (Walsh and Lynch 2018). We estimated  $h^2$  for  
253 each trait on the basis of the available measurements of R and S. Caution must be taken  
254 when interpreting the heritability parameter calculated by the breeder's equation in self-  
255 fertilizing plants because it might in fact estimate broad-sense heritability ( $H^2$ ).

#### 256 257 Analysis of DNA methylation changes after selection

258  
259 We tested *in silico* for changes in DNA methylation frequencies in response to phenotypic  
260 selection conducted *in vivo*. We constructed heat maps to visualize the changes in mean  
261 epi-allelic frequencies after selection for all the DMRs in both experimental populations  
262 by using the package ggplot2 (Wickham 2016). To this aim, we estimated in G1 the mean  
263 epi-allelic frequencies at each of the 126 DMRs, for each selection and control line, and at  
264 each selection intensity and trait, on the basis of the 1000 resampled data sets. Epi-alleles  
265 were defined as "0" when originating from the *DDMI* wild type Col-wt and "1" when  
266 originating from the *ddm1-2* mutant col-ddm1. As a result, the more *ddm1-2* mutant epi-  
267 alleles are present in a given line, the higher the frequency of DNA methylation.

268  
269 We condensed the epi-allelic variation of each experimental population into a reduced  
270 number of linearly independent dimensions by using a Principal Component Analysis.  
271 PCA was done using the function dudi.pca of the ade4 package (Dray and Dufour 2007).  
272 It summarized more than 90 % of epi-allelic variation in 16 dimensions for population 1  
273 and in 17 dimensions for population 2. As a result, every plant had a coordinate on each of  
274 those PCA dimensions. We estimated the average coordinate and its 95% CI based on the  
275 1000 resampled datasets, for each PCA dimension, selection or control line, selection  
276 intensity, and trait. We tested for significant differences in DNA methylation associated  
277 with selection by comparing the 95% CIs of high and low lines in G1. We used the lack of  
278 overlap between the 95% confidence intervals to test for a significant difference between  
279 high and low selection lines. As mentioned above, this is a conservative approach  
280 providing information on the statistical effects (range, direction, strength and reliability)  
281 that is not provided by p values (Ho et al. 2019).

#### 282 283 Analysis of molecular epigenomic integrity

284  
285 Molecular epigenomic sequence data covering the whole genome were used to confirm  
286 the integrity of the DMRs in the epiRILs that were used in the plant selection experiment.  
287 Tissue sampling of 24 epiRILs (12 per population) and Col-wt was done 24 days after  
288 germination. Two rosette leaves were sampled and put into a 2-ml tube that was directly  
289 dropped into liquid nitrogen for instant freezing to ensure DNA conservation. Samples  
290 were kept at  $-80^{\circ}\text{C}$ . DNA extraction was performed using DNeasy kit (Qiagen, Germany)  
291 following the manufacturer's protocol. Three replicates per EpiRIL were extracted  
292 separately and DNA content was quantified by using a Qubit fluorometer with the dsDNA  
293 HS Assay Kit (Invitrogen, USA). Quality was controlled using a NanoDrop  
294 spectrophotometer (Thermo Fisher Scientific, USA). The three DNA replicates were then

295 pooled together and a control of the possible degradation of the DNA was performed by  
296 using TapeStation automated electrophoresis (Agilent, USA). Enzymatic Methyl-  
297 sequencing (EM-seq) was then conducted on these DNA samples by the IGen Seq  
298 platform (ICM, Paris, France). EM-seq library was prepared with the NEBNext®  
299 Enzymatic Methyl-seq (EM-seq™) (New England Biolabs, USA) following the  
300 manufacturer's protocol. This method is known to provide accuracy and reliability at least  
301 equivalent to that achieved by Whole Genome Bisulfite Sequencing (WGBS) with similar  
302 global methylation levels indicating high resemblance in the overall detection of  
303 methylated Cs (Feng et al. 2020, Hoppers et al. 2020). Only the bisulfite conversion step  
304 differs between these two approaches; it is an enzymatic reaction in the EM-seq approach  
305 while it is a sodium bisulfite chemical treatment in the WGBS approach. The first EM-seq  
306 conversion step uses TET2 and an Oxidation Enhancer to protect modified cytosines from  
307 downstream deamination. TET2 enzymatically oxidizes 5mC through a cascade reaction  
308 into 5-carboxycytosine, protecting 5mC from deamination. The second enzymatic step  
309 uses APOBEC, which deaminates cytosines but does not affect 5caC. As a result,  
310 converted sequences are similar in the data generated by these two approaches and can be  
311 analyzed in the same way. Sequencing was done on an Illumina Novaseq 6000 sequencer  
312 (Illumina Inc, USA) with a flow cell S1 (300 cycles of 1600 million of reads (2\*53 million  
313 reads of 150 base pairs per sample).

314  
315 We verified the quality of the sequence reads by using the FastQC software Version  
316 0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). We then trimmed 10  
317 bp from each end of the paired-end reads and removed adapter sequences using Trim  
318 Galore software Version 0.6.5  
319 ([https://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)). Trimmed reads were  
320 subsequently aligned to the reference TAIR10 genome version of *Arabidopsis thaliana*  
321 (Assembly: GCA\_000001735.1) using BSMAP aligner Version 2.9 (Xi and Li 2009)  
322 specifying an allowed minimal insert size of 40 pb and a maximal insert size of 2500 bp.  
323 Only reads aligning uniquely to the reference genome were used for subsequent analyses.  
324 Cytosines with an average coverage below 8 (and above 100) across each line were  
325 removed to avoid a potential bias. Average genome coverage was 73.6 after filtering,  
326 which is well above most previous studies on DNA methylation in plants (Schmid et al.  
327 2018). Mapped reads were then used as input data in the BSMAP methylation caller  
328 Version 1.0.0 implemented in the Galaxy interface (<http://usegalaxy.org>) (Goecks et al.  
329 2010) to extract the methylation of each cytosine call and distinguish between the three  
330 contexts of methylation: CG, CHG and CHH. We used the methylKit package Version  
331 1.20.0 for DMR analysis (Akalın et al. 2012). False-positive methylation levels were  
332 measured by calculating methylation levels in the unmethylated chloroplast genome. The  
333 126 DMRs were identified in the epiRILs used in our experiment by calculating the mean  
334 methylation ratio of each marker found by filtering genomic positions (chromosome and  
335 start and stop bp) from the BSMAP methylation calling files with a custom script in R  
336 made publicly available using the packages tidyverse and dplyr (Mailund 2019, Wickham  
337 et al. 2019). These DMRs cover genomic regions of a size ranging from 200 base pairs to  
338 11 kb. The distribution of the Bisulfite Sequence signals was verified for the 126 markers  
339 by plotting the distribution of BS signals in boxplots of the WT-inherited (Methylated)  
340 and the *ddm1*-inherited (Unmethylated) markers with a custom script in R made publicly  
341 available on the ZENODO repository: <https://doi.org/10.5281/zenodo.7603324>.

342  
343 For each DMR marker, percentages of methylated cytosines were compared between Col-  
344 wt and each epiRIL by using Fisher's exact tests to confirm whether they were WT-



345 inherited (not significantly different from Col-wt) or *ddm1*-inherited (significantly  
346 hypomethylated in comparison to Col-wt). To this aim, we used the methylKit package.  
347 Parameters used to call for differentially methylated markers were defined as follows:  
348 differential methylation > 25% and q value < 0.01 as established by other work on the  
349 same species (Kim et al. 2017). This approach allowed us to identify statistical differences  
350 between Col-wt and each epiRIL for the 126 differentially methylated regions (DMRs)  
351 published in Colomé-Tatché et al. (2012), and then compare the DMRs found in our  
352 experiment with those already published in the literature. To this aim, we calculated the  
353 percentage of overlapping probe classification following the methods developed by  
354 Colomé-Taché et al. (2012) between published epiRIL DMR data and our EM-seq data for  
355 the plants used in our experiment. We considered percentages of similarity greater than  
356 80% as a signal of very strong molecular correspondence, > 70% as strong, > 60% as  
357 moderate, and < 50% as weak. We also evaluated the molecular correspondence between  
358 the available WGBS data published for 8 epiRILs (60, 92, 150, 193, 202, 232, 260 and  
359 480) (Bewick et al. 2016, Kooke et al. 2019, Lauss et al. 2018) and the EM-seq data of  
360 these same epiRILs used in our experiment by comparing directly the bisulfited  
361 sequencing signal. To this aim, we estimated the correlation between the 126 regions  
362 methylation ratios obtained from both techniques by using a Kendall rank correlation  
363 coefficient for each methylation context and for global methylation (cumulated  
364 information for all methylation contexts). We also conducted a hierarchical cluster  
365 analysis of the global methylation at the 126 DMRs of epiRILs and Col-wt, and then  
366 compared the hierarchical clusters by using cophenetic correlation coefficients. We also  
367 annotated the 126 DMRs using the PlantGDB database (Dong et al. 2004) and the  
368 Assembly version: AtGDB TAIR9/10 v171 (<https://plantgdb.org/AtGDB/>).

#### 369 Data and code availability

370  
371  
372 The EM-seq data used in this study are available on the NCBI bioproject online  
373 repository. The names of the repository/repositories and accession number(s) can be found  
374 below: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA773039>. Data and R protocols  
375 used for the statistical analyses are available from the ZENODO Digital Repository  
376 <https://doi.org/10.5281/zenodo.7603324>.  
377

## 378 Results

### 379 DNA methylation and phenotypic traits vary

380  
381  
382 We assessed and confirmed that the two *A. thaliana* experimental populations harbored  
383 DNA methylation polymorphism and trait variation in the *Columbia* wild type (Col-0)  
384 genetic background. DNA methylation is the most studied epigenetic mark that influences  
385 chromatin structure and the silencing of transposable elements (TE) and genes (19). Each  
386 experimental population (n = 180 plants per population; Fig. 1A) consisted of a different  
387 set of 60 distinct epigenetic recombinant inbred lines (epiRILs) out of the 123 available  
388 epiRILs produced in *A. thaliana* (17, see materials and methods for details).

389  
390 Molecular epigenomic sequence data covering the whole genome were used to confirm  
391 the integrity of the DMRs in the epiRILs on the basis of three biological replicates per  
epiRIL for 24 epiRILs. This molecular analysis confirmed that epiRILs differed as

392 expected by harboring DNA methylation polymorphism in the form of 126 Differentially  
393 Methylated Regions (DMRs) previously identified and whose stable inheritance is  
394 acknowledged (18, 20) (Figs. S1 and S2, Tables S1 and S2). Some of these 126 DMRs  
395 function as epigenetic quantitative trait loci (13) that underlie the variation of the four  
396 traits that we measured. Although we do not expect biomass, rosette diameter, flowering  
397 time and height to be independent at the phenotypic level, the extent to which their  
398 variation is associated with DNA methylation polymorphism was found to vary (20). In  
399 the two artificial populations, we found 14% to 25% variation (estimated by the  
400 coefficient of variation  $100 \times \text{SE}/\text{mean}$ ) in above-ground dry biomass (average  $\pm$  SE in  
401 populations 1 and 2, respectively;  $0.64 \pm 0.09$  g and  $0.60 \pm 0.15$  g), 12% to 23% variation  
402 in rosette diameter ( $9.7 \pm 1.12$  cm and  $7.7 \pm 1.80$  cm), 7% to 9% variation in flowering  
403 time ( $27.3 \pm 1.92$  d and  $28.3 \pm 2.4$  d), and 21% to 23% variation in height at first fruit  
404 ( $12.1 \pm 2.50$  cm and  $11.6 \pm 2.69$  cm). Thus, in accordance with the making of these  
405 experimental populations as different subsets of epiRILs, we found phenotypic trait  
406 variation and epigenetic variation in each of these two populations.

#### 407 DNA methylation stability across generations

408 The potential lack of stability of DNA methylation across generations has raised the very  
409 question of their evolutionary significance as a source of variation subject to selection (21-  
410 24). Here, we conducted molecular correspondence analyses that confirmed the broad  
411 stability of the 126 DMRs. Stability of DNA methylation polymorphism was assessed by  
412 comparing the 126 DMRs (Colomé-Tatché et al. 2012) between reference epiRILs  
413 established by Johannes et al. (2009) and the same epiRILs used in our selection  
414 experiments, therefore after regeneration. This comparison confirmed their molecular  
415 correspondence (Fig. S1). We used an Enzymatic Methyl-seq (EM-seq, 26) molecular  
416 sequencing approach that provided us with the methylation status of the whole genome  
417 cytosines of 24 epiRILs (12 per experimental population) with a high sequencing coverage  
418 (see methods and Table S3). In plants, DNA methylation can occur in Cytosine-  
419 phosphate-Guanine dinucleotides (CpG), CHG and CHH contexts (H = A, T or C) (19).  
420 We found high molecular correspondence by using the overlapping probe classification  
421 approach developed by Colomé-Tatché et al. (2012). This correspondence reached on  
422 average 87%, ranging from 69% to 97%, for the CpG methylation context and on average  
423 86%, ranging from 64% to 98%, for the CHG methylation context (Tables S1 and S2). We  
424 did not investigate the CHH methylation context because it presents a low methylation  
425 ratio, varies among cells, is often stochastic (27) and is less stable than CpG and CHG  
426 contexts (28), and was not available in the literature (Colomé-Tatché et al. 2012).  
427 Additionally, we compared the EM-seq data of 126 DMRs with the published WGBS data  
428 available for eight epiRILs (29-31) (Fig. S2 and Table S4). We found an average  
429 correlation of 0.65 (ranging between 0.49 and 0.79) for the CpG context, and an average  
430 correlation of 0.73 (ranging between 0.64 and 0.79) for the CHG context. Furthermore, the  
431 hierarchical clustering analysis indicated a high similarity of the methylation in epiRILs  
432 sequenced by both EM-seq and WGBS approaches, with a high (0.89) cophenetic  
433 correlation coefficient (Fig. S2). Our results were coherent with the knowledge that  
434 hypomethylation is stable across generations in the CpG context (32) and with the stability  
435 of hypomethylation in the CHG context where molecular mechanisms driving  
436 modifications differ (33). Although the methylation status is known to be reversible in  
437 DMRs (34, 35), we detected a very small number of changes in the 126 DMRs, illustrating  
438 the broad stability across generations of DNA methylation polymorphism (Tables S1 and  
439 S2).

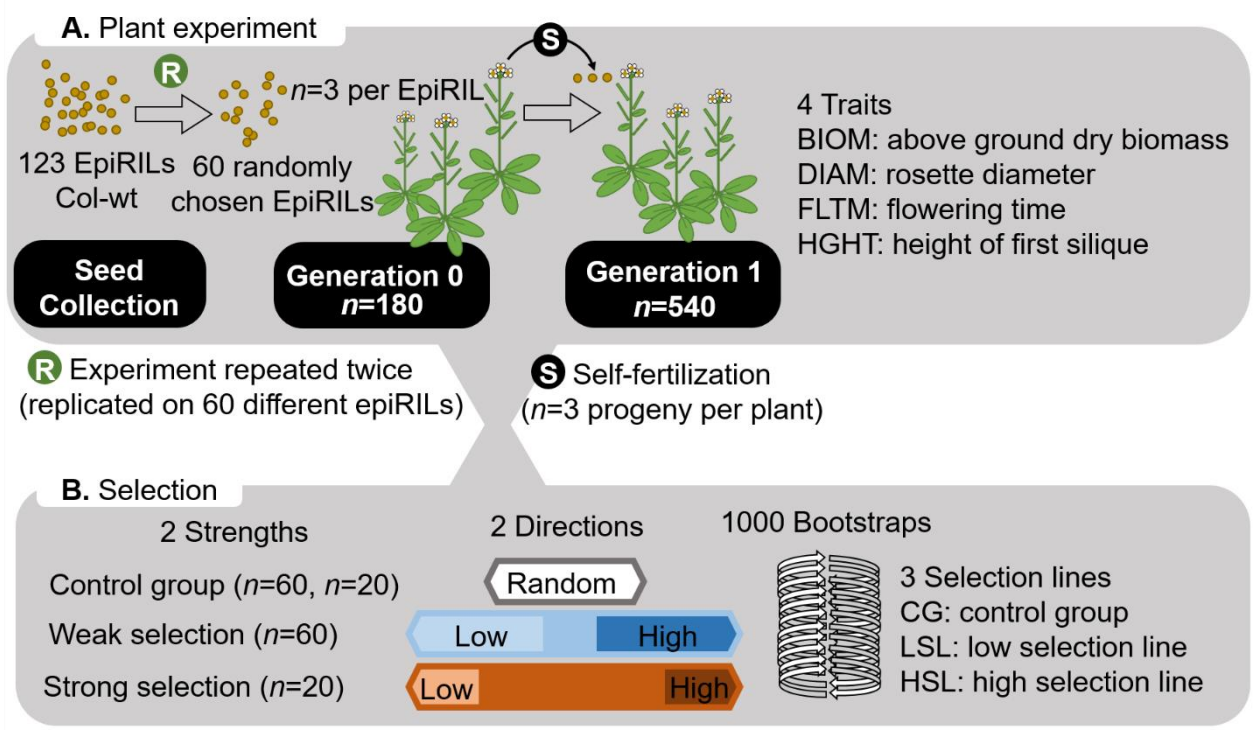
440 DNA hypomethylation can release TEs that can potentially impact gene expression and  
441 compromise genome integrity (18, 35, 36). Novel TE insertions have been shown to  
442 contribute to phenotypic variability in *A. thaliana* epiRIL populations (35, 37). However,  
443 several studies on *A. thaliana* epiRILs however did not find that novel TE insertions  
444 contributed to phenotypic variation (13, 18, 29, 35, 39). These studies qualified these  
445 insertions as rare and random; most of them were found to be neutral (13, 23, 24, 39).  
446 Most of these insertions are described as private, i.e., found in a single epiRIL, at the  
447 hemizygous state (24). TEs are often considered not to affect estimates of phenotypic  
448 variation and DNA methylation polymorphism estimated on the basis of multiple epiRILs  
449 because their effect would be diluted in a population made of multiple epiRILs as a result  
450 of DMRs differing by definition between epiRILs (13, 22, 39). Our *in silico* resampling  
451 approach also warrants our results against any bias produced by a given epiRIL by making  
452 any such potential effect infinitesimal. In the absence of resequencing approach, changes  
453 in copy numbers of private TE insertions affecting a given epiRIL phenotype cannot be  
454 strictly excluded. Our results showed that over the 126 DMRs, which included protein  
455 coding genes, intergenic regions, and TEs, only 25 DMRs included potentially mobile TEs  
456 (23) that could affect traits of interest (24) (Table S5). We therefore identified methylation  
457 changes occurring at these 25 DMRs between the 24 epiRILs sequenced by the EM-seq  
458 approach and the same epiRILs from the reference epiRIL data established by Johannes et  
459 al. 2009 (18). Most of these DMRs did not change. Very few new hypomethylation  
460 changes occurred in these DMRs, a mean of 0.2% (ranging from 0% to 4%) in CpG  
461 context, and of 0.3% (ranging from 0% to 4%) in CHG context. On average 6.3% (ranging  
462 from 0% to 32%) of these DMRs in the CG context and 9.6% (ranging from 0% to 36%)  
463 of these DMRs in the CHG context reverted from a hypomethylated state to the wild-type  
464 methylation state that is known to silence TEs and therefore keep the integrity of the  
465 genome structure (Tables S1 and S2). One can nevertheless not exclude that high copy  
466 numbers of private TEs might affect the phenotype of a given epiRIL even if as mentioned  
467 above, the likelihood is low and the potential effect greatly diluted by our resampling  
468 approach. Our selection experiment should therefore mostly target epiRIL phenotypic  
469 variation directly caused by DMRs but caution is necessary because it may act  
470 occasionally on genomic variants caused by high copy number TEs, which release would  
471 be due to hypomethylation.

## 472 Phenotypic response to selection

473 Despite the absence of genetic variation, we tested whether each experimental population  
474 responded to directional selection for higher and lower trait values. We artificially selected  
475 plants based on their morphological (above ground biomass, rosette diameter, flowering  
476 time) and reproductive trait (flowering time, height of first silique) values independently  
477 in each population, and for each trait. We selected for upper and lower trait values in G0  
478 plants and reproduced them to observe the response to selection in G1 plants. Typical  
479 selection experiments require to use different sets of plants; one for each selection trial  
480 based on a given trait. Here, we multiplied each G0 plant as if it had been positively  
481 selected for (three descendants per parent). As a result, we obtained a large number of  
482 plants in G1, so that we could build *in silico* the G1 dataset corresponding to a given  
483 selection intensity based on a given trait. For each population (n = 180 plants per  
484 population) and each trait, we randomly sampled a third of the plants to build a control  
485 group. We then selected the remaining upper and the lower third (weak selection line; n =  
486 60) of plants in terms of trait values to comprise high and low selection lines (HSL and  
487 LSL, respectively), in each population and for each trait. In parallel, also between G0 and

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G1, we applied a stronger selection pressure by randomly sampling ca. 11% of the plants to build a control group and selecting the upper and lower ca. 11% (strong selection line; n = 20) of the remaining plants (Fig. 1a, b).



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**Fig. 1. Selection experiment drives phenotypic trait changes.** **A**, Plant experiment: Two sets of 60 different epiRILs (*Arabidopsis thaliana* Col-ddm1) were randomly assembled to build the two different experimental populations that were cultivated at different periods in the cultivation chamber. Seeds from the collection were grown to produce a buffer generation without selection and generate the seeds used to grow G0 plants. G1 plants – the progeny of G0 – were produced by self-fertilization. **B**, Selection design: At G0, a control group was made of randomly selected plants. Plants with the highest and lowest trait values were respectively used to build the high and low selection lines. Weak and strong selection lines were respectively made up of 33% and 11% of the population. Corresponding progenies were used to build control groups and selection lines in G1, with differences between G1 lines reflecting responses to selection.

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In the progeny, we found significant divergence in response to selection between high and low selection lines in terms of rosette diameter and height in both populations (Table 1). We found trait changes in response to selection for size traits in both replicate populations of epiRILs, changes for flowering time, but not under every selection treatment, and no change for biomass. Statistical significance was established by lack of overlap between 95% Confidence Intervals (CIs) of trait means, which is a conservative approach (40). For each trait and selection strength, the 95% CIs were based on 1000 bootstraps generated in silico by 1000 independent random samples of the control group. For each randomly sampled control group, it was possible to establish the corresponding specific set of selection lines because we grew the progeny of every plant (Fig. 1A, see methods for details). As a result, potential biasing effects of random sampling, e.g., the weight of a given epiRIL, could not affect our analyses of selection lines and control groups. Divergence was also found in most cases for the flowering time, with the exception of population 2 where it was non-significant after weak selection. No significant response to

517 selection was detected for the above-ground biomass, in both populations at both selection  
518 intensities. The largest trait change after selection was found for height at first fruit, with a  
519 16% reduction observed in the LSL and a 13% increase in the HSL, but most trait changes  
520 were around 3 to 8 percent (Table 1). Strong selection logically generated larger trait  
521 changes in response to selection, but also more variable and therefore less predictable  
522 selection responses (41), as illustrated by larger 95% CIs (Table S6). Heritability was  
523 estimated as the proportion of phenotypic variation available for selection to act upon by  
524 using the breeder's equation (42). It was absent for biomass, which had not changed in  
525 response to selection, but relatively high for height at first fruit, rosette diameter and  
526 flowering time (ranging from 0.13 to 0.63, Table 1), all of which changed after selection.  
527 These results corroborate previous work on the inheritance of epiRIL trait variation (large  
528 heritability of height and flowering time) or lack thereof (negligible heritability of dry  
529 biomass) (20). We also found surprising results because rosette diameter, which was  
530 originally expected to lack heritability based on previous work (20), showed some ability  
531 to change under selection, which indicates heritability. Heritability is the heritable part of  
532 phenotypic variation that is available to selection. Yet it is not rare to observe discrepancy  
533 between heritability estimates based on the phenotypic variation of related individuals and  
534 those based on the actual response to selection (43, 44).

535 **Table 1. Selection differential (S), response to selection (R) and heritability (h<sup>2</sup>) for**  
536 **every selection line.**

Pop	Trait	Weak selection						Strong selection					
		LSL			HSL			LSL			HSL		
		S	R	H <sup>2</sup>	S	R	H <sup>2</sup>	S	R	H <sup>2</sup>	S	R	H <sup>2</sup>
1	Biomass	0.07	NS	N/A	0.07	NS	N/A	0.16	NS	N/A	0.14	NS	N/A
	Diameter	0.87	0.42 (5%)	0.48	0.89	0.41 (5%)	0.46	1.90	0.59 (7%)	0.31	1.74	0.66 (8%)	0.38
	Flowering time	1.36	0.85 (3%)	0.63	1.34	0.80 (3%)	0.60	2.65	1.28 (5%)	0.48	3.41	1.81 (6%)	0.53
	Height	1.85	0.41 (4%)	0.22	1.85	0.41 (4%)	0.22	3.80	1.28 (11%)	0.34	3.94	1.50 (13%)	0.38
2	Biomass	0.11	NS	N/A	0.11	NS	N/A	0.24	NS	N/A	0.23	NS	N/A
	Diameter	0.97	0.55 (6%)	0.57	1.69	0.72 (8%)	0.42	1.76	0.67 (8%)	0.38	3.90	1.13 (13%)	0.29
	Flowering time	1.63	NS	N/A	1.72	NS	N/A	2.77	0.87 (3%)	0.31	4.88	0.62 (2%)	0.13
		2.11	0.99	0.47	1.91	0.90	0.47	4.05	2.04	0.50	4.60	1.47	0.32

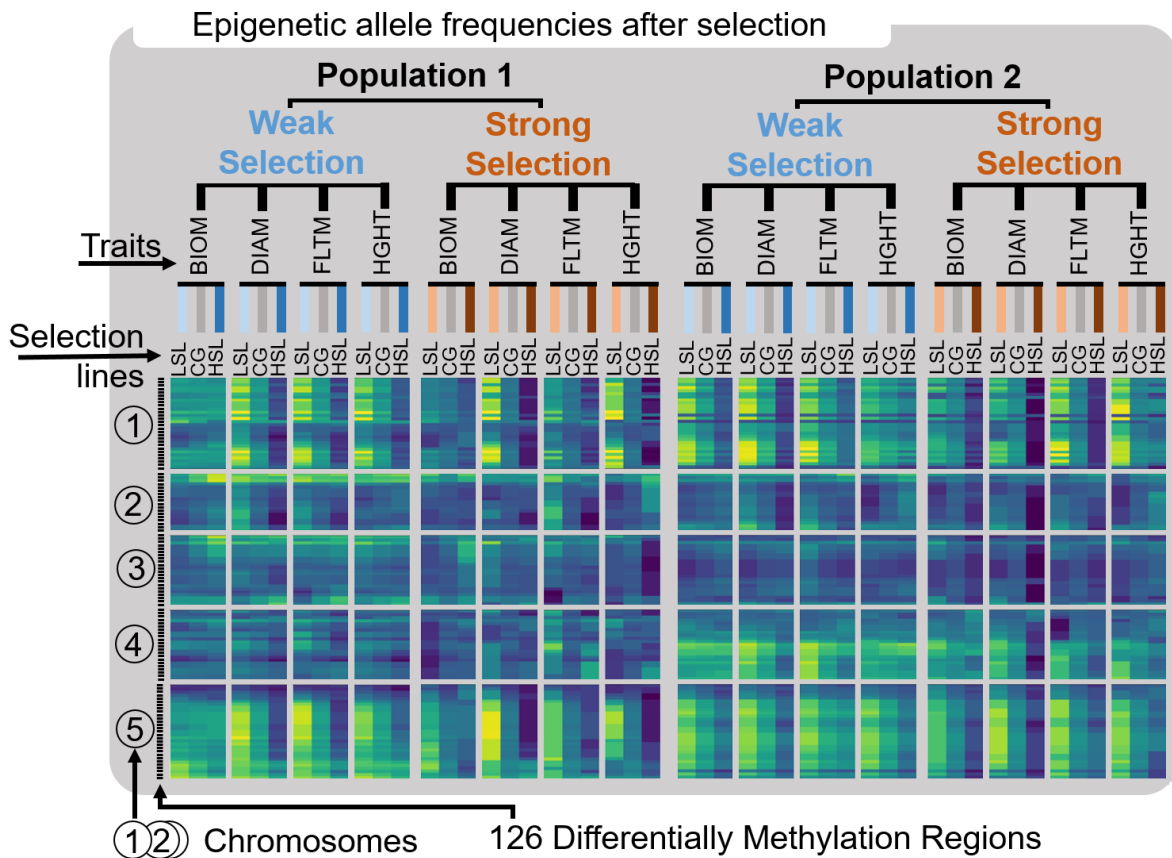
	Height	(8%)	(7%)	(16%)	(11%)
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538 Weak selection: selection by truncation of the upper and the lower third (n = 60) of  
539 *Arabidopsis thaliana* plants ranked by trait values to compose high and low selection lines  
540 (HSL and LSL, respectively). Strong selection: same method but selecting by truncation  
541 the upper and lower ca. 11% (n = 20) of plants. R is presented as trait change in trait units  
542 (g, cm, d) and percentage change. Narrow-sense heritability ( $h^2$ ) calculated by the  
543 breeder's equation is in fact broad-sense heritability ( $H^2$ ) in self-fertilizing plants.  
544 Selection was considered significant following a conservative approach avoiding sampling  
545 bias; when the 95% Confidence Intervals of average trait values estimated on the basis of  
546 1000 bootstraps did not overlap between upper and lower selection lines (Table S6).  
547 Biomass: above-ground dry biomass (g). Diameter: rosette diameter (cm). Flowering time  
548 (d). Height: Height at first fruit (cm)

549 Epigenetic signature of selection

550 We tested whether responses to selection are mirrored by changes in epigenetic allele  
551 frequencies at DMRs. After selection, we observed modifications of these frequencies (see  
552 Fig. 2 presenting the average frequencies based on 1000 *in silico* replicates for all  
553 selection and control lines in each experimental population). Estimating these frequencies  
554 on the basis of 1000 bootstraps excluded the potential bias caused by the weight of a given  
555 epiRIL in any given selection line or control group. These frequencies reflected divergent  
556 epigenetic changes in the progeny of high and low selection lines in each population,  
557 notably under strong selection (Fig. 2). Since sets of epiRILs differed between  
558 populations, selection lines and control groups, it is therefore legitimate to observe  
559 different frequencies for a given trait or chromosome region between populations, and to  
560 denote the signature of selection depicted by a similar enrichment of epigenetic alleles on  
561 the heatmap (Fig 2).

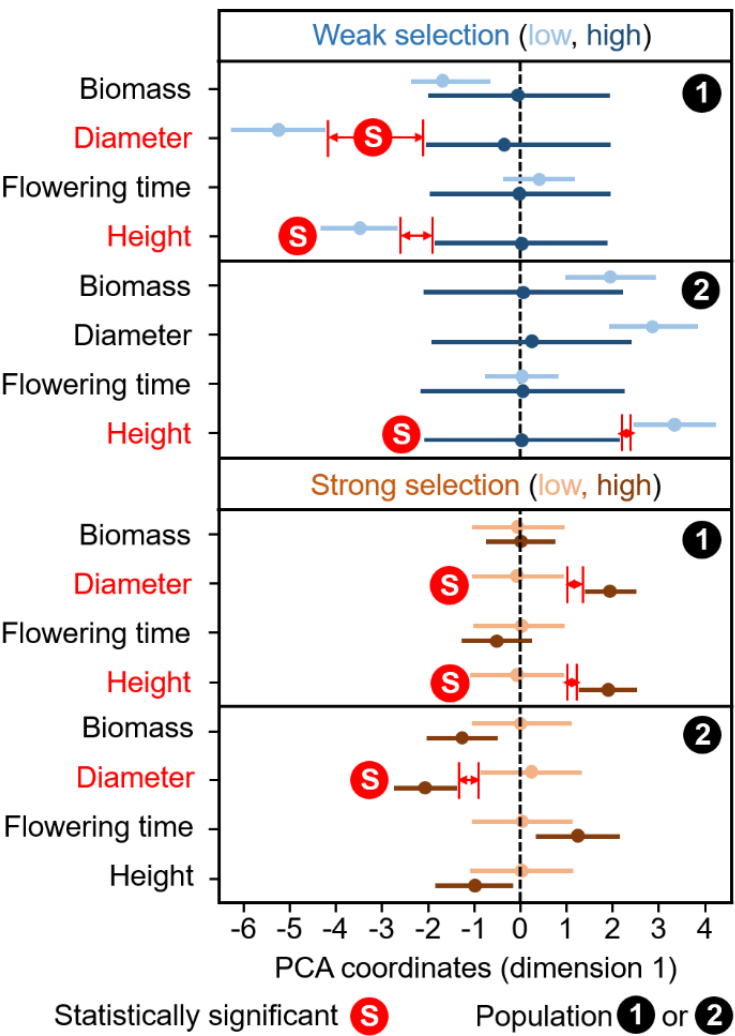


**Fig. 2. Selection experiment drives changes in epigenetic frequencies.** Changes in epigenetic allele frequencies after selection: The heat map presents DMR frequencies in G1 selection lines (LSL: low selection line and HSL: high selection line) and control groups (CG) for each of the four traits (BIOM: above ground dry biomass, DIAM: rosette diameter, FLTM: flowering time, HGHT: height of first silique) in populations 1 and 2. DMRs location on the five chromosomes are labelled on the left-hand vertical axis. Methylation frequencies of DMRs are represented by a colored band, with a gradient from blue (wild-type methylation status) to yellow (fixation of *ddm1-2* mutant methylation status). In each panel, DMR frequencies on a given chromosome are presented for the low, control and high selection lines. Each column corresponds to a trait. Frequencies were estimated on the basis of 1000 bootstraps.

We used a Principal Component Analysis (PCA) to reduce the dimensionality of epigenomic data and summarize its variation distributed over the whole genome into 16 independent variables that can be statistically analyzed. We found significant epigenetic divergence after selection on rosette diameter and height at first fruit in both populations (lack of overlap between 95% confidence intervals for PCA coordinates on the first dimension, representing 18% and 22% of the global DMR variation in populations 1 and 2, respectively, Fig. 3). Thus, the divergent selection for rosette wider and narrower rosette diameters led to opposite changes in the frequency of cytosine methylations in the same genetic background. A similar epigenetic response to selection was observed after divergent selection for smaller and higher plant height at first fruit. Phenotypic selection for either increased or decreased above-ground biomass did not trigger a significant phenotypic change in response to selection. It was therefore not surprising to observe the absence of significant epigenetic divergence in this trait between selection lines. Interestingly, selection for earlier and later flowering plants, which had resulted in

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moderate responses to selection in population 1 and almost no response in population 2, did not drive statistically significant epigenetic changes (Fig. 3). This lack of epigenetic response may reflect the conservative nature of our approach or the possibility that the epigenetic architecture of flowering time consists of one to few loci whose variation was not captured by our statistical approach. Our study in plants therefore provides direct experimental evidence for heritable epigenetic changes in response to selection based on size.



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**Fig. 3. Epigenetic divergence or its lack thereof between selection treatments.** Epigenetic divergence for each *Arabidopsis thaliana* epiRIL population (pop 1 and 2), each trait, each selection strength (n = 60 for each line under weak selection, n = 20 for each line under strong selection), and line (high and low selection lines) of plants. Principal Component Analysis coordinates for the first dimension (PC1), which synthesizes 18% and 22% of the global DMR variation in populations 1 and 2 respectively, are presented with their 95% confidence intervals. Divergence was considered significant following a conservative approach avoiding sampling bias: when 95% Confidence Intervals of PC1 values estimated on the basis of 1000 bootstraps did not overlap between upper and lower selection lines. Significant epigenetic divergent responses to selection are shown in red.

607 **Discussion**



608 Theory predicts that selection acts on transgenerational epigenetic variation presumably  
609 without a contribution of genetic variation (5, 7). Evidence for epigenetic divergence  
610 between populations sharing different histories of adaptation suggests such shaping of  
611 epigenetic trait variation by selection in both plants and animals (17, 45-51). Our selection  
612 experiment contributed to advancing this knowledge by providing a test confirming this  
613 expectation in real time over a single generation of selection, through the comparison of  
614 epiRIL experimental populations of *A. thaliana* surveyed before and after selection.  
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616 Our study did not aim to infer which causal epigenetic variants were responsible  
617 for which phenotypic trait value, but rather to test for an epigenetic response to selection.  
618 It is nevertheless interesting to note that the range of artificially induced epigenetic trait  
619 variation in *A. thaliana* epiRILs is similar to the heritable fitness-related trait variation  
620 observed in divergent natural populations (20). Although the DNA methylation  
621 polymorphism of epiRILs is larger than what can be observed in natural populations  
622 (Becker et al., 2011), our results provide us with evidence that a range of trait epigenetic  
623 heritabilities can be used to predict a range phenotypic changes in response to selection.  
624 Furthermore, as illustrated by Figure 2, LSLs tend to have higher DNA methylation state  
625 variability than HSLs, which appears to be mostly due to Chromosomes 1 and 5. In *A.*  
626 *thaliana* epiRILs, an increasing DMR frequency represents an increase in the proportion  
627 of *ddm1-2* mutant epigenetic alleles in the population. LSLs therefore harbor more  
628 hypomethylated loci than control and HSLs harbor more hypermethylated loci in  
629 comparison to control. This finding suggests that our selection for bigger epiRIL plants  
630 that flower later selected against the hypomethylated nature of epiRILs that was  
631 artificially created by using the *ddm1* mutation. The artificially induced global  
632 hypomethylation of the *A. thaliana* epiRIL genome produced smaller plants flowering  
633 earlier. In fact, we selected for epigenomes and phenotypes that are closer to wild-type  
634 plants.  
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636 Our experimental results show that selection can act on stable transgenerational epigenetic  
637 variation presumably without a contribution of genetic variation. Our findings therefore  
638 imply the contribution of DNA methylation polymorphism to short-term adaptation in  
639 natural *A. thaliana* populations. However, one of the limitations of our experimental  
640 setting is that it does not allow us to predict whether the epigenetic response to selection  
641 plays a predominant role in nature, where selection is a much more complex mechanism.  
642 In nature, selection will not target independently genetic and epigenetic components of  
643 fitness-related traits (43). Natural selection will affect simultaneously all components of  
644 fitness variation, which encompasses tightly linked genetic and epigenetic variation, some  
645 reversible methylation patterns and hypomethylation driven release of TEs modifying the  
646 integrity of the genome structure.  
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648 The scope for replication in other species of our proof of concept experimental approach is  
649 limited. This is because EpiRIL biological material is only available in very few species  
650 (20, 25). Heritable epigenetic variation for traits influencing fitness under environmental  
651 changes nevertheless exists in both plants and animals (18, 45-52), suggesting that our  
652 finding may be a common feature in many species. Other species harboring  
653 transgenerational epigenetic variation for fitness-related traits may benefit from this  
654 additional source of short-term adaptive or breeding potential (53, 54).  
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858 suggest following the CRediT model with each CRediT role given its own line. No  
859 punctuation in the initials.

860

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## 871 **Data and materials availability:**

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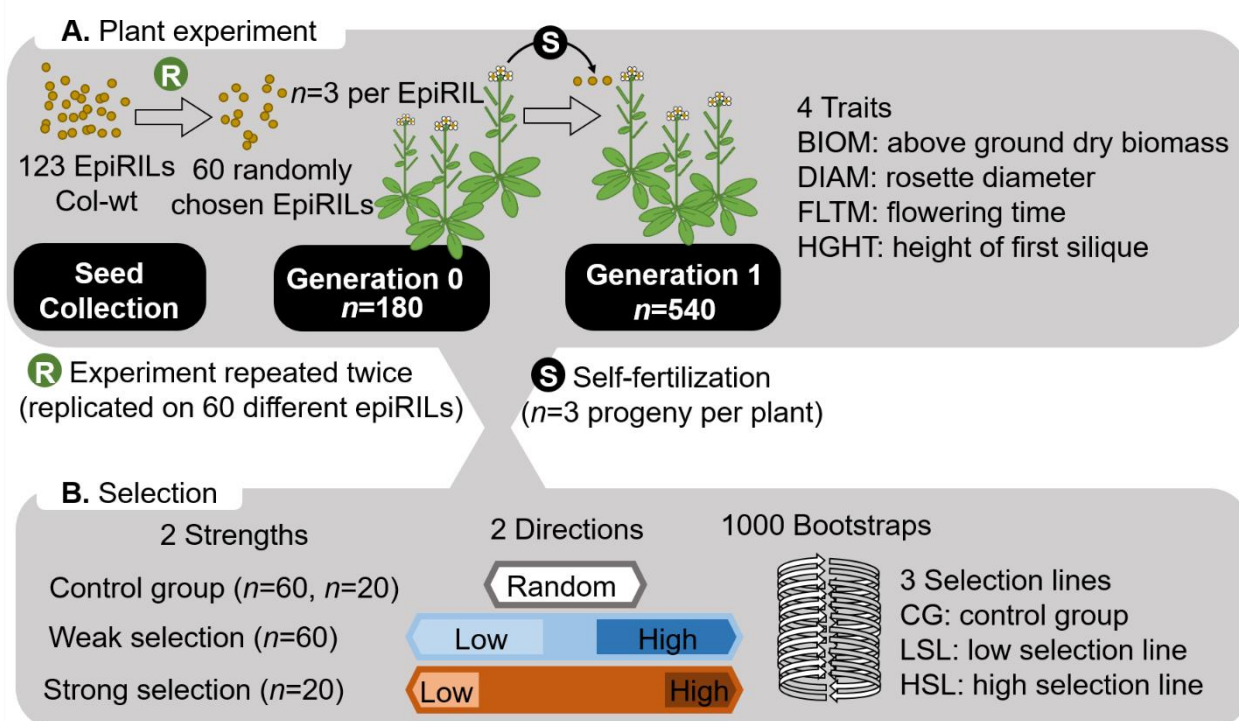
All data are available in the main text or the supplementary materials. The EM-seq data  
873 used in this study are available on the NCBI bioproject online repository. The names of  
874 the repository/repositories and accession number(s) can be found below:

875

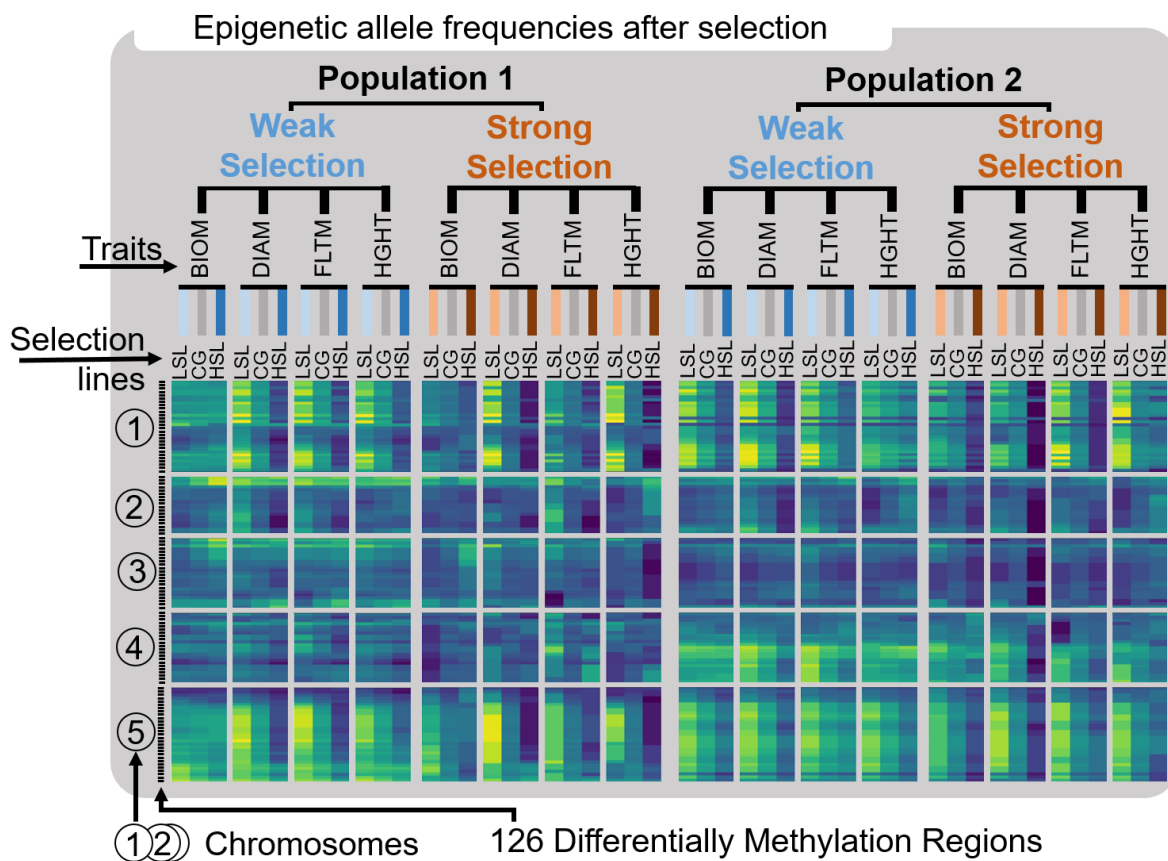
<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA773039>. Data and R protocols used for  
876 the statistical analyses are available from the ZENODO Digital Repository

<https://doi.org/10.5281/zenodo.7603324>. Correspondence and requests for the seed material of the 123 epiRILs - see Table S5 in Colomé-Thatché et al. (25) for a list of accession names - should be addressed to the Institute Jean-Pierre Bourgin (IJPB - UMR1318 INRAE-AgroParisTech, Versailles, France, <https://publiclines.versailles.inrae.fr/catalogue/epiril>). No material transfer agreement is necessary for scientific work on accessions of the model plant *Arabidopsis*.

## Figures and Tables

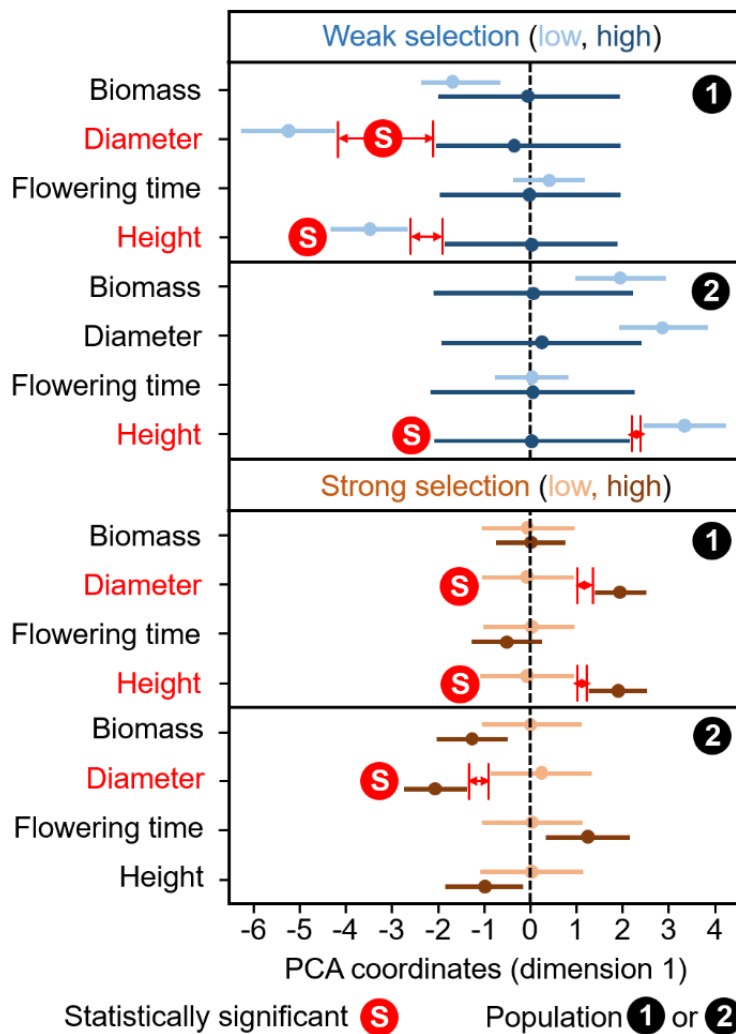


**Fig. 1. Selection experiment drives phenotypic trait changes.** (A), Plant experiment: Two sets of 60 different epiRILs (*Arabidopsis thaliana* Col-ddm1) were randomly assembled to build the two different experimental populations that were cultivated at different periods in the cultivation chamber. Seeds from the collection were grown to produce a buffer generation without selection and generate the seeds used to grow G0 plants. G1 plants – the progeny of G0 – were produced by self-fertilization. (B), Selection design: At G0, a control group was made of randomly selected plants. Plants with the highest and lowest trait values were respectively used to build the high and low selection lines. Weak and strong selection lines were respectively made up of 33% and 11% of the population. Corresponding progenies were used to build control groups and selection lines in G1, with differences between G1 lines reflecting responses to selection.



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 901 **Fig. 2. Selection experiment drives changes in epigenetic frequencies.** Changes in  
 902 epigenetic allele frequencies after selection: The heat map presents DMR  
 903 frequencies in G1 selection lines (LSL: low selection line and HSL: high selection  
 904 line) and control groups (CG) for each of the four traits (BIOM: above ground dry  
 905 biomass, DIAM: rosette diameter, FLTM: flowering time, HGHT: height of first  
 906 silique) in populations 1 and 2. DMRs location on the five chromosomes are  
 907 labelled on the left-hand vertical axis. Methylation frequencies of DMRs are  
 908 represented by a colored band, with a gradient from blue (wild-type methylation  
 909 status) to yellow (fixation of *ddm1-2* mutant methylation status). In each panel,  
 910 DMR frequencies on a given chromosome are presented for the low, control and  
 911 high selection lines. Each column corresponds to a trait. Frequencies were  
 estimated on the basis of 1000 bootstraps.





**Fig. 3. Epigenetic divergence or its lack thereof between selection treatments.**

Epigenetic divergence for each *Arabidopsis thaliana* epiRIL population (pop 1 and 2), each trait, each selection strength ( $n = 60$  for each line under weak selection,  $n = 20$  for each line under strong selection), and line (high and low selection lines) of plants. Principal Component Analysis coordinates for the first dimension (PC1), which synthesizes 18% and 22% of the global DMR variation in populations 1 and 2 respectively, are presented with their 95% confidence intervals. Divergence was considered significant following a conservative approach avoiding sampling bias: when 95% Confidence Intervals of PC1 values estimated on the basis of 1000 bootstraps did not overlap between upper and lower selection lines. Significant epigenetic divergent responses to selection are shown in red.

**Table 1. Selection differential (S), response to selection (R) and heritability ( $h^2$ ) for every selection line.** Weak selection: selection by truncation of the upper and the lower third ( $n = 60$ ) of *Arabidopsis thaliana* plants ranked by trait values to compose high and low selection lines (HSL and LSL, respectively). Strong selection: same method but selecting by truncation the upper and lower ca. 11% ( $n = 20$ ) of plants. R is presented as trait change in trait units (g, cm, d) and percentage change. Narrow-sense heritability ( $h^2$ ) calculated by the breeder's equation is in fact broad-sense heritability ( $H^2$ ) in self-fertilizing plants. Selection was considered significant following a conservative approach avoiding sampling bias; when the 95% Confidence Intervals of average trait values estimated on the

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basis of 1000 bootstraps did not overlap between upper and lower selection lines (Extended Data Table 3). Biomass: above-ground dry biomass (g). Diameter: rosette diameter (cm). Flowering time (d). Height: Height at first fruit (cm).

Pop	Trait	Weak selection						Strong selection					
		LSL			HSL			LSL			HSL		
		S	R	H <sup>2</sup>	S	R	H <sup>2</sup>	S	R	H <sup>2</sup>	S	R	H <sup>2</sup>
1	Biomass	0.07	NS	N/A	0.07	NS	N/A	0.16	NS	N/A	0.14	NS	N/A
	Diameter	0.87	0.42 (5%)	0.48	0.89	0.41 (5%)	0.46	1.90	0.59 (7%)	0.31	1.74	0.66 (8%)	0.38
	Flowering time	1.36	0.85 (3%)	0.63	1.34	0.80 (3%)	0.60	2.65	1.28 (5%)	0.48	3.41	1.81 (6%)	0.53
	Height	1.85	0.41 (4%)	0.22	1.85	0.41 (4%)	0.22	3.80	1.28 (11%)	0.34	3.94	1.50 (13%)	0.38
2	Biomass	0.11	NS	N/A	0.11	NS	N/A	0.24	NS	N/A	0.23	NS	N/A
	Diameter	0.97	0.55 (6%)	0.57	1.69	0.72 (8%)	0.42	1.76	0.67 (8%)	0.38	3.90	1.13 (13%)	0.29
	Flowering time	1.63	NS	N/A	1.72	NS	N/A	2.77	0.87 (3%)	0.31	4.88	0.62 (2%)	0.13
	Height	2.11	0.99 (8%)	0.47	1.91	0.90 (7%)	0.47	4.05	2.04 (16%)	0.50	4.60	1.47 (11%)	0.32

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