

DNA methylation in the wild: epigenetic transgenerational inheritance can mediate adaptation in clones of wild strawberry (*Fragaria vesca*)

Iris Sammarco¹ , Bárbara Díez Rodríguez^{2,3,4} , Dario Galanti^{5,6} , Adam Nunn^{7,8} , Claude Becker^{9,10} , Oliver Bossdorf⁶ , Zuzana Münzbergová^{1,11}  and Vít Latzel¹ 

¹Institute of Botany, Czech Academy of Sciences, Zámek 1, 252 43, Průhonice, Czechia; ²Natural Resources and Climate Area, CARTIF Technology Centre, Parque Tecnológico de Boecillo, parc. 205, 47151, Boecillo, Valladolid, Spain; ³Department of Biology, Philipps-University Marburg, Karl-von-Frisch Strasse 8, D-35043, Marburg, Germany; ⁴Department of Forest Genetics, Albert-Ludwigs-Universität Freiburg, Bertoldstraße 17, 79098, Freiburg i. Br., Germany; ⁵Royal Botanic Gardens, Kew, Richmond, UK; ⁶Plant Evolutionary Ecology, Institute of Evolution and Ecology, University of Tübingen, Auf der Morgenstelle 5, 72076, Tübingen, Germany; ⁷ecSeq Bioinformatics GmbH, Sternwartenstraße 29, 04103, Saxony, Germany; ⁸Department of Computer Science, University of Leipzig, Härtelstraße 16-18, Leipzig, 04107, Germany; ⁹Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Vienna BioCenter (VBC), Dr Bohr-Gasse 3, 1030, Vienna, Austria; ¹⁰LMU Biocenter, Faculty of Biology, Ludwig Maximilians University Munich, Grosshaderner Str. 2-4, 82152, Martinsried, Germany; ¹¹Department of Botany, Faculty of Science, Charles University, Benátská 2, 128 01, Prague, Czechia

Summary

Authors for correspondence:

Iris Sammarco

Email: iris.sammarco@ibot.cas.cz

Vít Latzel

Email: vít.latzel@ibot.cas.cz

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- Due to the accelerating climate change, it is crucial to understand how plants adapt to rapid environmental changes. Such adaptation may be mediated by epigenetic mechanisms like DNA methylation, which could heritably alter phenotypes without changing the DNA sequence, especially across clonal generations. However, we are still missing robust evidence of the adaptive potential of DNA methylation in wild clonal populations.
- Here, we studied genetic, epigenetic and transcriptomic variation of *Fragaria vesca*, a predominantly clonally reproducing herb. We examined samples from 21 natural populations across three climatically distinct geographic regions, as well as clones of the same individuals grown in a common garden.
- We found that epigenetic variation was partly associated with climate of origin, particularly in non-CG contexts. Importantly, a large proportion of this variation was heritable across clonal generations. Additionally, a subset of these epigenetic changes affected the expression of genes mainly involved in plant growth and responses to pathogen and abiotic stress. These findings highlight the potential influence of epigenetic changes on phenotypic traits.
- Our findings indicate that variation in DNA methylation, which can be environmentally inducible and heritable, may enable clonal plant populations to adjust to their environmental conditions even in the absence of genetic adaptation.

Introduction

Given their sessile nature, plants must be able to quickly adapt to changing environments. Among other mechanisms, plants can adjust their phenotypes through epigenetic alterations of gene expression, for example via DNA methylation (Riggs & Porter, 1996). In plants, DNA methylation occurs in three sequence contexts: CG, CHG and CHH (where H is A, C or T) (Finnegan *et al.*, 1998), which have different functions (Niederhuth & Schmitz, 2017). In all sequence contexts, DNA methylation represses transposon (TE) mobilization (Zemach & Zilberman, 2010), whereas methylation of gene promoters regulates gene expression (H. Zhang *et al.*, 2018). Importantly, part of DNA methylation is mitotically or even meiotically heritable, thus potentially affecting offspring phenotypes (Niederhuth &

Schmitz, 2014). As heritable phenotypes are the ultimate targets of natural selection (Darwin & Wallace, 1858), it is widely speculated that epigenetic variation, particularly that induced by environmental factors, may confer adaptive potential (Jablonka & Raz, 2009; Ashe *et al.*, 2021). However, establishing a direct link between epigenetic variation and adaptation is complex, particularly under natural conditions. A crucial step towards this goal would be to demonstrate a relationship between methylation variation related to variable environments, the heritability of such variation and its association with gene expression, connections that have been seldom established so far. Therefore, we need to quantify natural DNA methylation variation in plants grown in their natural habitats, determine the extent of heritability of environmentally induced DNA methylation variation and assess its impact on gene expression.

The exploration of environmentally induced epigenetic variation under natural conditions is a complex task, since epigenetic variation can have genetic, environmental and stochastic sources (Zhang *et al.*, 2013; Dubin *et al.*, 2015; Y-Y. Zhang *et al.*, 2018; Johannes & Schmitz, 2019; Díez Rodríguez *et al.*, 2022; Galanti *et al.*, 2022). Genetically determined DNA methylation variants are based on genetic modifications (Richards, 2006), which can be *cis*-acting (i.e. when a TE inserted upstream, a gene promoter drives the methylation of the promoter itself; Martin *et al.*, 2009), or *trans*-acting (i.e. when genetic mutations in genes involved in the DNA methylation machinery induce overall changes in DNA methylation patterns; Dubin *et al.*, 2015; Kawakatsu *et al.*, 2016; Galanti *et al.*, 2022). Environmentally induced DNA methylation variants, in contrast, are under the control of environmental cues (Medrano *et al.*, 2014; Kawakatsu *et al.*, 2016). They can thus arise quickly in response to environmental stimuli (Zhang *et al.*, 2013; Thiebaut *et al.*, 2019) and potentially contribute to rapid adaptation (Miryeganeh & Saze, 2020; Ashe *et al.*, 2021). However, most previous studies on natural plant populations were not able or did not attempt to detect the underlying sources of epigenetic variation, because of inappropriate experimental design, such as lack of field and common garden environments, or low-resolution molecular methods (Zoldoš *et al.*, 2018; Medrano *et al.*, 2020; Miryeganeh *et al.*, 2022). Clearly, the ability to discriminate between genetically determined and environmentally induced heritable epigenetic variation is key for understanding the role of epigenetic variation in the environmental adaptation of plants.

Clonality is a predominant reproductive strategy in many ecosystems, whereby genetically identical offspring are produced, resulting in a reduction in genetic diversity (Klimeš *et al.*, 1997). Nonetheless, epigenetic variation might be particularly relevant for the success and survival of clonal species, since it could to some degree compensate for their low standing genetic variation (Latzel & Klimešová, 2010; Verhoeven & Preite, 2014; Dodd & Douhovnikoff, 2016; Latzel *et al.*, 2016; Münzbergová *et al.*, 2019). In addition, the inheritance of environmentally induced DNA methylation variation may be particularly strong across clonal generations, which lack meiosis and the associated epigenetic resetting that typically leads to erasure of many environmentally induced epigenetic variants, especially in the CHH context (Feng *et al.*, 2010; Calarco *et al.*, 2012; Anastasiadi *et al.*, 2021). However, studies assessing the extent of epigenetic variation, and its heritability and environmental associations, in natural clonal plant populations are still scarce (Richards *et al.*, 2012; De Kort *et al.*, 2020, 2022; Díez Rodríguez *et al.*, 2022).

To fill this gap, we investigated the epigenetic variation, its heritability and its environmental associations in natural populations of a widespread clonal species, the wild strawberry (*Fragaria vesca*). We analysed the methylomes of 231 plants collected from 21 natural populations across multiple geographic regions, including different natural habitats in three European countries. We also examined the DNA methylation patterns of clonal offspring grown in a common garden to determine the extent of epigenetic inheritance and its potential impact on gene expression. Specifically, we formulated three hypotheses. First, we

hypothesized that natural populations of *F. vesca* exhibit DNA methylation variation in the wild, which is significantly associated with differences in local climatic conditions. Second, we hypothesized that climate-associated epigenetic variation is heritable across clonal generations. Lastly, we hypothesized that heritable epigenetic variation has a functional role by modulating gene expression.

Our hypotheses are based on our complementary study, where we conducted a reciprocal transplant experiment using a subset of the same populations (Sammarco *et al.*, 2022). This study provided preliminary clues, indicating that DNA methylation might indeed contribute to local adaptation.

Materials and Methods

Study species

Fragaria vesca L., Rosaceae, is an herbaceous perennial species with wide geographic distribution (Europe, northern Asia, North America and northern Africa; Darrow, 1966). It reproduces both clonally through stolons and sexually through seeds, although its sexual reproduction is very rare in natural conditions (Schulze *et al.*, 2012). While *F. vesca* is primarily a selfing species, outcrossing is also possible (J. Li *et al.*, 2012; Hilmarsson *et al.*, 2017).

Fragaria vesca has a diploid genome ($2n=2x=14$) of c. 240 Mb in size, which includes c. 34 000 genes (v.4.0.a1; Shulaev *et al.*, 2011; Edger *et al.*, 2018). Among these, nine genes encode DNA methyltransferases, including three genes for CMT3, one for MET1, four for DMR1/2 and one for DNMT2. Additionally, it contains four genes responsible for DNA demethylation, including one for DME and three for DML (Gu *et al.*, 2016).

Plant collection and growth

We selected 21 natural populations of *F. vesca* from three European countries, Italy, Czechia and Norway between May and July 2018 (Supporting Information Table S1). We chose these countries as they represented the southern limit (Italy), the core (Czechia) and the northern limit (Norway) of the native range of *F. vesca* distribution in Europe. To increase the environmental difference among the populations' sites, we sampled the populations following a climatic (mostly altitudinal) gradient within each country. The selected populations were geographically close within each country, with an average distance of c. 59 km. This proximity allowed us to focus on local environmental effects while minimizing within-country genetic variation.

For each population, we collected mature, fully developed leaves of four individuals directly from the field conditions ($n=84$) and we dried them in silica gel and used them for whole genome bisulphite sequencing (WGBS) analysis (see Fig. S1 for the experimental design). We then dug up the same ramets plus additional three ($n=147$) and planted them individually following a random block design in $70 \times 40 \times 20$ cm pots filled with a commercial mixture of compost and sand located in the common garden of the Institute

of Botany of the Czech Academy of Sciences in Průhonice, Czechia (49.994°N, 14.566°E) 1–10 d after their collection (see Table S1 for the climatic characteristics of the common garden). Plants were grown under a shading coverage reducing 50% of the light to simulate natural light levels at most of the localities. We let the plants propagate clonally for 1 yr. Then, we separated the biggest offspring ramet of at least the third generation from every clone and transplanted it into a new pot as indicated in Fig. S1. This allowed us to synchronize the age of the offspring ramets and terminate potential transfer of storages and molecules between the interconnected ramets of the clones. After 2 months, we collected mature, fully developed leaf samples and froze them immediately in liquid nitrogen. These samples were later used for WGBS ($n=147$). From a subset of three plants per population ($n=63$), we also collected mature leaf samples for RNA-sequencing in the same way as samples for WGBS.

WGBS library preparation and sequencing

We extracted genomic DNA from individual plants using the Qiagen DNeasy Plant Mini Kit, following the manufacturer's instructions with minor modifications. We prepared libraries for WGBS using the NEBNext Ultra II DNA Library Prep Kit and EZ-96 DNA Methylation-Gold MagPrep (Zymo, Irvine, CA, USA). See Methods S1 for more information.

Methylation and DMR calling

We used the EpiDIVERSE WGBS pipeline for bisulphite reads mapping and methylation calling (<https://github.com/EpiDiverse/wgbs>), which was specifically designed for nonmodel plant species (i.e. species that have not been extensively studied) (Nunn *et al.*, 2021). See Methods S2 for more information on methylation calling and Table S2 for mapping statistics.

To describe overall epigenetic variation within and among populations, and assess the proportion of DNA methylation variance explained by different predictors, we performed principal component (PCA) and redundancy (RDA) analyses on the methylation dataset. For these analyses, we used only the samples for which we had WGBS data for both conditions ($n=84$ per condition). We performed PCAs using custom scripts with the R function `prcomp` in the `STATS` package (v.3.5.1; R Core Team, 2021) and coloured the plots using either country of origin, mean temperature or precipitation averaged over 7 yr before the sampling year (2011–2018), as these were the only recent years available on the C3S Climate Data Store (CDS) website (<https://cds.climate.copernicus.eu/cdsapp#!/home>) (Cornes *et al.*, 2018). We performed RDA with the `RDA` function in the `VEGAN` package (v.2.6.4; Oksanen *et al.*, 2020). See Methods S2 for more information on PCA and RDA.

We identified differentially methylated regions (DMRs) using the EpiDIVERSE DMR pipeline (<https://github.com/EpiDiverse/dmr>) (Nunn *et al.*, 2021) and using the DMR caller METILENE with default parameters (Jühling *et al.*, 2016). We used populations as groups, and we called DMRs separately for all the pairwise comparisons between the populations from the field and

the populations from the garden (i.e. we never compared a field population with a garden population). See Methods S2 for more information on DMR calling.

We plotted methylation levels of DMRs overlapping with genes and TEs using the functions `computeMatrix` and `plotProfile` from DEEPTOOLS v.3.5.1 (Ramírez *et al.*, 2016). For genes, we used the gene annotations v.4.0.a2 downloaded from the Genome Database for Rosaceae (GDR; https://www.rosaceae.org/species/fragaria-vesca/genome_v4.0.a2) (Jung *et al.*, 2019), while for TEs we used an annotation carried out using the EDTA ANNOTATION pipeline v.1.9.6 (Ou *et al.*, 2019) on the substituted genome using default parameters, kindly provided by López *et al.* (2022).

SNP calling

We inferred single nucleotide polymorphisms (SNPs) from WGBS data using the EpiDIVERSE SNP pipeline with default parameters (<https://github.com/epidiverse/snp>) (Nunn *et al.*, 2021, 2022). For the DMR variance decomposition analysis (see below), separately for field and garden conditions, we then combined the output individual VCF files into multisample VCF files using BCFTOOLS (v.1.9; Danecek *et al.*, 2021). As above, we used only the samples for which we had WGBS data for both conditions ($n=84$ per condition). Using VCFTOOLS (v.0.1.16; Danecek *et al.*, 2021), we filtered the variants successfully genotyped in 80% of individuals, with a minimum quality score of 30 and a minimum mean depth of 3.

To describe overall genetic variation within and among populations, and to assess the proportion of genetic variance explained by different predictors, we performed PCA and RDA analyses on the genomic dataset. For both analyses, we combined the individual VCF files from both field and garden ($n=84$ per condition) into a multisample VCF file. We filtered for Minor Allele Frequency (MAF) ≥ 0.05 and pruned for linkage disequilibrium (LD) with an LD threshold (r^2) of 0.2 for SNP pairs in a sliding window of 50 SNPs, sliding by 5. After filtering, we were able to retain 76 669 SNPs. We plotted the PCAs with custom scripts with the R function `prcomp` in the `STATS` package (v.3.5.1; R Core Team, 2021) and using Hellinger-transformed SNPs. We performed RDA analysis similar to methylation, but only using country and climate of origin as predictors. In this analysis, we used Hellinger-transformed SNPs as dependent variables.

DMR variance decomposition analysis, heritability, GO and TE enrichment

To assess the amount of methylation variance explained by *cis*-genetic variants, *trans*-genetic variants and climatic variation, we performed a DMR variance decomposition analysis. For both field and garden samples, we ran three mixed models for each individual DMR (one for each predictor), and we classified each DMR according to what the strongest predictor of its variance was. If no predictor explained $>10\%$ of the variance, the DMR was classified as unexplained (Galanti *et al.*, 2022). See Methods S3 for more information on the models employed. The script

utilized for this analysis is available at: https://github.com/Dario-Galanti/popDMRs_refine_VCA/tree/main/DMRs_VCA.

We estimated the broad-sense heritability of epigenetic changes that were associated with climatic variation (Scheiner, 1993). Specifically, we considered only the DMRs that were found to be associated with climate in both field and garden conditions. First, we calculated the total epigenetic variance (VEpi) as the sum of genetic variance (VG), environmental variance (VE) and unexplained variance calculated in the DMR variance decomposition analysis ($VEpi = VG + VE + \text{unexplained variance}$). We estimated heritability (H^2) by adapting the traditional genetic formula $H^2 = VG/VP$ to $H^2 = VE/VEpi$. This calculation enabled us to determine the proportion of the total epigenetic variance attributable to environmental factors. Notably, this includes epigenetic changes that are environmentally induced, independent from genetic variation and transmitted to the clonal offspring in the garden conditions.

We plotted circo plots using the R package CIRCLIZE (v.0.4.9; Gu *et al.*, 2014). We performed the correlation analysis between number of *cis*-, *trans*-, climate-, unexplained DMRs and number of genes and TEs using the Pearson correlation method. We calculated the DMR, gene and TE counts assigned to 1-kb genomic bins and performed the correlation between DMR count and gene or TE count. We then ran a GO enrichment analysis for *cis*-, *trans*-, climate- and unexplained-predicted DMRs, separately for each sequence context and for field and garden conditions. We extracted DMR-related gene promoters with BEDTOOLS and performed a GO enrichment analysis using the R package CLUSTERPROFILER (v.3.18.1; Yu *et al.*, 2012) with an FDR-adjusted P -value < 0.05 .

We conducted an enrichment ratio analysis to assess the potential enrichment of *trans*-predicted DMRs in both CHG and CHH contexts, as well as unexplained-predicted DMRs within the CHH context, with various TE superfamilies. To determine the random distribution of DMRs across TE superfamilies, we employed Fisher's exact test. The results were statistically significant for all cases (P -value < 0.001), indicating that the distribution of DMRs across TE superfamilies was not random. Subsequently, we computed the enrichment ratio by dividing the proportion of DMRs within a specific TE superfamily by the proportion of that TE superfamily within the genome. We established an enrichment ratio threshold of 1 and classified TE superfamilies as enriched (ratio > 1) or depleted (ratio < 1).

Genome-wide association analysis

To assess the putative genetic basis of the DMRs assigned to climate as the strongest predictor, we ran genome-wide association (GWA) analysis for these DMRs in the garden conditions, including all the available samples to increase the statistical power of the analysis (seven plants per population, $n = 147$). This analysis allowed us to assess which DMRs were directly influenced by the climates of origin, as climate-predicted DMRs could in principle be under direct or indirect climate control. Direct environmental induction is when climate-predicted DMRs are associated only with environmental factors and not DNA sequence. However, if they are also associated with DNA sequence variation, the

link between environment and epigenetic variation could be indirect, through selection acting on genetic variants first.

We classified the DMRs displaying significant GWA peaks as *indirectly* associated with the environment and excluded them from the subsequent analysis, which aimed to examine the correlation between DMR methylation and gene expression. See Methods S4 for more information on the GWA analysis.

RNA-sequencing, correlation of climate-predicted DMRs with gene expression and differential gene expression analysis

We collected mature leaf samples from three randomly selected plants per population from garden condition ($n = 63$), and we snap-froze them in liquid nitrogen. We extracted mRNA using the Nucleospin RNA Plus kit (Macherey Nagel, Düren, Nordrhein-Westfalen, Germany), following the manufacturer's instructions with minor modifications. See Methods S5 for more information on RNA extraction, sequencing, reads alignment and quantification, and Table S2 for mapping statistics.

To describe overall transcriptome variation within and among populations, and to assess the proportion of transcriptome variance explained by different predictors, we performed PCA and RDA analyses on the transcriptome dataset. In these analyses, we employed read counts normalized with the R function DESeq in the DESeq2 package (v.1.30.1; Love *et al.*, 2014), followed by variance stabilizing transformation with the function vst from the same package. We performed PCA with custom scripts using the function plotPCA in the DESeq2 package using Hellinger-transformed read counts, and RDA with the RDA function in the VEGAN package, using Hellinger-transformed read counts as dependent variable and country and climate of origin as predictors. As above, we tested the statistical significance of the RDA analyses using a permutation test with 499 permutations.

For the correlation analysis between methylation and gene expression, for each sample, we normalized the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values and extracted the genes adjacent to the *directly* environmentally induced DMR-related promoters. These were the environmentally induced DMRs that were retained after excluding those that displayed significant peaks in GWA analyses. We performed Spearman correlation analysis with each of the remaining genes (572, 856 and 3955 genes in the CG, CHG and CHH contexts, respectively). We selected only those genes that showed a statistically significant correlation (P -value < 0.05). Given the relatively high number of tests conducted in this study, we chose not to adjust the P -values for multiple comparisons as this could be overly stringent. To assess whether the significant correlations could be due only to type I error, we compared the number of observed results with what we would expect to find by chance at P -value = 0.05. In the CG, CHG and CHH contexts, we would expect to find 29, 43 and 198 significant correlations occurring by chance, respectively. As the number of significant correlations observed in our data was more than twice the number expected by chance, we considered these correlations as real (Rothman, 1990; Zhu *et al.*, 2023).

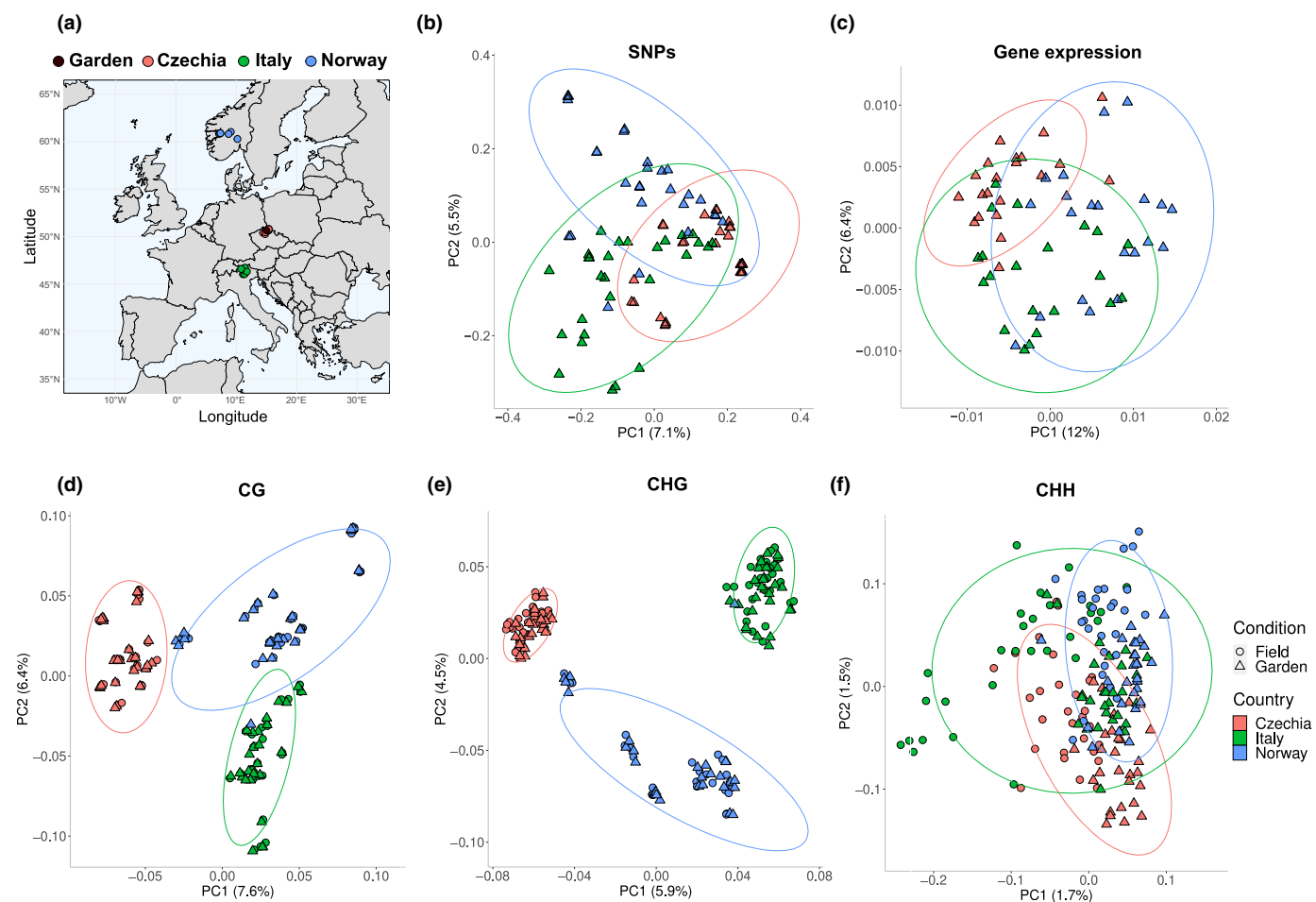


Fig. 1 Map of the sampling locations of *Fragaria vesca* populations, and principal component analyses (PCA) for DNA methylation, genetic variants (SNPs) and gene expression. (a) Sampling locations plotted using the R packages *SF* (v.1.0.9; Pebesma, 2018) and *RNATURALEARTH* (v.0.1.0; South, 2017). (b) Full dataset of SNPs shown only for garden plants, as these are clones of the field ones and thus are genetically identical (plants: $n = 84$). (c) Full dataset of gene expression (only available for garden plants; plants: $n = 63$). (d–f) Full datasets of CG, CHG and CHH methylated positions, respectively (field plants: $n = 84$, garden plants: $n = 84$).

We identified differentially expressed genes (DEGs) between populations using the DESeq2 package for R (v.1.30.1; Love *et al.*, 2014). We used an adjusted P -value < 0.05 (Benjamini–Hochberg) and an absolute value of fold change (FC) ≥ 1.5 as thresholds for statistical significance. To assess the proportion of DEGs that overlapped with environmentally linked DMRs, we merged the DEGs identified in all pairwise comparisons between populations into a single file. We then overlapped this list of DEGs with the list of environmentally linked DMRs that had significant correlations with gene expression.

Results

Hypothesis 1. Natural populations exhibit DNA methylation variation associated with differences in local climatic conditions

Genetic, epigenetic and transcriptome variation: PCA and RDA To describe overall genetic, transcriptome and epigenetic

variation within and among the studied populations, we performed a PCA for genetic variants (SNPs; Fig. 1b), gene expression (Fig. 1c) and DNA methylation (Fig. 1d–f). Overall, the plants appeared to cluster more clearly by country of origin than by temperature (Fig. S2a–e) or precipitation of origin (Fig. S2f–j). For DNA methylation, these geographic clusters were strong and almost equal in the field and garden, in the CG and CHG contexts (Fig. 1d,e). The separation was much weaker in CHH, which on the contrary seemed more strongly influenced by the growth conditions (field vs garden; Fig. 1f). However, DNA methylation levels were similar between field and garden samples even in the CHH context (Fig. S3).

Using RDA, we assessed the proportions of DNA methylation, genetic and gene expression variance explained by country and climate (temperature and precipitation) of origin and their joint effects. The analysis of DNA methylation also included growth conditions as a factor. We found that country explained the highest proportion of variance, followed by climate, and their joint effects (Fig. 2). Growth conditions generally explained very little variance.

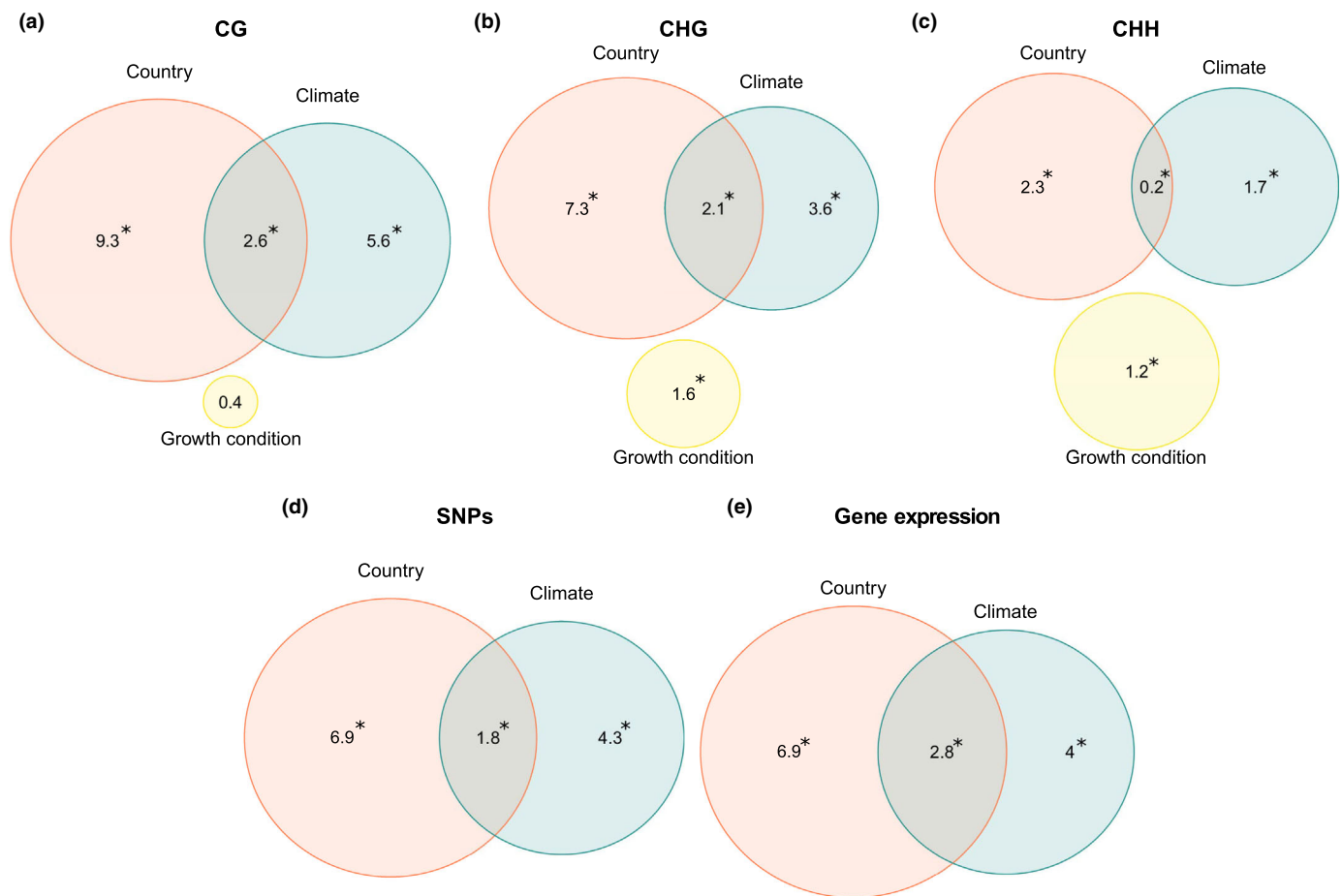


Fig. 2 Venn diagrams showing the percentage of variance for DNA methylation, genetic and gene expression variance explained by several predictors. The variance was calculated using redundancy analysis (RDA), with the country of origin, climate (mean temperature and precipitation averaged across 2011–2018) and growth conditions (field, garden) (only for methylation) used as predictors. (a–c) Percentage of CG, CHG and CHH methylation variance respectively (field plants: $n = 84$, garden plants: $n = 84$). (d) Genetic (SNPs) variance (field plants: $n = 84$, garden plants: $n = 84$). (e) Gene expression variance (garden plants: $n = 63$). Asterisks (*) represent significant values (P -value < 0.05).

Identification of DMRs in field and garden conditions To quantify methylation differences at the genomic-region level, we identified DMRs for all pairwise comparisons between populations from the same growth condition (i.e. we compared all the field populations to all the other field populations and all the garden populations to all the other garden populations). After merging overlapping or ‘book-ended’ DMRs identified in each pairwise comparison (see BEDTOOLS merge; Quinlan & Hall, 2010), we identified over 344 000 DMRs in the field (CG = 82 675, CHG = 49 600, CHH = 211 735) and almost 249 000 in the garden (CG = 71 972, CHG = 37 925, CHH = 139 097). We found substantial overlap of field and garden DMRs, with 76% of CG-DMRs, 63% of CHG-DMRs and 62% of CHH-DMRs in the field also present in the garden. In both growth conditions, CG-DMRs were most frequent in gene bodies, whereas CHG- and CHH-DMRs were predominantly associated with promoters and in particular TEs (Fig. S4). The numbers of CHH-DMRs in promoters and TEs were much higher in the field than in the garden, while CG- and CHG-DMRs were similarly abundant in the two datasets. We also

identified substantial methylation variations of these DMRs across samples within genes and TE regions (Fig. 3, field; Fig. S5, garden).

Hypothesis 2. Climate-associated epigenetic variation is heritable across clonal generations

Association of epigenetic variation with genetic and climatic variation In order to assess the association of epigenetic variation with genetic and climatic variation, we performed a DMR variance decomposition analysis. We found that in all sequence contexts and for both field and garden conditions, DMR variation was generally best predicted by *trans*-genetic variation, followed by climatic variation and *cis*-genetic variation (Fig. 4b). In the field, the fractions of climate-predicted DMR variation gradually increased from CG to CHG and CHH. However, under garden conditions, this increase was observed only from CG to CHG, as the fraction of climate-predicted DMRs decreased in CHH.

We then examined the genomic distribution of the genetic and climate-influenced DMRs. Overall, for both field and garden, the

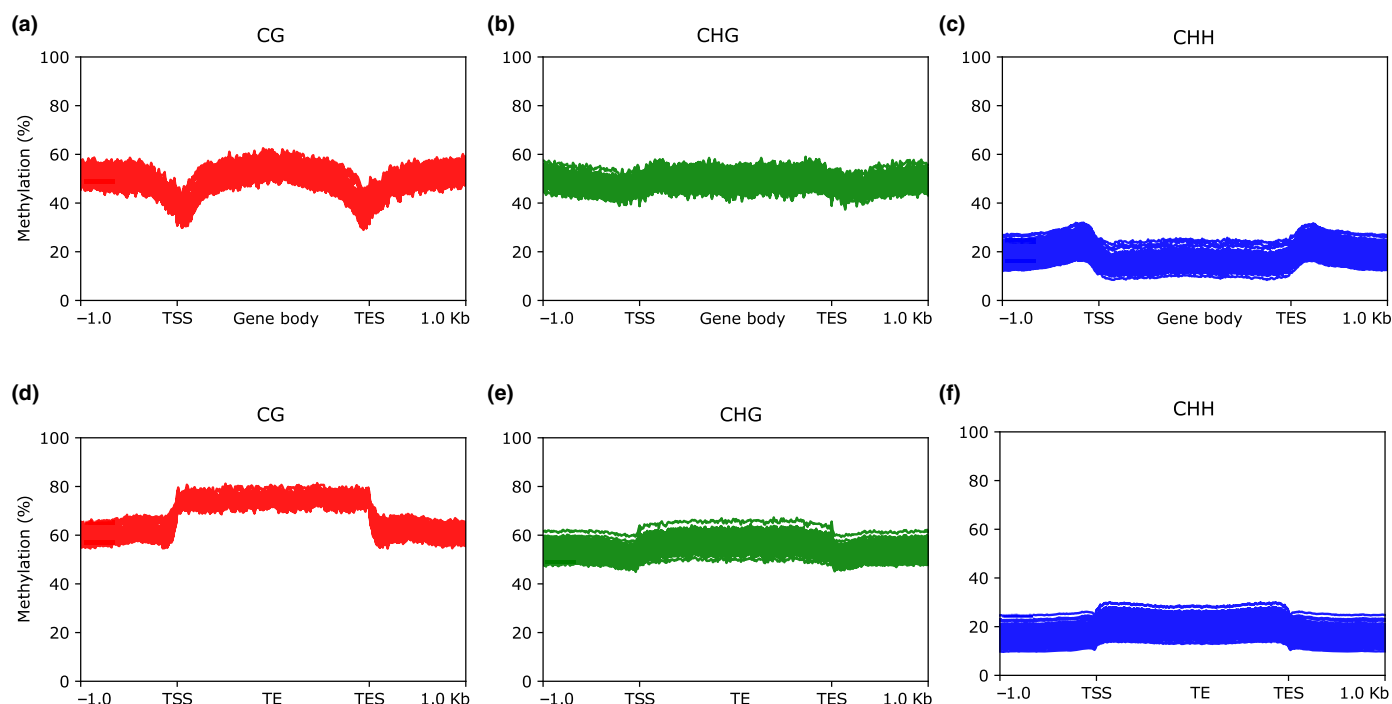


Fig. 3 Methylation level of differentially methylated regions (DMRs) in the field, overlapping with genes and transposable elements (TEs). (a) CG-DMRs, (b) CHG-DMRs and (c) CHH-DMRs overlapping with genes and their 1-kb-long upstream and downstream sequences. (d) CG-DMRs, (e) CHG-DMRs and (f) CHH-DMRs overlapping with TEs and their 1 kb-long upstream and downstream sequences. TES, transcription end site; TSS, transcription start site. Number of plants: 84.

genomic density of predicted CG-DMRs in *trans* largely followed the distribution of genes (Figs 4c, S6a; Table S3), while *trans*- and particularly climate-predicted DMRs in CHG and CHH mainly followed the distribution of TEs (Figs 4d,e, S6b,c; Table S3). Furthermore, unexplained DMRs predominantly mirrored the distribution pattern of TEs, with this trend being particularly prominent in the CHH context.

Accordingly, the number of predicted CG-DMRs in *trans* correlated positively with the number of genes and negatively with the number of TEs. Conversely, predicted CHG- and CHH-DMRs in *trans* and climate-predicted DMRs in all contexts negatively correlated with the number of genes and positively with the number of TEs (Table S3; see Fig. S7 for the raw number of DMRs). Finally, unexplained CHH-DMRs positively correlated with the number of TEs.

Upon closer examination, we observed that predicted CHG- and CHH-DMRs in *trans* DMRs seemed to be concentrated in restricted TE-rich regions of each chromosome, despite TEs being more widely distributed across the genome (Fig. 4d,e). In contrast, unexplained CHH-DMRs overlapped with the overall distribution of TEs. To determine whether the observed peaks corresponded to specific TE families, we performed a TE enrichment analysis. We observed that different TE superfamilies were either enriched or depleted in the CHG context, and this pattern was consistent in both field and garden conditions. However, in the CHH context, the enrichment or depletion of these superfamilies varied between field and garden conditions (Table S4). Specifically, we found that CHG-DMRs in *trans* displayed

enrichment in TE superfamilies, including DTA (hAT), DTT (Tc1/Mariner), Helitrons, and LTR Copia and Ty3, and depletion in DTC (CACTA), DTH (PIF/Harbinger) and DTM (Mutator). Similarly, CHH-DMRs in *trans* under field conditions showed enrichment in the DTC and Copia and Ty3 superfamilies, while showed depletion in DTA, DTH, DTM, DTT and Helitrons. In contrast, under garden conditions, CHH-DMRs in *trans* showed enrichment in DTA, DTC, DTH, DTM and Ty3, and depletion in DTT, Helitrons and Copia. Lastly, unexplained CHH-DMRs under field conditions displayed enrichment and depletion in the same TE superfamilies as the CHH-DMRs in *trans* under field conditions. Conversely, unexplained CHH-DMRs under garden conditions exhibited enrichment in DTC, DTM, Ty3, and depletion in DTA, DTH, DTT, Helitrons and Copia.

GO enrichment analysis of promoters with predicted DMRs in *cis*, *trans* and climate-predicted Since many DMRs overlapped with genes (Fig. S7), we performed a Gene Ontology (GO) enrichment analysis to functionally characterize genes containing predicted DMRs in *cis*, *trans* and climate-predicted in their promoters. We found enrichment for several GO terms, but only for CHG- and CHH- and not CG-DMRs (Fig. 5; Table S5). For CHG-DMRs, we found common terms between field and garden conditions, while no overlapping terms were identified for CHH-DMRs.

For the predicted DMRs in *cis*, we found enrichment for several terms related to biological process. However, we observed

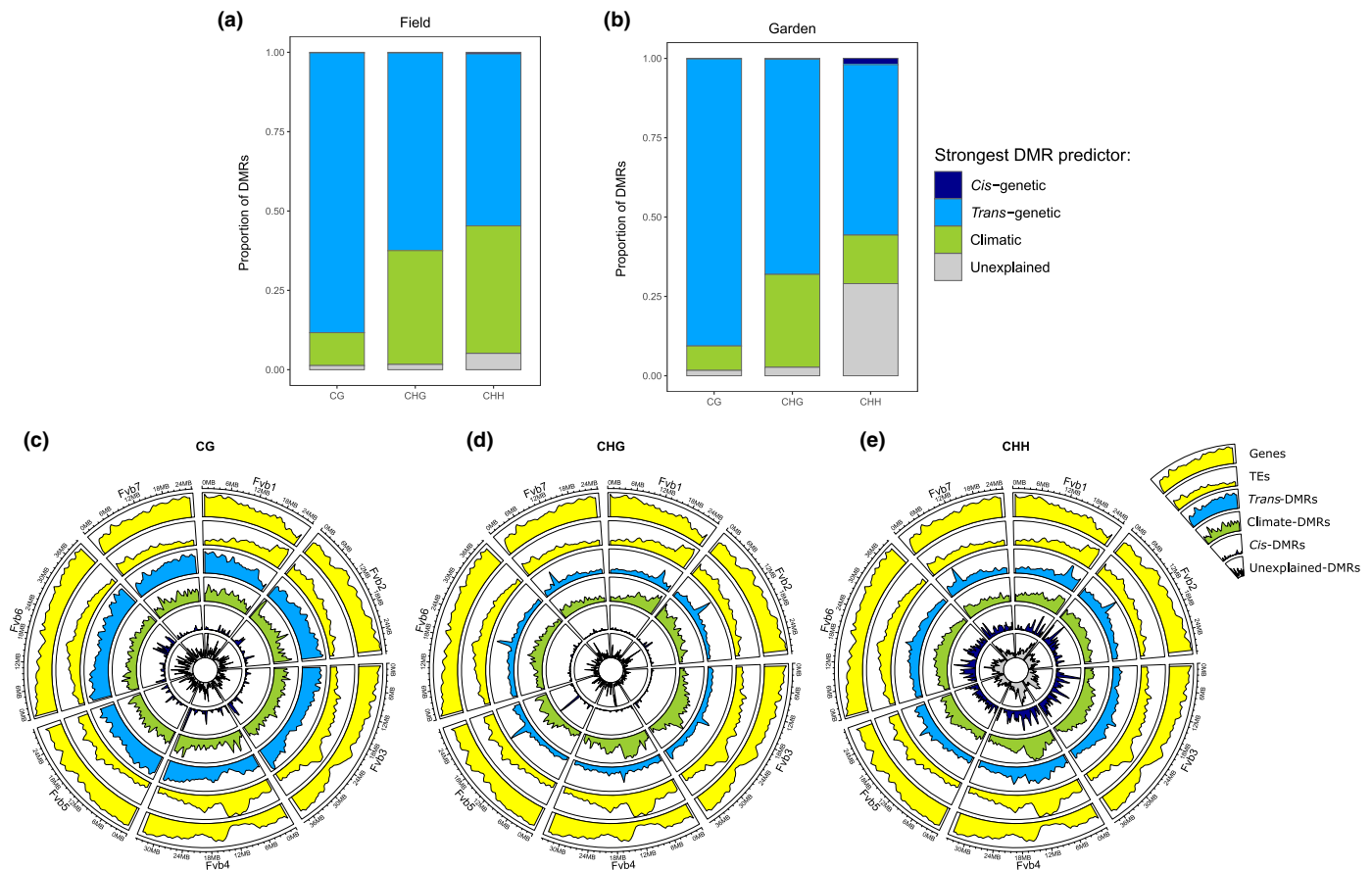


Fig. 4 Strongest predictors of differentially methylated regions (DMRs). The proportions of DMRs in different sequence contexts that were best predicted by either *cis*-genetic, *trans*-genetic or climatic variation. For each individual DMR, we ran three mixed models and we classified each DMR according to what the strongest predictor of its variance was (see Supporting Information Methods S3). DMRs with < 10% variance explained by all three predictors are classified as 'unexplained'. (a) Field, total DMRs: 82 546 CG, 49 459 CHG and 211 363 CHH ($n = 84$ plants). (b) Garden, total DMRs: 71 856 CG, 37 795 CHG and 138 807 CHH ($n = 84$ plants). (c–e) Circos plots visualizing the densities of field-DMRs, gene and transposable element (TE) annotations for all chromosomes (Fvb1–7) in CG (c), CHG (d) and CHH (e) contexts. From outer to inner circles: gene and TE annotations (yellow), predicted DMRs in *trans* (light blue), climate-predicted DMRs (green), predicted DMRs in *cis* (dark blue) and unexplained DMRs (grey). All the correlations calculated between number of *cis*-, *trans*-, climate-, unexplained DMRs and number of genes and TEs were significant at $P < 0.05$ (Table S3).

only a few enriched terms for both predicted DMRs in *trans* and climate-predicted DMRs, some of which were common between the two ('RNA–DNA hybrid ribonuclease activity'; 'retrotransposon nucleocapsid'). We also found 'retrotransposon nucleocapsid' among the unexplained DMRs.

When comparing field and garden conditions, we found overlapping terms for CHG-DMRs such as 'glucose metabolic process' for predicted DMRs in *cis*, 'retrotransposon nucleocapsid' and 'RNA–DNA hybrid ribonuclease activity' for predicted DMRs in *trans*. The term 'retrotransposon nucleocapsid' was also found for climate-predicted DMRs.

Hypothesis 3. Heritable epigenetic variation modulates gene expression

Identification of direct environmental associations of climate-predicted DMRs: GWA analysis To validate the overlap of climate-predicted DMRs between field and garden conditions, we compared the climate-predicted DMRs identified within these

growth conditions. Approximately 40% of DMRs showed overlap in CG, while *c.* 50% exhibited overlap in both CHG and CHH. To quantify the impact of environmental variation on inherited epigenetic changes, we calculated the broad-sense heritability (H^2) for these changes. Our results indicated a heritability of 0.03 for CG, 0.15 for CHG and 0.08 for CHH, highlighting the potential influence of climatic variation on inherited epigenetic changes.

It is important to note that the limited overlap of climate-predicted DMRs between field and garden conditions arises from numerous DMRs displaying nearly identical variance explained by *trans*-genetic and climatic factors. To distinguish the climate-predicted DMRs with a genetic basis and exclude them from further analysis, we conducted a GWA analysis. This analysis allowed us also to assess which DMRs were *directly* influenced by the climates of origin. We focussed on the *directly* climate-associated DMRs, as we aimed to explore the adaptive potential of epigenetic variation independent of genetic variation.

For this analysis, we used only the garden samples, to ensure that the selected climate-predicted DMRs were heritable and thus

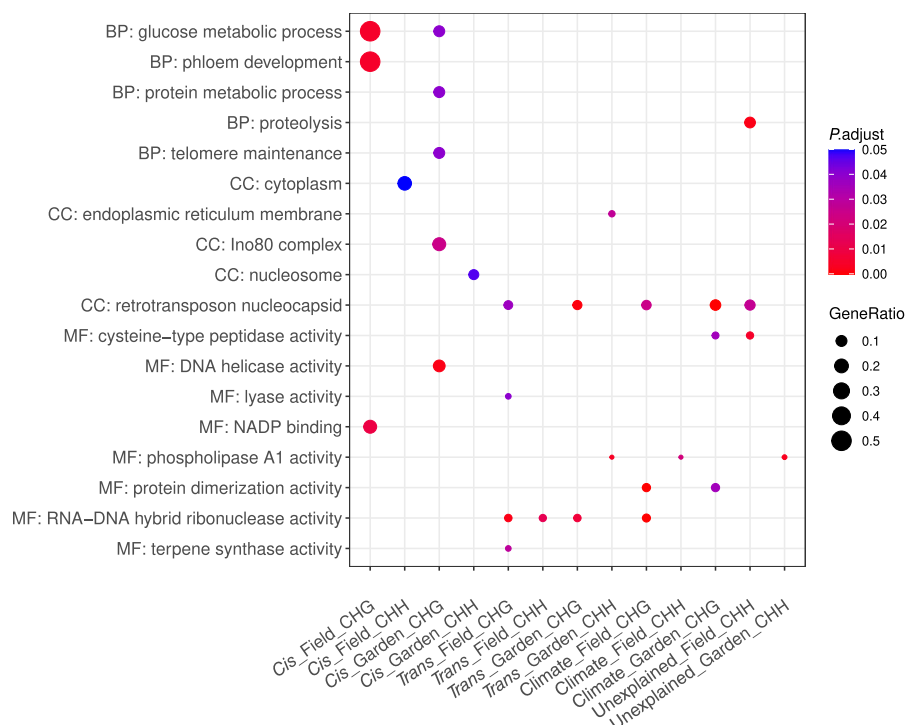


Fig. 5 Gene Ontology (GO) enrichment analysis of genes containing *cis*-, *trans*- and climate-predicted DMRs, as well as unexplained DMRs, in their promoters. The GeneRatio represents the proportion of these genes associated with each GO term. Only GO terms with an adjusted *P*-value < 0.05 are shown. BP, biological process; CC, cellular component; MF, molecular function.

of evolutionary potential. To increase the statistical power of the analysis, we included all the 147 garden samples. We performed GWA using individual DMR-promoter methylation as phenotypes and SNPs as predictors and selected the threshold *P*-value using Bonferroni correction. Out of 2092 CG-, 3049 CHG- and 8186 CHH-climate-predicted DMRs overlapping promoters, we found significant GWA hits for 62.8% of the climate-predicted DMRs in CG, 30.8% in CHG and 18.8% in CHH (dot plots for GWA in Fig. S8). We thus classified these DMRs as *indirectly* associated with the environment. In contrast, DMRs that showed no significant association with SNP variation in the GWA analyses were classified as *directly* environmentally linked.

As we focussed on garden offspring for GWA analysis, it is worth noting that detecting GWA hits in CHH may be constrained by the limited transgenerational stability of methylation in this context.

Correlation analysis of *directly* climate-predicted DMRs with gene expression and differential gene expression analysis To explore whether the putative *directly* environmentally linked DMRs likely had a functional role, we tested whether methylation levels of individual DMRs were correlated with the expression of their overlapping genes. For this analysis, we utilized the transcriptomic dataset available for a subset of the garden samples. We found statistically significant correlations in 11.4% of the cases in CG, 10.4% in CHG and 10.4% in CHH (corresponding to 65 genes in CG, 89 genes in CHG and 411 genes in CHH). We found both positive and negative correlations between promoter methylation of genes and their expression levels (Fig. 6; Table S6). A portion of these promoters were found to overlap with TEs (34% in CG and CHH and 35% in

CHG) (Fig. 6b–d). To complement our analysis, we applied the DIABLO method (Data Integration Analysis for Biomarker discovery using Latent cOmponents), an integrative approach that identifies key molecular drivers from multiomics data (Singh *et al.*, 2019). This analysis yielded numerous statistically significant associations, thus validating our initial findings (Fig. S9; Table S7).

To determine whether the environmentally linked DMRs that significantly correlated with gene expression also exhibited differential expression, we overlapped them with the list of DEGs identified between populations. We found that a subset of the DEGs overlapped with the environmentally linked DMRs, specifically 19.7% of genes in CG, 15.6% in CHG and 16.7% in CHH (corresponding to 13 genes in CG, 14 genes in CHG and 69 genes in CHH). These DEGs were mainly related to plant growth, response to pathogens and abiotic stresses. Moreover, we found that the DEGs that overlapped with both CHG- and CHH-DMRs were also related to TE mobilization. Lastly, the DEGs overlapping with CHH-DMRs demonstrated additional connections to protein turnover, carbohydrate, lipid and amino acid metabolism, the self-incompatibility system and the regulation of gene expression (Table S8).

Discussion

There is growing interest in the effects of environmental variation on plant DNA methylation, the inheritance of environmentally induced methylation variation across generations and its effects on phenotypes and plant adaptation (Jablonka & Raz, 2009; Thiebaut *et al.*, 2019; Ashe *et al.*, 2021). However, clear evidence is still scarce, particularly from natural conditions. Here, we

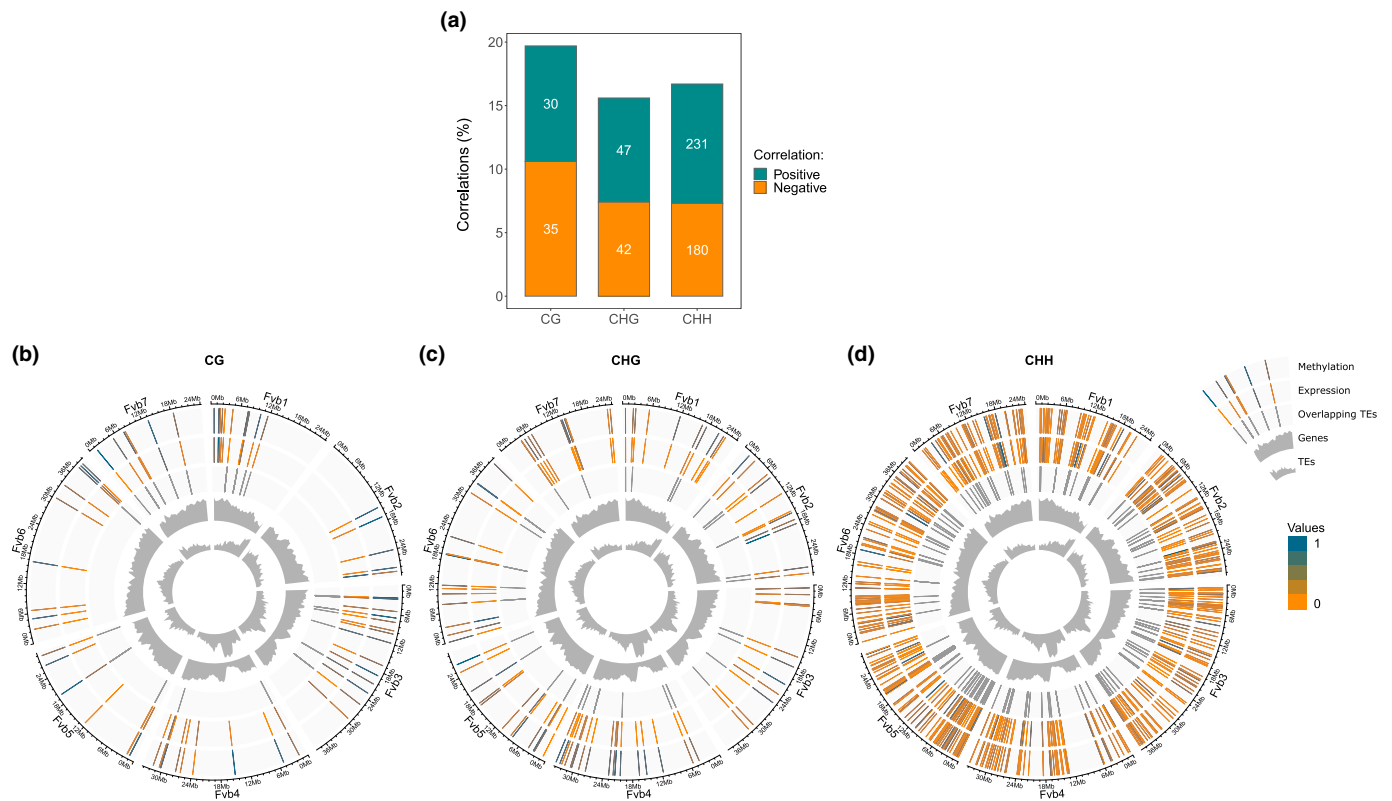


Fig. 6 Climate-associated DMRs that were significantly correlated with gene expression. (a) Percentage of genes with positive or negative correlation between promoter methylation of climate-predicted DMRs and expression of the adjacent genes. The numbers within the bars show the raw values of significant correlations. (b–d) Circos plots illustrating the methylation and gene expression levels of climate-associated DMR promoters significantly correlated with the expression of adjacent genes. These data were averaged across all 63 samples. Methylation and gene expression levels are depicted using a colour gradient, with orange representing 0 and blue representing 1. Similar colours between methylation and expression values indicate positive correlation, while opposite colours indicate negative correlation. Fvb1–7 denote all *Fragaria vesca* chromosomes. Different sequence contexts are displayed in separate panels: (b) CG, (c) CHG and (d) CHH. From outer to inner circles: methylation and gene expression levels (orange–blue colour gradient), presence of transposable elements (TEs) in the DMR promoters significantly correlated with expression (grey bars) and distribution of gene and TE annotations (grey).

tested whether climate of origin is associated with DNA methylation variation in natural plant populations of wild strawberry, whether such DNA methylation variation is stable across clonal generations and whether it correlates with gene expression.

Natural populations of *F. vesca* harbour DNA methylation variation, partly due to climatic conditions

The analysed populations harboured comparable genetic and epigenetic geographic structure (Fig. 1), suggesting that the observed epigenetic variation was largely genetically determined (as already reported for *F. vesca*) (De Kort *et al.*, 2020, 2022). However, upon closer inspection, the geographic distribution of CG and CHG methylation appears more defined than that of SNPs. This indicates that environmental factors, particularly climate, have a more substantial impact on CG and CHG methylation than on genetic variation. In contrast, gene expression patterns showed limited correlation with geographic location, suggesting that certain genes are broadly expressed across all locations and that they are not strongly associated with either genetic or climatic variation.

The RDA revealed that country of origin accounted for the largest proportion of methylation, genetic and expression variance, followed by climate and their synergic effects (Fig. 2). This emphasizes that country (including demographics) and climatic conditions contribute significantly to shaping the epigenetic structure of these populations. Moreover, aside from the DMRs related to DNA sequence variation, we also identified DMRs that were related to climatic variation (Fig. 4a,b). We observed an increase in the number of these DMRs from CG to CHG and CHH contexts. This pattern is consistent with previous studies that have reported an increase in the number of climate-associated DMRs from CG to CHG and CHH contexts and suggests that non-CG methylation may be particularly sensitive to climatic conditions (Díez Rodríguez *et al.*, 2022; Galanti *et al.*, 2022). A GO enrichment analysis of DMR-overlapping gene promoters showed that *cis*-genetic variants induced mainly DNA methylation variants in genes related to chromatin and chromatin remodelling, telomere maintenance and metabolic processes, while *trans*-genetic and climatic variation affected DNA methylation in genes related to RNA–DNA hybrid ribonuclease activity and retrotransposon nucleocapsid (Fig. 5).

Interestingly, since RNA–DNA hybrids and retrotransposon nucleocapsid are related to retrotransposon mobilization (Todd *et al.*, 2020), it is likely that both *trans*-genetic and climatic variations modulate transposition and that the environment might thus control TE mobilization in wild conditions (Rey *et al.*, 2016; Baduel *et al.*, 2021). Furthermore, we observed an enrichment of non-CG-DMRs induced by *trans*-genetic variation in pericentromeric regions (Fig. 4d,e). This could be due to *trans*-chromosomal interactions involving these regions. Such interactions could alter the methylation patterns within these regions, thereby contributing to the formation of these DMRs (Feng *et al.*, 2014). These DMRs induced by *trans*-genetic variation, in turn, might affect TE mobilization, particularly within pericentromeric regions.

Part of the climate-associated epigenetic variation is inherited across clonal generations

The comparison of epigenetic profiles of field-grown plants with their genetically identical clonal offspring in a common garden allowed us to assess the inheritance of environmentally associated DNA methylation and its role in gene regulation. Overall, DNA methylation levels were similar between field and garden samples (Fig. S3). CG and CHG methylation seemed to cluster similarly under both field and garden conditions, while CHH methylation displayed a less similar clustering between these two conditions (Fig. 1d–f). These findings can be attributed to the higher stability of CG and CHG methylation across clonal generations when compared to CHH methylation (Feng *et al.*, 2010; Calarco *et al.*, 2012).

We also found the greatest differences in the numbers of DMRs between field and garden conditions in CHH for both gene- and TE-related DMRs, with a generally higher methylation variation (Fig. 3) and number of DMRs in the field than in the garden (Fig. S4). DNA methylation in all contexts plays a crucial role in TE silencing and in the establishment of heterochromatin (Slotkin & Martienssen, 2007; Fultz *et al.*, 2015), and CHH also plays an important role in gene regulation in euchromatic regions (Zemach *et al.*, 2010; Gent *et al.*, 2013; Martin *et al.*, 2021). The higher methylation variation and number of gene- and TE-related DMRs in the field suggest a potential association between natural environmental conditions and the regulation of gene expression and TE mobilization. This is consistent with our previous finding of a possible link between the environment and TE mobilization in wild conditions (see above). However, it is important to clarify that our findings do not provide direct proof of connection between DNA methylation and TE reactivation. To establish a direct link, future studies could consider studying DNA methylation changes under natural environmental conditions coupled with assays to detect TE reactivation. Subsequently, measuring the fitness of these individuals could provide insights into whether TE reactivation can have evolutionary implications.

In order to distinguish whether the observed heritable DNA methylation variation was associated with DNA sequence variation and/or environments of origin, we performed the DMR variance decomposition analysis also for the plants grown in garden

conditions (Fig. 4b). We assumed that climate-associated DMRs maintained under common environmental conditions are likely adaptive, because otherwise one should not find such (nonrandom) patterns of climate association. Based on the DMRs that were found to be influenced by climate in both field and garden conditions, we found relatively high levels of broad-sense heritability (H^2) of environmentally induced epigenetic changes, suggesting that a significant proportion of the epigenetic changes under climatic control are heritable across clonal generations. Furthermore, they suggest a higher heritability of these changes in non-CG contexts compared with the CG context. This can be attributed to the higher contribution of climatic variation to non-CG methylation relative to CG methylation.

When comparing field and common garden conditions, we found similar amounts of climate-associated DMRs, especially in the CG and CHG contexts. The CHH context, in contrast, showed decreased climate-associated variation but a large increase in unexplained variation under garden conditions. 60% of these unexplained DMRs overlapped with DMRs associated with climate in the field (data not shown), suggesting that the unexplained DMRs in CHH were due to the environmental conditions in the common garden and that CHH methylation is the least stable across clonal generations and/or the most responsive to short-term environmental changes. On the contrary, the similar CG- and CHG-DMR variation for field and garden indicates that the climates of origin induced DMR variation in these contexts that was heritable across clonal generations.

Our findings are corroborated by a recent study of pennycress (*Thlaspi arvense*) where similar extents of climate-predicted DMRs were observed in natural accessions grown in glasshouse conditions (Galanti *et al.*, 2022). Also in pennycress the contribution of climatic variation to DMR variation increased from CG to CHG and CHH, and the CHH context harboured the greatest amount of unexplained variance. Although the pennycress study also found that *trans*-genetic variation explained most DMR variation, *cis*-genetic variation explained a greater fraction of DMR variation than in our study (*c.* 5–14% in *T. arvense* and 0.2–2% in *F. vesca*). This could be due to the higher standing genetic variation of the sexually reproducing *T. arvense* (Frels *et al.*, 2019) in comparison with the mostly clonally reproducing *F. vesca* in natural conditions (Schulze *et al.*, 2012).

Heritable climate-associated DNA methylation variation partly correlates with gene expression

Some of the climate-associated DMRs identified under common garden conditions (i.e. inherited) were significantly correlated with gene expression. Interestingly, our analysis found both positive and negative correlations with gene expression (Fig. 6). Although promoter methylation is usually negatively associated with gene expression (X. Li *et al.*, 2012), there are also some reports of positive effects of promoter methylation on expression (Lang *et al.*, 2017), especially in the CHH context (Gent *et al.*, 2013; Xu *et al.*, 2018; Rajkumar *et al.*, 2020; Wang *et al.*, 2020).

It is crucial to note that our study may not have been able to detect all correlations between methylation and gene expression. This could be due to the potential *trans*-acting effects of climate-associated DMRs on environmentally responsive genes, which might have limited our ability to identify such correlations. Alternatively, the observed correlations might be restricted to a specific subset of genes involved in TE regulation (Lloyd & Lister, 2021). Further research is needed to elucidate these dynamics.

Conclusions

Our study reveals that a portion of *F. vesca*'s DNA methylation is linked to climatic conditions, inherited across clonal generations, and affects gene expression, suggesting that it may be a target of natural selection. However, it is important to acknowledge that our study only considered temperature and precipitation as climatic variables, which could limit the understanding of the interplay between other environmental factors and adaptive epigenetic variation.

To further explore the role of epigenetic changes in local adaptation and evolutionary processes, we propose to test the fitness of plants that show climate-associated methylation patterns. This could provide significant insights into the evolutionary implications of epigenetic variation. Additionally, we propose to investigate other plant species in natural populations with different life-history traits, including clonal and sexual reproduction. Such studies could expand our understanding of how environmental and genetic variation shape natural plant populations.

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Competing interests









None declared.

Author contributions

IS, VL and ZM planned and designed the research. IS, ZM and VL conducted the fieldwork. IS, BDR, DG and AN designed computer programs. IS analysed the data and drafted the manuscript. IS, BDR, DG, AN, CB, OB, ZM, and VT contributed to

the interpretation of the results. IS wrote the first draft of the manuscript, and IS, BDR, DG, AN, CB, OB, ZM, and VT contributed substantially to revisions.

ORCID

Claude Becker  <https://orcid.org/0000-0003-3406-4670>
 Oliver Bossdorf  <https://orcid.org/0000-0001-7504-6511>
 Bárbara Díez Rodríguez  <https://orcid.org/0000-0003-3534-7197>
 Dario Galanti  <https://orcid.org/0000-0002-6567-1505>
 Vít Latzel  <https://orcid.org/0000-0003-0025-5049>
 Zuzana Münzbergová  <https://orcid.org/0000-0002-4026-6220>
 Adam Nunn  <https://orcid.org/0000-0002-9276-6243>
 Iris Sammarco  <https://orcid.org/0000-0002-4101-6223>

Data availability

The sequencing data that support the findings of this study are openly available in the European Nucleotide Archive (ENA, www.ebi.ac.uk/ena/), under the project PRJEB51609.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Experimental design of the study.

Fig. S2 Principal component analysis for genetic variants, gene expression and DNA methylation coloured by mean temperature and precipitation.

Fig. S3 Boxplots showing methylation distribution across different genomic features for field and garden conditions.

Fig. S4 Total numbers of differentially methylated regions identified in different genomic contexts.

Fig. S5 Methylation level of differentially methylated regions in the garden, overlapping with genes and transposable elements.

Fig. S6 Distribution of the strongest differentially methylated region predictor in the garden.

Fig. S7 Number of *cis*-, *trans*-, climate-predicted and unexplained differentially methylated regions overlapping promoters, gene bodies or transposable elements.

Fig. S8 Dot plots for genome-wide association analyses for climate-predicted differentially methylated regions overlapping promoters.

Fig. S9 Circos plots showing correlations identified through mixOmics analyses between CG, CHG and CHH-climate-associated DMRs and gene expression.

Methods S1 WGBS library preparation and sequencing.

Methods S2 Methylation and DMR calling.

Methods S3 DMR variance decomposition analysis.

Methods S4 Genome-wide association analysis.

Methods S5 RNA-sequencing.

Table S1 Characteristics of the sites of origin of the populations used for this study and of the common garden where the plants were cultivated.

Table S2 Mapping statistics for WGBS analysis, SNP calling and RNA-seq.

Table S3 Correlation between number of *cis*-, *trans*-, climate-, unexplained DMRs and number of genes and TEs.

Table S4 Enrichment ratio analysis of *trans*-predicted DMRs in CHG and CHH contexts and unexplained DMRs in the CHH context, across different TE superfamilies.

Table S5 Gene Ontology enrichment analysis of predicted DMRs in *cis*, *trans*, climate-predicted and unexplained DMRs.

Table S6 List of climate-predicted DMRs found in the garden condition with a statistically significant correlation between promoter methylation and expression of the adjacent gene.

Table S7 List of correlations identified through mixOmics analyses between climate-predicted DMRs and gene expression.

Table S8 List of overlapping differentially expressed genes and environmentally linked DMRs that had significant correlations with gene expression.

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