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RESEARCH ARTICLE

Long-term methylome changes after experimental seed demethylation and their interaction with recurrent water stress in *Erodium cicutarium* (Geraniaceae)

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ABSTRACT

- The frequencies and lengths of drought periods are increasing in subtropical and temperate regions worldwide. Epigenetic responses to water stress could be key for plant resilience to these largely unpredictable challenges. Experimental DNA demethylation, together with application of a stress factor is an appropriate strategy to reveal the contribution of epigenetics to plant responses to stress.
- We analysed leaf cytosine methylation changes in adult plants of the annual Mediterranean herb, *Erodium cicutarium*, in a greenhouse, after seed demethylation with 5-Azacytidine and/or recurrent water stress. We used bisulfite RADseq (BsRADseq) and a newly reported reference genome for *E. cicutarium* to characterize methylation changes in a 2 × 2 factorial design, controlling for plant relatedness.
- In the long term, 5-Azacytidine treatment alone caused both hypo- and hyper-methylation at individual cytosines, with substantial hypomethylation in CG contexts. In control conditions, drought resulted in a decrease in methylation in all but CHH contexts. In contrast, the genome of plants that experienced recurrent water stress and had been treated with 5-Azacytidine increased DNA methylation level by ca. 5%.
- Seed demethylation and recurrent drought produced a highly significant interaction in terms of global and context-specific cytosine methylation. Most methylation changes occurred around genic regions and within Transposable Elements. The annotation of these Differentially Methylated Regions associated with genes included several with a potential role in stress responses (e.g., PAL, CDKC, and ABCF), confirming an epigenetic contribution in response to stress at the molecular level.

INTRODUCTION

The climate is changing. The period 1983 to 2012 was likely the warmest 30-year period in the last 1400 years in the Northern Hemisphere (IPCC 2014). Strong regional and temporal variability and increased frequency of extreme events appear linked to global warming, together with a latitudinal redistribution of precipitation leading to increased flooding and drought events in different regions worldwide (Zhang *et al.* 2007). The Mediterranean Basin is especially vulnerable to predicted global changes, encompassing increased summer temperatures, reduced winter precipitation, and more frequent heatwaves and drought events (Giorgi & Lionello 2008). Plants in this region are generally adapted to inconsistent precipitation and drought (Cowling *et al.* 2005; Cook *et al.* 2016) and thus could have increased resilience to such stresses (Matesanz & Valladares 2014; Balao *et al.* 2018; López-Rubio *et al.* 2022). Analysing the mechanisms behind adaptation to recurrent drought in Mediterranean plants could thus be key to understanding potential plant responses to the predicted effects of climate change in this region and worldwide.

Mounting experimental evidence confirms that epigenetic mechanisms are involved in plant responses to stress (e.g., Gutzat & Mittelsten Scheid 2012; Sun *et al.* 2020). A comprehensive understanding of interrelationships between chromatin configuration, DNA methylation, histone tail modification, and activity of transposable elements in response to stress is available for model and crop species with well-annotated reference genomes (Mirouze & Paszkowski 2011; Gutzat & Mittelsten Scheid 2012; Bäurle 2018). However, research on non-model plants has largely focused on changes in cytosine methylation, providing evidence that this is a key element of plant epigenomes (Niederhuth & Schmitz 2017). In plants, methylation can be found in CG, CHG and CHH contexts (where H = A, C or T) because different families of DNA methyltransferases can be independently regulated (Lyko 2018). Experimental studies support the relevance of DNA methylation in response to several abiotic stressors, and particularly to water deficit and salt excess (Peng & Zhang 2009; Alonso *et al.* 2016; Banerjee & Roychoudhury 2017). In addition, experimental modification of DNA methylation profiles by

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applying inhibitors of DNA methyltransferases (DNMTs), such as Zebularine and 5-Azacytidine (Baubec *et al.* 2009; Lopez *et al.* 2016), has further confirmed the potential epigenetic regulation in phenotypic responses to stress in non-model plants (Verhoeven *et al.* 2010; Herman *et al.* 2016; Rendina González *et al.* 2016). Understanding the stability of epigenomic changes induced by artificial demethylation at the seed stage could be particularly relevant to evaluating use of epigenetic information and seed priming for crop improvement (Gallusci *et al.* 2017; Springer & Schmitz 2017).

Improving current understanding of the ecological importance of epigenetic variation in natural plant populations requires transitioning from use of anonymous markers and indirect evidence, to explicitly investigating DNA methylation changes in non-model species (Richards *et al.* 2017). This approach will also be useful for direct evaluation of epigenetic responses to environmental stress in non-model species. Intrinsic plant (epi)genomic features (Springer *et al.* 2016), life-history traits (Verhoeven & Preite 2014), and environmental factors associated with species' evolution (Balao *et al.* 2018; Herrera *et al.* 2019) may influence the extent of epigenetic modification in response to specific stressors. Consequently, extrapolation of findings in model and crop species to ecologically relevant organisms is complex (Richards *et al.* 2017). Restriction site-associated DNA sequencing (RADseq hereafter) has become a popular representation method in non-model organisms. RADseq uses restriction enzymes to guide complexity reduction towards sequencing only a representative fraction of the genome, and can be implemented with or without prior genomic resources (Andrews *et al.* 2016). Similarly, reduced representation bisulfite sequencing (RRBS) methods provide a cost-effective alternative to whole genome bisulfite sequencing (WGBS) for analysis of DNA methylation differences between groups of samples in species that lack a well-annotated genome (Paun *et al.* 2019). RRBS provides insights into the magnitude and genomic location of substantial methylation changes, which is relevant because the effects of DNA methylation on gene expression, transposon activation, and specific phenotypic traits depend on both sequence context and genomic location (Hirsch *et al.* 2012; Niederhuth & Schmitz 2017).

In this paper, we use BsRADseq (Trucchi *et al.* 2016) to investigate changes in DNA cytosine methylation after experimental seed demethylation and recurrent drought in leaves of adult *Erodium cicutarium*, an annual herb native to the Mediterranean region that has become invasive in many arid and semiarid regions (see e.g., Schutzenhofer & Valone 2006; Francis *et al.* 2012; Kimball *et al.* 2014). Addition of 5-Azacytidine at seed germination has proved effective in reducing global cytosine methylation in seedlings of several species, including *Erodium* (Alonso *et al.* 2017). However, detailed analysis of the genome-wide effects of such a demethylating agent is only available for the model species, *Arabidopsis thaliana*, which suggests a significant dose-dependent reduction in all cytosine contexts in 10-day-old seedlings (Griffin *et al.* 2016). To the best of our knowledge, no study has applied genomic tools to assess whether these early effects are maintained in later stages of the life cycle, although repeated foliar spraying and foliar injection of DNMT inhibitors have been proposed as alternatives to continuously altering DNA methylation profiles throughout the plant life cycle (see Puy *et al.* 2018; Herrera *et al.* 2019, respectively). Thus, evaluating magnitude and specific patterns of demethylation induced after seed treatment, and their stability up to the reproductive stage, is critical for assessing utility of this method in revealing associations between DNA methylation and plant phenotypic variance, and its contribution to stress responses in a broad range of plant species (Richards *et al.* 2017; Alonso *et al.* 2019b), as well as potential relevance for epigenetic priming of seed crops (Gallusci *et al.* 2017). In particular, only long-lasting demethylation effects that persist until the adult reproductive stage, ecompassing pollen maturation and seed formation, might be relevant for transgenerational transmission of induced epigenetic variants.

In this study we focused on recurrent drought, characteristic of Mediterranean ecosystems in which *E. cicutarium* is native, and because we were uncertain of the time lapse necessary for eliciting stress and subsequent recovery after a single extreme drought event (see e.g., Walter *et al.* 2011; López-Jurado *et al.* 2016). Exposure to stress may prime individuals and/or their offspring for improved response to similar or other stress events and prompt a stronger response (Walter *et al.* 2013; Pandey *et al.* 2015; Douma *et al.* 2017; Dangi *et al.* 2018). High water-use efficiency and rapid individual growth, particularly during dry years, characterize the invasive advantage of *E. cicutarium* in some annual plant communities (Kimball *et al.* 2014), suggesting that this species might be particularly adaptable to changes in water availability. Our specific questions were: (i) do genomes of adult plant leaves exhibit any signature of DNMT inhibition by 5-Azacytidine at seed stage; (ii) which genomic methylation changes are associated wiht recurrent water stress during *E. cicutarium* lifetime; and (iii) is there any interaction between the above two factors?

MATERIAL AND METHODS

Study species

Erodium cicutarium (L.) L'Hér. (Geraniaceae), is an annual herb native to temperate areas with hot summers in Mediterranean Europe, North Africa and Western Asia (Fiz-Palacios *et al.* 2010; Francis *et al.* 2012). It matures rapidly and has autonomous self-pollination, which makes it a good candidate for experimental greenhouse studies.

Experimental design

Demethylation and recurrent water stress were applied to offspring of plants cultivated in the greenhouse. A first generation was grown from seed to minimize effects of heterogeneous growing conditions of maternal wild plants, which could affect offspring phenotype and DNA methylation profiles (Latzel 2015). The parental generation (F_0) were adult plants from two *E. cicutarium* natural populations in the Cazorla mountains (Jaén province, SE Spain). Seeds were removed from fruits collected in the field, scarified, and germinated in universal substrate (COMPO SANA®) mixed 3:1 with perlite (substrate hereafter). The first generation (F_1) seedlings were grown in 1-l pots with the same substrate; pots were grouped in trays, watered twice per week, and trays periodically rotated within the greenhouse (16-h light; 25–20 °C) until completion of reproduction (ca. 6 months). Autonomously pollinated fruits were collected in paper bags, and stored at room temperature (see Alonso *et al.* 2017 for further details).

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After 4 months, seeds were removed from fruits, weighed and slightly scarified using sandpaper. A demethylation treatment was applied to half of the seeds as described in Alonso *et al*. (2017). In brief, scarified seeds were submerged in 150 µl of either Control (water + DMSO, 97:3 v:v) or a solution of 0.5 mM 5-Azacytidine (5-Aza; Sigma A2385, 100 mg) for 48 h at 4 °C. Immediately after, all seeds were individually transferred to seedling plug trays filled up with substrate. Trays were saturated in water and placed in a greenhouse (16-h light/8-h dark, 25/20 °C). After 20 days, seedlings were transplanted to 1-l pots. Extra seedlings were collected to confirm effects of demethylation treatment, detected as reduced global cytosine methylation of leaf DNA and shorter and fewer leaves compared to controls (Alonso *et al*. 2017).

After a further 3 weeks (6-week-old seedlings), recurrent water stress (WS hereafter) treatment started. For each maternal line, half of the offspring were watered to field capacity twice per week (i.e., optimal watering; WW hereafter) and half were watered to field capacity once every 10–11 days (WS) until the end of the experiment. Flowering started soon after transplantation and peaked (75% individuals with flowers) at week 10. The 5-Aza-treated plants tended to flower ca. 5 days later than untreated plants . The experiment ended when plants were 16 weeks old, with some showing signs of senescence.

Sample processing

Methylation analyses were conducted on DNA extracted from leaves of a subset of reproductive F_2 individuals from 2 provenances × 4 F_1-mothers × 2 demethylation levels × 2 watering levels = 32 F_2 individuals. Two to three fully grown leaves without signs of damage or senescence from each F_2 individual was collected, placed in labelled paper bags, and dried in silica gel at ambient temperature in sealed containers. Dried samples were homogenized to a fine powder using a Retsch MM 200 mill. Total genomic DNA was extracted using the Bioline ISOLATE II Plant DNA Kit and quantified with a Qubit fluorometer 2.0 (Thermo Fisher Scientific, Waltham, MA, USA).

RADseq and BsRADseq library preparation

The RADseq and BsRADseq libraries were prepared following the protocol of Trucchi *et al*. (2016), with minor modifications. In particular, we modified the restriction enzyme used and employed a more frequent cutter *Pst*I (6 bp restriction site) to maximize the density of markers for later differential methylation analysis.

We used 20 U *Pst*I HF restriction enzyme (New England Biolabs) to digest 800 ng DNA per individual and ligated 100 nM P1 barcoded methylated adapters overnight at 16 °C. Groups of samples barcoded with different P1 barcodes were pooled, cut to a target peak of 400 bp (with a Covaris E220 focused ultrasonicator) and ligated to methylated P2 adapters. We employed a set of methylated P1 and P2 barcoded adapters to protect sequences from modification during bisulfite treatment. Fragments were constructed with a double barcoding system: eight different 5-bp barcodes were inserted with the P1 adaptor, in combination with four different P2 adaptors with 6-bp long barcodes. Our barcodes differed from each other in at least four bases. At this step, one aliquot was separated and directly amplified by PCR as a standard RADseq library. For the other aliquot, bisulfite conversion of non-methylated Cs into Us was performed after P2 adapter ligation using MethylEdge Bisulfite Conversion System kit (Promega). PCR amplification was executed using KAPA HiFi HotStart Uracil+ MasterMix (Kapa) for 23 cycles to convert all the Us into Ts. The final two libraries were sequenced at VBCF Vienna (https://www.viennabiocenter.org/vbcf/next-generation-sequencing/) as paired-end 125 bp reads in two lanes of an Illumina HiSeq 2500 machine.

Assembly of *E. cicutarium* draft genome

The *E. cicutarium* genome is 1C = 1.20 pg and CG content = 0.4 (Pustahija *et al*. 2013). A draft genome for *E. cicutarium* was assembled from DNA extracted from a single individual after two events of autonomous selfing under greenhouse conditions using a paired-end strategy in Illumina HiSeq X (PE150) at AllGenetics (www.allgenetics.eu). KmerGenie 1.7038 (Chikhi & Medvedev 2014) was used to estimate the best kmer in the pair reads, and k = 71 was selected as the start point for de novo assembly with ABySS 2.02 (Simpson *et al*. 2009). Plastid and mitochondrial genomes were handled separately. Reads were mapped back to the assembled output to determine cover depth using BWA 0.7.12 (Li & Durbin 2010). SAMtools 0.1.19 (Li *et al*. 2009) was used to remove poor quality and secondary alignments. To avoid possible inconsistencies or the presence of contaminant sequences, contigs/scaffolds with a mean coverage <10x were discarded, as well as those >1,000 bp.

RepeatModeler 1.0.11 and RepeatMasker 4.0.7 were used to identify and mask the predicted repeats in the filtered assembly. Geneid 1.4 (Alioto *et al*. 2018) and SMA3s (Muñoz-Mérida *et al*. 2014) were used for predictions of genes and their subsequent functional annotation. The manually annotated protein database Swiss-Prot (taxonomic division: plants), available in the UniProt database (The Uniprot Consortium 2007), was used as reference. The predicted genes were also compared against the GenBank nr protein database, including only Embryophyta taxa entries, using BLAST+ 2.6.0 (Camacho *et al*. 2009). The draft genome assembly of *E. cicutarium* was submitted to NCBI under accession number JATABU000000000.

RADseq and BsRADseq data processing and alignment

Barcoded raw Illumina reads were processed with deML (Renaud *et al*. 2015) and STACKS v.2.0Beta8 (Rochette *et al*. 2019). To demultiplex individuals and remove low quality data, *process_radtags* was run with the following settings: PstI as restriction enzyme, restriction site check at the beginning of the reads disabled (as BS conversion affected sequence of the restriction site), discarding reads with low quality scores according to default parameters, and rescuing barcodes and RADtags with two sequencing errors (following Trucchi *et al*. 2016). The paired-end fastq files obtained were filtered to retain only full-length reads (i.e., P1 120 bp and P2 125 bp after barcode trimming), with no adapter contamination, and unambiguous barcode sites. Raw data can be accessed in the Sequence Read Archive (SRA) as BioProject ID PRJNA984161.

The standard RADseq sequences of each of 32 samples were mapped to the reference genome (see below) to identify

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variable sites (i.e., SNPs) involving a cytosine in the reference sequence. C/T polymorphisms were subsequently filtered and masked with vcftools for subsequent methylation analyses (see Trucchi *et al*. 2016 for details). BsRADseq reads of each of the 32 samples were mapped to the draft reference genome using the mapping routine in *Bismark* (Krueger & Andrews 2011) specifically designed to deal with bisulfite converted reads with the core aligner Bowtie2 (Langmead & Salzberg 2012) in a non-directional modus and allowing up to four mismatches for a 120 bp read (options: --non_directional -L 32 -D 10 -R 1 --score_min L,0,-0.2). The sodium bisulfite conversion efficiency rates were assessed by calculating cytosine methylation levels in the chloroplast genome (Schmidt *et al*. 2017) and found to be globally satisfactory, with conversion ≥99.2% in all cases (Table S1). After mapping, we checked the summary report for each individual, recording mapping efficiency, number of cytosines screened, distribution among the different contexts (CG, CHG, and CHH), and differential representation of original strands versus complementary to original strands. As expected, the reads mainly mapped (>99%) complementary to either the top or bottom strand of the reference genome because of the peculiarities of the bsRADseq protocol (Trucchi *et al*. 2016) and stochastic orientation of the contigs in the reference genome. Table S1 presents a summary of sequencing and mapping results for each individual sample. The next step was to extract methylation information of each cytosine position using the *Bismark_methylation_extractor* routine, ignoring the first 4 bp in the reads, including the "–no_overlap" flag to prevent counting the same cytosine if covered by both forward and reverse reads and producing cytosine reports (CX_report files in the Bismark output) for each individual sample with information on all cytosine contexts and all strands. Output was then merged by sequence context (CG, CHG, CHH) for downstream analyses.

Global methylation analyses

Differential methylation analyses were conducted in R software v. 4.2.1 (R Core Team 2022). First, the global effects of WS and 5-Aza on the genome-wide cytosine methylation level were evaluated using a generalized linear model (GLM) with binomial error and logit link. The dependent variable was the proportion of methylated Cs to unmethylated Cs in each sample (i.e., independent of their genomic position), and independent variables were WS and 5-Aza and their interaction effect (5-Aza + WS), as well as F_1-mother identity (as proxy of genotype), all considered as fixed factors. The three-way interaction was not assessed because of the absence of maternal replicates within each factor combination level; nevertheless, the experimental design controlled for any potential bias that maternal identity could introduce by always including one plant from each maternal family in the four factorial combinations. The analysis was run for the whole dataset and separately for each sequence context (CG, CHG, CHH).

Differential methylation analyses

To gain insight into specific effects of 5-Aza and WS on methylation patterns with an explicit genomic context, differentially methylated cytosines (DMCs) at CG, CHG, and CHH sites were estimated using the callDML command in DSS (dispersion shrinkage for sequencing data) package v. 2.34.0 (Feng *et al*. 2014) with a 2 × 2 ANOVA design including the effects of WS, 5-Aza and their interaction (DMLfit.multiFactor = WS + 5-Aza + WS:5-Aza). In brief, DSS allows complex experimental designs (including interaction effects) based on a beta-binomial regression model with arcsine link function, and takes advantage of using a shrinkage estimator of the dispersion parameter based on a Bayesian hierarchical model to reduce dependence of variance on the mean. *P*-values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate (FDR) procedure. For each effect (WS, 5-Aza, and their interaction), DMCs with a corrected $q < 0.05$ and >10% difference in methylation level were considered candidate DMCs.

To investigate methylation changes of the DMCs with significant interaction effects, we used the optimized K-means clustering, minimizing the sum-of-squares within cluster (WCSS; Witten & Tibshirani 2010), on the average methylation of each of the four levels of treatment combination (Control, 5-Aza, WS, 5-Aza + WS). Methylation of DMCs and clustered DMCs were visualized using a heatmap created with the R package *pheatmap* v. 1.0.12 (Kolde 2019).

Annotation of DMC-associated genes

The DMC-associated genes were defined as genes with DMCs within the gene body region (coding sequence region, CDS, and introns) and/or promoter regions (1 kb upstream the CDS). In addition, DMCs located within regions annotated as transposable elements were separately analysed.

RESULTS

Draft genome assembly and annotation

A total of 225 million pairs of reads were used in the assembly, giving a mean coverage of 33.8× (±SD = 33.5). The assembly of k = 97 was chosen as the best reconstruction. A total of 65,050 contigs/scaffolds were retained in the final assembly (total length 628,531192 bp; N50 = 12,445; L50 = 15,770; GC % = 41.0). The BUSCO 3.0 (Waterhouse *et al*. 2018) comparison to the Embryophyta gene set yielded 75.5% complete, 8.5% fragmented and 15.9% missing BUSCO orthologs. SMA3s was able to annotate a total of 43,537 genes with 99.9% associated with a specific GO term. Further, a total of 2,135 repeat families were predicted by RepeatModeler and 38.5% of the genome was masked as repetitive.

BsRADseq library output

We obtained between 2,654,652 and 31,840,142 pairs of reads for the individual samples. After quality filtering, between 1,133,391 and 11,417,213 paired sequences per individual were retained (Table S1), corresponding to 35.8–42.7% of raw reads. On average, the number of paired reads per sample was 4,486,477, GC content was 28.9% (±1.3), a low figure, as expected for bisulfite-treated samples. The mapping efficiency to our draft reference genome averaged 38.4%, ranging from 35% to 42.5% across studied individuals, resulting in an average coverage of 98.6x ± 2.1x (Table S1).

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Table 1. Genome-wide methylation levels in *Erodium cicutarium* as total and for each sequence context for the 32 samples.

treatments								
5-Aza	watering	family	sample	no. cytosines	mC (%)	mCG (%)	mCHG (%)	mCHH (%)
C	WW	PT_1031	CC_253	92353730	18.4	58.6	22.3	6.3
C	WW	PT_1001	CC_225	108547644	23.3	67.7	32.8	8.4
C	WW	PT_0969	CC_147	47011293	18.7	60.2	21.9	7.4
C	WW	PT_0925	CC_001	124533805	24.5	67.5	32.9	10.5
C	WW	CH_1307	CC_491	39099076	25.5	68.8	34.1	11.4
C	WW	CH_1245	CC_433	22052707	23.2	66.2	31.9	9.8
C	WW	CH_1233	CC_409	94508383	17.1	58.5	18.4	6.6
C	WW	CH_1187	CC_291	85841474	25.9	69.5	35.8	10.6
C	WS	PT_1031	CD_259	128071349	17.8	58.6	20.4	6.4
C	WS	PT_1001	CD_219	116121937	27.8	69.2	43.5	9.8
C	WS	PT_0969	CD_163	128356306	23.0	65.4	30.3	9.5
C	WS	PT_0925	CD_005	213718116	21.8	63.1	25.8	9.7
C	WS	CH_1307	CD_485	22299814	23.7	69.4	29.0	10.2
C	WS	CH_1245	CD_445	29859487	21.4	63.3	27.3	8.7
C	WS	CH_1233	CD_431	87893575	16.7	58.3	17.5	5.9
C	WS	CH_1187	CD_297	50225708	25.8	71.8	35.8	10.0
A	WW	PT_1031	AC_252	164753131	24.9	68.8	34.5	9.3
A	WW	PT_1001	AC_220	97316260	18.9	57.8	26	6.4
A	WW	PT_0969	AC_146	46070213	19.0	61.2	22.9	6.6
A	WW	PT_0925	AC_016	75973105	27.6	70.4	37.6	11.9
A	WW	CH_1307	AC_494	44354076	24.4	69.7	31.6	10.3
A	WW	CH_1245	AC_438	21496364	25.7	69.6	35.6	10.7
A	WW	CH_1233	AC_422	99564473	21.2	63.0	26.3	8.9
A	WW	CH_1187	AC_300	54846691	18.1	59.4	21.2	6.8
A	WS	PT_1031	AD_254	137114375	25.5	67.2	37.4	9.4
A	WS	PT_1001	AD_222	38996631	25.7	67.9	37.9	8.8
A	WS	PT_0969	AD_164	85184218	24.5	68.9	33.3	9.3
A	WS	PT_0925	AD_020	105676423	28.8	72.6	40	13.2
A	WS	CH_1307	AD_482	38691859	22.1	65.7	28.0	9.1
A	WS	CH_1245	AD_436	34188955	28.1	73.4	39.1	12.3
A	WS	CH_1233	AD_416	82021987	23.6	64.9	30.1	10.2
A	WS	CH_1187	AD_292	74545405	30.6	76.0	44.0	13.0

C = Control; A = Adult plants obtained after 5-Aza treatment of seeds; WW = Optimal watering; WS = Water stress.

Global DNA methylation changes

The global cytosine methylation level in genomes of adult *E. cicutarium* leaves, as estimated by *Bismark*, varied widely across studied plants and conditions, ranging from 16.7% to 30.6% (Table 1), and averaged 22.8% in genomes of untreated plants (i.e., controls). Furthermore, average cytosine methylation level was different for the three sequence contexts analysed: in CG context methylation was estimated as 66.0%, whereas 30.8% of cytosines were methylated in CHG, and only 9.3% in CHH contexts (Table 1). The CG sites were rarest in the genome but, because of their high mean methylation level, they represented a major proportion of methylated cytosines, contributing most to the overall cytosine methylation, which globally averaged 23.2% (±3.7%) in our full set of samples.

The wide range of variance in global methylation level across individuals was congruent with the significant effect of the F_1-mother factor (as proxy for genotype) on the proportion of methylated Cs (LR-χ^2 = 5421658, $P < 0.0001$; Figure S1) and the impact of the two treatments, whose effects exhibited a significant interaction on this variable (LR-χ^2 = 1036036, $P < 0.0001$. Whereas the 5-Aza seed exposure weakly affected Cs methylation in leaves of adult plants grown under optimal water conditions, a global and significant increment in methylation level (~5%) was observed in the genome of leaves that experienced recurrent WS and had been treated with 5-Aza at seed stage (Fig. 1). Changes in methylation level of Cs at the three sequence contexts followed similar patterns (Fig. 1). Interestingly, whereas drought stress appeared to decrease the global methylation level and that at CG and CHG contexts, it increased DNA methylation at CHH sites that tend to be associated with transposable elements (e.g., Martin *et al.* 2021).

Genomic methylation changes induced by 5-Aza and recurrent water stress in leaves

A total of 3.1% of the 222,835 analysed cytosine positions were identified as differentially methylated cytosines (DMCs) between control and plants experiencing only 5-Aza, only WS, or both treatments. There was an uneven distribution of DMCs along the genome: the number of DMCs per scaffold differed from null distribution (Wilcoxon test $P < 0.001$; Figure S2) mainly because the largest fraction of scaffolds only obtained one or two DMC, regardless of their size and Cs abundance.

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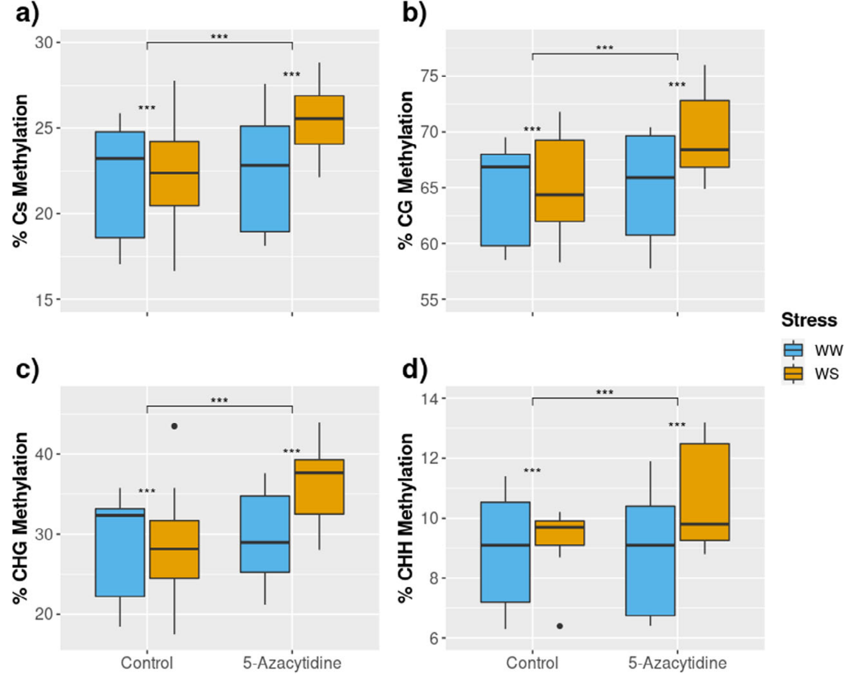

Fig. 1. Boxplots of percentage of methylated cytosines found on leaf genomes of adult *Erodium cicutarium* plants obtained from seed with no treatment (Control) and seed treated with 5-Azacytidine (5-Aza), grown with optimal watering (blue, WW) or recurrent water stress (yellow, WS). (a) Global methylation, (b) methylation percentage at CG, (c) methylation percentage at CHG, (d) methylation percentage at CHH. $^{***}P < 0.001$. N = 8 for each factor combination level. All cases the interaction between the two study factors was statistically significant (see Results for further details).

Further, the frequency of DMCs in the three sequence contexts did not resemble their relative abundance, with the highest proportion located in CHH (48.67%), followed by CG (33.31%), and the lowest in CHG (18.02%). Considering changes induced by the two treatments separately, seed exposure to 5-Aza had a broader impact on the adult leaf methylome in terms of positions modified (3,897 DMCs) than the WS treatment (1,417 DMCs). This trend was maintained across the three sequence contexts (Fig. 2a) with 1,323 versus 501 DMCs in CG context, 710 versus 235 in CHG, and 1,864 versus 681 in CHH for 5-Aza and WS treatments, respectively.

As regards direction of observed changes, rather unexpectedly the seed treatment with 5-Aza caused both hypo- and hyper-DMCs in adult leaves (Fig. 2). The number of hypo-DMCs (in comparison to control) was higher (773) than the number of hyper-DMCs (550) in the CG context, as expected from its predicted negative impact on the activity of the methylase enzyme, MET1, whereas the number of positions altered in the two directions was similar in both CHG and CHH contexts (Fig. 2a). Furthermore, WS caused a similar number of hypo- and hyper-DMCs at CG, CHG and CHH contexts (Fig. 2b, c).

Synergistic, antagonistic, and transgressive effects of combined 5-Aza and water stress on the leaf methylome

A total of 4,073 DMCs showed significant interactive effects between 5-Aza and WS treatments. Most DMCs were located in CHH (48.3%), followed by CG (33.6%), whereas the lowest proportion was identified in CHG context (18.1%). We clustered these DMCs by average methylation level across treatments, highlighting groups with distinct interaction patterns (Fig. 3a). The number of different patterns (i.e., k-means groups) was eight (which minimized the within group sum of squares; Figure S3). The eight patterns could be combined into two general trends (see dendrogram in Fig. 3a). The first included DMCs with a very low methylation level in most of the treatments (Clusters 1, 2, 3 and 4), usually including the control treatment. This "low-methylation group" represented most of the DMCs analysed with more than 600 DMC per cluster. The second group (Clusters 5, 6, 7 and 8) showed the opposite trend, including DMCs with a high methylation level (~100%) in most of the treatments.

Furthermore, the k-means clustering allowed us to reveal that many of the DMC were fully methylated or non-methylated in genomes of control plants identifying hypo- and hyper-methylation for the 5-Aza and WS treatments in comparison to the control (Fig. 3b; clusters 4 and 8, respectively). In addition, distinctive hyper- and hypo-methylation was detected for 5-Aza + WS treatment (Fig. 3b; clusters 3 and 7, respectively), singular hyper-methylation and hypo-methylation for the 5-Aza treatment (clusters 5 and 1, respectively), increased hyper-methylation (following methylation increment with 5-Aza) for the WS treatment (cluster 2), and the opposite trend (i.e., increased demethylation) for the WS treatment (cluster 6). From these patterns, we discerned synergistic effects of 5-Aza and WS on the methylation (clusters 2, 4, 6, and 8). The antagonist effects of both treatments were also shown in clusters 1 and 5. Furthermore, transgressive effects for treatments combination were detected (clusters 2, 3, 5, 6 and 7).

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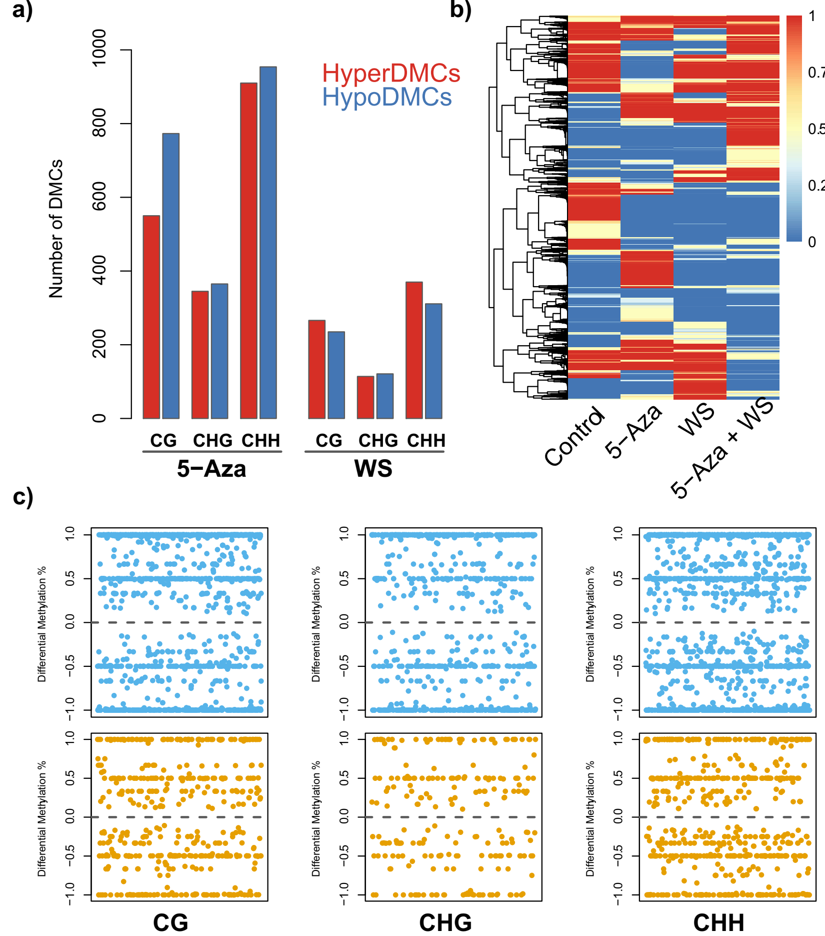

Fig. 2. (a) Number of differentially methylated cystosines (DMCs) by context induced by application of 5-Azacytidine at seed stage (5-Aza) and/or recurrent water stress (WS) that were hyper- or hypo-methylated compared to control plants. (b) Heatmap visualization of the 6848 DMCs obtained. Average methylation level for each experimental group (8 samples each) is shown. DMCs clustered using complete linkage clustering, and scale shown on the right, in which red and blue correspond to higher and a lower average methylation status, respectively. (c) Lower panels show methylation difference between the two conditions of each study factor, blue for 5-Aza and orange for WS, at every DMC evaluated on each of the three sequence contexts analysed. Each dot represents a specific DMC position for which a Y-axis value of 1 indicates complete lack of methylation in treated plants and full methylation in control condition.

Annotation of genomic regions overlapping DMC

We further annotated genomic regions found to be significantly differentially methylated (Fig. 4). For the 5-Aza treatment, 43% of the CG DMCs, 36% of the CHG DMCs, and 38% of the CHH DMCs overlapped with gene regions (exons, introns, and promoters). These proportions were slightly higher for the WS treatment: 46%, 41% and 41% for CG, CHG and CHH DMCs, respectively. Remarkably, the highest proportions of DMCs overlapping promoters and introns were in the CHH context for both treatments. In addition, on average, 21.3% of DMCs overlapped transposable elements (TEs). Moreover, 36% of the DMCs overlapping TEs appeared inserted in genic regions (introns and promoters; Figure S4). The percentage of TEs with DMCs (from total DMCs) inserted in genic regions increased from CG (4%) and CHG (6.5%) to the CHH context (11.5%). Furthermore, both treatments, 5-Aza and WS, had similar proportions of DMCs overlapping TE (without accounting for those inserted in genic regions) for each different context (Fig. 4). The proportions of TE-overlapped DMCs increased from the GC context (8% in 5-Aza) to the CHH context (19% in 5-Aza). In addition, although a large amount of TE-overlapped DMCs were uncategorized, the proportion of TE classes significantly differed from the genome distribution (χ^2 = 149.43, df = 6, $P < 0.001$). *Copia* elements containing DMCs (234) were higher than expected (47.5% versus 25.4%) whereas the LINE elements (61) showed a lower proportion (12.3% versus 27.9%; Fig. 5).

Finally, although the dispersed nature of the BsRADseq DMCs (Figure S2) limits the possibility of identifying genomic regions with multiple DMCs (i.e., Differentially Methylation Regions, DMR), we obtained 29 potential DMRs covering gene regions (i.e., overlapping >10 DMCs; Table 2). Eleven of these potential DMRs (37.9%) only overlapped intronic regions, whereas 31.0% appeared only in promotor regions, and 13.8% only in exonic regions. The annotation of these DMR-associated candidate genes included several with potential roles in stress response (Table 2).

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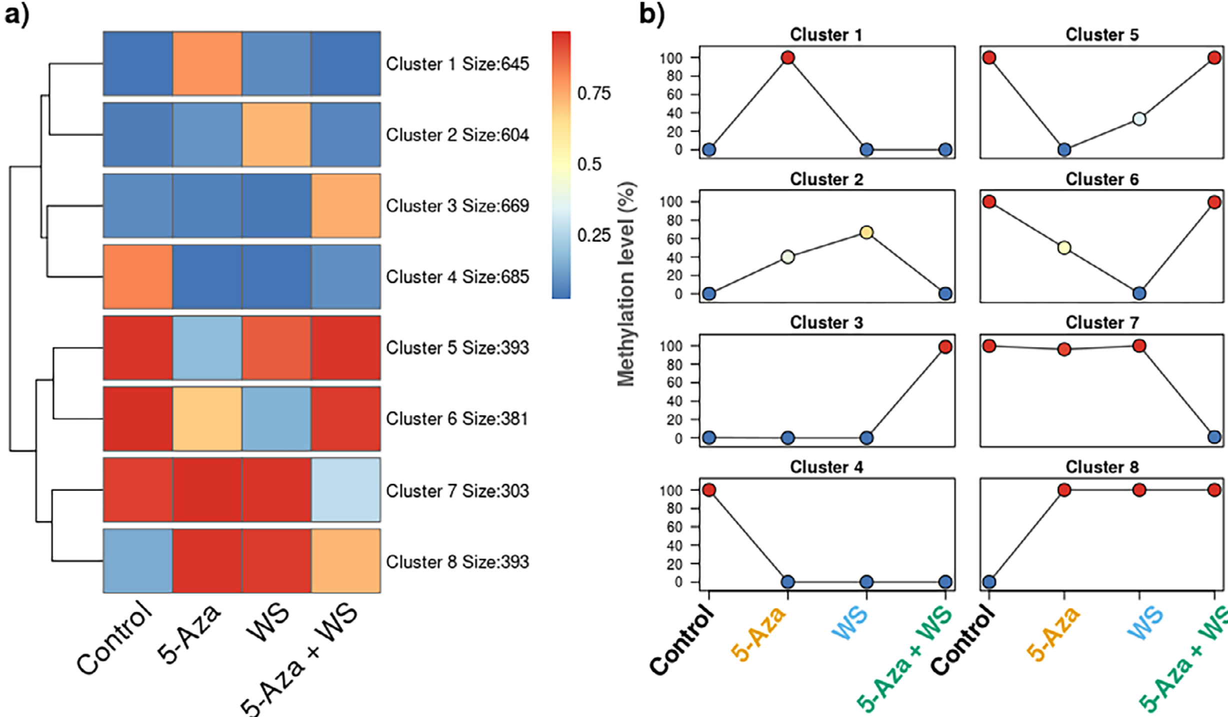

Fig. 3. Visualization of 5-Azacytidine and water stress treatment interaction effects on the methylome. (a) Hierarchical clustering of 4073 DMCs whose methylation changes exhibited a significant interaction between the two study factors and heatmap of K-means clusters (K = 8) showing common DMC patterns. The number of clusters minimized the within-cluster sum of squares. Size depicts number of DMC within each cluster. (b) Average methylation level for each experimental group on each K-means cluster.

DISCUSSION

The broad interspecific variation in global DNA methylation level, its phylogenetic signal, and its positive relationship with the haploid genome size (Alonso *et al.* 2016; Niederhuth *et al.* 2016; Alonso *et al.* 2019a, 2019b), together with the evolution of plant-specific methyltransferases (Jeddeloh *et al.* 1999; Law & Jacobsen 2010; Lyko 2018) support DNA methylation as a key component of plant epigenomes (Finnegan 2010). DNA methylation status is regulated by *de novo* DNA methylation, maintenance of methylation at DNA replication, and active demethylation. These three processes, together with the recruitment of histone modifiers and effectors, contribute to genome stability during development, and in response to genomic and environmental stress (Law & Jacobsen 2010; Boyko & Kovalchuk 2011; Fitz-James & Cavalli 2022). The deep molecular understanding of stress response in model plant species with well annotated reference genomes contrasts with the mostly indirect evidence gained for non-model plant species based on experimental alteration of DNA methylation and subsequent analysis of phenotypes, or from analysis of methylation changes of anonymous markers, such as MSAP (Richards *et al.* 2017). Molecular insights gained from RRBS methods provide a bridge between these two approaches (Paun *et al.* 2019) and could reveal whether epigenomic regulation in response to stress might act differently in species with contrasting epigenomic features relative to model organisms (Springer *et al.* 2016). In the following paragraphs we discuss how our results can aid understanding of the genomic signature of experimental demethylation conducted through application of 5-Aza at seed stage, and its impact on plant methylome responses to recurrent water stress in an annual plant.

Long-lasting methylation changes after seed demethylation detected by BsRADseq

The 5-Aza is a structural analogue of 5-methyl-cytosine that can be incorporated into DNA and RNA, where it establishes a covalent bond with a methyltransferase enzyme that cannot be easily reversed. The enzyme is trapped, thus reducing the number of active DNA methyltransferase enzymes in cells and therefore passively inducing DNA demethylation (Griffin *et al.* 2016; Lopez *et al.* 2016; Lyko 2018). Applied at seed stage, 5-Aza reduced global DNA methylation of seedlings in a dose-dependent manner (Griffin *et al.* 2016; Alonso *et al.* 2017). In *A. thaliana* seedlings the effect was more significant in CG context, where it reduced the frequency of fully methylated loci, particularly in highly methylated regions, whereas the loss of CHG and CHH methylation was mainly evident in highly methylated areas of the pericentromeric regions (Griffin *et al.* 2016).

Our analysis of changes in cytosine methylation in the DNA of *E. cicutarium* leaves collected at the onset of flowering is, to the best of our knowledge, the first attempt to characterize the long-lasting molecular signatures of seed demethylation treatment with 5-Aza. The abridged view of the methylome provided by BsRADseq analysis indicated a mild overall reduction in global cytosine methylation that was most evident in CHG

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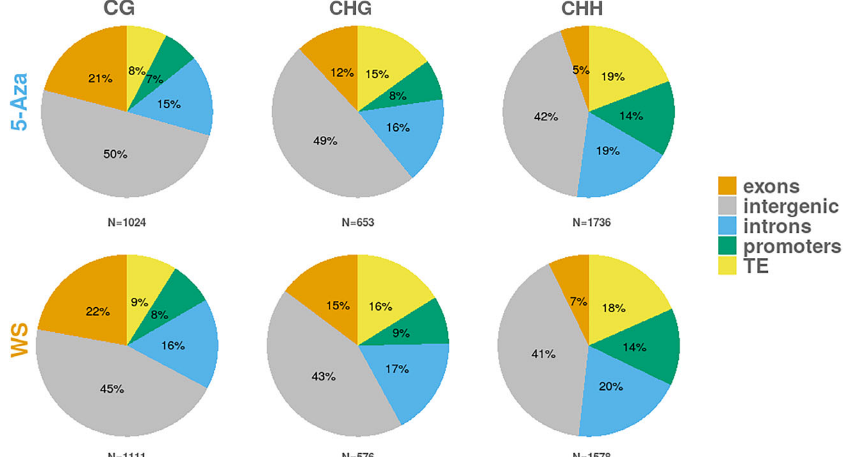

Fig. 4. Distribution of DMCs identified in 5-Azacytidine treatment (top) and water stress treatment (bottom). Exons, introns, and promoters corresponds to DMCs overlapping with genes; TE corresponds to DMCs overlapping with TEs. All other remaining DMCs are classified as intergenic.

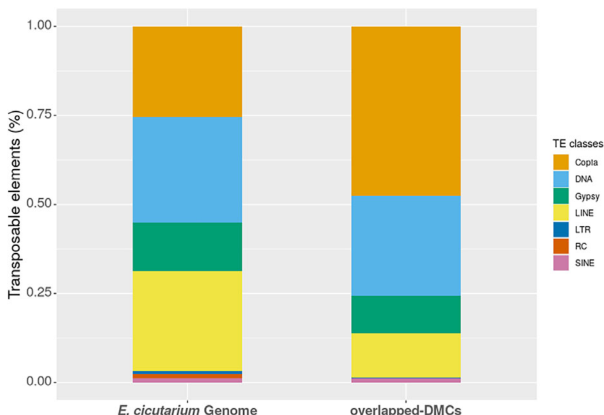

Fig. 5. Distribution of the TE classes along *Erodium cicutarium* genome and distribution of TE classes for the overlapped-DMCs (N = 285,236 and 492, respectively).

contexts (Fig. 1). More interestingly, both hyper- and hypo-DMC were obtained in leaf genomes of flowering plants treated with 5-Aza at seed stage in comparison to those from untreated seeds. In the CG context, the number of hypo-DMCs was slightly higher, whereas the number of positions altered in either direction was similar in both CHG and CHH contexts. These findings together suggest that 5-Aza at seed stage significantly altered the patterning of cytosine methylation in the genomes of adult plants and might at least partially erase the memory of past stress events (see e.g., Akkerman *et al*. 2016). Seed demethylation is, thus, a suitable tool for analysing the impact of deregulated epigenetic backgrounds on molecular and phenotypic response to stress, analysed either in treated individuals or in their progeny (Burton & Metcalfe 2014). It is also important to highlight here the significant family effect detected from analyses of global methylation level, which revealed that experimental demethylation does not necessarily equally impact all treated individuals, an aspect that has been reported in replicated ecological studies analysing phenotypic effects of seed demethylation using different genetic lines or provenances (Herman *et al*. 2016; Troyee *et al*. 2022), in contrast to a single accession or genetic line (e.g., Akkerman *et al*. 2016). Thus, a homogeneous impact of the treatment should not be taken for granted, and a screening of methylation changes across different genotypes, populations and species is highly desirable to assess the actual impact of 5-Aza seed treatment.

Genomic methylation changes in response to recurrent water stress across the individual life cycle

Epigenetic response to abiotic stress can involve complex regulation of histone markers, small RNA production, and DNA methylation (Banerjee & Roychoudhury 2017) that impinge on transcription of metabolic pathways and, in some cases, can be retained as memory markers of past events to improve individual (Fleta-Soriano & Munné-Bosch 2016) or transgenerational responses (Fitz-James & Cavalli 2022). In particular, significant DNA methylation changes in response to moderate to extreme water stress treatments have been reported for several plant species (see e.g., Pardo *et al*. 2020 for several grasses; Sow *et al*. 2021 for poplar), including variable responses of crop varieties expected to have contrasting drought tolerance in *Vicia faba* (Abid *et al*. 2017) and *Oryza sativa* (Wang *et al*. 2016), supporting its relevance as a mechanism for both long-term wild plant adaptation and crop improvement. Mild water stress applied at early seedling stage has been found to alter DNA methylation in leaves of *A. thaliana* Col-0, mainly at CHH contexts and predominantly within TE sequences, and significantly up- and down-regulates transcription of multiple genes (Van Dooren *et al*. 2020). Some stress-induced epigenetic variants are reversible, and recurrent stress is expected to promote molecular memory and increased tolerance in subsequent events (Wang *et al*. 2021). Our recurrent water stress, aimed at mimicking unpredictable rain in Mediterranean ecosystems, led to a significant overall increased in DNA cytosine methylation in all three contexts for genomes of *E. cicutarium* leaves at adult stage. Our analysis of DMCs indicated that a considerable fraction of cytosines across the genomes of plants experiencing WS were either hypo- or hyper-methylated compared to well-watered plants. A

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Table 2. Annotation of gene regions of *Erodium cicutarium* overlapping DMRs (i.e., ≥10 DMCs).

gene	annotation	DMCs promoter	exon	intron	treatment/cluster
Ec-S10188_3	CC-NBS-LRR resistance protein RGA3	28	0	0	C1
Ec-S25751_1	Methyltransferase-like protein 13	19	0	0	C5
Ec-S20359_1	ABC transporter F family member 4	17	0	0	C3
Ec-S27698_1	Phenylalanine ammonia-lyase PAL	17	0	0	C6
Ec-S71193_1	Uncharacterized protein	16	0	0	C1
Ec-S44030_4	Cyclin-dependent protein kinases 2 CDKC	13	0	0	C1
Ec-S06490_3	Uncharacterized protein	13	0	0	C2
Ec-S11263_1	Bpg2-like protein (Fragment)	11	0	0	5-Aza
Ec-S41184_1	1,4-alpha-D-glucan maltohydrolase	10	0	1	WS
Ec-S48520_2	Uncharacterized protein	6	6	0	C1
Ec-S09768_1	Uncharacterized protein	0	11	0	C8
Ec-S63962_1	Reverse transcriptase (RNA-dependent DNA polymerase)	0	10	0	5-Aza
Ec-S77906_2	Uncharacterized protein	0	10	0	C1
Ec-S53645_2	Apoptosis inhibitory protein 5 (API5)	0	10	0	WS
Ec-S29620_1	Amino acid permease 1	0	6	4	WS
Ec-S77915_1	ALE2 AtPERK1	0	1	18	WS
Ec-S76299_2	Agamous-like MADS-box protein AGL9 homologue	0	1	17	C6
Ec-S71479_1	Probable polygalacturonase	0	1	9	C3
Ec-S06731_4	DNA ligase 1	0	0	18	C2
Ec-S64428_1	(1- > 3)-beta-glucan endohydrolase 10	0	0	16	C1/C8
Ec-S64428_1	Glucan endo-1_3-beta-glucosidase 14	0	0	16	C8
Ec-S61275_5	Uncharacterized protein	0	0	14	WS
Ec-S08686_1	GDSL esterase/lipase At4g10955-like	0	0	13	WS
Ec-S63591_3	Uncharacterized protein	0	0	12	C4
Ec-S66538_1	Uncharacterized protein	0	0	12	C5
Ec-S76981_2	Hypothetical protein CCACVL1_13302	0	0	11	C1
Ec-S72001_2	60S ribosomal protein L37-3	0	0	10	5-Aza
Ec-S62651_1	Uncharacterized protein	0	0	10	C4
Ec-S28865_5	Transcription factor WER-like	0	0	10	WS

Shown are maximum number of DMCs, their genomic location, and most frequent treatment (either 5-Aza or WS) or interaction cluster (denoted by C1-C8 as in Fig. 3) associated with DMCs obtained. See Table S2 for details.

large proportion of DMCs in all contexts overlapped in intergenic regions (Fig. 4), an aspect potentially related to the reduced resolution of the annotation of the newly reported reference genome. Moreover, methylation changes at CG contexts mainly overlapped exons, and those in CHH were comparatively more frequently associated with TEs and introns.

Combined effects of 5-Aza and water stress on the leaf methylome

Significant interactions between DNA demethylating agents and experimental stress treatments have been proposed as reliable approaches to investigate the role of DNA methylation in regulating plant responses to stress in non-model species (Puy *et al*. 2018; Alonso *et al*. 2019a). Phenotypic evidence of significant interaction effects in response to water stress has been gained in several non-model plant species (Herman *et al*. 2016; Rendina González *et al*. 2016). Our study confirms the significant interaction at molecular level in leaf genomes of *E. cicutarium* adult plants between 5-Aza-induced demethylation and water stress. This interaction appears to produce a complex epigenomic instability, with each factor making unstable changes at a genome-wide scale. On the one hand, 5-Aza application to seeds triggers widespread changes in DNA methylation in adult leaves, but, importantly, it likely alters expression of transcriptional and post-transcriptional regulatory proteins, as suggested by the DMRs overlapping the orthologous Methyltransferase 13, RNA-dependent DNA polymerase, and 60S ribosomal protein (Lith°Ldo & Bousquet-Antonelli 2019; Sáez-Vásquez & Delseny 2019). Interestingly, 5-Aza promoted hypermethylation of the Ec-S10188_3 gene, which contains 28 differentially methylated cytosines in the promoter region (Table 2) and encodes a putative disease resistance protein, with high identity to Resistance genes analogs 3 (RGA3) in *Theobroma cacao* (Sekhwal *et al*. 2015). Likewise, the promoter of the Ec-S44030_4 gene, a Cyclin-dependent protein kinase (CDKC) ortholog, related to stress tolerance mechanisms (Kitsios & Doonan 2011), was also hyper-methylated. Although there was an antagonist effect of water stress and 5-Aza exposure for these genes (Cluster 1), the 5-Aza-induced hyper-methylation may resemble a priming state for more rapid future responses to disease attacks and abiotic stress (Latzel *et al*. 2016; Alonso *et al*. 2019b).

On the other hand, environmental stresses, such as cold and drought, can also affect the methylase and/or demethylase activity (Lucibelli *et al*. 2022) explaining the interaction with the demethylation agent 5-Aza. For example, heat stress induced downregulation of demethylases in *Triticum aestivum*

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(Gahlaut *et al.* 2022) but significantly upregulated them in *Camellia sinensis* under cold and drought (Zhu *et al.* 2020). Demethylases were also significantly induced by cold in *Dendrobium officinale*, where methylases were also affected by drought (Yu *et al.* 2021). In our study, water stress, as well as 5-Aza treatment, independently triggered promoter hypomethylation of methyltransferase 13, which may activate or upregulate its expression. However, the combination of treatments restored the control methylation level of this cytosine methylase. In addition, the methylation stage of many abiotic response genes was directly altered by water stress or its combination with 5-Aza treatment. For example, promoter regions of abiotic stress related genes, phenylalanine ammonia-lyase (PAL; Jeong *et al.* 2012) and ABC transporter F family (ABCF; Dahuja *et al.* 2021), were also affected. Most methylation changes were observed in CHH context potentially linked to a genome-wide decrease in repression of TE activity, as previously reported (e.g., Van Dooren *et al.* 2020).

Changes in methylation in transposable elements (TEs)

In plants, various TEs are involved in environmental stress adaptation (Casacuberta & González 2013; Sahu *et al.* 2013). We found significant differential methylation of *Copia* TEs in *E. cicutarium* under 5-Aza and WS treatments. The *Copia* superfamily of long terminal repeat (LTR) retrotransposon is known to be activated by biotic and abiotic stresses (Ito 2022). ONSEN elements are highly expressed under heat stress in Brassicaceae (Pietzenuk *et al.* 2016), whereas other *Copia* elements are activated by several environmental stresses in other species (Ito 2022). Transposon activation can affect gene regulation on a genome-wide scale by acting as *cis*-regulatory elements and conferring increased stress responsiveness to nearby protein-coding genes, which can facilitate acclimation or even adaptation to stressful conditions (Horváth *et al.* 2017; Dubin *et al.* 2018). Transposon activation under environmental stress can have negative effects on fitness (Horváth *et al.* 2017), but increasing TE methylation, especially in genomic regions neighbouring highly expressed stress-induced genes, could prevent TE transcription via RNA polymerase II and hence avoid deleterious effects to the plant (Secco *et al.* 2015).

CONCLUSIONS

By using a 2 × 2 factorial design, combining recurrent water stress and early life demethylation with a reduced representation method of surveying genome-wide methylation levels in adult plants, we show a significant interaction between methylation and plant stress response in the Mediterranean plant, *Erodium cicutarium*. We revealed extensive effects of both seedling demethylation and water stress on adult DNA methylation, with a large proportion of changes observed around genic regions and within TEs. In conclusion, our results provide useful information towards understanding the role of DNA methylation in plant drought responses.

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AUTHOR CONTRIBUTIONS

FB: Conceptualization, Investigation, Writing – original draft. **MM:** Methodology, Investigation. **PB:** Methodology, Investigation. **OP:** Conceptualization, Investigation, Writing – review and editing. **CA:** Conceptualization, Methodology, Investigation, Funding acquisition, Writing – original draft. All authors have read and agreed to the published version of the manuscript.

SUPPORTING INFORMATION

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

Table S1. Summary of sequencing and mapping results of the 32 individuals included in the BsRAD-seq library analysed.

Table S2. Details of every DMC detected within the 29 DMR obtained, defined as genomic regions with ≥10 DMCs, in leaf DNA of *Erodium cicutarium* plants with a 2 × 2 factorial design including seed demethylation with 5-Azacytidine (5-Aza) and recurrent water stress (WS).

Figure S1. Effects of maternal genotype (F1-mother identity) on genome-wise global methylation and estimates obtained for each cytosine context (CG, CHG, CHH).

Figure S2. Distribution of DMCs on scaffolds of the *Erodium cicutarium* genome.

Figure S3. Plot of within-groups sum of squares against number of clusters using the K-means algorithm using the average methylation level of the DMCs for each experimental group.

Figure S4. Relative distribution of the DMCs overlapping transposable elements according to sequence context (CG, CHG, CHH) and local annotation (promoter, exon, intron, nongenic).

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