DNA methylation dynamics in a coastal foundation seagrass species under abiotic stressors

Jessica Pazzaglia[1,2], Emanuela Dattolo[1], Miriam Ruocco[1], Alex Santillán-Sarmiento[1,3], Lazaro Marin-Guirao[1,4]*, Gabriele Procaccini[1]*

JP 0000-0002-8677-7712

ED 0000-0001-6005-5079

MR 0000-0002-3779-8373

AS 0000-0003-4663-0959

LMG 0000-0001-6240-8018

GP 0000-0002-6179-468X

[1] Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, 80121, Naples, Italy

[2] Department of Life Sciences, University of Trieste, 34127, Trieste, Italy

[3] Faculty of Engineering, National University of Chimborazo, Riobamba, Ecuador

[4] Seagrass Ecology Group, Oceanographic Centre of Murcia, Spanish Institute of Oceanography, Murcia, Spain

*These authors have contributed equally to this work

Abstract

DNA methylation (DNAm) has been intensively studied in terrestrial plants in response to environmental changes, but its dynamic changes in a temporal scale remain unexplored in marine plants. The seagrass *Posidonia oceanica* ranks amongst the slowest-growing and longest-living plants on earth, particularly vulnerable to sea warming and local anthropogenic pressure. Here, we analyzed the dynamics of DNAm changes in plants collected from coastal areas differentially impacted by eutrophication (i.e. oligotrophic, Ol; eutrophic, Eu) and exposed to abiotic stressors (nutrients, temperature increase and their combination). Levels of global DNA methylation (% 5-mC) and the expression of key genes involved in DNAm were assessed after one, two and five weeks of exposure. Results revealed a clear differentiation between plants, depending on environmental stimuli, time of exposure, and plants' origin. % 5-mC levels were higher during the initial stress exposure especially in Ol plants, which upregulated almost all genes involved in DNAm. Contrarily, Eu plants showed lower expression levels, which increased under chronic exposure to stressors, particularly to temperature. These findings show that DNAm is dynamic in *P. oceanica* during stress exposure and underlined that environmental epigenetic variations could be implicated in the regulation of acclimation and phenotypic differences depending on local conditions.

Keywords

Seagrasses, epigenetics, DNA methylation dynamics, gene expression, stress response

Introduction

Epigenetic mechanisms such as DNA methylation (DNAm), histone modifications and regulation by non-coding RNA (ncRNA) are important processes influencing chromatin structure and accessibility to genetic information, thus having a role in gene expression regulation [1]. Epigenetic variations may occur during organismal development and can be related to surrounding environmental conditions or arise stochastically [2]. These mechanisms can be flexible inducing short-term regulations in response to environmental stimuli or stable during the lifetime of an organism, being eventually heritable through multiple generations [3]. DNAm is a conserved mechanism, which occurs in both plants and animals [4]. This process is characterized by the addition of a methyl or hydroxymethyl group to the C5 position of cytosine to form 5-methylcytosine (5-mC). While in animals it mainly occurs in the context of CpG (or GC) dinucleotides, in plants it is also found in CHH and CHG contexts (where H = A, C or T) [5]. This reaction is mediated by methyltransferases, using S- adenosyl-l- methionine as donors of a methyl group, and the dynamics of its establishment, maintenance and removal is highly regulated through different pathways involving various enzymes [6]. DNAm can either activate or repress gene expression depending on the sequence context [7].

In plants, *de novo* DNA methylation is mediated by the RNA-directed DNA methylation (RdDM) pathway, which is based on small-interfering RNAs (siRNAs), scaffold RNAs, and many accessory proteins [8]. Once DNAm is established, its maintenance is regulated by different methyltransferases, depending on the sequence context (**Figure S1**). DNA methyltransferase 1 (MET1) regulates CG methylation during DNA replication, while chromomethylase 3 and 2 (CMT3, CMT2) mainly maintain CHG methylated. CHG methylation attracts H3K9-specific methyltransferases (i.e., SUVH4, SUVH5 and SUVH6) that favor the CMT3–H3K9me2 interaction [9]. Their recruitment induces histone H3 lisyne9 di-methylation (H3K9me2) and facilitates CMT3 and CMT2 functions in a cross-talk mechanism between CHG methylation and H3K9 methylation [10]. In model plant species, this interaction is known to be crucial for maintaining methylation, as mutations in SUVH4 destabilize CMT3–H3K9me2 interactions, prevent H3K9me2, and reduce CMTs activities, decreasing CHG methylation (e.g., in *Arabidopsis thaliana* [11]). The removal of 5-mC can be a passive process associated to a lower expression of methyltransferases (i.e. MET1) [12] or it can be regulated by the activity of bifunctional 5-mC DNA glycosylases including the repressor of silencing 1 (ROS1), the transcriptional activator demeter (DME), the demeter-like protein 2 (DML2) and DML3 [13]. Interestingly, DNAm and histone modification mechanisms can “store” environmental information, contributing to the establishment of a stress memory in many plant species [14]. Since the epigenetic landscape influences gene regulation and thus the phenotype, the interplay between genome-wide DNAm and gene expression is suggested to be the basis of stress –tolerance [15] and phenotypic adjustments to environmental conditions, eventually resulting in local adaptation [16].

Being sessile organisms, plants are frequently exposed to chronic or recurring disturbances in natural environments. The storage of past stress events can occur through the regulation of a specific set of genes known as stress memory genes [17]. For instance, plants exposed to thermal stress showed the activation of specific heat shock factors (i.e., heat-stress memory genes) involved in transcriptional memory as they can be easily regulated during the recurring stress [18]. Their induction is maintained by epigenetic mechanisms (including histone methylation) and by the interaction of specific genes (i.e., stress-memory genes) with the chromatin structure, such as FORGETTER1 in *A. thaliana* [19]. The ability to acquire a stress memory for enhancing resilience against further stress has also been

shown in seagrasses [20]. In particular, the co-variation of DNAm and photosynthetic performance in plants previously exposed to heat stress could be linked with gene expression regulation to improve their plasticity under changing conditions, emphasizing the importance to explore these epigenetic mechanisms also in marine plants [21]. The dynamic regulation of DNAm in response to stressful conditions is a key process to be addressed in the era of global environmental changes, especially in marine clonal plants that are particularly vulnerable to environmental shifts. Although great progress in the understanding of epigenetic processes in plants has been achieved by using well established terrestrial model species, little is known about the dynamics of epigenetics mechanisms in aquatic or marine non-model plants, such as seagrasses.

Analyzing DNAm variation at different time scales and its role in short-term stress responses and stress memory is fundamental, especially in foundation species such as marine macrophytes, which are declining worldwide due to climate changes and local pressures [22]. Seagrasses can have different reproductive strategies and clonal persistence and include some of the earth's largest and oldest plant species, such as *Posidonia oceanica* [23]. This species is endemic to the Mediterranean Sea and is among the slowest-growing and long-living species, structuring complex and highly valuable ecosystems [24]. The degree of phenotypic plasticity observed among seagrass species reflects the interaction between genotypes and the surrounding environments, which is considered a crucial property for their survival to environmental shifts [25]. While genetic diversity has the potential to increase resilience capacity of seagrass meadows under long-term environmental changes [26], epigenetic modifications such as DNAm may contribute to the regulation of phenotypic plasticity favouring short-term responses (i.e., acclimation) to rapid environmental changes [27]. However, the physiological, morphological and transcriptomic response to single or multiple stressors can also depend on the environmental conditions locally experienced by plants in their native habitats [27–30]. Recently, Entrambasaguas *et al.* (2021, [31]) explored the gene body DNAm (gbM) patterns in different *P. oceanica* ecotypes, revealing the existence of a relationship between gbM and gene expression flexibility depending on the origin of plants. Hence, the genetic–epigenetic control already described in terrestrial plant species, could also regulate the interaction of seagrass genotypes with the surrounding environment, affecting their resilience to environmental changes.

Most of the epigenetic research in plants exposed to abiotic stressors such as drought, cold or heat stress and salinity, has provided important evidence of stress-induced DNAm and demethylation both at the genome level and at specific loci [32]. Furthermore, as observed in terrestrial species, the non-stressed progeny can inherit the DNAm landscape from parental plants exposed to stress with the potential to improve its stress tolerance [33].

In this study, we aimed to investigate the dynamics of DNAm by analysing the expression profiles of key genes involved in *de novo* and maintenance DNAm and demethylation, as well as histone methylation in adult plants of *P. oceanica* with a different history of nutrient loads. This analysis paralleled the assessment of global DNA methylation level (% 5-mC) of plants at different time of the exposure to multiple stressors (nutrients addition, temperature increase and their combination). We also aimed to investigate on pre-acquired memory by analysing a specific gene (i.e. FORGETTER1) involved in heat-stress memory. According to previous observations in terrestrial plants and in seagrasses, our initial hypothesis is that DNAm can be influenced by specific environmental stressors and local pressures at the site of origin of plants.

2. Methods

2.1 Experimental design and plant collection

Leaf material of *P. oceanica* used for this study was collected during the experiment performed by [23] in 2020. Briefly, large plant fragments were collected by SCUBA diving from shallow-water meadows growing in two locations with different history of nutrient loads: Spiaggia del Poggio (Bacoli) in the Gulf of Pozzuoli (Italy, 40 47.9300 N; 14 05.1410 E), and Castello Aragonese in the Island of Ischia (Italy, 4044.1140N; 1357.8660 E). The former site presents eutrophic conditions due to local anthropogenic pressure, in contrast to Ischia site, which is in a marine protected area and more oligotrophic (for a detailed description of sampling sites see [28]). Because of their proximity, both locations experienced similar Sea Surface Temperature (SST) regimes. Two plant fragments (a rhizome portion bearing a minimum of eight vertical shoots) for each eutrophic (Eu) and oligotrophic sites (Ol) were allocated in each tank of the indoor mesocosm system at Stazione Zoologica Anton Dohrn (SZN, Naples - Italy) [34] and exposed to single and multiple stressors. The system consisted of 12 glass aquaria (500 L) filled with natural seawater. The experiment was designed including four treatments as follows: Control (C), Nutrients (N), Temperature (T) and Nutrients + Temperature (NT) (**Figure S2**). After a first acclimation phase (see the experimental design in Pazzaglia et al., 2020 for more detail), temperature was gradually increased (0.5 C° day^{-1}) in the T and NT treatments to 30 °C, whereas temperature in the C conditions was maintained at 24°C. In N and NT treatments, a nutrient solution prepared using Osmocote Pro® fertilizer pellets (170 mM total nitrogen) was added weekly to maintain a nutrient enrichment (DIN = 26.8 ± 4.0 mM). A total of 72 leaf samples (n = 3 replicates) were collected for gene expression and % 5-mC analysis from both Eu and Ol plants, after one week (W1), two weeks (W2) and five weeks (W5) from different treatments (C, N, T and NT; i.e., 2 plants x 3 replicates x 4 treatments x 3 time points). Leaf tissue was cleaned from epiphytes with a scalpel and immediately submerged in RNA later© collection solution (Ambion, life technologies) for gene expression analysis, whereas leaf samples for DNA extraction were stored in silica gel.

2.2 Gene expression analysis

RT-qPCR (Real Time-quantitative Polymerase Chain Reaction) analysis was used to assess differences in expression level of target genes in control vs. treatments (N, T and NT) in both Eu and Ol plants during the course of the experiment (W1, W2 and W5). Total RNA was extracted with Aurum™ Total RNA Mini Kit (BIO-RAD) following manufacturer's instructions. RNA purity and concentration were checked using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and RNA quality was assessed through 1.0% (w/v) agarose gel electrophoresis. Total RNA (500 ng) from each sample was retro-transcribed into cDNA with the iScript™ cDNA synthesis kit (BIO-RAD), according to manufacturer's protocol. Five genes of interests (GOIs) were selected as a subset of key regulators of DNAm in terrestrial plants [4] and representatives of the DNA methylation machinery: *de novo* DNAm (DNA cytosine-5-methyltransferase, DRM, and argonaute, AGO), DNAm maintenance (DNA methyltransferase 1, MET1, and chromomethylase 2, CMT2), DNA demethylation (repressor of silencing 1, ROS1), Histone methylation (histone-lysine n-methyltransferase, SUVH4) and stress memory (forgetter 1, FGT1) (**Table S1**). Primers for GOIs were specifically designed using a *P. oceanica* transcriptome [35] with the primer analysis software Primer3 v. 0.4.0 [36,37] setting primer length to 18-20 bp, product size to 100-250 bp and Tm = 59-61°C. Three putative reference genes (elf4A, GAPDH and 18S) already developed for *P. oceanica* were tested for stability in our experimental conditions**.** The best reference genes (RGs) were identified by using the web-based tool RefFinder (http://blooge.cn/RefFinder/, [38]) that integrates

three major computational programs for RG assessment (geNorm, Normfinder, BestKeeper). Accordingly, two reference genes (elf4A and GAPDH) were selected and used for target gene-expression normalization, based on their stability values and according to previous works with the same species under abiotic stress [39,40]. Primer sequences, efficiencies (*E*) and regression coefficients (R^2) of GOIs and RGs are shown in **Table S1**. Primers for GOIs with *E* within the range 90-110% and R^2>0.95 were used in the study (**Table S1**). RT-qPCR efficiencies for all primer pairs were calculated from the slopes of standard curves of the threshold cycle (C_T) of five cDNA dilutions according to the equation $E = 10^{-1/slope}$. A 1:5 cDNA template dilution was used for the analysis.

RT-qPCR reactions were performed in triplicates in a Viia7 Real Time PCR System (Applied Biosystems) using Fast SYBR® Green MasterMix (Applied Biosystems) as fluorescent detection chemistry and MicroAmp Optical 384-well reaction plates (Applied Biosystems). Reactions were carried out in a 10µl final volume with 5µl MM SYBR® Green, 2µl of 1.4 pmol μl^{-1} primers and 1µl of 1:5 cDNA as template. The thermal profile of the reactions was as follows: 95°C for 20 s, 40 times 95°C for 1 s and 60°C (for 20 s. Relative quantification of gene expression was obtained following [41]. The amplification data were analyzed using the ViiA7™ Software v.1.0 (Applied Biosystems) and the differential expression parameters were manually calculated as follows: the cycle threshold (C_T), the negative difference in cycles between the reference genes (RGs) and the respective GOI ($-\Delta C_T = C_T$ RGs - C_T GOI), the fold expression change = ±2 ($|(-\Delta C_T$ treatment)-$(-\Delta C_T$ control)|).

2.3 Global DNA methylation assessment

Genomic DNA was isolated using the NucleoSpin® Plant II kit (Macherey–Nagel). DNA quality was checked through 1.0% agarose gel electrophoresis and the concentration was accurately determined by the Qubit dsDNA BR assay kit with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Global DNA methylation (% 5-mC) was assessed colorimetrically in duplicate by an ELISA-like reaction with the 5-mC DNA ELISA Kit (Zymo Research) starting from 50 ng DNA per sample and reported as % 5-mC relative to the standard input of DNA quantity. Absorbance at 450 nm was read using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific).

2.4 Data analysis

To investigate the effects of different experimental conditions on DNAm, including both % 5-mC and gene expression of methylation-related genes, a Permutational Multivariate Analyses of Variance (PERMANOVA) was carried out. The model consisted in three fixed factors, ‘plants’ (P) with two levels (Eu and Ol), ‘nutrients’ (N) and ‘temperature’ (T) both with two levels (control and high), and one random factor, time (W). The analysis was performed on Euclidean distances of data, using 9999 permutations of the residuals under a reduced model. A repeated-measures ANOVA (3-way repeated measures generalized linear models, RM-GLM) was conducted to investigate the effect of single stress factors (N and T) and their interaction on gene expression and % 5-mC data in both Ol and Eu plants. The model utilized for the analysis included ‘time’ (W) as a within-subject factor, ‘plants’ (P) with two levels (Eu and Ol), ‘nutrients’ (N) and ‘temperature’ (T) both with two levels (control and high). To assess the dynamics of gene expression and % 5-mC for each group of plants (Eu and Ol), a second repeated measures ANOVA (2-way RM-GLM) excluding P as a factor was performed using only treatments (N and T) as fixed factors with two levels (control and high) and time (W) as a within-subject factor. In addition, to test the effect of different treatments for each time point a factorial 2-way ANOVA (2-way ANOVA) was performed separately for Ol and Eu plants, considering N (high,

control) and T (high, control) as factors. Data were checked for the assumptions of normality and homoscedasticity and transformed when necessary. In the case of RM-GLMs, the assumption of sphericity was assessed using Mauchly's sphericity test. A *post-hoc* mean comparison test (Student-Newman-Keuls, SNK) was performed when significant differences were found ($P < 0.05$). To further assess most interesting patterns Spearman's Rank correlations were performed. The analysis was conducted first to link gene expression with % 5-mC in Ol and Eu plants across all treatments (C, N, T, N+T) separately, and then to correlate expression of single genes under different treatments between Ol and Eu plants (n = 9). Only significant correlations between Eu and Ol plants undergoing the same treatment were taken into consideration and reported. PERMANOVA analysis was performed with the Primer v.6.1.12 & PERMANOVA + v.1.0.2 software package (PRIMER-E Ltd). All the other statistical analyses were performed using the statistical package STATISTICA (StatSoft, Inc. v. 10).

3. Results

3.1 Dynamics of global DNA methylation and gene expression

Overall, the % 5-mC levels measured for Ol plants were higher than those observed for Eu plants during the whole experiment (factor P, $p < 0.01$, **Table S2**). This was particularly evident considering both the averaged values of % 5-mC across all treatments and for only experimental controls of Ol and Eu plants (**Figure 1, Figure S4**). In both plant typologies, values varied along the experiment (Ol, factor W, $p < 0.05$; Eu, factor W, $p < 0.01$; **Table S3**), decreasing from W1 to W2 and W5 (**Figure 1**). Notably, when the % 5-mC levels were normalized to controls, a steeper decrease was observed in W2 in both Ol and Eu plants, increasing again after five weeks of exposure to treatments, especially for Eu plants (**Figure S3**). In Eu plants, although both nutrients (N) and temperature (T) altered significantly the % 5–mC in W1 (**Table S4**), only nutrients were the main driver of % 5-mC differences over time (W × N, $p < 0.05$; **Table S3**), where the % 5-mC measured in treatments with high nutrients decreased significantly from W1 (1.35%) to W2 (0.95%). In Ol plants, the % 5–mC changed significantly over time, but independently from treatment (W, $p < 0.05$; **Table S3, Figure 1**). In order to assess changes in both % 5-mC and gene expression profiles in *P. oceanica* plants exposed to different abiotic stressful conditions, a PERMANOVA analysis was carried out. Time (W, week) and temperature were the main factors driving significant differences independently from plants' origin (W and T, $p < 0.001$, **Table S5**).

3.2 Gene expression

According to the 2-way RM-GLM, GOIs were upregulated at W1 and decreased over time in Ol plants, while an opposite trend was observed in Eu plants that increased or maintained high expression values during the exposure to stress in five of the seven genes analysed (AGO, MET1, ROS1, SUVH4 and FGT1, **Table S3, Figures 2 and 3**). The expression of single genes is reported below for Ol and Eu plants separately.

3.2.1 Oligotrophic plants (Ol)

Ol plants overexpressed genes involved in *de novo* DNAm (DRM and AGO) in W1 especially in temperature treatments (T and NT) (**Figure 2**, **Table S3 and S4**), showing a significant variation in gene expression modulation over time (**Table S2, Figure 2**). Similarly, genes involved in DNAm maintenance (MET1 and CMT2) were also overexpressed in W1 under temperature treatments (T

and NT, **Table S4**). The expression of the gene involved in histone methylation (SUVH4) changed over time, showing an opposite expression pattern at W1 compared to Eu plants, (Spearman's r = -0.53; **Table S2, Figure 2**). The temporal pattern observed for ROS1 significantly differed between Ol and Eu plants (W x P, $p < 0.05$; **Table S2**). Ol plants increased ROS1 expression over time in all treatments, especially in response to temperature treatments (T and NT, **Figure 2**, **Table S3** and **S4**). FGT1 expression patterns were influenced by the interaction between N and T (N x T, $p < 0.05$; **Table S3, Figure 2)**, with no significant changes over time.

3.2.1 Eutrophic plants (Eu)

Contrary to Ol plants, Eu plants repressed the expression of genes involved in *de novo* DNAm (DRM and AGO) and its maintenance (MET1 and CMT2) in W1 (**Figure 2**, **Table S3 and S4**). In particular, MET1 and CMT2 were significantly different between Ol and Eu plants depending on temperature (P x T, **Figure 2**; P<0.05, **Table S2**). As for Ol plants, SUVH4 expression levels changed over time and significant differences were observed especially under N and T interactions at W2 (N x T, $p <$ 0.05 **Table S4**). Eu plants increased also the expression of ROS1, especially from W1 to W2 and W5 (**Figure 2, Table S3**). Significant changes over time were also observed for FGT1 that was particularly influenced by temperature treatments (W x N x T, $p < 0.05$; **Table S3, Figure 2)**.

3.3 DNA methylation and gene expression correlations between Ol and Eu plants

Temporal patterns of gene expression differed between Ol and Eu plants (**Figure 2**, see **"**Temporal patterns" in **Table S6**), with the only exception of SUVH4 where positive correlations were observed under the exposure to single treatments (Spearman's n = 9, N: r = 0.75; T: r = 0.78), and DMR, where positive correlations were observed only under the exposure to temperature (Spearman's n = 9, T: r = 0.75, see "Temporal patterns" in **Table S6**). A positive correlation for % 5-mC changes in Ol and Eu plants was observed only under N treatment (Spearman's n = 9, r = 0.75; see **"**Temporal patterns" in **Table S6**). % 5-mC changes were negatively correlated with the expression of CMT2, FGT1 and ROS1 and positively correlated with the expression of AGO in Eu plants exposed to NT (see "gene expression vs % 5-mC" in **Table S6**).

Discussion

Here we assessed, for the first time in seagrasses, the dynamics of DNA methylation (DNAm) in *P. oceanica* plants collected from environments with a different history of nutrient loads. Our results revealed that DNAm, considering both gene expression and % 5-mC, is influenced by the time of exposure to stress conditions and by plants' local environmental conditions, with temperature (T) being the main driver of the observed differences. Thus, the present study highlights that the modulation of DNAm could play a fundamental role in the regulation of physiological and gene expression responses to single and multiple stressors. Environmental conditions experienced by plants in their home environment alter the expression of key genes involved in DNAm [27,28] and global DNA methylation levels (% 5-mC). Below, the complexity of plants responses in terms of % 5-mC and gene expression is discussed separately.

Global DNA methylation levels change dynamically over time and depends on plant origin

The total DNA methylation levels (% 5-mC) measured in both Ol and Eu plants after one week from the initial exposure to stressors were higher than the levels measured at the end of the experiment, suggesting the higher implication of DNAm during the initial response to stress conditions. Studies

performed on terrestrial plants exposed to abiotic stressors, also showed a reduction of % 5-mC levels over time [41]. Here, the % 5-mC levels strongly decreased after two weeks from the initial exposure and tended to increase again or remained constant after five weeks of exposure. This suggests that besides the involvement of DNAm changes at the initial phase of the stress perception, it could be dynamically involved in the regulation of stress response under prolonged exposure.

In agreement with our hypothesis, values of % 5-mC varied between plants collected in the two experimental sites, confirming that local environmental conditions affect plant stress response [27,28] and highlighting the potential relationship between DNAm and response to stress. Plants with different history of stress exposure showed also a different temporal pattern of % 5-mC changes in presence of nutrients, indicating that DNAm could induce different responses in *P. oceanica* plants along chronic stress exposure.
Plants collected in oligotrophic environments (Ol plants) were more responsive to experimental stress conditions, showing higher % 5-mC in comparison to plants that have already experienced stress conditions in their home environment (eutrophic, Eu plants). This evidence is in line with previous observations found at transcriptomic level after two weeks of stress exposures, where Ol plants showed the largest gene regulation, especially in the leaf [27]. A study performed on *Brassica napus,* revealed a different degree of DNAm levels between heat-tolerant and heat-sensitive genotypes; in that case, tolerant genotypes showed lower levels of DNAm, contrary to sensitive ones that displayed an increase in methylation levels [42]. Changes in DNAm levels in response to temperature increase were also observed in different plant model species as a stress response mechanism regulated by the expression of specific genes (i.e., *Arabidopsis thaliana* [43]; *Gossypium hirsutum* [44]). Additionally, a recent study performed on clonally-propagated ramets of *Fragaria vesca* (wild strawberry) collected from natural conditions and then transplanted to their home localities and to climatically distinct localities (i.e. Italy, Norway and Czechia, [45]), revealed DNAm changes depending on local environmental adaptation. In that case, the removal of epigenetic memory of the original environment using the application of 5-azacytidine reduced DNAm levels and changed plant's ability to survive their shift to climatically different localities.

It is worth to mention that DNAm levels vary widely among angiosperms, [46], including seagrasses, according to species and populations life history [31]. Methylome variation was also recently observed among ramets of the same genet in the seagrass *Zostera marina* under heat stress, and appeared to be linked with photosynthetic performance and thus phenotypic plasticity [21]. In *P. oceanica*, % 5-mC changes were also observed in relation to different leaf developmental stages and temperature increase [34], and light conditions [47]. Here we observed that DNAm is a dynamic process, changing during stress exposure and varying between genotypes with different history of chronic exposure to stress. In addition, while nutrients influenced mostly % 5-mC changes over time, temperature was the main driver for significant % 5-mC changes independently from plants' origin and time exposure, suggesting that stressor typology can be responsible for specific stress signatures in plants [48,49]. Similar evidence supports that the integration of epigenetics analysis in multifactorial experiments is of fundamental importance for better exploring plants' responses in more realistic and complex future scenarios[50].

Nevertheless, it remains difficult to correlate our results with plants' performance previously observed only at the end of the experiment [28]. To address this question, further studies based on advanced techniques, such as genomic sequencing-based methods, can help to identify specific

DNAm sites and regions involved in stress-responses, rather than global DNAm changes. However, in our case of study Ol and Eu plants did not show differences in genetic or genotypic variability [28] and considering that different DNAm patterns exists in response to abiotic stressors in plants, it is reasonable to assume that the strong differentiation in the levels of % 5-mC between Ol and Eu observed in this study could have a role in the different regulation of the transcriptome machinery, modulating distinct gene pathways and thus driving different phenotypic responses in these plants. In this context, the integration of -omic epigenetic approaches with transcriptomics could result in a high-resolution approach for better exploring responses to future environmental changes.

The expression of DNA methylation, demethylation and maintenance related-genes depends on exposure time to stress and plants' origin

Evidence of different gene expression responses to stress based on local acclimation/adaptation to different environments was already described for different seagrass species [51–53]. In particular, different epigenetic-related genes were found to be differentially regulated in *P. oceanica* plants under thermal stress, revealing higher vulnerability to temperature in more sensitive plants (i.e., cold-adapted [54]). In addition, deep *P. oceanica* ecotypes showed much higher expression levels of key genes involved in the heat stress response [51] than shallow ones, pointing out that local conditions affect gene expression plasticity to thermal events [51].

Here, the expression patterns of genes involved in *de novo* DNAm and its maintenance were in line with trends of variation over time of % 5-mC. We analysed the expression of DRM and AGO genes whose interaction catalyses *de novo* DNAm in plants through the RNA-directed DNA methylation (RdDM) pathway [55]. In *A. thaliana*, the RdDM pathway is involved in the production of small-interfering RNAs (siRNAs) produced by DNA polymerases that are subsequently loaded onto AGOs, mediating the recruitment of DRM for methylation of target loci [56]. In Ol plants, both genes were overexpressed in W1, especially in treatments with temperature increases, together with ROS1, which catalyses the removal of DNAm (**Figure 3**). These findings are in line with previous observations in terrestrial plants exposed to temperature increase [43]. In that case, the authors reported a simultaneous increase of ROS1 and DRM2 genes that could result in a target-specific deposition and removal of DNAm. In plants, the ROS1 gene promoter includes a sequence termed DNAm monitoring sequence (MEMS), which allows the coordination between DNAm and active demethylation through the transcription regulation of ROS, functioning as a "methylstat" [57]. The increase of DNAm at the MEMS favours the increase of ROS1 expression. Since the DNAm at the MEMS is also regulated by RdDM and MET1 based on the ROS1 activity, this system could monitor DNAm state regulating and maintaining the dynamics of DNAm and demethylation [33]. Eu plants showed an opposite pattern to Ol plants, downregulating genes involved in DNAm and its maintenance in W1, whereas ROS1 was overexpressed. This evidence demonstrates a different DNAm regulation depending on local environmental conditions. Plants that were already impacted by local disturbances had probably already activated genes involved in DNAm and its maintenance prior to the exposure to experimental stress conditions. Indeed, in our experimental conditions DNAm appeared to be especially important during the first phase of the stress exposure, with a different modulation of key genes involved in the DNAm machinery. However, only the analysis of DNAm (both gene expression and % 5-mC) before the exposure phase could fully support this hypothesis.

In line with the expression patterns observed in Ol plants for genes involved in *de novo* DNAm, MET1 was highly overexpressed in treatments with high temperatures (NT and T). A similar

regulation was already demonstrated in *Arabidopsis thaliana* exposed to thermal stress, where the overexpression of MET1 controls the maintenance of cytosine methylation at symmetrical CG positions, while the downregulation of MET1 induces a strong reduction of cytosine methylation marks [58]. In contrast, MET1 showed an opposite trend in Eu plants, which was similar to that observed for the other genes. This suggests that high transcript abundance of MET1 could be necessary for maintaining high DNAm levels. However no significant correlations were observed between the expression of MET1 and % 5-mC, suggesting that the regulation of global DNA methylation levels probably function in coordination with other proteins not included in our analysis.

As already reported above, heat-tolerant genotypes in plants tend to show lower DNAm levels in response to temperature stress [42]. The lower expression of genes involved in DNAm and its maintenance reported for Eu plants at the early insurgence of stress, could be related to the local disturbance experienced by plants in their natural conditions, which already imposed the regulation of the epigenetic machinery. Hence, only a prolonged exposure to further stress induces the modulation of DNAm-related genes, contrary to Ol plants that promptly activated these genes after one week of exposure. The different stress perception of plants depending on the time exposure to stress conditions is corroborated by the evidence that no correlations were observed between plants in terms of gene expression, except for SUVH4 and DMR under nutrients and heat stress, respectively. Recently, the transcriptomic analysis carried out on Ol and Eu plants in W2 revealed a larger gene regulation in leaves of Ol plants in respect to Eu plants. However, Eu plants showed a larger number of genes and the activation of different metabolic processes especially in the shoot-apical meristem (Eu SAM), which is considered one of the most sensitive plant tissue [35]. This response anticipated the high shoot mortality observed in Eu plants several weeks after the prolonged exposure to stressors [28]. The higher % 5-mC observed for Ol and EU plants after one week of exposure, and the different behaviour of target genes during the experiment, emphasise the effect of local environmental conditions on the response to future stressors. Therefore, the observed DNAm changes seemed to anticipate the stress response perceived by *P. oceanica* plants and could be the link between phenotypic changes and the environment [25]. These new findings can have important implications for understanding the degree of stress perception in seagrasses, and since their regulation was strongly dependent on local environmental conditions, these genes could be also tested and used as molecular biomarkers of stress response in *P. oceanica*.

We also investigated a specific plant DNA methyltransferase (CMT2) involved in both maintenance and *de novo* DNAm [59]. Chromomethylase can also be targeted by H3K9me2, deposited by SUVH4, due to the dual recognition mechanism mediated by its bromo adjacent homology (BAH) and chromo domains [60]. High expression levels of chromomethylases were already observed in *P. oceanica* plants exposed to cadmium toxicity [61]. In that case, DNA hypermethylation was associated with chromatin condensation, increasing the heterochromatic nuclear fraction. In Ol plants, CMT2 and SUVH4 showed higher expression values in W1 and W5, contrary to Eu plants that repressed these genes in W1. It is important to emphasize that SUVH4 is also known as suppressor of variegation 3–9 homolog (SUVH) family H3K9 MTases kryptonite (KYP), which is a plant-specific protein that methylates H3K9 forming a feedback mechanism that maintains epigenetic silencing in plants [9]. Our results suggest that DNAm and histone modifications could cooperate in regulating stress responses, especially at the initial exposure to stressors.

While % 5-mC patterns observed for Ol plants were similar to the expression of target genes involved in *de novo* DNAm, in Eu plants the increased expression of methyltransferases did not correspond with an increase of % 5- mC (Figure 1 and 2). It is important to underline that % 5-mC estimates the total methylated cytosine in different sequence contexts (CG, CHG, CHH). Since DNAm is catalysed by various enzymes that are targeted by distinct regulatory pathways, the % 5-mC observed for Ol and Eu plants likely depends on the activity of different genes in addition to those selected in this study. To this end, a transcriptomic analysis performed during the experimental exposure to stress over time could provide an overview of the regulation of different genes and functions, improving our understanding of the temporal evolutions of stress responses in seagrasses.

Does plants' response depend on stress-memory genes?

In addition to genes involved in DNA modifications, we also analysed the expression levels of FORGETTER1 (FGT1) which was identified as a relevant gene for heat stress memory in *A. thaliana* [19], interacting with chromatin remodelers [62]. In this study, the expression of FGT1 changed over time and was particularly sensitive to heat stress in both Ol and Eu plants. However, in Eu plants, FGT1 was repressed in treatments with high nutrients addition and overexpressed in T only after one week of the initial stress exposure. Thus, its regulation appeared to be significantly influenced by temperature and nutrients interaction at the early phase of the exposure to stressors. Generally, memory genes show lower abundance before the occurrence of a stress [17]. During the exposure to stress conditions, they actively regulate the transcription of genes involved in the stress response [17]. Thus, with the occurrence of another stress event they can be more quickly re-induced [14]. It is worth to notice that a similar regulation strategy could have been activated in Eu plants, where the presence of elevated temperature induced high expression levels that remained constant over time, whereas the exposure to nutrients activated FGT1 later, as the exposure time to stress increased. This new finding revealed the potential role of FGT1 in regulating nutrients-memory responses in plants that already experienced high nutrient conditions in their home environment. Similar evidence of a transcriptional memory in *P. oceanica* plants was previously observed in seedlings, where the exposure to an anomalous warming event (i.e., priming treatment) activated genes related to stress responses and epigenetic modifications, conferring higher tolerance to further stressful conditions [63]. However, this evidence is not fully supported by our data as it requires the analysis of other genes involved in stress-memory responses (i.e., HSPs).

Conclusions and perspectives

In this study, the dynamics of DNA methylation of a keystone seagrass species, living in different environments and experimentally exposed to nutrient and temperature stress, was assessed for the first time through the analysis of global DNA methylation and the expression of epigenetic-related genes. We showed that DNA methylation is a dynamic process influenced by environmental stressors and local environmental conditions, which may have important implications in regulating seagrass stress response.

We demonstrated that the DNAm machinery was regulated mostly during the first exposure to stressors with differences in the expression of epigenetic-related genes between plants living in environment with a different history of exposure to stress. These findings represent a significant step forward in the understandings of epigenetic regulation dynamics in seagrasses. In fact, our results pointed out that environmentally induced epigenetic variations can have an important role in driving

differential responses in highly clonal marine plants such as *P. oceanica*. The in-deep characterization of genome-wide epigenetic marks (e.g., context-specific DNAm) in plants from contrasting environments is necessary to better explore seagrasses' ability to withstand and acclimate to future environmental changes. Considering that environmental changes are occurring so fast, understanding the epigenetic contributions to rapid responses to changing environments, including the local environmental effects, can contribute to developing more accurate predictions of seagrass performance and survival to future environmental changes, in particular sea warming. Our results pointed out that performing a multifactorial experiment and exposing seagrasses to stressful conditions, that reflect future realistic scenarios, can reveal specific stress signatures.

Further empirical evidence is required to corroborate the potential link between epigenetic regulation and gene expression, which could provide new insights on potential markers of seagrass vulnerability to abiotic stress. Moreover, understanding epigenetic regulation and the regulation of genes involved in stress memory could shed light on the molecular mechanisms behind phenotypic plasticity in foundation species, improving conservation and restoration efforts.

FIGURE LEGENDS

Figure 1. Global % of methylated cytosine (% 5-mC) measured in Ol and Eu plants (P) during the course of the experiment (W1 = one week; W2 = two weeks; W5 = five weeks of the exposure) for the different treatments (N = nutrients; T = temperature; NT = nutrients + temperature). Significant differences resulting from 3-way RM-GLM are reported in the central square, while results of 2-way RM-GLM and *post-hoc* tests performed individually for Ol and Eu plants, are shown in the bottom left corner of each graph. Data are mean ± SE (n = 3).

Figure 2. Expression dynamics of GOIs involved in *de-novo* DNAm (DRM and AGO) and its maintenance (MET1 and CMT2), DNA demethylation (ROS1), and histone methylation (SUVH4) measured in both Ol and Eu plants under different stress conditions (N = nutrients; T = temperature, NT = nutrients + temperature) compared to control (dashed line). Significant differences resulting from 3-way RM-GLM are reported in the central square, while results of 2-way RM-GLM performed individually for Ol and Eu plants are shown in the bottom left corner of each graph. Data are mean ± SE (n = 3).

Figure 3. Graph showing gene expression patterns observed in Ol and Eu plants during the exposure phases (W1 = one week; W2 = two weeks; W5 = five weeks). **a)** *De novo* DNAm involves a plant specific RNA polymerase V (Pol V) which produces noncoding RNA transcripts that act as a scaffold to recruit AGOs and DRM through base-pairing of associated siRNAs; **b)** DNAm maintenance operated by DNA methyltransferase 1 (MET1) and chromomethylases (CMTs) through the interaction with H3K9-specific methyltransferases (SUVHs); **c)** DNA active demethylation is mediated by repressor of silencing 1 (ROS1) which is a bifunctional 5-mC DNA glycosylase inducing the excision of the 5-mC base from the DNA backbone. Blue (Ol plants) and red (Eu plants) lines refer to the general expression patterns of analysed genes under different experimental conditions over time. The horizontal dashed line represents the control (expression of reference genes).

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Author contributions

JP: Formal analysis, Writing original draft; JP and AS: Investigation; JP, MR and ED: Conceptualization; GP and LMG: Supervision. All authors: Writing review and editing.

Data Availability

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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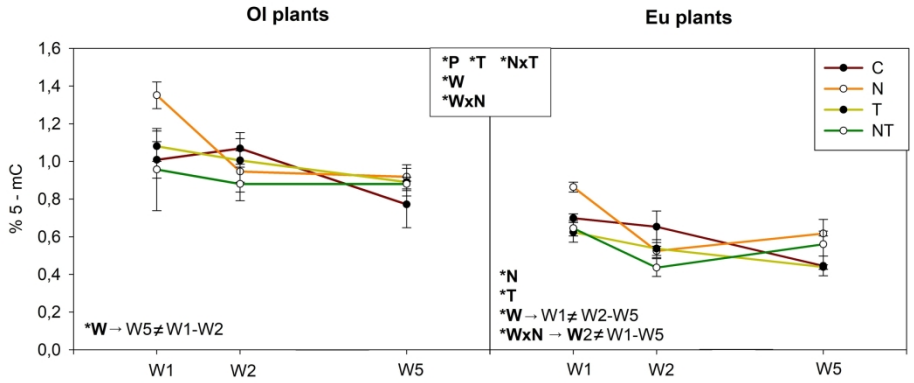

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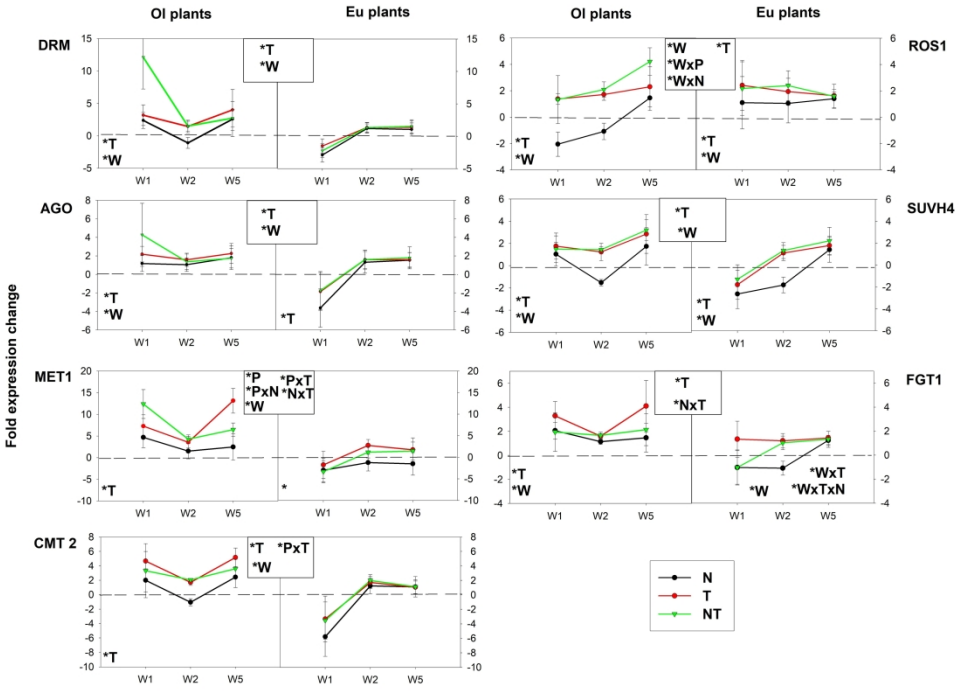

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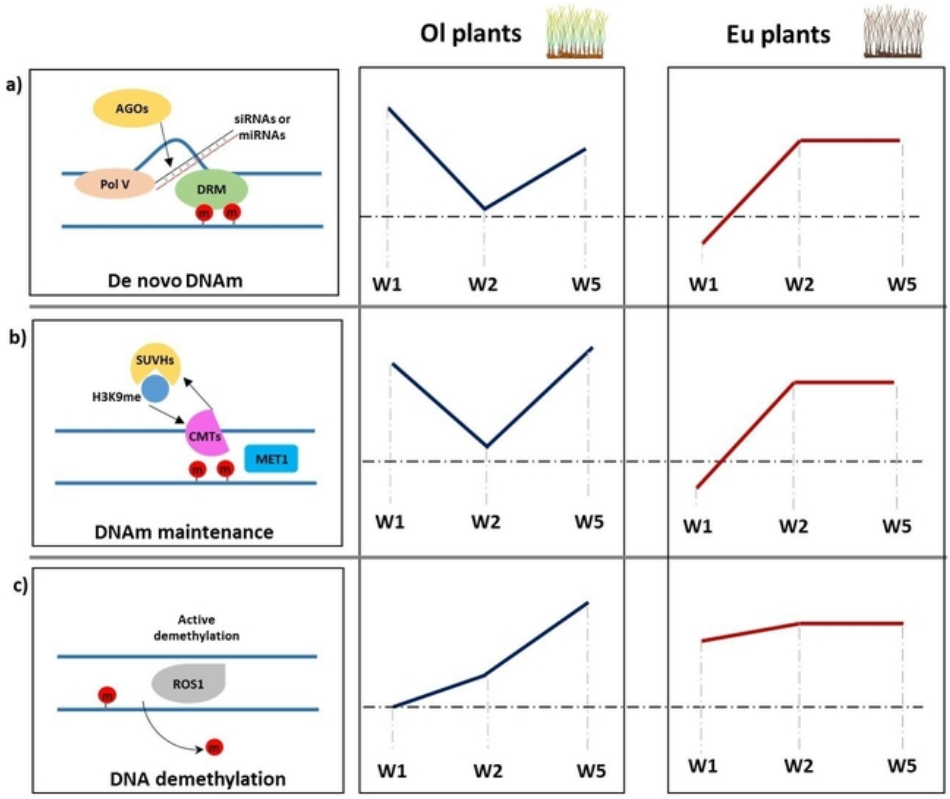

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57x46mm (300 x 300 DPI)