Local environment modulates whole-transcriptome expression in the seagrass Posidonia oceanica under warming and nutrients excess

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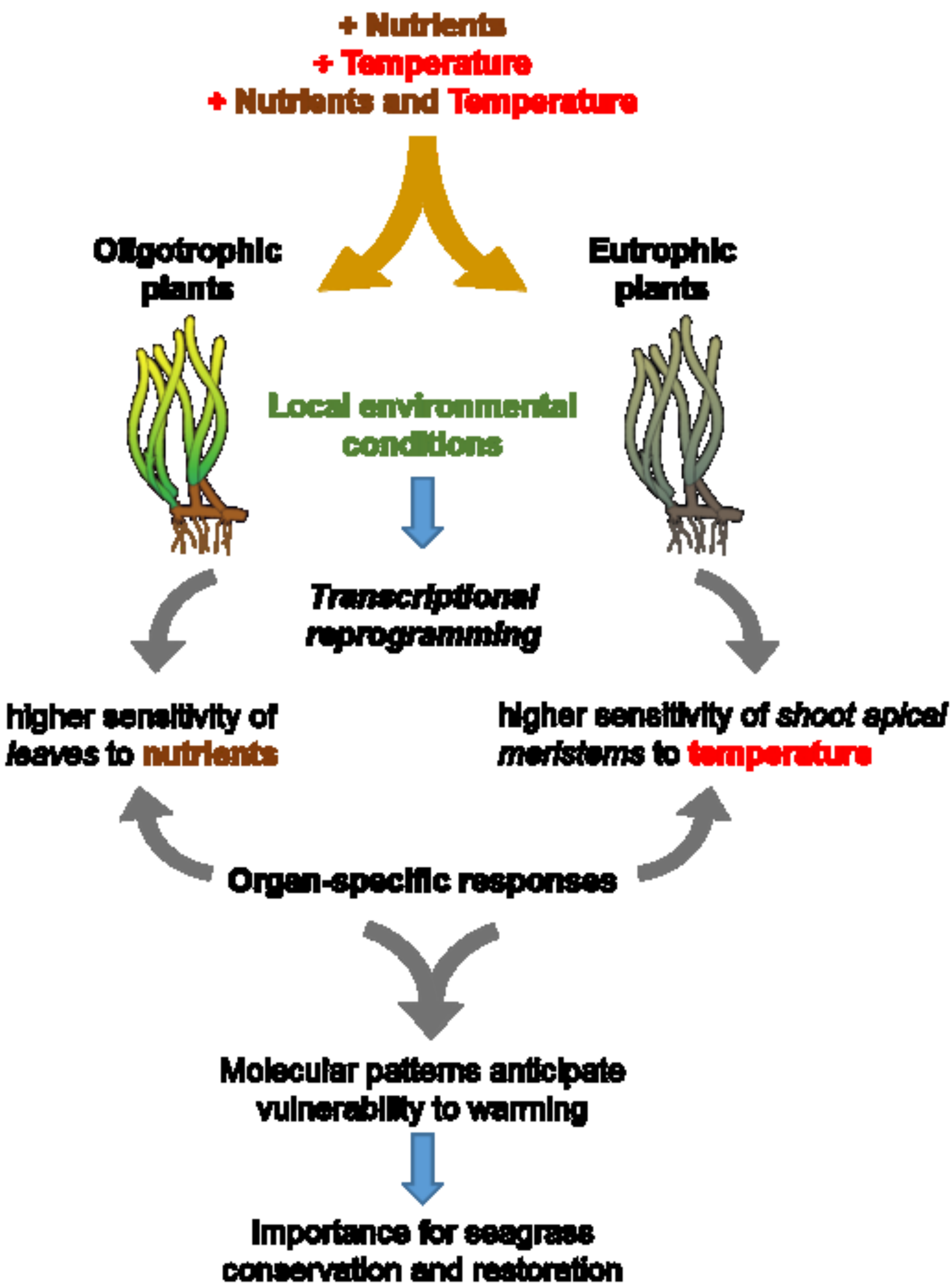
Highlights

- Local pressure influence plants' transcriptional responses to stress
- Plants in eutrophic sites will be more impacted by seawater temperature increase
- Organ-specific vulnerability to single and multiple stresses
- Potential epigenetic regulation of transcriptional responses to stress

Graphical Abstract



Local environment modulates whole-transcriptome expression in the seagrass *Posidonia oceanica* under warming and nutrients excess

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Abstract

The intensification of anomalous events of seawater warming and the co-occurrence with local anthropogenic stressors are threatening coastal marine habitats, including seagrasses, which form extensive underwater meadows. Eutrophication highly affects coastal environments, potentially summing up to the widespread effects of global climate changes. In the present study, we investigated for the first time in seagrasses, the transcriptional response of different plant organs (i.e., leaf and shoot apical meristem, SAM) of the Mediterranean seagrass *Posidonia oceanica* growing in environments with a different history of nutrient enrichment. To this end, a mesocosm experiment exposing plants to single (nutrient enrichment or temperature increase) and multiple stressors (nutrient enrichment plus temperature increase), was performed. Results revealed a differential transcriptome regulation of plants under single and multiple stressors, showing an organ-specific sensitivity depending on plants' origin. While leaf tissues were more responsive to nutrient stress, SAM revealed a higher sensitivity to temperature treatments, especially in plants already impacted in their native environment. The exposure to stress conditions induced the modulation of different biological processes. Plants living in an oligotrophic environment were more responsive to nutrients compared to plants from a eutrophic environment. Evidences that epigenetic mechanisms were involved in the regulation of transcriptional reprogramming were also observed in both plants' organs. These results represent a further step in the comprehension of seagrass response to abiotic stressors pointing out the importance of local pressures in a global warming scenario.

Keywords: Seagrasses, multiple stressors, global warming, eutrophication, gene expression, epigenetics

Introduction

Coastal marine environments are among the most threatened marine habitats (Worm et al., 2006). The continuous increase of human urbanization along the coastline, with the extensive use of marine resources and services, has amplified the number and diversity of anthropogenic stressors. Among different local pressures, eutrophication due to nutrient inputs from human activities (e.g., agriculture, urban/industrial development and aquaculture) is one of the greatest concerns for coastal habitats, especially for environments characterized by dense urbanization such as most of the Mediterranean basin (Liquete et al., 2016). The dominant components of nutrient inputs are nitrates and phosphorus, which are considered the main nutrient sources intensifying water hypoxia and acidification, as a consequence of phytoplankton and microbial proliferation (Gobler and Baumann, 2016). Additionally, different indirect effects are linked to nutrient increase such as the reduction of light penetration along the water column, which compromises biological performances of photosynthetic organisms and in general the benthic production (Touchette and Burkholder, 2000). In an era of global warming, the effects induced by these local disturbances can be much more complex depending on their interaction with ongoing climate changes, which are globally threatening marine ecosystems (He and Silliman, 2019; Nguyen et al., 2021). The intensification of anomalous events of seawater warming and the increase of sea surface temperature at unprecedented rates can induce synergic or antagonistic effects when more eutrophic conditions occur (Ceccherelli et al., 2018; Paerl and Scott, 2010). Thus, local pressures may have the potential to exacerbate or buffer the effects of climate change on marine habitats (Bowler et al., 2020). Understanding how marine organisms can overcome the potential cumulative impacts by multiple stressors is becoming of fundamental importance especially for sessile organisms such as marine plants (Micheli et al., 2013).

Seagrasses are marine angiosperms belonging to the order *Alismatales*, representing a unique group of higher plants that re-colonized marine environments, forming extensive underwater meadows (Les et al. 1997). These habitat-forming species provide important services and benefits to ecosystems and human livelihoods (Nordlund et al., 2018). Similarly to their terrestrial counterpart, seagrasses have a high carbon storage capacity, which underlines their potential contribution to climate change mitigation (Duarte et al. 2013; Gattuso et al. 2018). Despite their importance, seagrasses are declining globally at alarming rates (Waycott et al., 2009). New projections estimate a massive reduction of marine habitat-forming species as a consequence of global warming by the end of 2050, stressing that environmental changes are occurring too fast, preventing their capacity to react properly (Trisos et al. 2020).

The evolutionary success of marine plants derives from their extraordinary adaptation capacity, which allowed them to colonize heterogeneous environments including temperate and tropical regions with different environmental conditions (Short et al., 2007). Single species display peculiar strategies from physiological to gene expression rearrangements for adapting along wide bathymetric and latitudinal gradients (Dattolo et al., 2017; Jahnke et al., 2019). These emerging plastic properties that characterize some seagrass species are at the basis of the appearance of different phenotypes according to local environmental settings (Bergmann et al., 2010; Franssen et al., 2011; Pazzaglia et al., 2020; Soissons et al., 2017). Among seagrasses, *Posidonia oceanica* (L.) Delile is an iconic species widely distributed in the Mediterranean basin, forming large meadows across the photic zone (Telesca et al., 2015). Featuring among the oldest living genotypes on our planet, due to the prominent clonal propagation, *P. oceanica* is an ideal target species for studying plasticity of phenotypic response to environmental changes (Arnaud-Haond et al., 2012).

Molecular signatures at the basis of phenotypic responses to single stressors have been explored in seagrasses, especially in relation to different light and thermal regimes (e.g., Dattolo et al., 2017; Marín-Guirao et al., 2016; Massa et al., 2011; Ruocco et al., 2021). In general, large-scale gene expression studies in response to abiotic stresses have revealed the regulation of specific stress genes that modulate different phases of the cellular stress response, such as protein folding and degradation (Franssen et al., 2011; Reusch et al., 2008; Traboni et al., 2018). Particularly, warming can induce oxidative stress, enhancing the accumulation of reactive oxygen species (ROS) able to damage membranes, proteins and DNA. Under such conditions, seagrasses activate their antioxidant system, which includes key ROS-scavenging enzymes (Franssen et al., 2014; Purnama et al., 2019; Traboni et al., 2018; Tutar et al., 2017; Winters et al., 2011). Additionally, photosynthesis is one of the most heat-sensitive processes and the modulation of genes encoding for crucial enzymes of the photosynthetic apparatus is part of the machinery that regulates primary metabolism under heat stress (Marín-Guirao et al., 2017; Ruocco et al., 2019a; Wang et al., 2018). In seagrasses, the analysis of transcriptional profiles in populations experiencing diverse thermal regimes in their home environments has revealed differential responses, reflecting the contribution of local adaptation to gene expression divergence (e.g., Franssen et al., 2011). Thus, plants living in more dynamic and variable environments (e.g., southern regions and/or shallow intertidal waters) showed higher thermal tolerance and can be more resilient to environmental changes than plants living in more stable environments such as the tropics (Ashander et al., 2016; Botero et al., 2015; Chevin and Hoffmann, 2017; Pazzaglia et al., 2021; Tomasello et al., 2009).

While modulation of gene expression in seagrasses under thermal stress has been extensively investigated (for a review see Nguyen et al., 2021), considerably less emphasis has been given to gene-expression changes in response to high nutrients conditions. Most of the literature is focused on nutrient assimilation and physiology, pointing out the importance of leaf tissues in nutrient uptake (Touchette and Burkholder, 2000). Direct effects induced by the excess of nutrients on growth and survival have been shown in seagrasses (Burkholder et al., 2007), while the mechanisms behind nutrient toxicity and gene expression regulations are still unclear.

NH_4^+ is the primary form of nitrogen that can be assimilated by seagrasses, through high- or low-affinity transporters, depending on external nutrient concentrations. Since the assimilation of nutrients differs among above- and below-ground tissues, this is also reflected in the regulation of specific responsive genes that tend to be activated earlier in the leaf in respect to below-ground tissues (Pernice et al., 2016). In *P. oceanica*, the regulation of genes playing a key role in nutrient assimilation is influenced by the co-occurrence with other types of stressors, such as herbivory (Ruocco et al., 2018) and acidification (Ravaglioli et al., 2017). All this highlights that interactions among different stressors and local disturbances need to be considered for a complete understanding of the effects of global changes on seagrasses. However, only a few studies have investigated the effects of nutrients in a global warming scenario, focusing mainly at plant physiological responses (Artika et al., 2020; Campbell and Fourqurean, 2013; Mvungi, 2011; Pazzaglia et al., 2020).

Epigenetic mechanisms, such as chromatin modifications, have recently been recognized to play a crucial role in gene regulation in response to abiotic stressors (Bhadouriya et al., 2021; Lindermayr et al., 2020). Chromatin accessibility can be regulated by the exclusion or inclusion of different histone variants and various histone modifications (e.g., acetylation/deacetylation, methylation/demethylation) can be influenced by environmental variations. In plants, chromatin modifications induced by specific environmental stress can regulate the transcriptional machinery at somatic level (within the same generation), and have the potential to be stored or memorized for future reoccurring events (Bäurle and Trindade, 2020; Dai et al., 2017; Kumar et al., 2017; Tasset et al., 2018). While epigenetic changes have been extensively investigated in terrestrial plants, they remain mostly unexplored in seagrasses. Indeed, only few studies have recently analysed epigenetic responses to abiotic stressors, especially DNA methylation marks (*P. oceanica*, Greco et al., 2012; Greco et al., 2013; Ruocco et al., 2019b; Entrambasaguas et al., 2021; *Zostera marina,* Jueterbock et al., 2019; *Posidonia australis* and *Zostera muelleri*, Nguyen et al., 2020).

The present study aims to investigate the transcriptome rearrangements occurring in *P. oceanica* plants with a different history of nutrient loads and exposed to single and multiple stressors. Starting from previous physiological assessments (Pazzaglia et al., 2020), here we proceeded with a further step, exploring the whole transcriptome profile of leaf and shoot-apical meristem (SAM) in plants with a different origin, and provided a functional characterization of biological processes activated in response to temperature increase, nutrients addition, and their combination. In general, the SAM is considered the most sensitive plant organ with the lowest tolerance threshold, playing a crucial role in the maintenance of growth and survival under abiotic and biotic stresses (Fulcher and Sablowski, 2009). Recently, a gene expression study performed on SAM revealed the activation of an early molecular response in respect to the leaf, besides a much more complex and specific response (Ruocco et al., 2021). We hypothesize that leaves and SAMs of plants growing in environments with a different history of nutrient loads would show a divergent gene expression signature and the activation of specific biological processes in response to the same stress conditions. We also expect different effects induced by nutrients and thermal stressors, which should modulate the transcriptional profile of *P. oceanica* plants. Furthermore, since epigenetic mechanisms are involved in gene regulation, we also predict a differential activation of related processes. Overall, we aim to assess plant response in a future scenario of local human-driven pollution and global increase of seawater temperature.

2. Methods

2.1 Plant collection and experimental design

The sampling sites and the experimental design for this study are the same of Pazzaglia et al. (2020). Briefly, large fragments of *P. oceanica* bearing 10-20 vertical shoots were collected by SCUBA diving on May 15 – 16th 2019 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 (Bacoli) is considered an impacted site as it is close to a highly urbanized area with more eutrophic conditions in respect to the latter site (Ischia), which is in a marine protected area (for a comprehensive description of sampling sites see Pazzaglia et al., 2020). The N leaf content value which is an indicator of the nutrient status, in fact, was almost twice in Bacoli (%N leaves = 1.89 % ± 0.2; C/N ratio = 16.7 ± 0.9) than in Ischia (%N leaves = 0.97% ± 0.2; C/N ratio = 33.2 ± 2.4, supplementary data in Pazzaglia et al., 2020). Additionally, nutrients concentrations measured in the sediment pore water revealed almost double values in the Bacoli site than the Ischia site (DIN [µM] = 47.9 ± 4.4 in Bacoli, and 26.7 ± 8.9 in Ischia site; PO_4 − [µM] = 4.3 ± 1.0 in Bacoli, and 2.1 ± 0.4 in Ischia. As plants growing in the two sites were exposed to different anthropogenic pressures, here we refer to plants collected in Bacoli as relatively eutrophic (Eu plants), and plants collected in Ischia as relatively oligotrophic (Ol plants). After sampling, plants were exposed to multiple stressors in an indoor mesocosm facility at Stazione Zoologica Anton Dohrn (SZN, Naples, Italy) (Ruocco et al. 2019b) following a multi-factorial design, including four treatments: Control (C), Nutrients (N), Temperature (T) and Nutrients + Temperature (NT). The experimental set-up consisted of 12 glass aquaria (500 L) filled with natural seawater. Two plant fragments for each Eu- and Ol- plants were allocated in the same tank using a basket filled with coarse sediment. Stress levels were set according to a previous mesocosm experiment and different environmental observations at the sampling sites (Pazzaglia et al., 2020). The temperature treatments (T and NT) consisted in the gradual increase (0.5 °C day^{-1}) of temperature from control conditions (measured during the sampling, 24°C) to 30°C, which is 4–5 degrees above the summer average. The nutrient treatments (N and NT) consisted in the increase of nutrient concentrations adding a stock solution (170 mM total nitrogen) that was prepared using Osmocote Pro fertilizer pellets (6 months release: 19% N – 3.9% P – 8.3% K, ICL Specialty Fertilizers). The solution was added every week in order to maintain a nutrient enrichment condition in N and NT treatments (DIN = 26.8 ± 4.0 mM).

2.2 RNA extraction and 3'Tag sequencing

After two weeks from the initial exposure to stress conditions (T2), three samples per treatment of *P. oceanica* leaf and shoot-apical meristem (SAM) were collected (n = 3). A portion of 6 cm of the second leaf was cleaned from epiphytes and immediately submerged in RNA later© tissue collection solution (Ambion, life technologies). Leaf samples were kept at 4 °C overnight to let the solution penetrate into the tissue, and finally stored at - 20 °C. The first most apical 0.5 cm of the rhizome tip, containing the SAM, were also collected from the same shoots and preserved in liquid N_2, since previous trials demonstrated that RNA later solution does not permeate appropriately in the meristem tissue. Total RNA was extracted with the Aurum™ Total RNA Mini Kit (BIO- RAD). RNA purity and concentration was assessed by using NanoDrop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies) and 1% agarose gel electrophoresis, while RNA integrity was assessed by means of 2100 BioAnalyzer (Agilent). Twenty-four libraries (3 replicates × 4 treatments × 2 different plant conditions) were constructed for each tissue (24 leaf and 24 SAM) with the QuantSeq 3' mRNA-Seq Library Prep Kits (Lexogen) and sequenced using Ion Torrent technology (Ion Torren GeneStudio). The QuantSeq protocol produces only one fragment per transcript, generating reads towards the poly (A) tail. In contrast to the traditional RNA-Seq, TagSeq approach directly reverse transcribed cDNAs from the 3′ end of the mRNAs, without a fragmentation step. It represents a cost-effective approach applicable to model species and it has also been successfully applied to non-model species for which reference transcriptomes are available (Marx et al., 2020; Moll et al., 2014). Hereinafter, we refer to leaf and SAM of Ol plants as 'Ol leaf' and 'Ol SAM', respectively, and to leaf and SAM of Eu plants as 'Eu leaf' and 'Eu SAM', respectively.

2.3 Data filtering and functional annotation

Raw reads were quality checked using FASTQC (Andrews, 2010) and then subjected to a cleaning procedure using Trimmomatic (Bolger et al. 2014), setting the minimum quality per base at 15 phread score and minimum length of the read after cleaning at 50bp. All cleaned reads were then mapped, independently, on the reference transcriptome of *P. oceanica* (Ruocco et al., 2021) using the Bowtie2 aligner (default settings, Langmead and Salzberg, 2012). Reads count and FPKM (fragments per kilobase of exon model per million reads mapped) calculation per transcript for each replicate were performed using the eXpress software (Roberts et al., 2011). Functional annotation of the reference transcriptome was carried out through sequence similarity search against the Swiss-Prot database using the BLASTx software (Camacho et al., 2009), setting as minimum E-value threshold $1e^{-3}$ and getting only the best hit detected.

2.4 Differentially Expressed Genes (DEGs) and Gene Ontology (GO) enrichment analysis

DEGs analysis was performed using two tools implementing two different statistical approaches: DESeq2 (Love et al., 2014) and edgeR (Robinson et al., 2010). For each transcript, the mean of the $\log_2$ fold change values (Log_2FC) obtained with the two tools was calculated. The thresholds for the DEGs calling were FDR ≤0.05 or *P*-adjusted ≤0.05, and Log_2 fold change ≤|1.5|. Differential gene expression profiles resulted from the comparison between all treatments (N, T and NT) *vs* control in both organs and plant conditions. A graphical representation of shared and unique DEGs across samples was obtained using DiVenn 2.0 interactive tool (Sun et al., 2019). DEGs-related GO-terms were retrieved by using InterProScan (version 5.33, Jones et al., 2014) and GO enrichment analysis was performed using the Ontologizer software (Bauer et al., 2008). The threshold used to identify significantly enriched functional terms was *P* ≤0.05. DEGs and GO enrichment results are discussed separately for leaf and SAM, comparing Ol and Eu plants. GO enriched terms for both Ol and Eu plants are reported in Tables S3 and S4. Additionally, GO enriched terms related to epigenetic mechanisms (epi-GOs) were screened for leaf and SAM independently from the treatments, and unique/shared biological processes and molecular functions for Ol and Eu plants are described separately.

3. Results

3.1 General overview of transcriptomic responses

Different transcriptomes obtained for both organs of *P. oceanica* plants collected in different environmental conditions (Ol leaf, Ol SAM, Eu leaf and Eu SAM) showed a comparable number of transcripts and significantly matched to Swiss-Prot database (**Table 1**). Full DEGs results are included in **Table S1**, whereas GO terms associated with biological processes, cellular components and molecular functions obtained for all treatments are reported in the **Table S2**.

Table 1. *Summary description of the number of transcripts within each dataset (N = Nutrients, T = Temperature, NT = Nutrients + Temperature). The % of annotated transcripts for each dataset via BLASTx is also shown.*

Unique datasets	**N. transcripts**				**Annotated transcripts**	**% of annotated transcripts**
	N	**T**	**NT**	**Tot.**		
Ol leaf	108,022	108,594	110,649	124,077	70,722	57.0
Ol SAM	110,119	112,831	112,163	125,401	71,380	56.9
Eu leaf	102,831	105,067	105,329	112,473	66,909	59.5
Eu SAM	107,489	108,442	107,724	121,807	70,599	58.0

3. 2 Leaf-specific transcriptomic responses

3.2.1 Differentially expressed genes (DEGs) and GO enrichment analysis

Leaf showed the largest transcriptomic response in treatments with nutrients addition (N and NT), whereas a less severe effect was observed under the increase of only temperature (T), which is similar between Ol and Eu plants (**Fig. 1**). However, while Ol leaf showed the highest percentage of DEGs in N treatment, Eu leaf appeared more responsive to NT (**Fig. 1**). The comparison of up and down-regulated DEGs among treatments, highlighted a larger and unique transcriptome rearrangement occurring in the leaf under nutrients addition, in particular in Ol plants exposed to N (Fig. 2 and Fig. 3), where most of the unique DEGs were up-regulated (**Fig. 2a; Table S1**). Contrarily, T treatment induced only a limited and less specific response (**Fig 2a**). Eu leaf displayed a distribution pattern of

DEGs similar to Ol leaf, with higher number of unique DEGs under N and NT (higher in NT), in comparison to T treatment (**Fig. 2b, Table S1**).

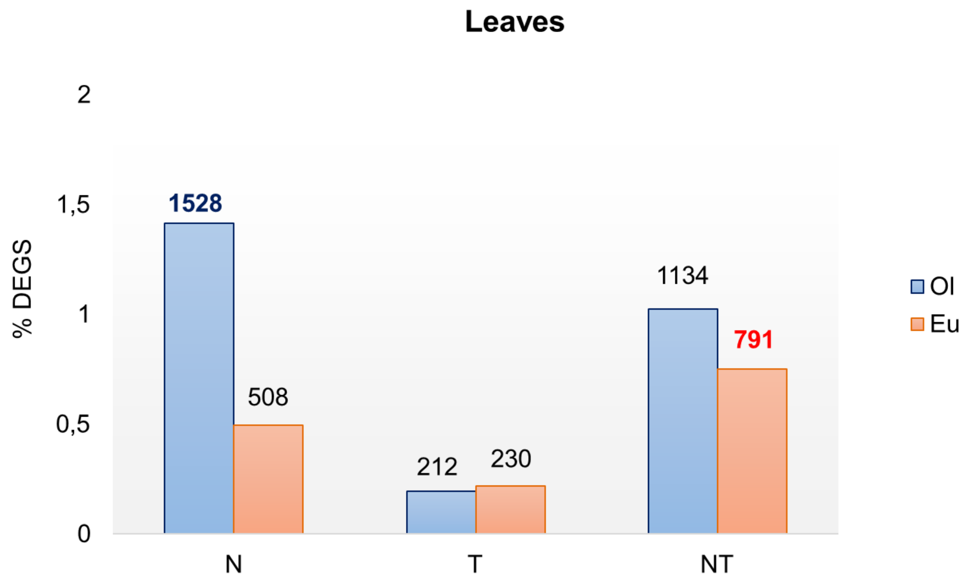

Figure 1. *Percentages of DEGs (down- and up-regulated) over the total number of transcripts counted for each unique dataset (Ol leaf and Eu leaf). The total n° of DEGs is shown on the top of each histogram. The greatest n° of DEGs are highlighted in bold with different colours for Ol (blue) and Eu plants (red).*

The GO enrichment analysis of the leaf revealed similar patterns in both Ol and Eu plants, activating more processes under nutrients addition (N and NT, **Fig. 3**; **Table S2**). However, unique GO enriched terms found in Ol leaf under N conditions were twice of those counted in Eu leaf for the same treatment (**Fig. 3a**, **Table S3**). In Ol leaf, different transcripts belonging to the transport category like *Nuclear transport factor 2B (NTF2)* and *Zinc transporter 4 (ZIP4)* were overexpressed in presence of nutrients (N and NT) (**Table S1**). One of the most significant GO enriched term in the N treatment was related to "protein kinase activity" including enzymes involved in protein degradation such as *Putative U-box domain- containing protein 50 (PUB50)* and the *RING-H2 finger protein (ATL13)* that were up- and down-regulated, respectively. Ol leaf activated also defence processes regulating e.g., *Leucine-rich repeat-like serine/threonine/tyrosine protein kinase (SOBIR1)* and the *Stromal cell-derived factor 2-like protein (SDF2).* In addition, DEGs of NT and N treatments shared different GO terms including "photosynthesis", pointing out the down-regulation of genes that play a crucial role in photosystem assembly and functions (*HCA6-Chlorophyll a-b binding protein CP26, PSBS-Photosystem II 22 kDa protein 1*). The presence of nutrients activated also processes related to metabolism like "nitrogen cycle metabolic process" and "reactive nitrogen species metabolic processes", where key genes of nitrate assimilation were down-regulated (*NR2-Nitrate reductase [NADH] 2* and *NRT2.5-High affinity nitrate transporter 2.5*). Several transcripts within this category were also up-regulated in NT, including key enzymes involved in the lipid biosynthesis pathway like *Allene oxide synthase 1 (AOS), Delta(8)-fatty-acid desaturase 2 (SLD2)* and S*NF1-related protein kinase regulatory subunit beta-1 (AKIN subunit beta-1)* (**Table S1**). In this treatment (NT), Ol leaf activated also processes related to flavonoid synthesis (i.e., *Chalcone and Squalene synthase*). The exclusive exposure to temperature (T) induced the lowest activation of specific biological processes (**Fig. 3a; Table S2**). In this case, Ol leaf regulated processes related to defence mechanisms and Ubiquitin-conjunctions ("regulation of biological quality", "chaperone binding") that include transcripts encoding for positive regulators of basal defence such as *Protein SGT1 homolog A and B* that were down-regulated. In general, few processes were shared among all treatments, mostly including categories related to metabolism ("oxidoreductase activity", "small molecule metabolic process") and flavonoids ("flavonoid biosynthetic process" and "flavonoid metabolic process").

Similarly, Eu plants showed the highest counts of GOs uniquely enriched in treatments with nutrients addition, especially in the combined treatment (NT, **Fig. 3b**; **Table S3**). In this case, "structural constituent of chromatin", "oxidoreductase activity" and "generation of precursor metabolites and energy" were the most significant categories (**Table S3**). Genes belonging to these terms are involved in the modulation of chromatin structure (*HMGBs*, *high mobility group proteins*), mitochondrial electron transport chain (*Cytochrome c oxidase subunit 1*, *COX1* and *Ubiquinol oxidase 1b, AOX1B*), and starch synthesis (*Glucose-1-phosphate adenylyltransferase small subunit 1, AGPC*), and were highly down-regulated. In contrast to Ol plants, in Eu leaf different processes related to transcriptional regulation were also activated in the presence of only nutrients (N, "regulation of nucleobase-containing compound metabolic process" and "transcription"*).* Different Transcription factors (TFs) belonging to these categories were differentially regulated, including transcriptional activators such as *WRKY22-transcription factor 22* and *MED16- Mediator of RNA polymerase II transcription subunit 16* that were down-regulated, and the *SARD1- Protein SAR DEFICIENT 1*, which was up-regulated. The exposure to T treatment induced a less pronounced response activating processes involved in stress response and photosynthesis ("photosystem", "phosphoprotein binding" and "carbohydrate derivative binding"). Associated genes encoded for chaperone proteins (*HSP70-1-Heat shock 70 kDa protein 1*) and photosystem proteins (*PSBS1-Photosystem II 22 kDa protein 1*). Overall, treatments shared common processes related to transport and defence activities ("nitrate transport", "small molecule metabolic process", "reactive nitrogen species metabolic process") down-regulating genes involved in the response to nitrate (*Protein NRT1/ PTR FAMILY 6.4, NIA2- Nitrate reductase [NADH] 2*) and oxidation (*DOX1- Alpha-dioxygenase 1*).

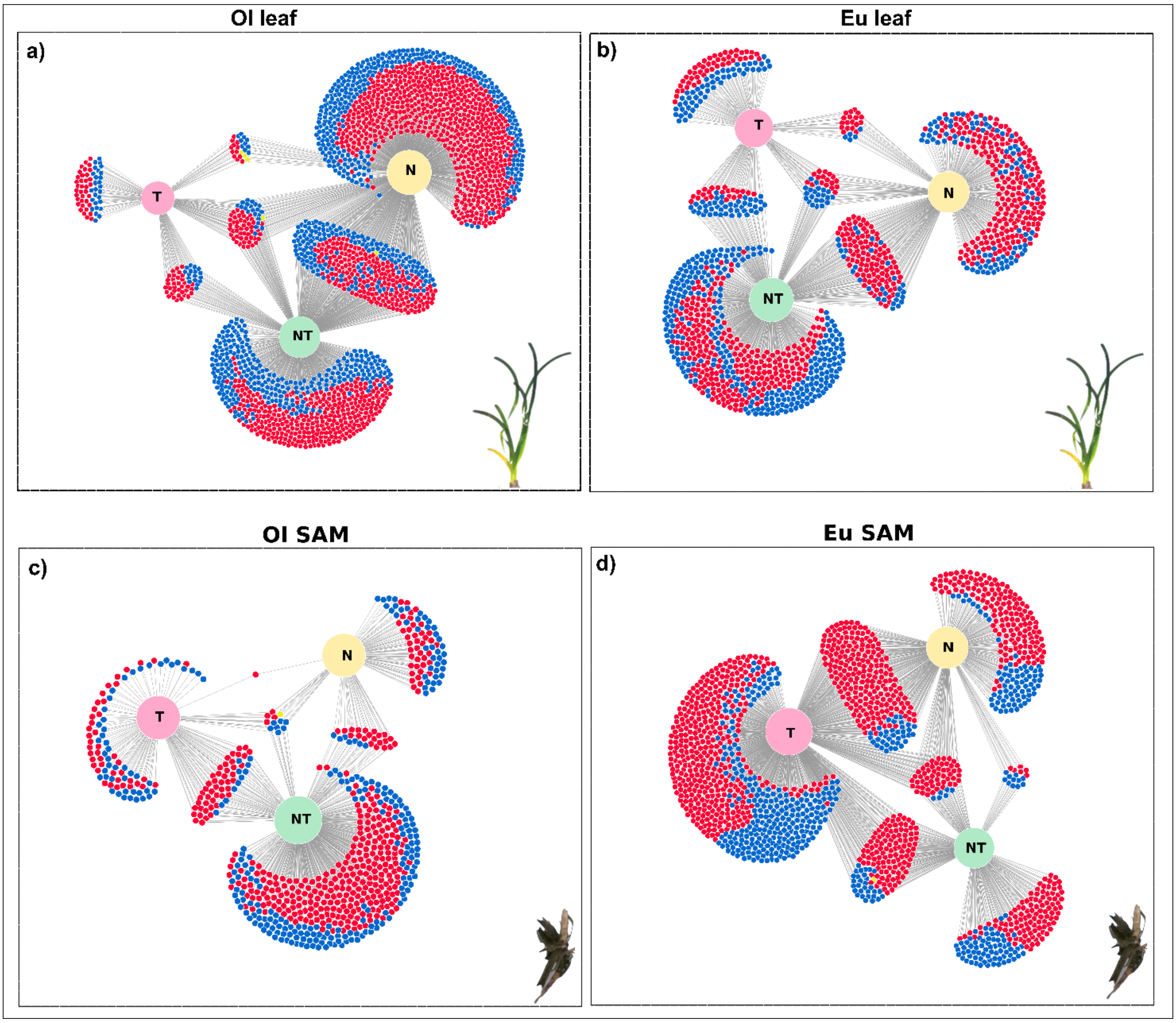

Figure 2. *DiVenn diagrams showing unique and shared differentially expressed genes (DEGs) among treatments (N = Nutrients, T = Temperature and NT = Nutrients + Temperature) in Ol leaf (a), Eu leaf (b), Ol SAM (c) and Eu SAM (d). Red and blue nodes refer to up- and down-regulated DEGs respectively, whereas yellow nodes refer to shared DEGs among treatments that were up-regulated in one sample but down-regulated in another one.*

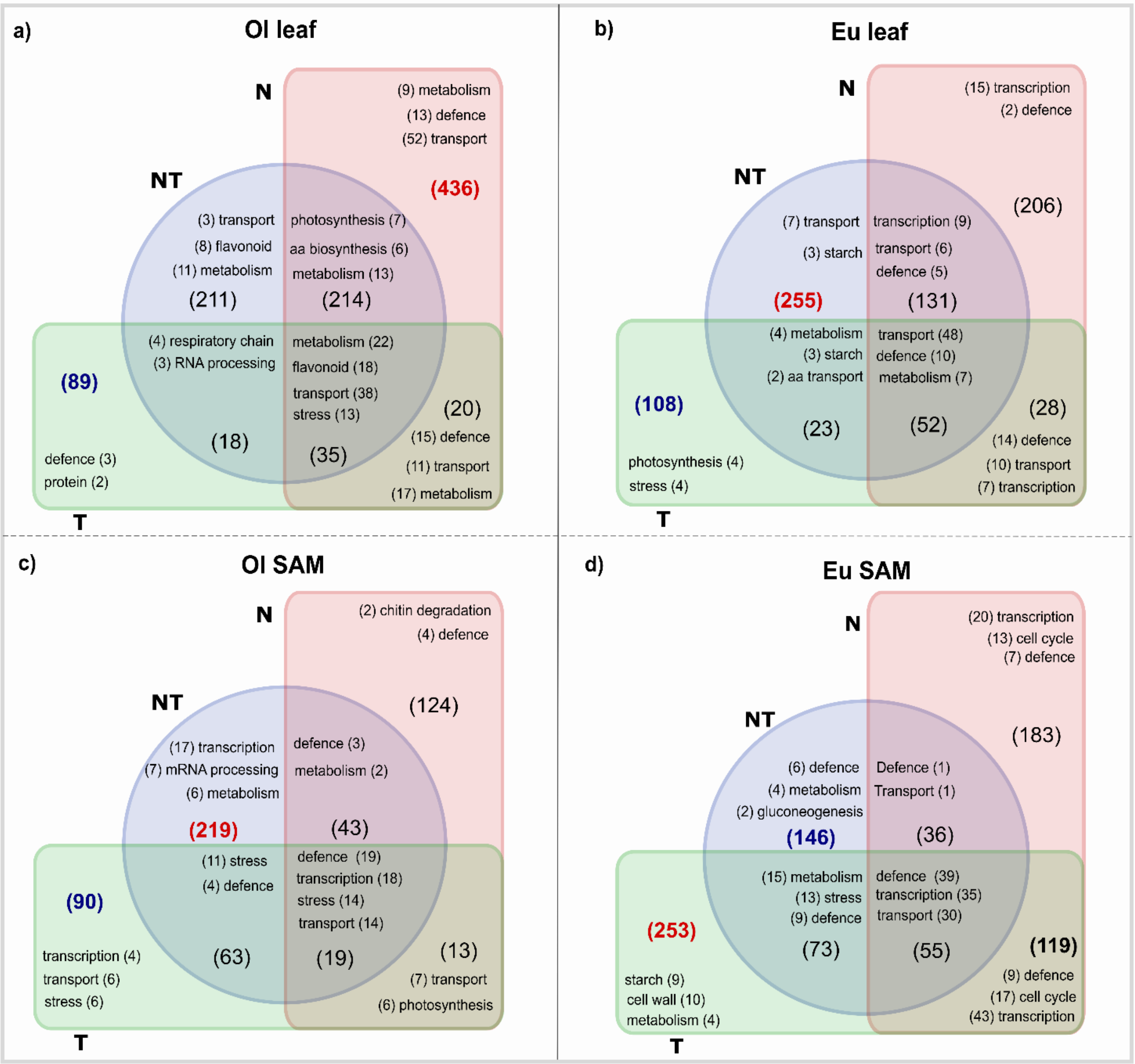

Figure 3. *Venn diagrams showing unique and shared GO enriched terms in Ol leaf (a), Eu leaf (b), Ol SAM (c) and Eu SAM (d). The number of unique and shared GOs is shown in brackets. Red and blue numbers identified the largest and lowest counts, respectively. The number of DEGs associated to the most significant GOs were also reported in brackets with the associated category, which corresponds to keywords derived by the Retrieve/ID mapping tool of UNIPROT database.*

3.3 SAM-specific transcriptomic responses

3.3.1 Differentially expressed genes (DEGs) and GO enrichment analysis

Contrary to leaf, SAM showed a greater response to temperature treatments (T and NT) with clear differences between Ol and Eu plants (**Fig. 4**). While Ol plants showed the higher counts of DEGs under the combined treatment (NT), Eu plants revealed a huge gene activation under the exposure to only temperature (T), followed by N and NT treatments (**Table S1)**. Differences in terms of DEG distributions among treatments in Ol and Eu plants were more evident for SAMs (**Fig. 2**).

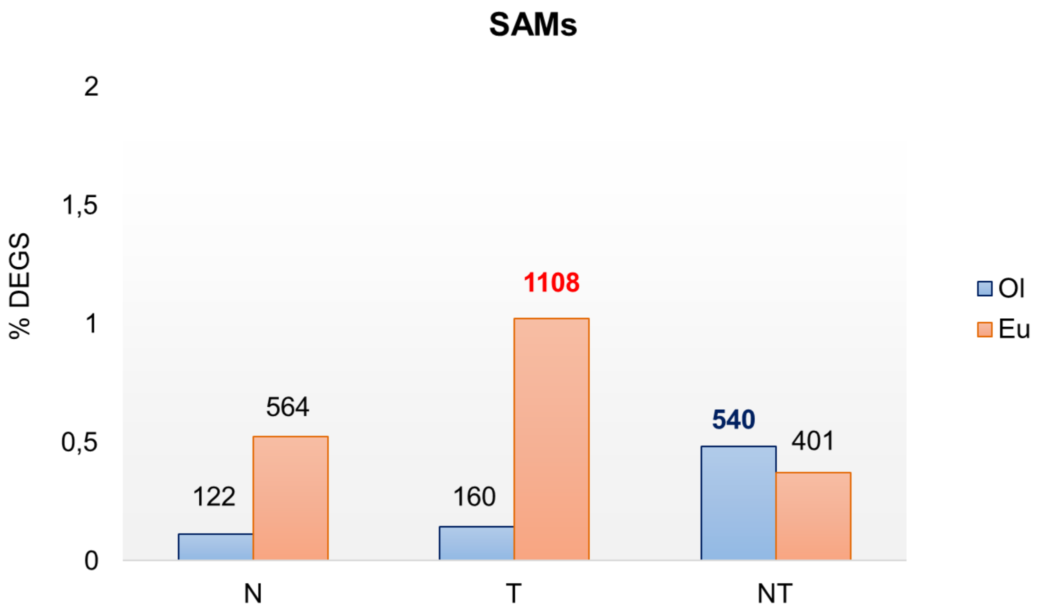

__Figure 4__. Percentages of DEGs (down and upregulated) normalized by the total number of transcripts counted for unique datasets (Ol SAM and Eu SAM). The total n. of DEGs is shown on the top of each histograms. The greatest counts of DEGs are underlined in bold with different colors for Ol (blue) and Eu plants (red).

Ol SAM showed a higher number of DEGs under NT treatment that were mostly up-regulated (**Fig. 2c**; **Table S2**). On the other hand, T treatment induced the highest transcriptomic response in Eu SAM, sharing most of DEGs with N treatment (**Fig. 2d; Table S2**). Eu plants expressed a lower number of DEGs in the combined treatment (NT), that were mostly shared with T treatment.

Surprisingly, SAM response to treatments was less pronounced with respect to the leaf, with a general lower number of distinct enriched GOs terms (**Table S2**). However, GO terms and related processes in the SAM were significantly different between Ol and Eu plants (**Fig. 3**; **Table S2**). In detail, Ol SAM responses were more pronounced in treatments with nutrients (N and NT), highlighting the down-regulation of different transcripts mostly related to defense mechanisms, like *Alpha-dioxygenase (DOX1)* and *Nodulin-related protein 1 (NRP1)* (**Table S1**). In Ol SAM, "aminoglycan metabolic process", "cell wall macromolecule metabolic process" and "chitinase activity" were the most significantly enriched terms in N treatment, where other similar processes related to nutrient-induced stress ("cellular response to nitric oxide") were shared with NT treatment (**Fig. 3c; Table S3**). Notably, distinct processes related to transcription were activated in NT ("gene expression") modulating TFs involved in gene expression regulation like *Transcription factor MYB7*, which was up-regulated, and *Protein LNK1* and *SWI/SNF complex component SNF12* that were repressed. Different processes related to stress response were also shared between NT and T treatments ("unfolded protein binding" and "heat shock protein binding") with the expression of key genes encoding for chaperone proteins (*HSP83, HSP90-5* and *Chaperonin CPN60-1*). T treatment induced a less pronounced response, which is in contrast to Eu SAM where the presence of temperature alone showed the largest number of unique GO enriched terms (**Table S3**). Under these conditions, Eu SAM activated processes mainly related to starch synthesis ("glucose-1-phosphate adenylyltransferase activity" and "starch biosynthetic process") and cell wall biogenesis ("cellular carbohydrate metabolic process"). DEGs related to these categories, all overexpressed, are key genes involved in starch synthesis (*AGPP-Glucose-1-phosphate adenylyltransferase small subunit 2*, *WAXY - Granule-bound starch synthase 1* and *ISA3-Isoamylase 3*) and cell wall construction (*XTH28-*

Probable xyloglucan endotransglucosylase and *CSLD5- Cellulose synthase-like protein D5*) (**Table S1**). Contrarily to Ol SAM, Eu SAM shared most of the GO enriched terms with N treatment, where the most representative categories were related to transcription ("protein-DNA complex", "DNA binding" and "chromatin"). Here, associated DEGs included different histone variants (*H2B, H3.2, H3.3*) and several TFs belonging to different families (*MYBS2, BHLH35, NFYB5, HHO5*) (**Table S1**).

3.4 Insights into epigenetic regulation

Different unique epigenetic-related GO terms (epi-GOs) were found in treatments with nutrients in both Ol and Eu leaves (**Table 2**). In Ol plants, leaf and SAM activated unique epigenetic-related functions (**Fig S1a** and **b**). In detail, Ol leaf regulated processes related to "RNA methylation activity" and "methylated histone binding" that included the largest count of associated transcripts (**Table 2**). Here, important chromatin remodelers and RNA methyltransferases were over-expressed, especially under nutrient stress conditions (*Chromatin remodeling protein, Putative tRNA (cytidine(32)/guanosine(34)-2'-O)-methyltransferase*). In Ol SAM, different unique epi-GOs related to terms such as "chromatin organization" and "histone modification" were the most representative biological processes, including the largest counts of transcripts (**Table 2**). Associated DEGs included DNA methyltransferase (*DNA (cytosine-5)-methyltransferase DRM1*) and chromatin remodelers (*CH5-Protein CHROMATIN REMODELING 5*), which were up-regulated under T treatment.

Contrarily to Ol plants, Eu leaf and Eu SAM shared several processes related to DNA binding functions. Regulated genes in Eu leaf belonged to the category of "sequence-specific DNA binding", which showed the largest counts of transcripts (**Table 2**). In such a case, different DEGs involved in transcription regulation were regulated in treatments with nutrients like *WRKY transcription factor 22* and *SARD1-Protein SAR DEFICIENT 1* that were highly overexpressed, and *ALKBH10B-RNA demethylase,* which was repressed in the treatment with only nutrients (N, **Table S1**). In Eu SAM, "chromatin binding" was the most representative molecular function considering the number of associated transcripts (**Table 2**). Here, genes involved in transcription regulation were differentially expressed such as *AHL16-AT-hook motif nuclear-localized protein 16,* which was overexpressed under single treatments (N and T), and DNA methylation including *MET1-DNA (cytosine-5)-methyltransferase*) that was up-regulated in N and NT (**Table S1**).

Table 2. *Unique and shared GO enriched terms related to epigenetic mechanisms in Ol plants (leaf – SAM) and Eu plants (leaf – SAM). The GO identification (GO ID), category (GO cat.), description, P value and the number of associated transcripts are reported.*

Ol leaf					**Eu leaf**				
GO ID	**GO cat.**	**GO description**	**P value**	**N. Transcripts**	**GO ID**	**GO cat.**	**GO description**	**P value**	**N. Transcripts**
GO:0102741	MF	paraxanthine:S-adenosyl-L-methionine 3-N-methyltransferase	4.10E-08	6	GO:0031062	BP	positive regulation of histone methylation	2.42E-02	137
GO:0004161	MF	dimethylallyltranstransferase activity	9.65E-03	20	GO:0070989	BP	oxidative demethylation	9.51E-03	34
GO:0002128	BP	tRNA nucleoside ribose methylation	9.81E-03	37	GO:0070734	BP	histone H3-K27 methylation	3.03E-02	126
GO:1990258	BP	histone glutamine methylation	1.09E-02	9	GO:0061087	BP	positive regulation of H3-K27 methylation	4.42E-02	46
GO:0035064	MF	methylated histone binding	2.29E-02	192	GO:0031058	BP	positive regulation of histone modification	2.60E-02	203
GO:1990259	MF	histone-glutamine methyltransferase	2.39E-02	9	GO:0035513	BP	oxidative RNA demethylation	1.38E-04	28
GO:0008898	MF	S-adenosylmethionine-homocysteine S-methyltransferase	2.42E-02	43	GO:0043982	BP	histone H4-K8 acetylation	3.29E-02	22
GO:0008173	MF	RNA methyltransferase	3.96E-02	618	GO:0043565	MF	sequence-specific DNA binding	2.34E-04	4743
-	-	-	-	-	GO:0035515	MF	oxidative RNA demethylase activity	4.66E-04	28
-	-	-	-	-	GO:0043984	BP	histone H4-K16 acetylation	1.30E-02	14
-	-	-	-	-	GO:0080182	BP	histone H3-K4 trimethylation	4.02E-02	68
Ol SAM					**Eu SAM**				
GO ID	**GO cat.**	**GO description**	**P value**	**N. Transcripts**	**GO ID**	**GO cat.**	**GO description**	**P value**	**N. Transcripts**
GO:0016576	BP	histone dephosphorylation	1.58E-04	13	GO:0035404	BP	histone-serine phosphorylation	2.64E-02	16
GO:0006325	BP	chromatin organization	3.63E-03	2963	GO:0009008	MF	DNA-methyltransferase activity	2.65E-02	71
GO:0031498	BP	chromatin disassembly	4.52E-03	6	GO:0003682	MF	chromatin binding	9.49E-03	946
GO:0032986	BP	protein-DNA complex disassembly	5.04E-03	7	GO:0006342	BP	chromatin silencing	5.39E-04	273
GO:0140658	MF	ATP-dependent chromatin remodeler activity	5.49E-03	361	GO:0000819	BP	sister chromatid segregation	3.22E-02	515

GO:0009008	MF	DNA-methyltransferase activity	1.33E-02	71
GO:0051052	BP	regulation of DNA metabolic process	1.56E-02	645
GO:0000018	BP	regulation of DNA recombination	2.17E-02	204
GO:0006304	BP	DNA modification	2.71E-02	663
GO:0008172	MF	S-methyltransferase activity	2.95E-02	67
GO:0016570	BP	histone modification	2.98E-02	1628
GO:0016569	BP	covalent chromatin modification	3.48E-02	1649
GO:0003886	MF	DNA (cytosine-5-)-methyltransferase activity	3.50E-02	47
GO:0000792	CC	Heterochromatin	3.14E-02	114
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GO:0061712	MF	tRNA (N(6)-L-threonylcarbamoyladenosine(37)-C(2))-methylthiotransferase	9.00E-05	15
GO:0006346	BP	DNA methylation-dependent heterochromatin assembly	4.86E-02	51
GO:0071824	BP	protein-DNA complex subunit organization	1.57E-03	776
GO:0035600	BP	tRNA methylthiolation	2.72E-04	18
GO:0035174	MF	histone serine kinase activity	3.99E-02	14
GO:0071204	CC	histone pre-mRNA 3'end processing complex	4.23E-02	16
GO:0065004	BP	protein-DNA complex assembly	1.34E-04	617
GO:0070828	BP	heterochromatin organization	1.98E-02	204
GO:0034401	BP	chromatin organization involved in regulation of transcription	1.51E-02	441
GO:0000785	CC	chromatin	8.92E-03	1910
GO:0006306	BP	DNA methylation	4.39E-02	509
GO:0031938	BP	regulation of chromatin silencing at telomere	9.09E-03	1
GO:0003886	MF	DNA (cytosine-5-)-methyltransferase activity	3.50E-02	47

Eu Leaf – Eu SAM

Go ID	**GO cat.**	**GO description**	**P value**	**N. Transcripts**
GO:1903231	MF	mRNA binding - posttranscriptional gene silencing	1.92E-02	5
GO:0044815	CC	DNA packaging complex	7.35E-04	239
GO:0032993	CC	protein-DNA complex	1.32E-02	471
GO:0150100	MF	RNA binding - posttranscriptional gene silencing	1.23E-02	5
GO:0003677	MF	DNA binding	3.92E-02	11285
GO:0006333	BP	chromatin assembly or disassembly	1.07E-02	431
GO:0030527	MF	structural constituent of chromatin	2.58E-07	16

4. Discussion

Here we describe, for the first time in seagrasses, the whole-transcriptome response of different organs (leaf and shoot apical meristem) of *P. oceanica* plants living in two contrasting environments with a different history of nutrient loads and exposed to single and multiple stressors. Our comparative transcriptomic analysis provides clear evidence for an effect of the local (native) environment in determining/influencing the ability of the species to cope with global stress factors, in agreement with previous physiological and morphological evidences (Pazzaglia et al., 2020). The exposure to single and multiple stressors differentially affected plants' transcriptomic response and highlighted an organ-specific vulnerability of plants depending on their origin. Leaf was more responsive in presence of nutrients whereas SAM showed more vulnerability to temperature treatments. Below, the principal outcomes from leaf and SAM analyses are discussed separately, considering the effects of treatments and plant origin.

4.1 The effects of local environment in driving differential responses to stress

4.1.1 Leaf vulnerability to stress conditions

A large transcriptomic reprogramming was observed in leaves of plants coming from both oligotrophic (Ol) and eutrophic (Eu) environments, when exposed to high nutrient loads alone or in combination with warming (Fig. 5). The exposure to only warming, induced instead a less pronounced response, which is in line with physiological responses reported in Pazzaglia et al. (2020), where the presence of nutrients induced the greatest effects on both Ol and Eu *P. oceanica* plants. This is probably due to the high nutrient affinity of leaves, which bear the primary responsibility for the assimilation of dissolved inorganic nitrogen (e.g., NH4+ and NO3-) in the species (Lepoint et al., 2002; Romero et al., 2006). Contrary to terrestrial plants, seagrasses live in more oligotrophic environments and the maintenance of high productivity through high nutrient incorporation is operated by Na+-dependent nitrate, phosphate and amino-acids transport systems that favour nutrient assimilation from the surrounding environments, regulating plants' nutrient budget (Alcoverro et al., 2000; Rubio et al., 2018). In our study, transcriptomic responses to nutrient enrichment also differed in plants according to their origin. Thus, leaves of plants from oligotrophic conditions (Ol) showed a more complex transcriptome reprogramming under nutrient enrichment than leaves from eutrophic conditions (Eu). The number of DEGs was indeed more than four times higher in Ol leaves than in Eu leaves.

Ol plants required a considerably higher level of transcriptome regulation in treatments with nutrients, activating processes related to transport activities to cope with the new stress condition. These plants down-regulated high-affinity nitrate transporters (NRTs and NIAs), which can be interpreted as a need to prevent the excess of nutrient assimilation. Similar strategies have already been observed in terrestrial plants, where the excess of nutrients modulated the assimilation of nitrate through an inhibitory mechanism that temporally blocks its activity, favouring the subsequent adaptation to stressful conditions (Reyes et al., 2018; Stitt et al., 2002). Moreover, different modulation of NRTs has already been observed in *P. oceanica* plants exposed to different temporal regimes of nutrient loading (Ravaglioli et al., 2017; Ruocco et al., 2018). Ruocco et al. (2018) showed that the leaves of plants under discrete/pulse nutrient addition enhanced the activity of genes involved in nitrate uptake and reduction (NRT2 and NR); while the leaves of plants chronically exposed to nutrient additions repressed the expression of these genes. This regulatory mechanism allowed plants to take advantage of pulse nutrient events, while their down-regulation was considered as a strategy adopted by plants to avoid excessive nitrogen uptake and assimilation. Other low-affinity nitrate transporters were overexpressed in both Ol and Eu leaves, which could explain the higher nitrogen content previously measured at the end of the experiment (Pazzaglia et al., 2020). The excessive assimilation of nitrates by Ol leaf induced the modulation of processes related to reactive nitrogen species, activating defence mechanisms that are typically involved in plant responses to abiotic stresses. Genes functioning as E3 ubiquitin ligase like PUB50 and ATL13 were up- and down-regulated, respectively, under high nutrient conditions. These genes are reported to participate in many cellular functions, playing a role in the regulation of abiotic and biotic stressors and in the modulation of hormone signalling (Seo et al., 2012; Sharma and Taganna, 2020; Yee and Goring, 2009). In addition, Ol leaf specifically regulated processes related to flavonoid synthesis that are representative of stress-induced conditions in *P. oceanica* plants (Migliore et al., 2007). In this experiment, leaves exposed to the combination of nutrients addition and temperature increase showed an up-regulation of Squalene and Chalcone (CHL) synthases, which could reveal a different degree of sensitivity by leaves in comparison with the exposure to only nutrients. Chalcones are key enzymes of the flavonoid biosynthesis pathway in angiosperms (Heglmeier and Zidorn, 2010; Hu et al., 2019; Mannino and Micheli, 2020). They play important roles in plant defence against biotic and abiotic stress factors (e.g., UV light and pathogens; Dao et al., 2011). The induction of CHLs expression depends on environmental stimuli resulting in the accumulation of secondary metabolites (Besseau et al., 2007). The over-expression of these genes suggests the presence of an altered natural metabolism in Ol plants that could be the result of the accumulation of reactive oxygen species (ROS) (Fini et al., 2011). In line with this evidence, high nutrient levels impaired the photosynthetic performance of Ol plants, down-regulating components of light harvesting complexes (e.g., LHCA6) and subunits of the photosystem II (e.g., PSBS). For these genes, a differential regulation was already observed in *P. oceanica* plants from meadows with different light regimes and exposed to reciprocal light conditions (Dattolo et al., 2017). In that case, the variation in light availability induced plants to adopt contrasting photo-acclimatory strategies to improve the utilization of the available light, maintaining a high photosynthetic efficiency (Dattolo et al. 2014, 2017). Ultimately, Ol plants experiencing for the first time acute eutrophic conditions, suffered more than Eu plants that have faced direct and indirect effects of eutrophic waters during their life history (Pazzaglia et al., 2020).

By contrast, leaves of Eu plants were less responsive to the presence of only nutrients, while the largest transcriptome modulation was observed in the combined treatment. Since these plants already experienced nutrient stress conditions in their local environments, they appeared more vulnerable when nutrients were combined with temperature increases, and thus in the presence of a new stress typology that required a large transcriptomic response. However, the variation in nutrients availability induced a substantial transcriptomic reprogramming of different transcription factors, as already reported in model plant species (Brumbarova and Ivanov, 2019). On the other hand, in the combined treatment, Eu leaf regulated processes related to the generation of precursor metabolites and energy, where a key gene involved in starch synthesis (AGPC) was down-regulated. This gene synthetizes ADP-glucose from glucose 1-phosphate and ATP, which is required as a glucose donor for starch synthesis in the plastid (Patron et al., 2004). Starch synthesis plays an important role in plant metabolism supporting growth and productivity under abiotic stresses (Thalmann and Santelia, 2017). The regulation of starch biosynthesis observed in Eu leaf suggests that these plants instead of activating large metabolic processes to counteract stress from nutrient excess modulated their energetic reserves to provide more energy for sustaining growth (Marín-Guirao et al., 2018; Krasensky and Jonak, 2012). Eu leaf also regulated genes with oxidoreductase activity (COX1 and AOX1) under the combined treatment. In *P. oceanica* plants, heat stress modulated the expression of Alternative oxidase 1a (AOX1), which plays a key role in the maintenance of the redox homeostasis in the mitochondrial respiratory chain (Marín-Guirao et al., 2017; Ruocco et al., 2019a; Tutar et al., 2017). Furthermore, other transcripts involved in the regulation of salicylic acid (SARD1), which is a defence hormone for local and systemic acquired resistance in plants (Zhang et al., 2010), were up-regulated in the presence of nutrients. All these evidences support the existence of regulatory defence machineries in plants that had already experienced stress conditions in their local environments, giving prominence to different strategies adopted by plants to counteract stress conditions previously observed in Pazzaglia et al. (2020).

4.1.2 SAM response to single and multiple stressors depends on plants' origin

The transcriptomic response of shoot apical meristems (SAMs) was less pronounced and differed substantially from the response of leaves in the experimental treatments, which contrasts with the pattern observed for the same species under severe light limitation (Ruocco et al., 2021). In addition, while the leaf transcriptomic response was mostly triggered by nutrients, the SAM mainly responded to warming with differences between Ol and Eu plants (Fig. 5). Eu SAM was more responsive to temperature alone, while in Ol SAM the strongest transcriptomic response was observed in the combined treatment (NT). Transcriptional profiles followed opposite patterns in Ol SAM and Eu SAM, especially in terms of activated processes. While Ol SAM was more responsive to NT, showing a lower vulnerability to T, Eu SAM showed a huge activation of specific processes in T, whereas NT induced the lowest response.

Stress categories related to chaperon activities ("unfolded protein binding" and "heat shock protein binding") were among the most representative ones in Ol plants under temperature treatment, and in Eu plants under both T and NT treatments, where also metabolic processes were highly differentially regulated. In Ol SAM, temperature induced the over-expression of Heat shock proteins (HSPs) that are a group of highly conserved proteins involved in the protection of cells against harmful consequences of a diverse array of stressors (Beere, 2004). This evidence is in line with previous studies performed on *P. oceanica*, where HSPs were upregulated in response to heat stress (Marín-Guirao et al., 2016; Ruocco et al., 2021; Ruocco et al., 2019b; Traboni et al., 2018). Different HSPs were also regulated in Eu SAM as a stress response shared between N and T treatments. Particularly in this case, more transcripts encoding for HSPs were highly regulated, confirming the higher vulnerability to temperature increase of Eu plants. Although heat stress signals are particularly evident in Eu plants, important processes related to cell wall construction and starch metabolism appeared to be modulated under warming conditions. In Eu SAM, different enzymes involved in starch metabolism were over-expressed (e.g., AGPC, ISA3 and WAXY). Their regulation in Eu plants suggests that these plants were energetically active to contrast thermal stress and therefore they modulated carbohydrate metabolism to provide more energy. This evidence could also explain carbohydrate modulation previously observed at the rhizome level only in Eu plants (Pazzaglia et al., 2020).

In agreement with the above evidence, Eu SAM also overexpressed key genes involved in cell wall biogenesis and organization, including Cellulose synthase (CSLD5) and Xyloglucan endotransglucosylase/hydrolase (XTH28). In terrestrial plants, these genes have a fundamental role in load-bearing cell wall framework, showing also different regulations to environmental stimuli (Sasidharan et al., 2014; Xu and Huang, 2000; Yan et al., 2019). In fact, the integrity of cell wall provides important mechanical strengths to counteract abiotic stresses (Kesten et al. 2017). These findings support the fact that Eu plants were metabolically active, especially in the presence of a new stress factor. However, this strategy probably implied large energetic costs, especially under chronic exposure to stress conditions that could explain the huge increase of shoot mortality observed in the T treatment several weeks later, at the end of the experiment (-40%, Pazzaglia et al. 2020). Stress responses observed in SAMs also confirmed the high sensitivity of the shoot apical meristem to acute stresses already detected in *P. oceanica* under different experimental conditions (Ruocco et al., 2021). Furthermore, the transcriptomic profiles of the SAMs observed in the present study revealed different levels of response, which depends on the stress typology. The molecular pattern observed after two weeks from the initial exposure to stresses may also be considered as an anticipatory signal of physiological and morphological responses observed at the end of the experiment. Similarly, the altered expression of stress-related genes anticipated morphological changes and population collapse in *P. oceanica* under eutrophication and burial stress (Ceccherelli et al., 2018).

4.2 Evidence of gene-expression regulation due to epigenetic mechanisms

In seagrasses, little is known about the role that epigenetic mechanisms have in driving gene expression responses to environmental stimuli. Only few studies have suggested that epigenetic mechanisms are involved in the regulation of stress responses in marine plants, pointing out their potential role in the regulation of phenotypic plasticity to environmental changes (Entrambasaguas et al., 2021; Jueterbock et al., 2019; Marín-Guirao et al., 2017, 2019; Nguyen et al., 2020; Pazzaglia et al., 2021; Ruocco et al., 2019b). Additionally, epigenetic marks could also be linked to the ability for creating a stress-memory in plants pre-exposed to stress (Nguyen et al., 2020), and different epigenetic states exists among different plant tissues, as well as among portions of different age of the same tissue (Ruocco et al., 2019b). Here, Ol and Eu plants showed a substantial regulation of processes related to chromatin modifications in both leaf and SAM. In particular, epigenetics mechanisms were mostly activated in organs where Ol and Eu plants showed the largest transcriptomic modulation, suggesting a potential epigenetic regulation of gene expression responses.

Ol leaf mainly regulated genes involved in the modification of the chromatin structure. Chromatin remodelling complexes are conserved proteins that harbour ATPase/helicase of the SWITCHING DEFECTIVE2/SUCROSE NON-FERMENTING2 (SWI2/SNF2) to control DNA accessibility regulating gene expression (Clapier and Cairns, 2009). Recently, these complexes were also found to regulate nitrate responsive genes in maize (Meng et al., 2020). In that case, the core subunit of the SWI/SNF-type ATP-dependent chromatin remodelling complex interacted with high affinity nitrate transporters repressing their expression in the presence of nitrate supply. Similarly, Ol leaf increased the expression of transcripts encoding for chromatin remodelling proteins under high nutrient conditions. As mentioned above, an excess of nutrients induced the greatest transcriptomic response in Ol leaf and most of the genes involved in epigenetic modifications were differentially expressed under such conditions. Although it is hard to find a functional relation between gene expression changes and epigenetic variations, this study provides new insights into the potential key role played by chromatin modifications in the regulation of target genes under environmental disturbances. Likewise, different GO enriched terms related to chromatin remodelling and modifications were also observed in Eu plants. These plants showed a great transcription regulation under stress conditions, especially in the SAM, where different transcription factors were shared between N and T treatments. Notably, processes related to protein-DNA binding and chromatin modifications were modulated in response to single stressors. In this case, the gene encoding for AT-hook motif nuclear localization (AHL) proteins, which belongs to a family of transcription factors, was overexpressed in N and T. The AT-hook motif is a small DNA-binding motif, which recognizes specific DNA structures activating or inhibiting the expression of different genes (Nagano et al., 2001). In plants, it is over-expressed under various abiotic stresses, including drought, salinity and temperature (Zhou et al., 2016). Furthermore, in Eu SAM, different histone variants were mostly regulated under single stressors (H2B, H3.2, H3.3), where a larger number of DEGs was observed. In *Arabidopsis thaliana*, histone proteins, especially H3.3 was found to be preferentially enriched in the 3’ end of the transcribed regions, which was also related to gene body methylation (Wollmann et al., 2017). Further observations revealed that the recruitment of these complexes induced transcriptional reprogramming during the differentiation of plant cells in response to biotic and abiotic stresses (Tripathi et al., 2015). In this study, eutrophic (Eu) plants activated transcriptional reprogramming to contrast nutrient stress for counteracting also the negative effect induced by the exposure to a new stress factor, which was temperature. A similar regulation involving physiological, genetic and epigenetic responses was previously observed in *P. oceanica* plants during warming (Marín-Guirao et al., 2019). In that case, plants showed altered expression levels of genes involved in epigenetic modifications that are at the intersection between stress tolerance and flowering processes. As stated by the authors, this regulation could be related to different response mechanisms adopted by plants to survive warming conditions. Moreover, it is worth underlining that stable epigenetic states regulating phenotypic variations can be inherited across generations favouring stress memorization (Bruce et al., 2007). Since plants previously exposed to stress stimuli can store stress information to be primed and more active to cope with the reoccurrence of stress events (Bäurle and Trindade 2020; Friedrich et al., 2019), this study provides epigenetic signatures that could suggest the existence of a transcriptional memory in plants that had already experienced stressful conditions due to local pressures.

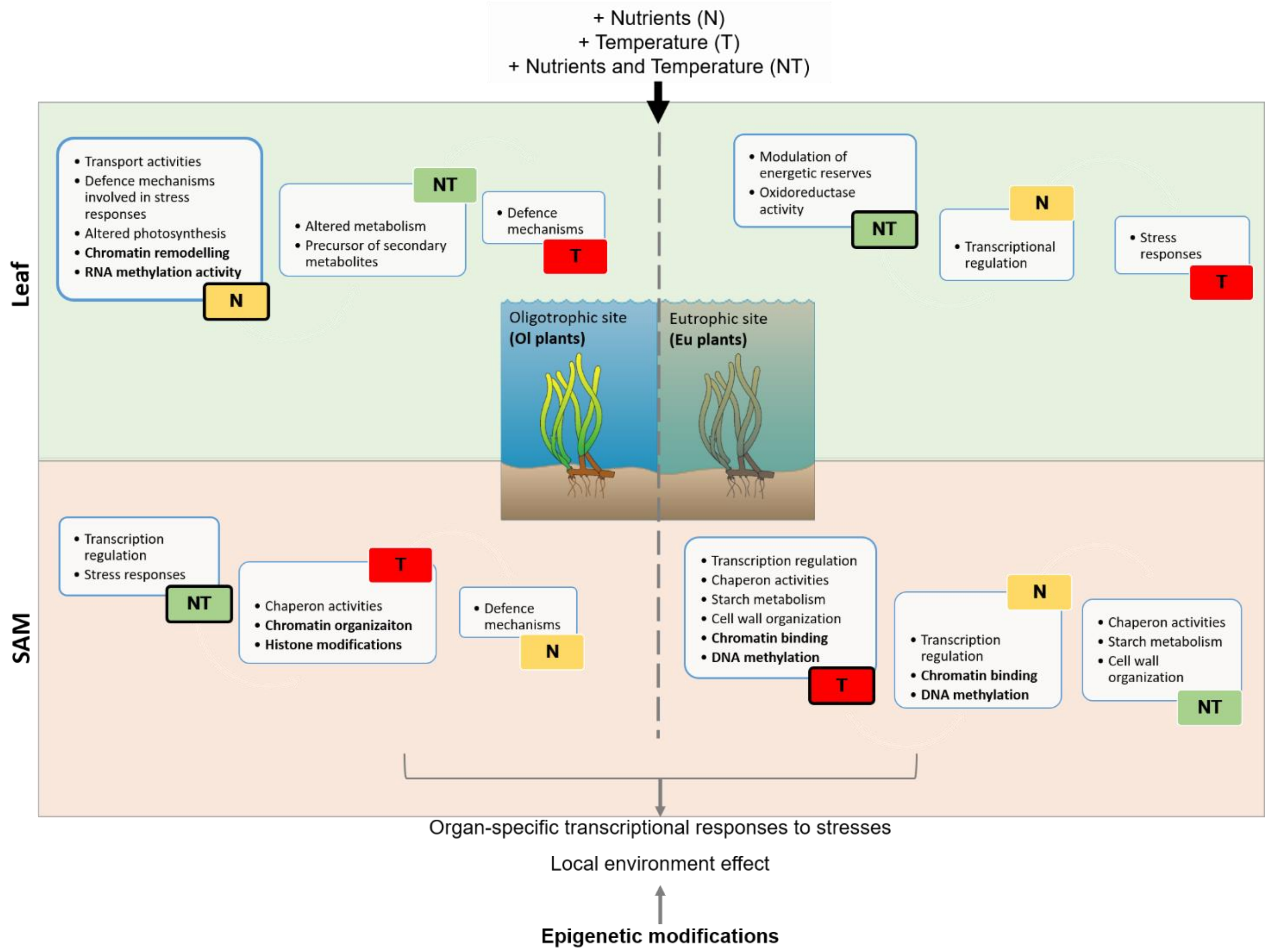

Figure 5. *Summary description of main results for leaf and SAM in Ol and Eu plants exposed to single (nutrients addition and temperature increases) and multiple stressors (nutrients addition plus temperature increase). In the leaf of Ol plants, N induced the greatest transcriptomic reprogramming followed by NT and T, contrary to the SAM, where NT induced the larger transcriptomic regulation. In Eu plants, leaf showed a greatest reprogramming under NT followed by N and T, while the SAM showed a larger transcriptomic regulation in T. Transcriptomic data revealed an organ-specific vulnerability to stressors, which depends on local environmental conditions, with the potential role of epigenetic regulation (see the main text for more detail).*

5. Conclusions and perspectives

The present work represents a further step in the comprehension of *P. oceanica* responses to single and multiple stressors. The transcriptomic profiles of plants under single and multiple stress conditions provide a valuable playground for further studies and future insights on the response of marine plants to realistic and complex scenarios, as those already occurring under the framework of climate change. Local pressures experienced by plants in their home environment have a marked influence on plants' transcriptional responses under unprecedented stress conditions, influencing their ability to withstand current and future challenges. This study also highlighted an organ-specific vulnerability to stress, with a higher sensitivity of the leaf to high nutrients addition, in contrast to SAM, which was more responsive to temperature increase. This contrasting sensitivity/responsiveness opens the possibility to improve our ability to manage and protect seagrass meadows by monitoring the response of appropriate plant organs with specific responsiveness to particular stressful conditions. Plants that experienced for the first time eutrophic waters needed to be more active to cope with the nutrient excess conditions expressing different genes related to metabolic, detoxification and photosynthesis processes, contrary to plants pre-exposed to eutrophic waters that only required the activation of basic processes to withstand high nutrient levels. In the latter, the activation of specific processes related to starch synthesis and its degradation and cell wall organization suggests that eutrophic plants invested energy to counteract the exposure to a new stress condition (i.e., high temperature), increasing shoot mortality in the case of a chronic stress exposure. The pre-exposure to local environmental conditions influences the degree of transcriptomic responses of the SAM to single and multiple stressors. In this case, plants already experiencing local pressures at their home site resulted more vulnerable to temperature increases. In a global warming scenario, these results suggest that meadows that are already impacted by local pressures (e.g., eutrophic conditions) will be compromised by future temperature increases.

Chromatin remodelling seems to be involved in plant responses to different stressors, since a different regulation of epigenetic-related genes was observed among plants and treatments. However, more studies on chromatin modifications are required to better understand the function of epigenetic changes in driving stress responses in seagrasses and to identify specific "actors" involved in the process. This could also provide new insights into the mechanisms that regulate the transcriptional memory of the SAM, which is fundamental for understanding seagrass survival to future environmental changes. Moreover, the molecular pattern observed in the SAM differed according to the stress typology and plants' origin, and anticipated the high shoot mortality observed several weeks later after chronic exposure to warming, suggesting its strong potential as a sentinel-organ to monitor seagrass meadows under direct and indirect human pressures. Since *P. oceanica* is widely distributed along the Mediterranean coasts, from pristine to highly disturbed sites, it is important to bear in mind that local conditions could play an important role in their ability to withstand regional and global climate change-related stressors. In the framework of the UN decade of ecosystem restoration, similar studies are necessary to improve conservation and restoration management of seagrasses and marine natural resources in general.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: