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A king and vassals' tale: molecular signatures of clonal integration in *Posidonia oceanica* under chronic light shortage

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Abstract

1. Under unfavourable conditions, clonal plants benefit from physiological integration among ramets, sharing resources and information. Clonal integration can buffer against environmental changes and let the plant clone work as a "macro" organism. Molecular signals that regulate this phenomenon are completely unknown in marine plants.

2. Here, we present a first comprehensive study providing insights into the metabolic role of different types of ramets (i.e., apical *vs* vertical) in the foundation species *Posidonia oceanica*. Plants were exposed to 80% diminishing irradiance level (LL) in a controlled-mesocosm system. Subsequent multi-scale variations in whole transcriptome expression, global DNA methylation level, photo-physiology, morphology and fitness-related traits, were explored at different exposure times. We tested the hypothesis that vertical shoots (the "vassals") can provide vital resources to apical shoots (the "kings") under energy shortage, thus safeguarding the whole clone survival.

3. Whole transcriptome analysis of leaves and shoot-apical meristems (SAMs) emphasised signatures of molecular integration among ramets, which strongly correlated with higher organisation level responses. In both shoots types, the exposure to LL resulted in a growth slowdown throughout the experiment, which started from immediate signals in SAMs. In apical shoots, this was linked to an acclimative response, where they were suffering a mild stress condition, while in vertical ones it fell in a more severe stress response. Yet, they suffered from sugar starvation and showed a clear cellular stress response in terms of protein refolding and DNA repair mechanisms. Several epigenetic mechanisms modulated the observed gene-expression patterns and the cross-talk between DNA methylation and the cellular energetic status appeared to regulate shoot metabolism under LL.

4. *Synthesis*. Our results demonstrate a high level of specialisation of integrated ramets within seagrass clones and a "division of labour" under adverse conditions. Vertical shoots appear to do "most of the job" especially in terms of resource providing, whereas activated functions in apical shoots were restricted to few important processes, according to an "energy-saving" strategy. The response of vertical shoots could be seen as a "sacrificing response" allowing the survival of "the king" that is key for ensuring propagation and population maintenance, and for the colonisation of new environments.

Keywords: clonal integration, DNA methylation, energy shortage, molecular indicators, RNA-Seq, marine angiosperms, shoot apical meristem

1. Introduction

Plants are modular organisms. When such modules are capable of iterating themselves in an independent manner, thus producing offspring through vegetative propagation, the plant is referred to as clonal (Liu F, Liu J, & Dong, 2016). Clonally formed offspring are called 'ramets', whereas the whole plant, which can comprise a number of clonal ramets, is referred to as a 'genet' (Harper, 1977). Different ramets belonging to the same genet share the same genotype (Harper, 1977). Within a genet, each ramet has the potential to perform all biological functions, and can be regarded as an independent individual.

Clonal plants dominate diverse terrestrial and marine ecosystems as primary producers, and include many of the most important crops and invasive plants, and some of Earth's largest, tallest and oldest plant species (Douhovnikoff & Dodd, 2015). Clonal integration, i.e., the physiological integration taking place among the different ramets for sharing resources and information, is a striking attribute of clonal plants. It plays a crucial role in their ecological and evolutionary success, enabling them to act as a cooperative system (Liu et al., 2016). This is possible since ramets are physically linked to each other through horizontal structures (e.g., rhizomes or stolons) allowing the translocation of various material, including external resources absorbed by plants (e.g., water and nutrients), hormones, photosynthates and secondary metabolites, via interconnected vascular structures (Liu et al., 2016). Clonal integration permits plants to cope with spatio-temporal heterogeneity of the environment. For example, within a single genet, donor ramets situated in favourable microsites (e.g., with abundant resource supply) can help resource-poor or otherwise adversely placed ramets, to alleviate their shortages (e.g., shading, nutrient depletion and drought) and/or to tolerate abiotic and biotic stressors (Liu et al., 2016). This has often been observed from parent ramets (older) to offspring ramets (younger/developing), although reciprocal exchange of resources between neighbouring ramets growing in differing-quality patches, has also been described (Alpert, 1999). Ultimately, resource sharing through clonal integration results in an increased performance of the recipient part without decreasing that of donor parts (at least in the short-term), thus leading to an increased performance of the whole clone (Song et al., 2013). Numerous studies have shown that clonal integration can support ramets to survive in stressful environments, for instance under high salinity (Evans & Whitney, 1992; Pennings & Callaway, 2000) and soil alkalinity stress (Zhang W, Yang, Sun, Chen, & Zhang Y,

2015), or to withstand defoliation by herbivores (Schmid, Puttick, Burgess, & Bazzaz, 1988; Wang et al., 2017).

Seagrasses are clonal rhizomatous plants sharing a similar morphology to that of terrestrial monocotyledons. All seagrass species present a highly organised growth based on the reiteration of ramets, which are composed of a bundle of leaves, a piece of rhizome, and a root system. Rhizomes are stems extending either horizontally on (or below) the sediment surface or vertically, raising the leaves towards (or above) the sediment surface (Marbà, Duarte, Alexandre, & Cabaço, 2004). Besides providing mechanical support and nutrient storage, rhizomes are responsible for the extension of the seagrass clone in the space, as well as for connecting adjacent ramets, thus enabling physiological integration (Marbà et al., 2004). Shoots growing vertically and horizontally are called orthotropic (vertical) and plagiotropic (or apical if terminal) shoots, respectively. Seagrass beds typically have wide spacing between many vertical shoots with few horizontal apices and are able to spread through those apices (Terrados, Duarte, & Kenworthy, 1997a), which grow horizontally until space has been completely colonised. Plagiotropic shoots can revert into vertical, which leads to the cessation of their horizontal growth, or vertical shoots can branch to produce horizontal ones, when the apical meristem of the original horizontal rhizome dies (Marbà et al., 2004). Rhizome elongation rate, leaf production and turnover are far higher in apical shoots than vertical ones (Marbà & Duarte, 1998). The Mediterranean seagrass *Posidonia oceanica* has dimorphic rhizomes; hence, it possesses both horizontal (plagiotropic or apical) and vertical (orthotropic) rhizomes, whereas other species such as *Zostera* spp. have only horizontal rhizomes.

Clonal integration has been demonstrated in seagrasses, for example in the form of nitrogen and carbon translocation among neighbouring ramets (Marbà et al., 2002). Photosynthates and nutrients can be re-allocated within seagrasses mainly toward organs with high metabolic activity, including growing leaves, flowering shoots and remarkably apical shoots, thus resulting in an enhanced clone growth and meadow spreading (Harrison, 1978; Libes & Boudouresque, 1987; Marbà, Hemminga, & Duarte, 2006; Marbà et al., 2002; Terrados, Duarte, & Kenworthy, 1997b; Schwarzschild & Zieman, 2008ab). Clonal integration supports seagrass persistence, ameliorating adverse effects of environmental stressors. For example, Tuya and colleagues (Tuya, Espino, & Terrados, 2013a; Tuya, et al., 2013b) demonstrated that the preservation of clonal integration in *C. nodosa* buffered its physiological performance against small-scale burial events and nutrient enrichment, similarly to what observed for *T. testudinum* under localised light limitation (Tomasko & Dawes, 1989). The importance of clonal traits was also revealed in *Z. noltii* grown under low

light conditions and organic matter enrichment (Olivé, García-Sánchez, Brun, Vergara, & Pérez-Lloréns, 2009). Specifically, a differential plant response was observed when contrasting levels of organic matter and light were established between the plant apex and distal parts, with harmful effect of organic matter being alleviated when the apex was grown in high light. This demonstrated that apical shoots represent the leading parts of the plant, and are highly sensitive to light deprivation (Olivé et al., 2009).

Light availability is by far the most important factor controlling seagrass growth, survival, and depth distribution (Lee, Park, & Kim, 2007; Ralph, Durako, Enríquez, Collier, & Doblin, 2007). This is attributed to the fact that the minimum light requirement for seagrasses is one of the highest among all angiosperms. Underwater irradiance attenuation occurs naturally along several gradients, namely the bathymetric, the canopy, and the leaf-epiphytic gradients. In addition, light attenuation may occur indirectly through excess anthropogenic nutrients leading to eutrophication, increased sediment accretion and resuspension, aquaculture and dredging, as well as regional weather and oceanic swell patterns (Ralph et al., 2007). Light shortage, due to natural and/or anthropogenically-driven processes, can compromise the photosynthetic process and ultimately lead to seagrass loss, as already documented worldwide (Ralph, Tomasko, Moore, Seddon, & Macinnis-Ng, 2006; Short & Wyllie-Echeverria, 1996). Seagrass responses to light limitation at multiple level of organisation, from molecular to physiological and morphological levels, and across various spatial scales, from leaf to meadow scale, has been deeply addressed (Dattolo, Marín-Guirao, Ruiz, & Procaccini, 2017; Dattolo et al., 2014; Davey et al., 2018; Kumar et al., 2017; Olesen, Enríquez, Duarte, & Sand-Jensen, 2002; Ralph et al., 2007). Nonetheless, the differential response to light limitation of specific shoot types, and its fundamental implications for the survival of the whole clone, has never been addressed so far, especially in terms of molecular and cellular rearrangements.

The present study aims at disentangling the relationship between apical and vertical shoots in the foundation species *P. oceanica* undergoing energy deprivation. To this end, we analysed photo-physiological properties, morphology and fitness-related traits (i.e., leaf growth rate and carbohydrate content) of those shoots under chronic low light (LL). Molecular mechanisms underlying such responses were explored through whole transcriptome profiling and global DNA methylation analyses of the different ramets. Our hypothesis is that molecular signals of clonal integration would be seen when the transcriptome profile of the two shoot types is compared under energy shortage, emphasizing unique biological roles for apical shoots ("the kings"), those

responsible for colonisation and population maintenance through clonal extension, and vertical shoots ("the vassals"), which should provide vital resources for ensuring the whole clone survival to the stress event.

2. Materials and Methods

2.1 Experimental design and light treatment

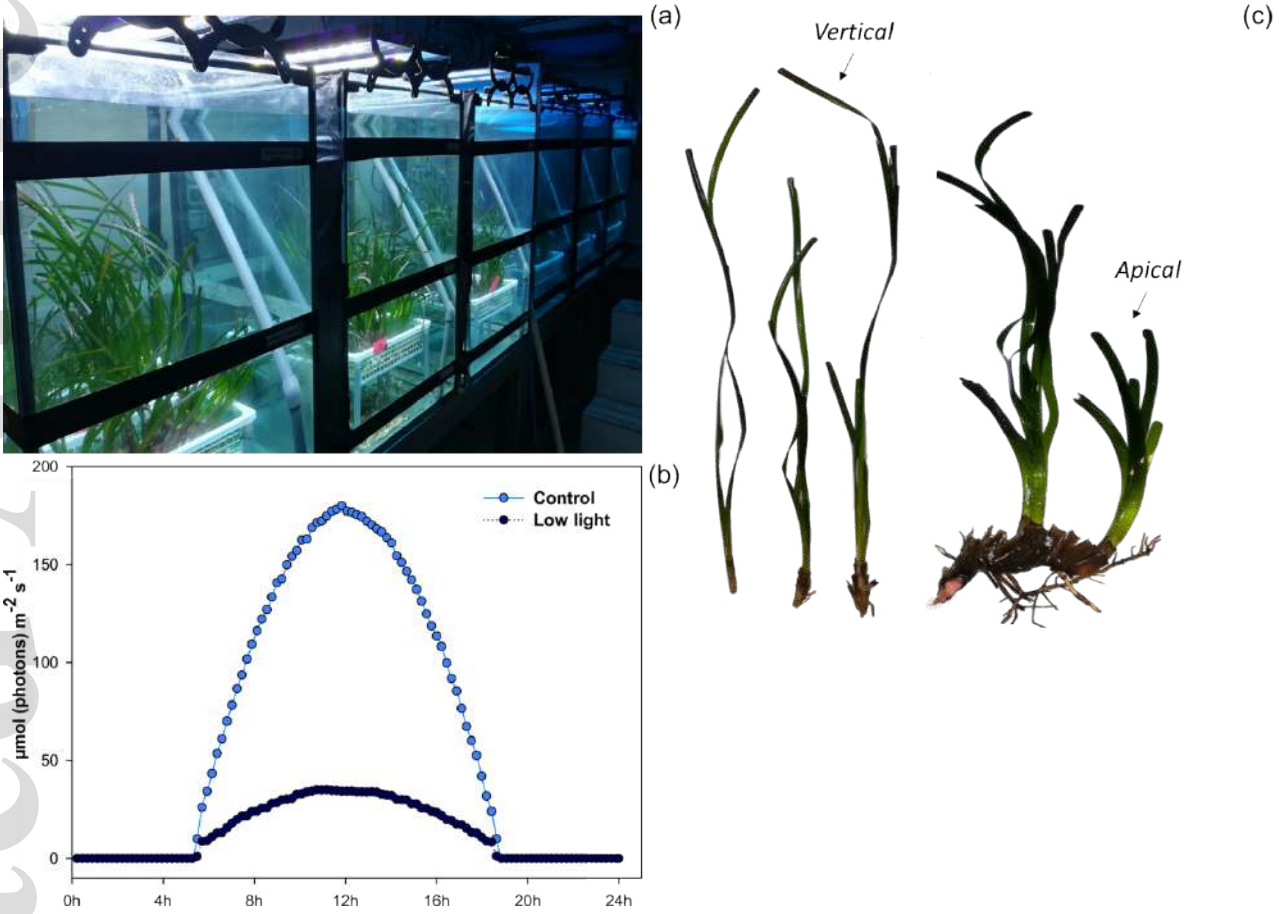
For this study, large *P. oceanica* fragments bearing several vertical shoots and at least one apical shoot, were collected by SCUBA diving from a shallow-water meadow (8–10 m depth) located around the island of Ischia (Gulf of Naples, Italy 40°43.849' N, 13° 57.089' E) on 16th February 2018 (11:00–12:00 pm). Winter *P. oceanica* plants were chosen to intensify stress responses and shorten response times, as they possess little stored energy in the form of carbohydrates. Plant material was kept in darkened coolers filled with ambient seawater and rapidly transported to the laboratory (within 1–2 h) to be transplanted in the indoor mesocosm facility of Stazione Zoologica Anton Dohrn (Naples, Italy) (Fig. 1a) (see Ruocco, De Luca, Marín-Guirao, & Procaccini, 2019a for an in-depth description of the system). Twenty-four plant fragments of similar size and shoot number (15-25 connected shoots) were selected to standardise the experiment, and individually attached to the bottom of twelve plastic net cages (40x30x10 cm) filled with coarse sediment (two fragments per pot). Two randomly selected cages were then placed in each of six glass aquaria (500L). Large fragments of *P. oceanica* were used to ensure unaltered clonal integration and healthy conditions of plants and to resemble the canopy structure of the meadow (Ruocco et al., 2019a).

Prior to start the experimental treatment, plants were acclimated for 10 days (t_0) to mean prevailing environmental conditions of the sampling site during the study period (temperature: ca. 16.5 °C; salinity: 37.5; max. noon subsurface irradiance: ca. 200 µmol photons m^{-2} s^{-1}; 11h:13h light:dark photoperiod). Subsequently, irradiance level in half of the tanks (n=3) was lowered to 40 µmol photons m^{-2} s^{-1} for simulating a strong shading event (80% light reduction), while lamps of control tanks (n=3) were maintained at ca. 210 µmol photons m^{-2} s^{-1} (Fig. 1b). Both values represent max. noon irradiance levels. Temperature and salinity levels were left as in the acclimation phase (T: ca. 16.5 °C; salinity: 37.3–37.7). Continuous light and temperature measurements were performed by means of LI-COR LI-1400 quantum sensor and HOBO® Pendant® UA-002-64 data loggers (Onset Computer Corporation), respectively. Salinity was measured daily in each aquarium using a WTW Cond 3310 portable conductivity meter and kept within the range indicated above by adding freshwater to compensate for evaporation.

The low-light (LL) exposure lasted 40 days. Molecular, physiological and morphological assessments were carried out at definite time points, according to their expected response timing

(Macreadie, Schliep, Rasheed, Chartrand, & Ralph, 2014). Morphological parameters and leaf growth rate were assessed throughout the experiment (i.e., after 15 days - t_1, 30 days - t_2, and 40 days of exposure - t_3), whereas carbohydrate content was only determined at the end of the experiment (t_3). Shoot photosynthetic performance and global DNA methylation were determined at t_1 and t_2. Genome-wide transcriptome analysis was performed on leaves and shoot-apical meristems (SAMs) of apical and vertical shoots at t_1, in order to capture the early activation of plant molecular stress signals, likely anticipating physiological and ultimately morphological changes (Ceccherelli et al., 2018; Macreadie, Schliep, Rasheed, Chartrand, & Ralph, 2014). To remove age-related effects, vertical shoots employed for the analyses were selected at least three positions after the apical one/s. Examples of ramets used for this experiment are shown in Fig. 1c.

Figure 1. (a) View of the experimental system at Stazione Zoologica Anton Dohrn; (b) Daily irradiance at the top of the leaf canopy measured with LI-COR LI-1400, in control (light blue) and LL (dark blue) tanks over a 24h cycle; (c) Example of vertical and apical shoots of *P. oceanica* used for this experiment



2.2 Shoot morphology and fitness-related traits

A set of vegetative variables (i.e., shoot size, number of leaves per shoot, max. leaf length and width) and fitness-related traits (i.e., leaf growth rate and non-structural carbohydrate content) were determined at the selected time points (see above) on apical and vertical shoots under control and chronic LL. To determine the leaf growth rate, all apical and vertical shoots of one rhizome per tank (at least one apical and three vertical shoots) were marked at the beginning of each experimental phase (t_0, t_1 and t_2) following the Zieman method (Zieman, 1974). Marked fragments were harvested at the end of each experimental phase (t_1, t_2 and t_3) to determine the surface of newly formed tissue below the needle mark (as cm^2 $shoot^{-1}$ day^{-1}).

The total content of non-structural carbohydrates (TNC) (soluble sugars and starch) was analysed in leaf and rhizome tissues, to assess the energetic status of the shoots under light shortage. Analyses were conducted following the phenol-sulphuric method modified from DuBois, Gilles, Hamilton, Rebers, and Smith (1956). Briefly, leaf samples (central sections of 2^{nd}-rank leaves) and the first 2 cm of the rhizome apexes were dried and finely ground (ca. 50 mg) with a Mixer Mill MM300 (QIAGEN) and tungsten carbide beads (3 mm). TNC were then solubilised by three sequential extractions with 80% (v/v) ethanol at 80 °C for 15 min. After centrifugation (3000 rpm in an Eppendorf 5810 R centrifuge - rotor A-4-62 for 10 min.), the ethanol extract was used for the determination of soluble sugars content, while the pellet was hydrolysed for starch determination (24h at RT) with 3 mL NaOH 0.1M. For both soluble sugars and starch, 3% aqueous phenol (0.25 mL) and 95-97% H_2SO_4 (2.5 mL) were mixed with 1mL sample in glass tubes and the solution was allowed to rest for 30 min. Absorbance was then read at 490 and 750 nm with an Agilent 8453 UV-Vis spectrophotometer. TNC content was calculated using sucrose calibration curves (standard sucrose 99 %, Biorad). Apical and vertical shoots were only analysed for TNC content at t_3 (n=3).

2.3 Effective quantum yield

Chlorophyll *a* fluorescence measurements were performed with a diving-PAM fluorometer (Walz, Germany). The saturation pulse method was used to measure F and Fm' in apical and vertical shoots of plants after 5h of illumination in aquaria. The effective quantum yield of PSII ($\Delta F/Fm' = Fm'-F/Fm'$) was calculated as a proxy of plant productivity since it reflects the photosynthetic performance of plants. Chlorophyll *a*-derived photosynthetic measurements were determined on two apical and two vertical shoots per tank, then values were averaged to be used as

individual replicates (i.e., n° of replicates used in statistical tests *n*=3; total biological replicates N=6).

2.4 Global DNA methylation

Leaf material for DNA extraction was obtained from the middle section of 2nd-rank leaves of one apical and one vertical shoot per tank (*n*=3). Leaf tissue (about 5-7 cm) was accurately cleaned of epiphytes and dried with silica gel. Genomic DNA was subsequently isolated and quality-checked following Ruocco et al. (2019a). DNA concentration was accurately determined by the Qubit dsDNA BR assay kit with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Global DNA methylation was assessed colorimetrically in duplicate by an ELISA-like reaction with the Methyl Flash™ Methylated DNA Quantification Kit (Epigentek Inc.) and reported as % methylated DNA relative to the input DNA quantity for each sample (50 ng). Absorbance at 450 nm was assayed using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific).

2.5 Statistical analysis

Two-way GLM repeated measures ANOVAs (RM-ANOVAs) were conducted to detect the effects of shoot type and treatment on plant morphological characteristics, leaf growth rate, effective quantum yield and global DNA methylation, along the course of the experiment. The analysis consisted of two fixed factors: "Shoot Type" (ST), with two levels (apical and vertical) and "Light" (L), with two levels (control and low light), and "Time" as a within-subject factor. This allowed us to avoid the potential non-independence of measurements from the same aquaria. Carbohydrate contents at the end of the experiment (t_3) was assessed by two-way ANOVAs with the same fixed factors. Normality of data was tested using the Shapiro-Wilk test and variance homogeneity was verified using Levene's test. In the case of RM-ANOVAs, the assumption of sphericity was assessed using Mauchly's sphericity test. When parametric assumptions were not met, data were Box-Cox transformed. Student-Newman-Keuls post-hoc test was used whenever significant differences were detected. All ANOVAs were performed using the statistical package STATISTICA (StatSoft, Inc. v. 10).

2.6 Genome-wide transcriptome sequencing and analysis

RNA extraction, library preparation and sequencing: Leaf sub-samples (ca. 5 cm) for RNA extraction were obtained from middle section of mature leaves (3rd-rank leaf) of vertical and apical

shoots (*n*=3). In addition, the first most apical 0.5 cm of the rhizome tip, containing the SAM, were also collected from the same shoots (*n*=3). Leaf material was gently cleaned from epiphytes and submerged in RNAlater© tissue collection (Ambion, life technologies), stored one night at 4°C, and finally stowed at -20°C. Rhizome fragments were cleaned from leaf sheaths and sediment particles and then preserved in LN2 to be definitely stored at -80 °C. For both plant organs, total RNA was extracted and checked for purity following Ruocco et al. (2019a). RNA concentration was accurately determined by Qubit® RNA BR assay kit using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). RNA quality was assessed by measuring the RNA Integrity Number (RIN) with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.); only high-quality (RIN ≥ 7) RNA was used for RNA-Seq analysis. Twenty-four indexed cDNA libraries (2 shoot types × 2 organs × 2 treatments × 3 biological replicates) were constructed with the Illumina TruSeq® Stranded mRNA Library Prep Kit, and sequenced with an Illumina NextSeq 500 platform (single-ends 1×75 cycles) at Genomix4life s.r.l. (Salerno, Italy).

Data filtering and transcriptome assembly: Raw sequencing data were checked using FastQC v0.11.5 (Andrews 2010), and then cleaned for Illumina adaptors and trimmed for quality using Trimmomatic v0.36 (Bolger, Lohse, & Usadel, 2014). Only reads with a minimum length of 50 bp were retained. Subsequent transcriptome assembly was conducted using the Trinity pipeline v.2.5.0 (Haas et al., 2013) with default parameters. To achieve the most comprehensive transcriptome as possible, this newly assembled transcriptome was combined with three previously published *P. oceanica* transcriptomes (D'Esposito et al., 2017; Entrambasaguas et al., 2017; Marín-Guirao, Entrambasaguas, Dattolo, Ruiz, & Procaccini, 2017) into one merged assembly. Intra-assembly redundancy was decreased by using CD-hit-EST v4.6.7 (Huang, Niu, Gao, Fu, & Li, 2010).

Functional annotation, differential expression and GO enrichment analysis: Assembled contigs were annotated through sequence similarity search against UniProtKB/Swiss-Prot and NCBI non-redundant sequence (Nr) protein databases using BLAST+ tool v2.6.0 (Altschul et al., 1997) (e-value cutoff $1e^{-6}$). Subsequently, results were loaded on Blast2GO v.5 (Conesa et al., 2005) to retrieve Gene Ontology (GO) terms (e-value cutoff $1e^{-6}$). Enzyme code (EC) annotation and KEGG maps were also retrieved. Full description of transcriptome assembly and annotation results can be retrieved from Supporting information (section 1).

For the differential gene-expression analysis, reads from each biological replicate were individually mapped to the assembled transcriptome using Bowtie v1.1.1 (Langmead et al., 2009),

and the expression of each transcript was quantified using the Expectation-Maximization method (RSEM) (Li & Dewey, 2011). Finally, differentially expressed genes (DEGs) for each pairwise comparison were determined using a Generalised Linear Model (GLM) in the edgeR package (Robinson, McCarthy, & Smyth, 2010). The bulk of low-abundance transcripts were removed keeping those having at least a ≥ 1 cpm (read/count per million) for at least three samples. Transcripts were considered significantly differentially expressed (up- and down-regulated) if FDR-corrected *P* value < 0.05. Due to the high number of DEGs, a more stringent cut-off of logFC > ±2 and FDR < 0.05 was also applied.

Expression values generated by edgeR were used for examining profiles of expression across different samples through a hierarchical clustering. A heatmap of DEGs was generated using the heatmap3 package in R v3.2.2 (R Core Team, 2015). To assess overall similarity across samples, their relationships were also explored through a PCA on the transposed normalized expression matrix with R v3.2.2. Venn diagrams to identify shared and unique DEGs between different contrasts were performed with http://bioinformatics.psb.ugent.be/webtools/Venn/. GO-term enrichment analysis of DEGs was performed through the Fisher's exact test approach by using the GO enrichment analysis function provided by Blast2GO v.5 with a threshold FDR of 0.05. Due to a large list of enriched GO terms was obtained in SAM comparisons, a further reduction to most specific terms (FDR < 0.01) was carried out. Summarisation and visualisation of GO terms were performed by using the REVIGO web service (http://revigo.irb.hr/) (Supek, Bošnjak, Škunca, & Šmuc, 2011).

3. Results

3.1 Morphological and photo-physiological responses of apical and vertical shoots to chronic LL

On average, total shoot size was higher in vertical than apical shoots (Table 1). Apical shoots generally contained a significantly higher number of leaves per shoot and a lower maximum leaf length (Tables 1). Both variables decreased in apical and vertical shoots under chronic LL (Table 2), although such variations were not significant at any sampling time points. LL had mild effect on max. leaf width, with a significant reduction observed at t_1 (~ 5%) and t_3 (~ 6-7%) (Table 1 and 2), and no significant differences between shoot types. LL exposure caused a global reduction in shoot size that was especially evident after 30 and 40 days of exposure, for both apical (~ 31%) and vertical (~ 26-27%) shoots (Table 1 and 2).

At photo-physiological level, effective photochemical efficiency ($\Delta F/Fm'$) was significantly reduced by 30 and 39% in LL plants at t_1 and t_2, respectively, without any differences between apical and vertical shoots (Fig. 2 and Table 2).

Table 1. Plant morphological characteristics in apical and vertical shoots at t_1, t_2 and the end of the exposure (t_3). Values are means (SE) for *n*=3.

	Shoot size (cm^2 $shoot^{-1}$)	**Leaves per shoot**	**Max leaf length (cm)**	**Max leaf width (cm)**
15 days (t_1)				
Apical				
Control	171.88 (34.71)	9.33 (0.67)	38.43 (9.47)	0.95 (0.03)
LL	134.87 (8.86)	8.33 (0.88)	31.67 (3.00)	0.90 (0.00)
Vertical				
Control	166.48 (17.24)	5.26 (0.30)	54.31 (9.05)	0.94 (0.00)
LL	145.84 (28.25)	4.69 (0.14)	42.42 (5.88)	0.90 (0.03)
30 days (t_2)				
Apical				
Control	182.26 (13.51)	7.83 (1.83)	45.33 (5.95)	0.94 (0.02)
LL	125.68 (12.15)	5.00 (0.58)	42.83 (6.22)	0.92 (0.02)
Vertical				
Control	259.05 (37.14)	5.32 (0.24)	83.09 (12.10)	0.98 (0.01)
LL	192.46 (26.93)	4.60 (0.40)	68.27 (1.99)	0.95 (0.03)
40 days (t_3)				
Apical				
Control	154.73 (14.91)	6.67 (0.88)	41.50 (5.97)	0.95 (0.03)
LL	106.84 (11.14)	7.33 (1.20)	28.83 (1.17)	0.88 (0.02)
Vertical				
Control	230.38 (8.55)	5.17 (0.33)	71.88 (6.46)	0.97 (0.00)
LL	167.95 (31.73)	4.58 (0.36)	61.33 (9.88)	0.91 (0.04)

Figure 2. Changes in effective quantum yield (ΔF/Fm') of apical and vertical shoots at t_1 and t_2. Data are mean ± SE (n=3). Black and grey bars represent controls and LL plants, respectively. Asterisks indicate significant differences between control and LL conditions. Full results of 2-way RM-ANOVAs are reported in Table 2. *$P < 0.05$

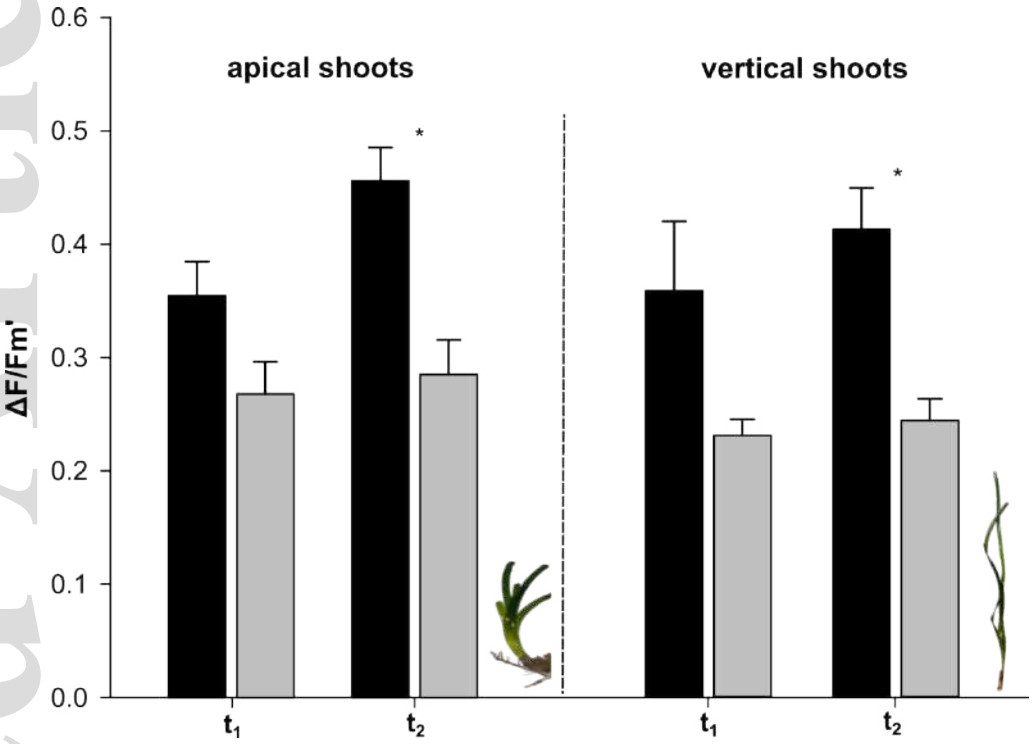


Table 2. Two-way RM-ANOVAs to assess the effect of shoot type (ST) and low-light treatment (L) on plant morphological and photo-physiological characteristics along the course of the experiment. $P < 0.05$ are in bold, $P < 0.1$ are underlined. Results of Mauchly sphericity test (*M*) and Levene's test (*L*) are reported below

		Shoot size				*Leaves per shoot*				*Max. leaf length*		
Effect	***df***	**MS**	**F**	***P***	***df***	**MS**	**F**	***P***	***df***	**MS**	**F**	***P***
Light (L)	1	21189.038	14.274	**0.005**	1	0.002	8.835	**0.018**	1	875.979	3.488	0.099
Shoot type (ST)	1	20434.641	13.765	**0.006**	1	0.012	45.649	**0.000**	1	5829.704	23.215	**0.001**
L×ST	1	16.690	0.011	0.918	1	0.000	0.105	0.755	1	58.731	0.234	0.642
Error	8	1484.497			8	0.000			8	251.115		
Time	2	3910.766	2.448	0.118	2	0.001	2.331	0.129	2	990.812	9.405	**0.002**
Time×L	2	904.119	0.566	0.579	2	0.001	1.196	0.328	2	7.132	0.068	0.935
Time×ST	2	4537.912	2.841	0.088	2	0.001	1.864	0.187	2	331.244	3.144	0.071
Time×L×ST	2	209.043	0.131	0.878	2	0.000	0.743	0.491	2	39.166	0.372	0.695
Error	16	1597.369			16	0.000			16	105.351		
		M=0.55; *L*>0.05 t_1,t_2,t_3				*M*=0.23; *L*>0.05 t_1,t_2,t_3				*M*=0.86; *L*>0.05 t_1,t_2,t_3		
		Transform=none				Transform=Box-Cox				Transform=none		

		Max. leaf width				*Leaf growth rate*				*ΔF/Fm'*		
Effect	***df***	**MS**	**F**	***P***	***df***	**MS**	**F**	***P***	***df***	**MS**	**F**	***P***
Light (L)	1	0.019	23.534	**0.001**	1	10.399	276.299	**0.000**	1	2.959	27.001	**0.001**
Shoot type (ST)	1	0.003	3.664	0.092	1	0.816	21.672	**0.002**	1	0.188	1.711	0.227
L×ST	1	0.000	0.018	0.896	1	0.013	0.340	0.576	1	0.044	0.404	0.543
Error	8	0.001			8	0.038			8	0.110		
Time	2	0.002	1.069	0.367	2	0.742	7.898	**0.004**	1	0.269	5.329	0.050
Time×L	2	0.001	0.527	0.600	2	0.314	3.342	0.061	1	0.068	1.339	0.281
Time×ST	2	0.001	0.755	0.486	2	0.880	9.364	**0.002**	1	0.007	0.134	0.723
Time×L×ST	2	0.000	0.015	0.985	2	0.043	0.454	0.643	1	0.005	0.098	0.762
Error	16	0.002			16	0.094			8	0.051		
		M=0.49; *L*>0.05 t_1,t_2,t_3				*M*=0.33; *L*>0.05 t_1,t_2,t_3				*L*>0.05 t_1,t_2		
		Transform=none				Transform=none				Transform=Box-Cox		

3.2 Effects of chronic LL exposure on fitness traits of apical and vertical shoots

LL greatly slowed down leaf growth rate, as it was significantly reduced at all sampling time points, in shaded with respect to control plants (Fig. 3 and Table 2). Specifically, already after 15 days of exposure to 40 μmol photons m^{-2} s^{-1}, leaf growth was reduced by 50% and 41% in apical and vertical shoots, respectively (Fig. 3). After one-month exposure, a further decline of up to 62% and 55% in apical and vertical shoots, was observed (Fig. 3). At the end of the experiment (t_3, 40 days of exposure) leaf growth rate of apical shoot was 78% lower than controls, whereas for vertical shoots the decrease was still around 50%, similarly to what observed at t_2 (Fig. 3). Overall, the reduction in leaf growth rate was always greater in apical than vertical shoots (Fig. 3).

The factor "shoot type" had a significant effect on total non-structural carbohydrate (TNC) accumulation in leaf tissues, yet there was a significant interaction with the factor "light" (ST×L), as showed by 2-way ANOVAs (Table 3). Specifically, at t_3 while TNC content in leaf tissue of vertical shoots exposed to LL fell down dramatically (42%, $P < 0.05$ SNK), in apical shoots it showed values significantly higher than vertical shoots (50%, $P < 0.05$ SNK) and even slightly higher than those displayed by their own control (*ns*) (Fig. 4 and Table 4). The same pattern was not visible in rhizomes (Fig. 4).

Figure 3. Changes in leaf growth rate of apical and vertical shoots at t_1, t_2 and t_3. Data are mean ± SE (n=3). Black and grey bars represent controls and LL plants, respectively. Asterisks indicate significant differences between control and LL conditions. Different letters indicate significant differences between shoot types, for each light treatment. Full results of 2-way RM-ANOVAs are reported in Table 2. $^{**}P < 0.01$, $^{***}P < 0.001$

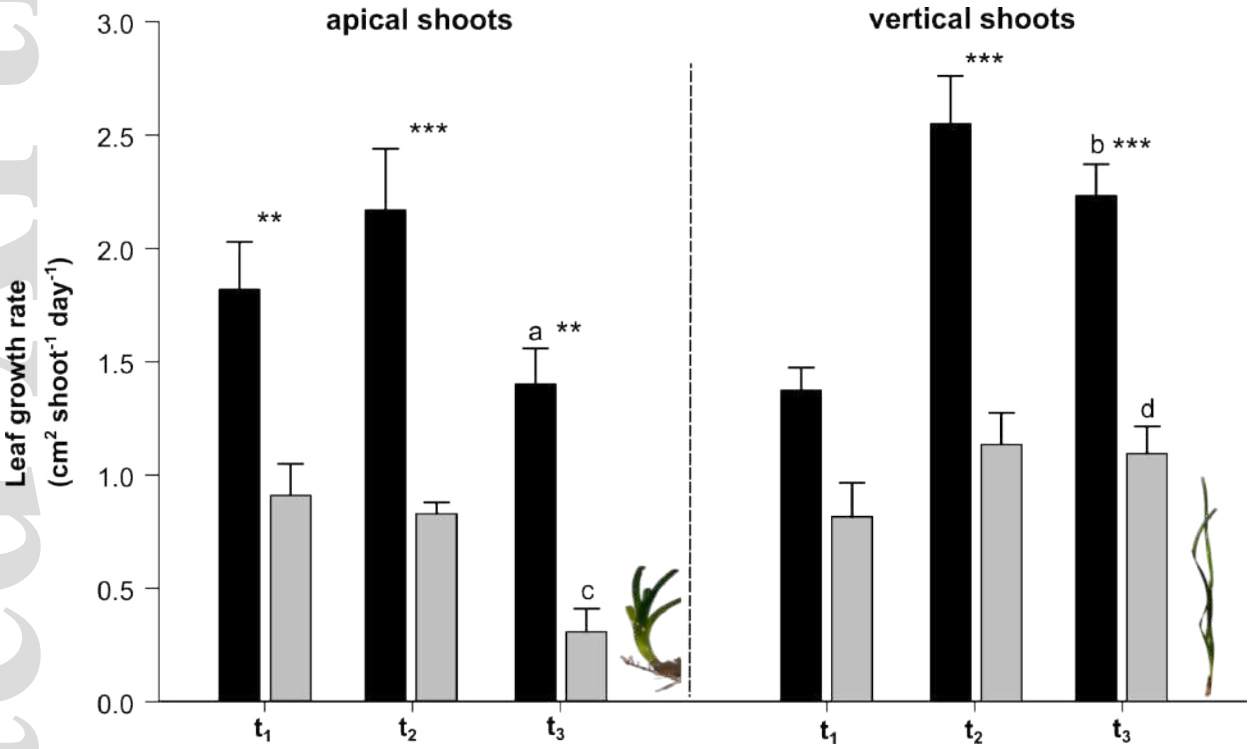


Figure 4. Changes in total non-structural carbohydrates (soluble sugars and starch) in leaves and rhizomes of apical and vertical shoots at the end of the experiment (t_3). Data are mean ± SE (n=3). Black and grey bars represent controls and LL plants, respectively. Asterisks indicate significant differences between control and LL conditions. Different letters indicate significant differences between shoot types, for each light treatment. Full results of 2-way ANOVAs are reported in Table 3. *$P < 0.05$

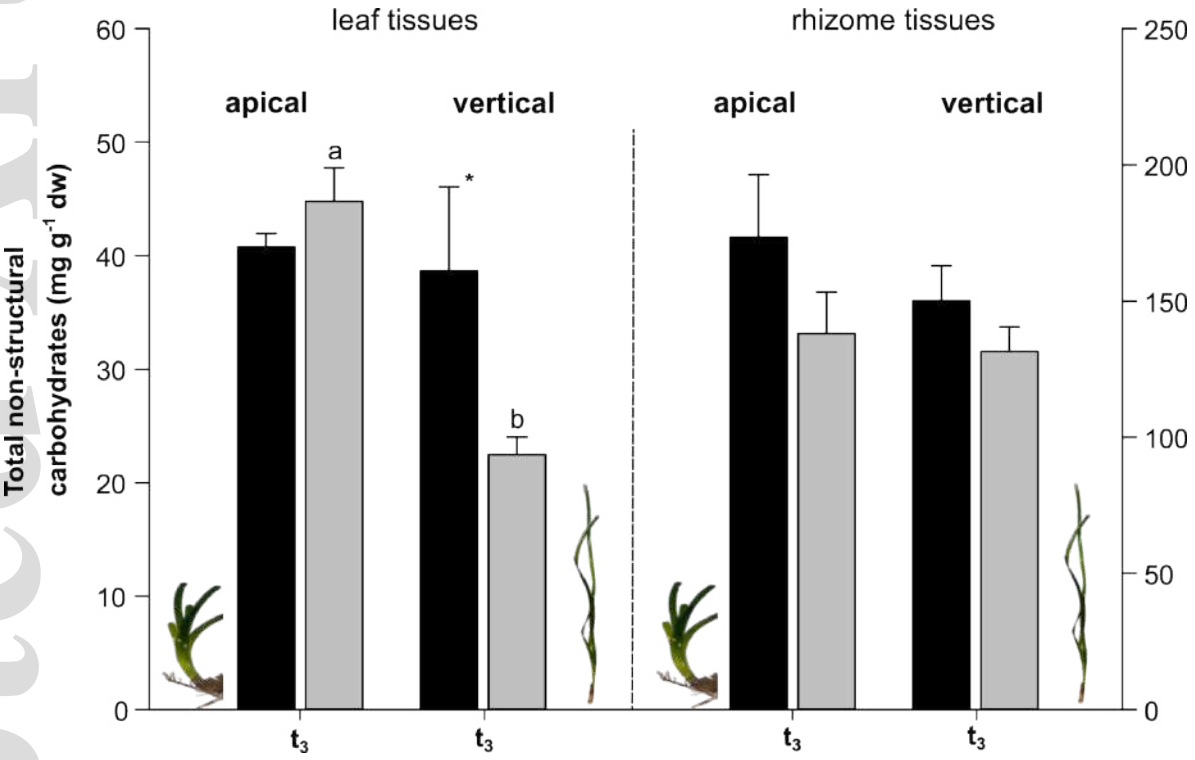


Table 3. Factorial two-way ANOVAs to assess the effect of low-light treatment (L) and shoot type (ST) on total non-structural carbohydrate content (TNC) of apical and vertical shoots at the end of the experiment (t_3). $P < 0.05$ are in bold. Results of Levene's test (L) are reported below

Total non-structural carbohydrates				
Effect	***df***	40 days (t_3)		
Leaves		**MS**	**F**	***P***
Shoot type (ST)	1	449.424	8.828	**0.018**
Light (L)	1	112.208	2.204	0.176
ST×L	1	306.743	6.025	**0.040**
Error	8	50.908		
		L=0.09		
		Transform=none		
Rhizomes				
Shoot type (ST)	1	675.058	0.887	0.374
Light (L)	1	2182.268	2.869	0.129
ST×L	1	213.221	0.280	0.611
Error	8	760.762		
		L=0.27		
		Transform=none		

3.3 Effects of chronic LL on global DNA methylation levels of apical and vertical shoots

Overall, the two-way RM-ANOVA highlighted a significant Time×L×ST interaction ($P < 0.05$; Table 4). At t_1 an increase in % of methylated DNA was detectable in both shoot type under chronic LL (Apical: control = 5.95 ± 1.07, LL = 8.41 ± 1.12; Vertical: control = 4.29 ± 0.09, LL = 5.92 ± 0.07). On the contrary, after 30 days of exposure to LL (t_2), global DNA methylation level decreased in vertical shoots while increasing in apical ones (Apical: control = 4.19 ± 0.47, LL = 5.06 ± 0.33; Vertical: control = 4.17 ± 0.67, LL = 2.94 ± 0.17). Consequently, a significant difference was found in their response to LL (apical *vs* vertical: $P < 0.05$ SNK). In general, strongest variations in DNA methylation level were found at the first sampling time (t_1), while such variations tend to homogenise after one-month exposure (t_2). Yet, apical shoots under LL always possess higher % methylated DNA than vertical ones.

Table 4. Two-way RM-ANOVAs to assess the effect of low-light treatment (L) and shoot type (ST) on global DNA methylation level in leaves of apical and vertical shoots along the course of the experiment. $P < 0.05$ are in bold. Results of Levene's test (L) are reported below

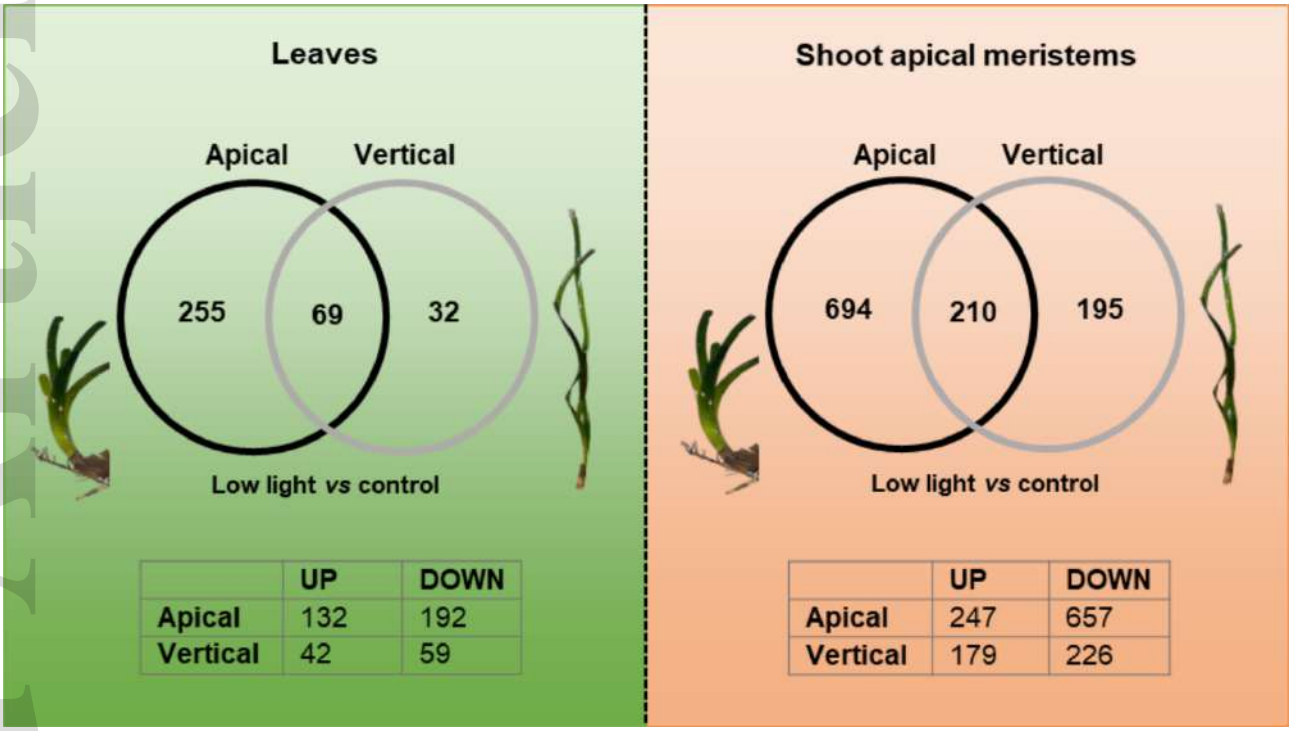
Global DNA methylation				
Effect	***df***	**MS**	**F**	***P***
Light (L)	1	0.046	1.784	0.218
Shoot type (ST)	1	0.277	10.775	**0.011**
L×ST	1	0.065	2.536	0.150
Error	8	0.026		
Time	1	0.495	53.163	**0.000**
Time×L	1	0.126	13.521	**0.006**
Time×ST	1	0.000	0.028	0.871
Time×L×ST	1	0.065	6.978	**0.030**
Error	8	0.009		
		L>0.05 t_1,t_2		
		Transform=Box-Cox		

3.4 General description of differential gene-expression patterns in apical and vertical shoots

The profile of expression across different samples at gene level was firstly explored through a hierarchical clustering (Fig. S3a). A clear differentiation was present between leaf and SAM samples, where most DEGs were up-regulated in one organ and down-regulated in the other, or vice-versa. The PCA (Fig. S3b) confirmed this pattern, revealing a greater contribution of the factor "organ type" in respect to "light" in modulating global transcriptomic responses. Specifically, the PC1 explained 46.23% of the total variance, and segregated two well-distinct sample groups, corresponding to leaves and SAMs. Vertical segregation along the PC2 occurred between LL and control samples (5.91% total variance) (Fig. S3b).

Overall, in the "LL *vs* control" comparisons of both SAMs and leaves, a higher number of DEGs (logFC > ±2; FDR < 0.05) was always identified for apical shoots (SAMs = 904; leaves = 324) in respect to vertical ones (SAMs = 405; leaves = 101) (Fig. 5; Table S3). Considering the different analysed plant organs, LL exposure had a greater effect on the transcriptomic response of SAMs, rather than leaves, as revealed by the larger number of DEGs identified in the former contrasts in both shoot types (Fig. 5). In both plant organs, a reduced number of DEGs was shared between apical and vertical contrasts (SAMs = 210; leaves = 69), whereas the most part of them was exclusively associated to the LL response of apical shoots (SAMs = 694; leaves = 255). Unique DEGs in LL-exposed vertical shoots were 195 and 32, for SAMs and leaves, respectively (see Venn diagrams in Fig. 5).

Figure 5. Summary of DEG analysis with relative number of up- or down-regulated genes (logFC > ±2; FDR < 0.05) for each plant organ (leaves and SAMs), and Venn diagrams depicting shared and unique DEGs in the "LL *vs* control" contrasts for apical and vertical shoots



	UP	DOWN
Apical	132	192
Vertical	42	59

	UP	DOWN
Apical	247	657
Vertical	179	226

3.5 Transcriptomic response of apical and vertical leaves to LL

Even though a reduced total number of DEGs was identified in vertical leaves in respect to apical ones under LL (see above), a substantial higher number of enriched GO terms (as biological processes, GO-BPs) was associated with the former contrast. With a total of 36 GO-BP terms, the transcriptome reprogramming observed in leaves of vertical shoots under LL appeared to be much more complex and multifaceted than that of apical shoots, which was restricted to a total of 13 GO-BP enriched terms (Table S4). Yet, few enriched biological functions were in common between the two contrasts, while the large part of them was specifically associated to the response of apical or vertical shoots (Table S4).

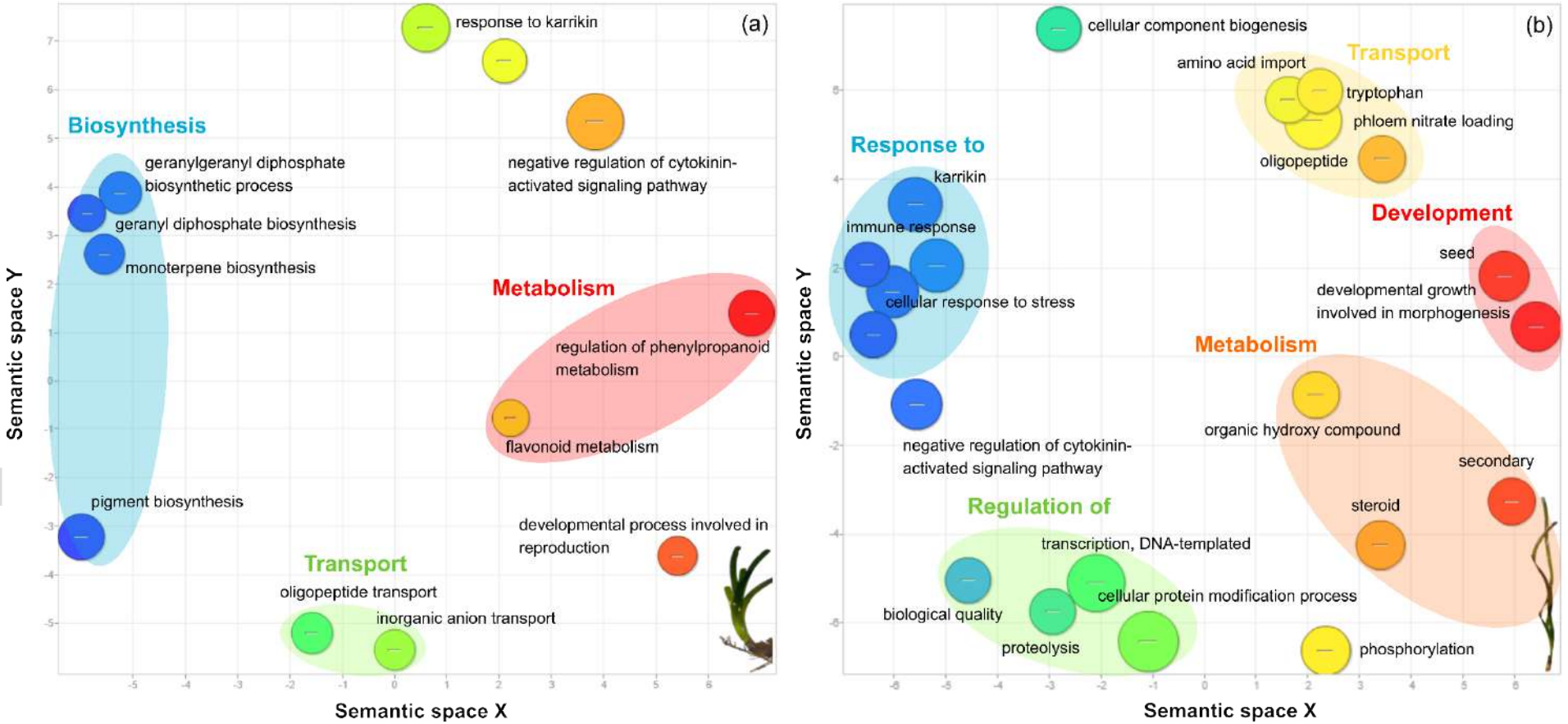
Shared response of apical and vertical leaves: Among shared DEGs identified between apical and vertical contrasts, it is worth noticing the presence of transcripts involved in key plant metabolic processes, such as photosynthesis, chlorophyll biosynthesis, and glycolysis/gluconeogenesis, as significantly down-regulated under LL (Table S3). Similarly, some transcripts encoding for amino acid, oligopeptide and nitrate transporters were among top down-regulated ones (Table S3). Shared up-regulated genes included some transcripts for stress-related proteins and interestingly *GALACTINOL-SUCROSE GALACTOSYLTRANSFERASE 6*, which is known to be induced by dark (Table S3).

Specific response of apical leaves: Exclusive GO enriched BPs in apical leaves included plant hormone-related signalling pathways and response to plant hormones (*negative regulation of cytokinin-activated signalling pathway* and *response to abscisic acid*), and secondary metabolite-related metabolic processes (*pigment biosynthetic process*, *regulation of phenylpropanoid metabolism*, *flavonoid metabolic process* and *geranylgeranyl diphosphate biosynthetic process*) (Fig. 6a and Table S4). Most transcripts specifically associated with hormone signalling pathways were transcriptional factors, and were generally up-regulated in LL. In addition, hormone receptors like *ABSCISIC ACID RECEPTOR PYL 8* and some genes related to the auxin-activated signalling pathway (e.g., *AUXIN EFFLUX CARRIER 8*) were also over-expressed. The same was observed for proteins involved in secondary metabolite biosynthesis, such as terpenes and anthocyanins (Table S3). Among down-regulated genes, it is worth remarking the presence of two transcripts involved in phototropism and photoperiodism (Table S3), and of the enzymes *NITRATE REDUCTASE,* involved in the first step of nitrate assimilation, and *SUCROSE PHOSPHATE SYNTHASE 4*, which plays a fundamental role in photosynthetic sucrose synthesis.

Specific response of vertical leaves: As commented above, a higher number of GO enriched BPs was recognized in vertical leaves under LL. Among these, the most significant ones (FDR < 0.01) were *cellular protein modification processes*, *regulation of transcription*, *oligopeptide transport*, *cellular response to stress* and *negative regulation of cytokinin-activated signalling pathway*" (Fig. 6b and Table S4). Several other GO-BPs were enriched at FDR < 0.05 e.g., *amino-acid import*, *phloem nitrate loading*, *developmental growth involved in morphogenesis*, *organic hydroxy compound metabolic process*, *positive regulation of proteolysis*, *secondary metabolic process* and *regulation of response to external stimulus* (Fig. 6b and Table S4).

Overall, transcripts involved in phloem nitrate loading, oligopeptide/amino acid transport, as well as carbohydrate transport, were down-regulated in LL (Table S3). Up-regulated transcripts were mostly included in the GO categories *regulation of transcription*, *cellular protein modification*, *cellular stress response* and *response to ethylene*. Several transcripts with a role in protein repair were found over-expressed in LL, including many chaperones and chaperone regulators, members of the universal stress and LEA protein families, as well as some proteins involved in DNA damage response (Table S3). Lastly, the enzyme *SUCROSE SYNTHASE 4*, a fundamental sucrose-cleaving enzyme, was found among over-expressed transcript in LL.

Figure 6. Graphical depiction of enriched GO-BPs in leaves of apical (a) and vertical (b) shoots under LL (FDR < 0.05). GO-BP terms are coloured by semantic similarity to other GO terms and bubble size reflects the abs_log10_pvalue of the GO-term in the Fisher test. The two-dimensional semantic space was generated by the REVIGO web service



3.6 Transcriptomic response of apical and vertical SAMs to LL

Similarly to what observed for leaves, a higher number of DEGs was recognised in the SAM of apical rather than vertical shoots (Fig. 5), but this did not translate in a higher number of GO enriched terms in this shoot type. In fact, a considerable higher number of enriched BPs was associated with the response of vertical shoots. Specifically, only 108 enriched GO-BPs (FDR < 0.05) were identified for apical SAMs, in respect to the 283 GO-BPs (FDR < 0.05) found for vertical ones. For simplicity, only BPs enriched at FDR < 0.01 are reported in Tables S5 and S6; GO-BP subsets are also depicted in Fig 7. A total of 14 GO enriched biological functions (FDR < 0.01) were shared between the two contrasts, where the remaining part was specifically associated with the response of apical or vertical shoots (Tables S5 and S6).

Shared response of apical and vertical SAMs: Surprisingly, many structural and functional components involved in the photosynthetic process, chlorophyll biosynthesis and carbon-assimilation pathways, were identified as differentially expressed in the transcriptome of SAMs. The vast majority of these DEGs were strongly down-regulated under LL in both shoot types. Among down-regulated transcripts were photosystem subunits, electron transport-related proteins, and proteins assisting photosystem assembly and repair (Table S3). Equally down-regulated were transcripts involved in chlorophyll biosynthesis and carbon fixation (e.g., *RUBISCO ACTIVASE)*. Transcripts for proteins responsible of carbohydrate biosynthesis and transport (e.g., *SUCROSE PHOSPHATE SYNTHASE 4* and *SUGAR PHOSPHATE/PHOSPHATE TRANSLOCATOR*) were also generally down-expressed under LL, with some exceptions. Similarly to what observed for leaves, shared enriched BPs in LL-exposed SAMs were associated to main phytohormones signalling pathways, namely *gibberellic acid mediated signalling pathway, regulation of jasmonic acid mediated signalling pathway, negative regulation of abscisic acid-activated signalling pathway* and *auxin efflux* (Tables S5 and S6), and transcripts associated with these pathways were generally over-expressed.

Interestingly, many functions associated to the epigenetic regulation of gene expression were enriched in SAMs under LL e.g., *DNA methylation*, *histone H3-K9 methylation*, *nucleosome organisation* and *chromatin silencing by small RNA* (Tables S5 and S6). DE transcripts included in these categories were generally down-regulated, and belonged to five main groups: 1) histone proteins; 2) protein argonaute involved in RNA-mediated gene silencing; 3) DNA-binding factors involved in RNA-directed DNA methylation (RdDM); 4) transcriptional factors; and 5) enzymes like histone methyltransferases, demethylase and acetyltransferase (Table S3).

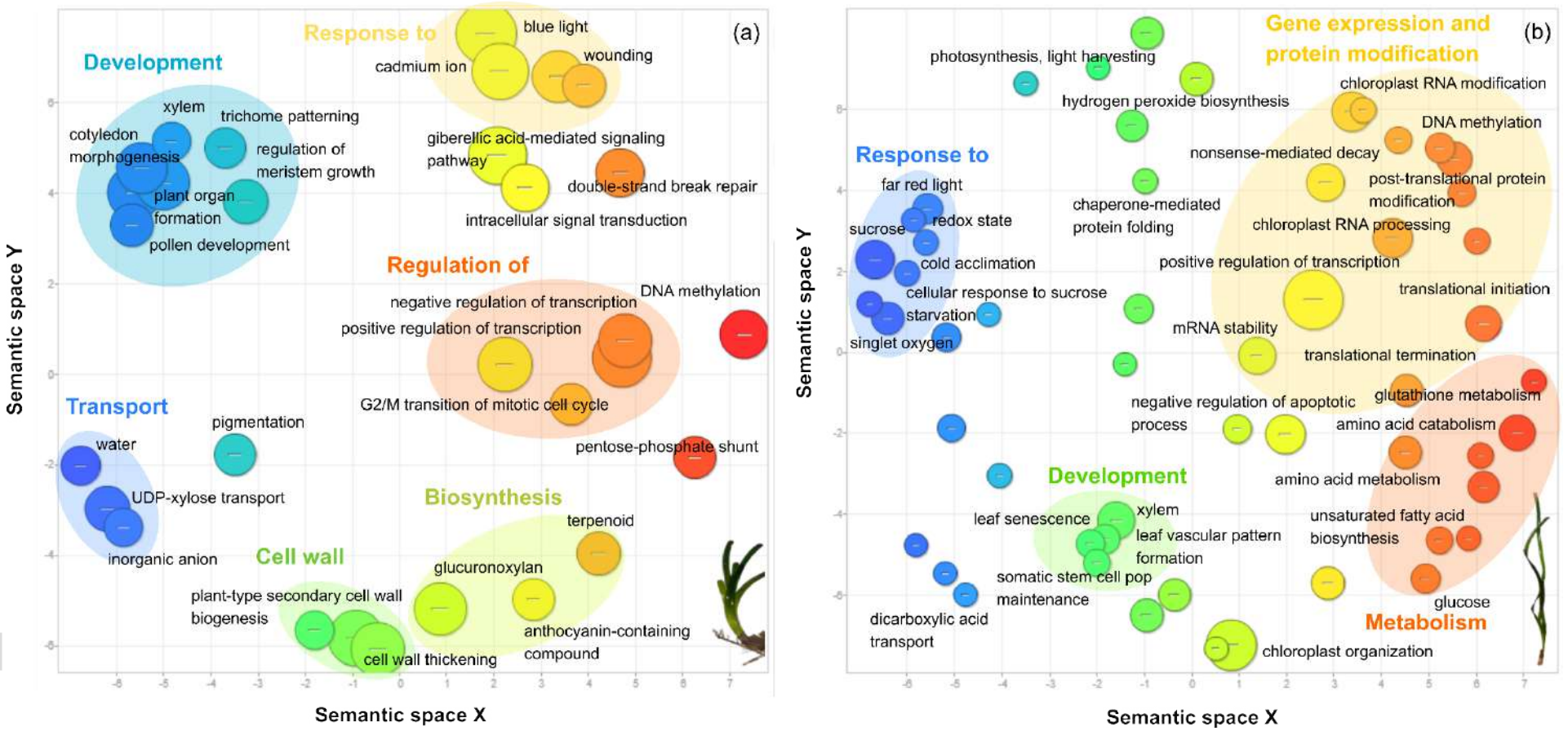
Among shared down-regulated genes, it is worth mentioning some RNA-binding proteins involved in leaf development and phloem/xylem histogenesis (Table S3). A fundamental light-responsive gene was also strongly down-regulated in the meristem of both shoot types, namely *LIGHT-DEPENDENT SHORT HYPOCOTYLS 3,* which is a developmental regulator required for SAM maintenance and formation of lateral organs. Other shared enriched BPs were *positive regulation of transcription, response to sucrose*, *plant-type secondary cell wall biogenesis* and *response to far red light* (Tables S5 and S6).

Specific response of apical SAMs: In the meristem of apical shoots, fundamental responsive functions were enriched, as e.g., those related to plant development (*plant organ formation*, *cotyledon morphogenesis*, *cell wall modification involved in multidimensional cell growth, regulation of meristem growth*, *plant-type cell wall assembly*) (Fig. 7a and Table S5). Notably, many transcripts falling in abovementioned categories showed a reduced expression in LL, such as *PROTEIN G1-LIKE4* (GIL4) that acts as a developmental regulator by promoting cell growth in response to light. BP categories related to gene transcription and signalling (e.g., *negative regulation of transcription DNA-templated*, *intracellular signal transduction),* DNA replication and repair, and cell cycle (e.g., *regulation of G2/M transition of mitotic cell cycle* and *double-strand break repair via homologous recombination*), were also among top GO enriched terms (Fig. 7a and Table S5). Among DEGs included in these categories, many of them were down-regulated, including transcripts encoding for cyclins and transcriptional factors (Table S3).

Specific response of vertical SAMs: Under LL, a significant higher number of BPs was enriched in the meristem of vertical shoots (see Fig. 7b and Table S6). Top enriched functions included those related to chloroplast assembly and arrangement of constituent parts (*chloroplast organisation, chloroplast RNA modification and chloroplast RNA processing*) that were not identified in apical shoots. A vast majority of transcripts involved in these processes were down-expressed in LL, for example the *PALE CRESS* protein, which is required for chloroplast differentiation, *RNA POLYMERASE SIGMA FACTOR SIGE* or *TOC75-3*, which is an essential protein required for the import of protein precursors into chloroplasts. Other unique GO-BPs were those related to sugar responses and signalling (e.g., *cellular response to sucrose starvation*, *sugar mediated signalling pathway* and *glucose metabolic process*) and amino acid metabolism (*regulation of cellular amino acid metabolic process* and *branched-chain amino acid catabolic process*). Enzymes with a role in sucrose starvation were generally over-expressed in LL, as were genes involved in sugar mediated signalling pathway (Table S3).

Stress-related biological functions were particularly represented and included processes related to protein repair/degradation (*proteasomal ubiquitin-independent protein catabolic process* and *chaperone-mediated protein folding*), DNA damage (*DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest*) and apoptosis (*negative regulation of apoptotic process*). Curiously, many transcripts encoding for subunits of the proteasome complex were identified as down-regulated under LL*,* whereas proteins involved in DNA repair were over-expressed (Table S3). DEGs involved in the blue light-signalling pathway had a mixed behaviour; however, fundamental photoreceptors like *PHOTOTROPIN 1A* and cryptochromes were up-regulated in LL. The same was observed for some genes involved in long-day photoperiodism (Table S3). One enriched BP was particularly relevant, namely the *somatic stem cell population maintenance,* which included both up and down-expressed transcripts.

Figure 7. Graphical depiction of enriched GO-BPs in SAMs of apical (a) and vertical (b) shoots under LL (FDR < 0.01). GO-BP terms are coloured by semantic similarity to other GO terms and bubble size reflects the abs_log10_pvalue of the GO-term in the Fisher test. The two-dimensional semantic space was generated by the REVIGO web service



4. Discussion

Here, we explored for the first time at the molecular level, the differential behaviour of apical and vertical shoots in the foundation seagrass species *P. oceanica* under chronic light shortage. Our hypothesis was that under an energetic crisis, metabolic rearrangements occurring in vertical shoots ("the vassals") would be devoted to provide resources for the apical one ("the king"), representing the leading part of the clone, to withstand the unfavourable event (Liu et al., 2016). Following this view, the response of vertical shoots could be seen as a "sacrificing response" allowing the survival of "the king" that is key for ensuring propagation and population maintenance, and for the colonisation of new more favourable environments (i.e., "escape" strategy). Our multi-scale analysis of physiological, morphological and molecular plasticity under LL exposure, allowed finding signatures of clonal integration between the two shoot types, in support of our initial hypothesis.

4.1 Physiological and morphological evidences supporting the "king and vassals" hypothesis

Under chronic LL, apical and vertical shoots showed a similar response in terms of photosynthetic performance, with a large reduction (about 30-40%) in the effective quantum yield of PSII (ΔF/Fm') that was especially evident after 1-month exposure. This is a typical response of *P. oceanica* to diminishing irradiance level, in agreement with previous observations (Dattolo et al., 2017; Dattolo et al., 2014; Procaccini et al., 2017). However, the response of apical and vertical ramets starts to diverge when analysing shoot morphology and fitness-related traits under light shortage. Although both shoot types greatly slowed down their leaf growth rate throughout the experiment, for apical shoots this was in a much greater extent in respect to vertical ones, reaching around the 80% reduction after 40 days of exposure. Ultimately, this resulted in a larger reduction in their max. leaf length and width and overall shoot size at the end of the experiment. Structural changes as the reduction of plant size are considered the main adaptive mechanisms to offset light reductions in large-sized seagrass species, aimed at maximising light exposure of photosynthetic tissues and minimise respiratory demands (Collier, Lavery, Ralph, & Masini, 2008; Dattolo et al., 2017; Olesen et al., 2002; Ralph et al., 2007). Here, LL-induced growth arrest and decrease in leaf biomass would allow apical shoots to save fundamental resources (e.g., sugars) needed to withstand the temporary stress event (Kosovà, Vítámvás, Prášil, & Renaut, 2011; Ruocco, Marín-Guirao, & Procaccini, 2019b). This is coherent with our assessment of the

energetic status of the two shoot types by measuring the total content of non-structural carbohydrates. After 40-days of exposure to extreme LL, TNC content in leaf tissue of apical shoots was even slightly higher than the control, while in vertical shoots it dropped down dramatically, being around half of that measured in apical ones. Although this pattern was not confirmed in rhizomes, probably due to the duration of the experiment was too brief to see an effect in these organs, this suggests the possibility of a translocation of photosynthates between ramets, as previously demonstrated in seagrasses (Harrison, 1978; Marbà et al., 2006; Marbà et al., 2002; Terrados et al., 1997b) and terrestrial clonal plants (e.g., Duchoslavová & Jansa, 2018; Qian, Li, Han, & Sun, 2010). As apical shoots did not increment their photosynthetic performance under LL, the "conservative" strategy they put in place in terms of growth arrest and reduction of leaf biomass, together with a possible translocation of photosynthates from neighbouring vertical ramets, would have allowed maintaining their sugar reserves constant all along the experiment.

4.2 Molecular signatures of clonal integration under energy shortage

Our comparative transcriptome analysis revealed different BPs enriched in apical and vertical shoots exposed to chronic LL, and only a small portion of shared BPs. Surprisingly, although a higher number of DEGs was generally found in both leaves and SAMs of apical shoots, those were associated to a reduced number of GO-BPs, in respect to vertical ones. This clearly demonstrates a high level of specialisation of integrated ramets within seagrass clones and a "division of labour" of different shoot types under adverse conditions, as observed in terrestrial plants (Stuefer, During, & de Kroon, 1994). Overall, under light shortage, the response of apical shoots appeared to be less complex and restricted to few important functions, whereas that of vertical shoots was more heterogeneous and involved a wide variety of processes. Below, we discuss the main gene categories/cellular functions associated to physiological integration mechanisms among ramets.

Hormone response/signalling: In the leaf transcriptome of apical shoots, there were only a few enriched BPs under LL. Top GO terms were those associated with the regulation of phytohormone signalling pathways and response to hormones (e.g., *negative regulation of cytokinin-activated signalling pathway*) and many transcripts within these categories were up-regulated. In addition, several transcripts involved in the auxin-activated signalling pathway and transport, were also among top over-expressed genes. Cytokinin-related signalling pathway was also enriched in vertical leaves under LL. In terrestrial systems, there is considerable evidence that

hormones can cause differences in biomass of plant parts in response to different resource availability, and can also regulate translocation between branches (Voesenek & Blom, 1996). In particular, two major types of plant hormones, auxins and cytokinins, can direct the transport of carbohydrates and nutrients between plant parts (Alpert, Holzapfel, & Benson, 2002; Cole & Patrick, 1998; Javid, Sorooshzadeh, Modarres-Sanavy, Allahdadi, & Moradi, 2011; Morris & Arthur, 1987).

At the level of meristems, GO terms associated to phytohormone response and signalling were particularly represented in both shoot types, in particular those related to auxin (e.g., *auxin efflux*, *basipetal auxin transport*, *auxin homeostasis*). Our findings thus indicate that hormones (e.g., auxins or cytokinins) could be responsible for modifying patterns of resource sharing between ramets in *P. oceanica* under light shortage and eventually enhance resource concentration in particular ramets, as apical shoots. Although our experimental design cannot provide direct evidence supporting this hypothesis, it is worth mentioning that the other few GO terms enriched in apical leaves were those associated to secondary metabolism, as *regulation of phenylpropanoid metabolism* and *flavonoid metabolic process*. Similarly, in apical SAMs, GO terms related to this class of secondary metabolites (e.g., *flavonol biosynthetic process*) were equally present. Flavonoids, in particular, a subgroup of phenylpropanoid compounds, are involved in modifying the rate of auxin transport, thus leading to altered auxin distribution and accumulation (Bielach, Hrtyan, & Tognetti, 2017; Kuhn, Geisler, Bigler, & Ringli, 2011; Peer & Murphy, 2007). By controlling the processes of phytohormone transport and distribution, flavonoids could indirectly modulate patterns of resource accumulation in *P. oceanica* ramets under LL (Bielach et al., 2017; Peer & Murphy, 2007).

Stress response: In agreement with our hypothesis, GO-terms related to the cellular stress response (CSR) (*sensu* Kültz, 2005) were particularly over-represented in the leaf transcriptome of vertical shoots (e.g., *cellular response to stress*, *positive regulation of proteolysis*), whereas no stress-related terms were found in apical leaves. Similarly, in the SAM analysis of apical shoots only 1 BP was specifically associated to CSR (i.e., *double-strand break repair via homologous recombination*), while enriched BPs in vertical SAMs included several processes related to different phases of the CSR, including protein repair/degradation, DNA damage responses and ultimately apoptosis. Interestingly, transcripts with a role in protein repair, as chaperones and chaperone regulators, were generally overexpressed in LL, as were those related to the response to DNA damage, whereas transcripts related to proteolysis were down-regulated. This mirrors what

previously reported in *P. oceanica* under heat stress, where CSR appeared to bypass the intermediate proteolysis-related pathway, suggesting that molecular chaperoning, DNA repair and apoptosis inhibition processes are the instant stress signals exerted by the species (Traboni et al., 2018).

Growth arrest and sucrose starvation: The negative regulation of functions related to cell growth and proliferation is one of the key responses of plants to non-lethal abiotic/biotic stressors and it has a high adaptive value, as the resultant plant is more likely to survive (Kitsios & Doonan, 2011). Our study revealed that LL slowed-down shoot size and leaf growth rate in both shoot types, although with a different extent. Notably, LL-induced growth arrest started at the level of meristems, as clearly highlighted from the underlying gene-expression responses. In general, the decrease of plant size can be attributed both to a reduction in cell number as well as cell growth (Kitsios & Doonan, 2011). In apical shoots, both ways can be postulated, as many GO-terms related to these processes were enriched (e.g., *regulation of G2/M transition of mitotic cell cycle*, *mitotic metaphase plate congression*, *cytokinesis by cell plate formation*, *cell wall modification involved in multidimensional cell growth)*. In particular, the reduction in cell number can be attributed to the observed suppression of cell cycle-related transcripts (e.g., cyclins), resulting in cell-cycle arrest at the G1/S and G2/M checkpoints, prolonged S-phase progression and/or delayed entry into mitosis (De Veylder, Beeckman, & Inzé, 2007).

In vertical shoots, we found a similar pattern, although this did not seem to be related to an acclimative response. The inactivation of genes required for cell-cycle progression can arise from the activation of DNA stress checkpoints, which also induces DNA-repair related genes. This coordinated action ensures that cells repair their damaged genome before they proceed into mitosis (De Veylder et al., 2007). The analysis of vertical SAMs revealed the presence of many GO terms associated with DNA damage, and many genes related to these processes were found over-expressed in LL (see above). The inhibition of cell proliferation and cell-cycle arrest observed in these shoots could actually results from the interplay between CSR mechanisms and sucrose starvation (Riou-Khamlichi, Menges, Healy, & Murray, 2000; Yu, 1999).

Sucrose is the major organic carbon form exported from source to sink organs (Rosa et al., 2009) and a key modulator of cell division rates, as its availability to proliferating meristematic cells reflects the overall photosynthetic capacity and prevailing environmental conditions (Koch, 1996). In addition, sucrose acts as a signalling molecule modulating gene-expression (Koch, 1996; Yu, 1999). In SAMs of vertical shoots, these functions were particularly represented (e.g., *cellular*

response to sucrose starvation and *sugar mediated signalling pathway*), and this is consistent with our assessment of TNC content, although the results obtained for rhizomes might indicate an asynchrony between SAM signals and the actual carbohydrate content in storage organs. Regarding specific sugar related gene-expression, transcripts encoding for proteins involved in carbohydrate biosynthesis/metabolism exhibited a mixed behaviour in LL. The same was observed for sugar transporters, as some of them were over-expressed, while others were down-regulated. As the specific function and directionality of these transporters are still unknown in seagrasses, it is hard to find a definitive molecular pattern responsible for regulating sugar translocation between shoots.

However, what appears to be very clear is the effect of sucrose starvation on nitrate and amino acid metabolism. In sugar-starved cells, a decrease in enzymatic activities related to nitrate reduction/assimilation and protein synthesis is generally observed to protect cells against nutrient stress, together with an over-expression of genes related to amino acid catabolism, as an alternative way to sustain respiration and metabolic processes (Yu, 1999). In our analysis, transcripts involved in nitrate loading (e.g., nitrate transporters) were always found among top down-regulated ones in SAMs and leaves under LL. In addition, *ASPARAGINE SYNTHETASE*, which is considered a marker of stress conditions under sucrose depletion, was among the top expressed transcripts in vertical SAMs. Asparagine considerably accumulates under sugar starvation, accounting for most of the N released by protein degradation. It works as a detoxification product, acting as N storage compound under high ammonium concentration (Borek, Paluch-Lubawa, Pukacka, Pietrowska-Borek, & Ratajczak, 2017; Brouquisse, James, Pradet, & Raymond, 1992; Downs & Somerfield, 1997).

4.3 Epigenetic regulation of gene expression in apical and vertical shoots under LL

Many functions associated with the epigenetic regulation of gene expression were over-represented in both shoot types, especially in the transcriptome of SAMs, where the GO terms *DNA methylation* and *chromatin silencing by small RNA* were found among the top enriched BPs. Other functions related to histone modifications and small RNA-based epigenetic changes were enriched at lower significance level (FDR < 0.05), e.g., *histone H3-K9 methylation*, *histone H3-K36 dimethylation*/*trimethylation* and *regulation of histone acetylation* (data not shown). DEGs included in these categories exhibited a variable behaviour, although most of them were down-expressed in LL. Epigenetic mechanisms listed above play an essential role in modulating

chromatin structure and function and subsequent gene activity, and are associated to both developmental processes and stress responses (Gutzat & Mittelsten Scheid, 2012; Mirouze & Paszkowski, 2011). The significance of these epigenetic marks differs depending on the location of the modified sites, and on the type of chemical modification (Liu, Lu, Cui, & Cao, 2010; Niederhuth & Schmitz, 2017), hence it is difficult to unambiguously link them with transcriptional repression or activation.

Here, we analysed global DNA methylation level in leaves of apical and vertical shoots under LL, revealing a general increase in % 5mC at least in the short-term (t_1), mirroring what previously found in other seagrass stress studies (Greco, Chiappetta, Bruno, & Bitonti, 2012, 2013; Ruocco et al., 2019b). More interestingly, apical shoots always displayed a genome hyper-methylation in respect to vertical ones. This well fits with the general "energy-saving" strategy adopted by these shoot types under light shortage. Increase in DNA methylation might be an attempt to down-regulate transcriptome expression to slow down the overall metabolism, which would allow these ramets to preserve fundamental energy needed to overcome the temporary challenge (Saraswat, Yadav, Sirohi, & Singh, 2017). The role DNA methylation and other epigenetic mechanisms have in the regulation of energy metabolism in plants and animals has been recently highlighted (Donohoe & Bultman, 2012; Marsh & Pasqualone, 2014; Shen, Issakidis-Bourguet, & Zhou, 2016). In plants, the coordination of epigenetics with metabolism seems to be essential for cells to rapidly adjust metabolism and gene expression to changing environmental conditions (Shen et al., 2016). Research exploring this energetic/epigenetic cross-talk, and thus the mechanisms of mutual regulation between metabolite homeostasis and epigenetics, is ongoing in terrestrial plants and should be taken into consideration also in seagrasses.

4.4 Response of the shoot-apical meristem to LL: a new early warning indicator in seagrass research?

Under LL, the transcriptomic response of apical and vertical SAMs was always much greater than that identified in leaves. Surprisingly, fundamental processes such as photosynthesis, carbon assimilation and carbohydrate biosynthesis, were enriched in SAM transcriptomes, and the associated DEGs were in much higher numbers than leaves. Transcripts for proteins assisting photosystem assembly, DNA binding factors such as the SIGE factor, which recruits plastid-encoded RNA polymerase to specific initiation sites (e.g., *psbA* and *psbD*) (Chi, He, Mao, Jiang,

& Zhang, 2015), as well as regulators of Calvin cycle enzymes, such as *RUBISCO ACTIVASE 1-2*, were among key down-expressed genes. These results demonstrate that the expression of constituents of the photosynthetic machinery, which correlates directly with chloroplast development, starts already in the SAM of *P. oceanica*. In further support of this, it is worth mentioning that, at least in the meristem of vertical shoots, the GO terms *chloroplast organisation*, *chloroplast RNA modification* and *chloroplast RNA processing* were found among the top-enriched ones. This was not an obvious observation, as for instance in maize very few photosynthetic-related genes are found to be expressed in the SAM and leaf primordia (Brooks III et al., 2009). On the contrary, our results are quite similar to those found for the shoot apex of tomato, where the presence of transcripts for different chloroplast functions was already detected in the stem cell-containing region of the SAM, revealing an early acquisition of photosynthetic capacity (Dalal et al., 2018). Another important consideration is that LL exposure seems to significantly impair the SAM transcriptional machinery responsible for chloroplast biogenesis and later for the establishment of photosynthetic competence, and these gene-expression changes likely anticipated leaf-related responses.

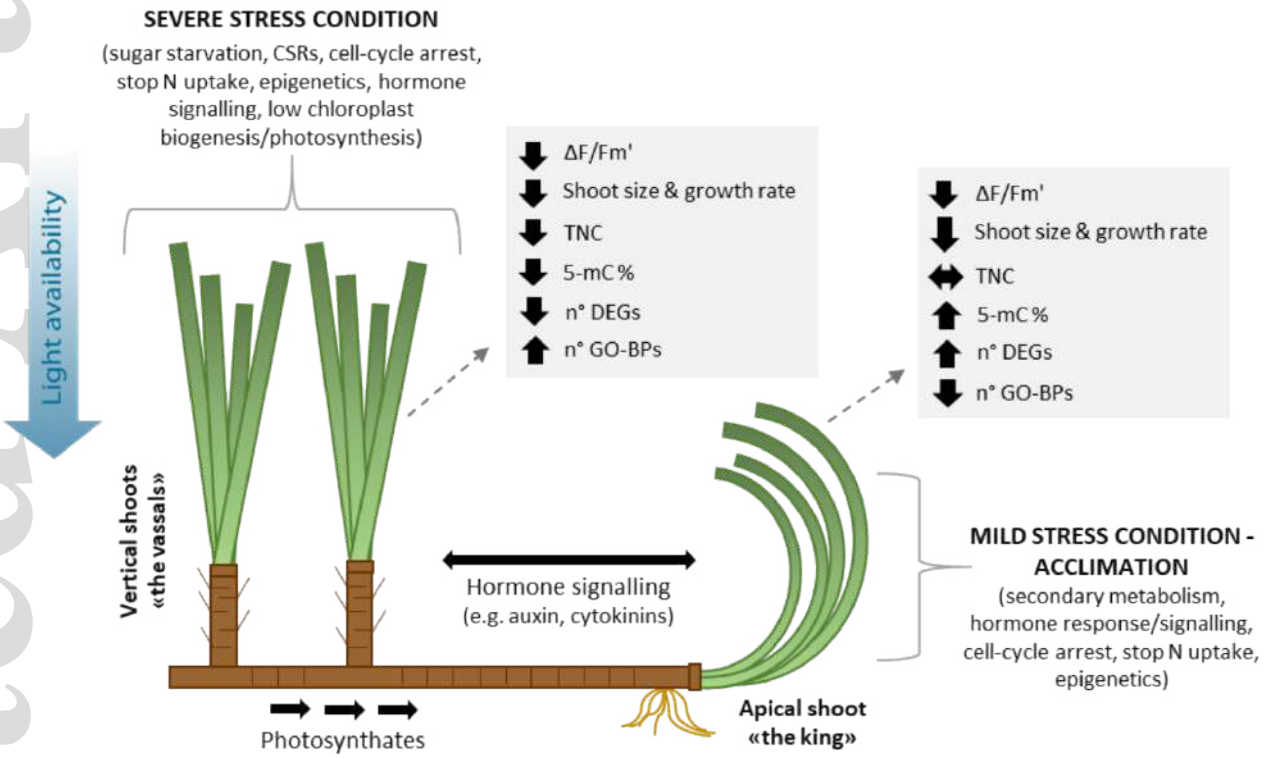
Other enriched functions in SAMs included those related to meristem growth and maintenance, development of plant organs/tissues (e.g., phloem/xylem histogenesis), as well as DNA damage/repair. These functions were only marginally represented in leaves. Ultimately, this experiment revealed that the stress response in *P. oceanica* exposed to chronic LL starts primarily at the level of meristems, which appeared to be the most sensitive plant parts, with the lowest tolerance threshold. SAMs are fundamental structures ensuring organogenesis over the whole plant's life. Accordingly, plants evolved special mechanisms to safeguard the genome integrity of these cell niches through cell-cycle arrest, DNA repair and at last selective programmed cell death programs that are different from those of differentiated cells (Fulcher & Sablowski, 2009; Hefner, Huefner, & Britt, 2006).

From an ecological perspective, this opens a new view where the SAM-related response could be considered a fundamental indicator of seagrass stress status. If further studies will demonstrate that the molecular response of SAM to other abiotic/biotic stressors occurs not only in a greater extent, but also in much earlier than leaves, the role of the latter should be reconsidered. Specific protocols should be developed to exploit this key plant organ and to identify suitable target genes to be used as early-warning molecular monitoring tools (Macreadie et al., 2014).

Conclusions

This research sheds first light on the role of apical and vertical shoots under energy shortage in seagrasses and proposes some molecular, physiological and morphological responses that may underline clonal integration mechanisms to safeguard the whole clone survival under stress events (Fig. 8). Our results clearly demonstrate a high level of specialisation of the different ramets within seagrass clones and a "division of labour" under adverse conditions. Although further investigations are needed, it appears that vertical shoots do "most of the job" especially in terms of resource providing, whereas enriched functions in apical shoots were restricted to few important processes, according to an "energy-saving" strategy. Communication and eventually resource (e.g., sugar) translocation among ramets appeared to be based on phytohormone release (e.g., auxins and/or cytokinins). In both shoot types, the exposure to LL resulted in a leaf growth slowdown all along the experiment, which started from immediate signals produced in shoot apical meristems. In apical shoots, this was linked to an acclimative/resistance response, where they were suffering a mild stress condition (eustress-prevalent; Jansen & Potters, 2017), while in vertical shoots it fell in a more severe stress condition (distress-prevalent; Jansen & Potters, 2017). The latter suffered from sugar starvation and showed a clear cellular stress response in terms of protein refolding and induction of DNA repair mechanisms. Several epigenetic mechanisms were involved in modulating the observed gene-expression patterns and the cross-talk between DNA methylation and the cellular energetic status appeared to have an important role in the regulation of shoot metabolism under LL. Finally, our experiment strongly highlighted the fundamental role that shoot apical meristems, more than leaves, can have as early-warning molecular monitoring tools in stress-related studies.

Figure 8. Schematic (qualitative) representation of main leaf and SAM-related responses of vertical and apical shoots in *P. oceanica* under chronic light shortage. Overall signatures of photo-physiological, morphological and molecular variables are outlined in the grey boxes. Specific transcriptomic rearrangements (as biological processes) for apical and vertical shoots are synthetized in round brackets



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Authors' contributions

MR, LMG and GP conceived the ideas and designed the experiment; MR, LMG, GP and ED participated in mesocosm maintenance and sample collection; MR performed molecular, morphological, biochemical and physiological assessments with a significant help from LMG and AM; LE performed all the bioinformatics work. MR and LMG analysed the data; MR led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability

RNA-Seq reads from this study are accessible at the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) under the accession numbers SRR11593205- SRR11593228 (BioProject ID PRJNA627562). All other supporting data used in this study are publicly available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.x3ffbg7g9 (Ruocco et al., 2020).

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