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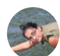

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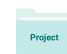

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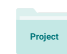

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Genome-wide transcriptional reprogramming in the seagrass *Cymodocea nodosa* under experimental ocean acidification

Running head: *Cymodocea nodosa* transcriptome in high CO_2

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Abstract

Here we report the first use of massive scale RNA-Sequencing to explore seagrass response to CO_2-driven ocean acidification (OA). Large-scale gene expression changes in the seagrass *Cymodocea nodosa* occurred at CO_2 levels projected by the end of the century. *C. nodosa* transcriptome was obtained using Illumina RNA-Seq technology and *de novo* assembly, and differential gene expression was explored in plants exposed to short-term high CO_2 / low pH conditions. At high pCO_2, there was a significant increased expression of transcripts associated to photosynthesis, including light reaction functions and CO_2 fixation, and also to respiratory pathways, specifically for enzymes involved in glycolysis, in the tricarboxylic acid cycle and in the energy metabolism of the mitochondrial electron transport. The up-regulation of respiratory metabolism is probably supported by the increased availability of photosynthates and increased energy demand for biosynthesis and stress-related processes under elevated CO_2 and low pH. The up-regulation of several chaperones resembling heat stress-induced changes in gene expression, highlighted the positive role these proteins play in tolerance to intracellular acid stress in seagrasses. OA further modifies *C. nodosa* secondary metabolism inducing the transcription of enzymes related to carbon-based-secondary compounds biosynthesis, in particular the synthesis of polyphenols and isoprenoid compounds that have a variety of biological functions including plant defense. By demonstrating which physiological processes are most sensitive to OA, this research provide

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a major advance in the understanding of seagrass metabolism in the context of altered seawater chemistry from global climate change.

Introduction

Ocean acidification (OA) is a direct consequence of the oceanic uptake of atmospheric CO_2 (Caldeira & Wickett 2003) that is causing fundamental ecological transformations as a result of changes in physical, chemical and biological environments (Gruber 2011; Hoegh-Guldberg & Bruno 2010). A drop of ocean pH of about 0.1 pH units from ≈ 8.21 to 8.10 has already been recorded (Royal Society 2005) and a further reduction of 0.3–0.5 units is predictable by the end of the century (Caldeira & Wickett 2005; Feely *et al.* 2009). In this process, the relative proportion of the inorganic carbon species is being altered, shifting away from carbonate (CO_3^{2-}) towards more bicarbonate (HCO_3^-) and aqueous carbon dioxide ($CO_{2(aq)}$) (Rhein *et al.* 2013).

Marine organisms vary broadly in their individual responses to OA, as a result of differences in their physiological and ecological characteristics (Hendriks *et al.* 2010; Kroeker *et al.* 2010). The meta-analysis of published data suggests that there is greater sensitivity among heavily calcified organisms and a higher tolerance among more active and mobile organisms such as crustaceans and fishes (e.g. Orr *et al.* 2005; Doney *et al.* 2009; Hofmann *et al.* 2010). Non-calcifying marine photoautotrophs are expected to benefit from enhanced CO_2 and HCO_3^- supply for photosynthesis (Koch *et al.* 2013; Kroeker *et al.* 2013; Kroeker *et al.* 2010).

In both temperate and tropical shallow coastal systems marine angiosperms (seagrasses) act as foundation species (Larkum *et al.* 2006). Seagrasses sequester carbon and nutrients and the habitat complexity within their meadows enhances the diversity and abundance of associated organisms (Hughes *et al.* 2008) and influences the physical environment by reconfiguring

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water flow, trapping and stabilizing sediments (Hemminga & Duarte 2000). Because of the key ecological services they provide to the coastal zone, seagrass-based ecosystems rank amongst the most valued ecosystems on earth, surpassing the economic value of coral reefs and tropical rainforests (Barbier *et al.* 2010; Costanza *et al.* 1997; Costanza *et al.* 2014).

The distribution of seagrass ecosystems is declining worldwide, as a direct consequence of human-induced factors that negatively impact the meadows, such as eutrophication, mechanical destruction, and aquaculture (Orth *et al.* 2006; Waycott *et al.* 2009). An open question is whether current climate changes exacerbate seagrass threats. Establishing if these key habitat-forming species possess the physiological plasticity or adaptation capacity to adjust in a rapidly changing environment is a central question for current research (Reusch 2014; Short & Neckles 1999).

Seagrasses, unlike macroalgae, are commonly considered to be DIC (Dissolved Inorganic Carbon) limited under current CO_2 conditions because they are relatively inefficient in utilizing bicarbonate (HCO_3^-) for photosynthesis (Beer 1989; Beer & Koch 1996; Invers *et al.* 2001). Under actual increasing CO_2 conditions seagrass species are expected to increase their use of CO_2 (Beer & Koch 1996), enhancing photosynthesis, growth rates and biomass (Koch *et al.* 2013; Russell *et al.* 2013). Despite this assumption, results from laboratory and mesocosm experiments conducted so far, over different time periods, are not consensual. For example, short-term CO_2 enrichment of *Zostera marina* increased photosynthetic rate and shoot productivity decreasing the species daily light requirements (Zimmerman *et al.* 1997). On the other hand, a long-term experiment (1 year) on the same species showed that increasing CO_2 did not alter biomass-specific growth rates, leaf size, or leaf sugar content of aboveground shoots, but significantly enhanced reproductive output, belowground biomass and vegetative proliferation of new shoots (Palacios & Zimmerman 2007). Similarly, *Thalassia hemprichii* responded to high CO_2 increasing maximum relative electron transport

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rate, minimum saturating irradiance, leaf growth rate, and non-structural carbohydrates in belowground tissues (Jiang *et al.* 2010). Alexandre *et al.* (2012) found higher maximum photosynthetic rate and photosynthetic efficiency in *Zostera noltii*, after 5 months of exposure to high CO_2, but they did not observe any significant effects on leaf growth rates, probably due to nitrogen limitation. More recently, Ow and coauthors (2015) examined the physiological responses of three tropical seagrasses to a range of seawater CO_2 levels for 2 weeks. Despite increases in net productivity and photosynthetic parameters in all three species, a differential growth response was observed, probably due to varying carbon allocation strategies among species.

Studies conducted in the proximity of natural CO_2 vents revealed that seagrasses can be adapted to live under permanently high-CO_2 levels (Fabricius *et al.* 2011; Hall-Spencer *et al.* 2008; Takahashi *et al.* 2015) and are able to exploit CO_2 of volcanic origin (Vizzini *et al.* 2010), but can also exhibit a stress-like response, putatively related to their level of adaptation to CO_2 and other compounds of volcanic origin (Lauritano *et al.* 2015; Olivè *et al.* 2017). In *Posidonia oceanica*, Hall-Spencer *et al.* (2008) found no difference in the photosynthetic performances of individual shoots between control and naturally acidified sites, but seagrass production and shoot density was highest in an area of lower pH (7.6). Differently, the photosynthetic activity of *Cymodocea nodosa* was stimulated by acidified conditions at a shallow volcanic CO_2 vent, as shown by the significant increase in Chl*a* content, maximum electron transport rate and compensation irradiance (Apostolaki *et al.* 2014). Low pH promoted *C. nodosa* community productivity, but without a corresponding increase in plant biomass, possibly resulting from nutrient limitation, grazing or poor environmental conditions (Apostolaki *et al.* 2014). In contrast, Olivè *et al*. (2017) found a significant decrease in *C. nodosa* net plant productivity in the volcanic CO_2 vents at Vulcano Island. Globally, these results indicate that seagrass responses in naturally acidified

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conditions are highly dependent upon species and environmental characteristics of the site, and cautious must be taken when using data derived from vent sites as a proxy for future ocean acidification scenario.

The application of high-throughput gene-expression profiling, primarily transcriptomics, is increasingly recognized as a powerful tool for physiological investigation in response to environmental change (Evans & Hofmann 2012; Hofmann *et al.* 2008; Hofmann *et al.* 2005). For organisms experiencing environmental stress, the modulation of gene expression is indeed one of the most rapid and versatile ways to react. Hence, examining the expression levels of many thousands of genes simultaneously provides a broad view of molecular changes that accompany alterations in physiological states (Evans & Hofmann 2012; Gracey 2007). Yet, the advent of fast and cost-effective Next Generation Sequencing (NGS) technologies has shown great potential for expanding transcriptome resources also in non-model species (Ekblom & Galindo 2011; Unamba *et al.* 2015).

Transcriptomic approaches have been successfully applied to assess the effects of OA in several marine taxa, such as corals (Barshis *et al.* 2013; Kaniewska *et al.* 2012; Moya *et al.* 2012; Moya *et al.* 2015; Vidal-Dupiol *et al.* 2013), sea-urchins (Evans *et al.* 2013; Evans & Watson-Wynn 2014; Todgham & Hofmann 2009) and crustaceans (Harms *et al.* 2014).

In seagrasses, several studies have published whole transcriptomes of *Zostera* spp. (*Z. marina* and *Z. noltii*), obtained via sequencing cDNA libraries and 454 pyrosequencing (Franssen *et al.* 2011; Franssen *et al.* 2014; Gu *et al.* 2012; Massa *et al.* 2011; Reusch *et al.* 2008; Wissler *et al.* 2011; Wissler *et al.* 2009) or, more recently, via Illumina sequencing technology (Kong *et al.* 2014), under a range of environmental conditions (e.g. temperature, salinity, light intensity and quality). However, the effects of either short- or long-term exposure to elevated CO_2 and low pH have not been assessed so far.

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Here, the whole transcriptome of the seagrass *Cymodocea nodosa*, the only temperate species of a tropical widespread genus, was sequenced and *de novo* assembled using Illumina RNA-Seq technology, with the goal of exploring the large-scale differential expression of transcripts in response to short-term CO_2-driven acidification. This work represents the first transcriptome assembly and annotation for *C. nodosa*, thus significantly expanding the molecular resources available for this species. Furthermore, by demonstrating which biological processes are most sensitive to OA, this research constitutes a major advance in the understanding of seagrass metabolism, in the context of altered seawater chemistry from global climate change.

Materials and methods

Model species and experimental system

Cymodocea nodosa (Ucria) Aschers. is a dioecious, rhizomatous seagrass (Hemminga & Duarte 2000), widely distributed in the Mediterranean basin, and also occurring along the Southern Portuguese and Northwestern African coasts (den Hartog 1970). It grows on both sandy and muddy substrates, and most commonly occurs in shallow waters, although it can reach depths of about 30-40 m (den Hartog 1970). *C. nodosa* is a perennial species, that reproduces both clonally by vegetative propagation (stolonization) and sexually by seed germination (Buia & Mazzella 1991).

For this study, *C. nodosa* ramets (i.e. morphological individuals) were randomly collected in Cadiz Bay Natural Park (SW Spain, 36°32′N 6°17′W) at the end of January 2014, from a shallow-water meadow (1-3 m depth). The distance between sampled plants within the meadow was 4-5 m to ensure sufficient genetic diversity. Special care was taken to limit breakage of rhizome connections. Plants were then transported to the CCMAR (Centre of Marine Sciences) field station at Faro, Portugal in darkened containers filled with seawater.

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On the 1st of February shoots were planted (within 24h after uprooting), in an outdoor facility composed of 10 independent mesocosm tanks (250L each), operating in open-circuit (water flow set to 800 mL/min), each with a 10cm-high sand bottom layer (Fig. S1). About fifteen *C. nodosa* ramets were planted per tank. Each experimental tank was coupled with a header-tank where running seawater was strongly bubbled with either ambient or CO_2-enriched air. The CO_2-enriched air was prepared by injecting pure CO_2 in a 5000L closed mixing tank. The CO_2 partial pressure (pCO_2) in the mixing tank was set at 1200 ppm and continuously monitored by a non-dispersive infrared gas analyzer (IRGA), connected to a PID (Proportional-Integrative-Derivative) digital controller that in turn regulated the operation of a solenoid valve for the injection of CO_2. This type of regulation (using PID control instead of the common on/off switches) allowed for an extremely stable pCO_2 in the mixing tank along the experimental period. The CO_2-enriched air was being prepared in continuous mode and pumped to the header tanks by an industrial grade air blower. The partial pressure of CO_2 in the experimental tanks was allowed to naturally rise at plant's respiration, and to decrease during the day under the effect of photosynthesis (Fig. S1).

High-CO_2 exposure and sampling for transcriptome analysis

Prior to the start of the CO_2 treatment, plants were left to recover from transportation and monitored for five days after transplantation (i.e. tank acclimation). At the end of the acclimation period (t_0), *C. nodosa* was either kept under present day pCO_2 conditions (400 µatm), or under elevated pCO_2 (1,200 µatm), i.e. close to the IPCC 'business as usual' scenario for 2100 (Ipcc 2014). Plant material for RNA extraction and subsequent transcriptome sequencing was sampled at t_0 and after 15 days of exposure to current or elevated pCO_2 (t_1) from three of the five experimental tanks per treatment (Fig. S2). All samples were collected between 11 and 13h to avoid variability from the circadian

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modulation of gene expression, rapidly cleaned of epiphytes and entirely submerged in RNAlater© tissue collection (Ambion, *life technologies*), to inhibit RNA degradation. After allowing the solution to penetrate throughout the tissue for one night at 4°C, leaf samples were definitely stored at -20°C until RNA extraction.

Seawater chemistry

Dissolved oxygen concentration (O_2) in the water (Optode MiniDO2T, PME) and environmental irradiance (Licor LI-190) were continuously measured throughout the experimental period. Water flow, temperature, salinity, pH (NBS scale) and alkalinity were monitored daily at 10-11 am in each mesocosms and header-tank. Alkalinity was estimated by linearization from potentiometric titration following Gran (1952) method. Accuracy (~ 9 $\mu mol \cdot KgSW^{-1}$) was checked using Certified Reference Material (CRM batch #126 and #129; Scripps Institution of Oceanography, La Jolla, CA, USA). DIC system parameters were calculated with the CO2sys.xls program (Pierrot *et al.* 2006) using the dissociation constants of Mehrbach *et al.* (1973) as refitted by Dickson & Millero (1987). Daily data of physico-chemical parameters are shown in Fig. S3, average values for the whole experimental period are reported in Table S1 and S2.

Genotyping and RNA extraction

Before proceeding to RNA extraction, *C. nodosa* samples were genotyped by using seven species-specific polymorphic microsatellite markers (Ruggiero *et al.* 2004). To ensure a sufficient number of distinct genotypes to be used for gene expression analysis, we randomly collected a total of 10 plants at t_0, and 10 plants per each individual tank at t_1, for a total of 70 samples. DNA samples were obtained from individual shoots by cutting off a 4cm-long leaf piece. Leaf tissue was then dried with silica gel (AppliChem) and ground through a Mixer

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Mill MM300 (QIAGEN). Subsequent DNA extraction was carried out using the NucleoSpin® 96 Plant II kit (Macherey-Nagel) following manufacturer's instructions. Microsatellites primers were combined for amplification in a single 7-plex PCR reaction (for details see Table S3). Multiplex PCRs were conducted in 25µL reaction volumes containing 12.5µL Multiplex PCR Master Mix (QIAGEN) and 0.5µL of genomic DNA (6-10ng). Thermal cycling consisted of 15min at 95°C, 35 cycles of 60 s at 94°C, 90 s at 58°C, and 90 s at 72°C, followed by a final extension of 30min at 72°C. PCR products were analyzed on an Automated Capillary Electrophoresis Sequencer 3730DNA Analyzer (Applied Biosystems). Distinct Multi Locus Genotypes (MLGs) were determined with the software Gimlet (Valière, 2002).

Total RNA from the youngest fully developed leaves (second-rank leaves) of genotyped ramets was extracted with Aurum™ Total RNA Mini Kit (BIO-RAD), following manufacturers protocol. About 7cm-long leaf sections were ground to a fine powder with mortar and pestle containing liquid N_2. 700µL of lysis solution (supplemented with 2% (w/v) polyvinylpyrrolidone-40 (PVP) and 1% β-mercaptoethanol) were added to about 100-120mg of powdered tissue. Samples were then homogenized through a Mixer Mill MM300 (QIAGEN) and tungsten carbide beads (3mm) for 3min at 20.1Hz. The quantity and purity of the total RNA was checked using NanoDrop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies) and 1% agarose gel electrophoresis. RNA was used when Abs260 nm/Abs280nm and Abs260nm/Abs230nm ratios were >1.8 and 1.8<x<2, respectively. RNA quality was calculated by measuring the RNA Integrity Number (RIN) with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.); only high quality (RIN >7) RNA was used.

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Library preparation and sequencing

For *C. nodosa* transcriptome sequencing and assembly, 3 genotypes (as biological replicates) at t_0 and 3 genotypes per treatment at t_1, for a total of 9 distinct genotypes, were used. Quality checked RNA samples were sent to Personal Genomics s.r.l. (Verona, Italy) for libraries preparation and sequencing. The nine cDNA libraries were constructed according to the Illumina TruSeq® Stranded mRNA library protocol outlined in "TruSeq® Stranded mRNA Sample Preparation Guide" (Part # 15031047; Rev. E; October 2013), from 2.5µg total RNA. The libraries were subsequently size selected using the Pippin Prep automated gel electrophoresis system (Sage Science) for 350 to 550bp. Paired-end sequencing (100bp × 2) was performed on two separate lanes of the HiSeq™1000 Illumina platform, with an estimated depth of about 160-180millions of reads per lane. Demultiplexing to FASTQ files was performed with CASAVA ver. 1.8.2 using default parameters. Reads not passing the Illumina chastity filter were removed, prior to proceed with the subsequent analyses.

Data filtering and de novo assembly

Raw sequencing data were checked using FastQC (http://bit.ly/1aNGclw), and then cleaned for adaptors and trimmed for quality using Trimmomatic (Bolger *et al.* 2014) with the following parameters: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:1:true SLIDINGWINDOW:3:25 MINLEN:50. Data were then normalized *in silico* and *de novo* assembled using the Trinity pipeline (ver. Trinity_201407) (Haas *et al.* 2013) using the following parameters: SS_lib_type RF --normalize_reads --inchworm_cpu 24 --bflyHeapSpaceInit 24G --bflyHeapSpaceMax 240G —bflyCalculateCPU --CPU 24 --jaccard_clip --min_kmer_cov 2. Jaccard clip was used to mitigate false fusion of transcripts resulting adjacent/overlapping on the genome. The assembling resulted in 171,105 contigs which were then used as a reference to map back the single reads using the software Bowtie

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(parameters: -p 20--chunkmbs 10240--maxins 500--seedlen 20--tryhard-a –S) (Langmead *et al.* 2009). The –a parameter in Bowtie allows to retain all the valid alignments which guarantees that all alternative transcripts from the same gene in a common exon are reported. In order to remove sequences that did not have enough representative reads, and to count reads aligned to each transcript only those transcripts having a count per million (cpm) greater than 1 for at least 2 samples (replicates) were retained.

Functional annotation, differential expression and functional enrichments analysis

Functional annotation of *C. nodosa de novo* generated transcriptome was conducted using the Annocript pipeline (Musacchia *et al.* 2015). The software allows the alignment of the transcripts against known proteins from the UniProt database (The UniProt 2009; The UniProt Consortium 2013). Specifically, Swiss-Prot and UniRef90 proteins (ver. 2014_08) were used with the blastx with parameters: -word_size 4 -evalue 1E-5 -num_threads 20 -num_descriptions 5 -num_alignments 5 -threshold 18. Rpstblastn against the Conserved Domains Database (CDD) (Marchler-Bauer *et al.* 2013) was performed to annotate the domains composition of the putative proteins with the following parameters: -evalue 0.00001 -num_descriptions 20 -num_alignments 20. A blastn against Rfam to align against non-coding RNAs permitted to find possible contaminations. Annocript executes also dna2pep and Portrait to get respectively, from each sequence, the longest ORF (Open Reading Frame) and a non-coding potential score. Based on the best hit, Annocript also associates GO terms (Ashburner *et al.* 2000; The Gene Ontology Consortium 2008), Enzyme Commission identifiers from the ExPASy database (Bairoch 2000), and UniPathways (Morgat *et al.* 2012). The R package “edgeR” (Robinson *et al.* 2010) was used to normalize the expression levels of the transcripts obtained and to select the differentially expressed transcripts between *C. nodosa* under elevated relative to control CO_2 condition. Transcripts were considered

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significantly differentially expressed if the false discovery rate (FDR) ≤ 0.05 and the fold change (FC) $> \pm 2$. A plot of Biological Coefficient of Variation (BCV) to determine variability among biological replicates within our RNA-Seq experiment was also calculated using edgeR. Gene Ontology (GO) and Pathways enrichment analyses were conducted exploiting the Fisher exact test and the FDR correction of the p-values. We selected as significant only GO terms and Pathways showing a different proportion among the differentially expressed genes with respect to the whole transcriptome, showing at least 5 representative transcripts in the whole transcriptome and at least 2 among the differentially expressed ones and an adjusted p-value smaller than 0.05 (FDR 5%) for GO or 0.1 (FDR 10%) for Pathways.

RT-qPCR validation

To verify RNA-Seq results, Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) using Fast SYBR® Green Master Mix (Applied Biosystems) and Viia7 Real Time PCR System (Applied Biosystems) was conducted as described in Mazzuca *et al.* (2013). Six replicates per treatment at t_1, comprising at least 4 distinct genotypes, and independent from those used for the transcriptome analysis, were assayed. Five hundred nanograms of RNA from each sample were retro-transcribed in cDNA with the iScript™ cDNA synthesis kit (BIO-RAD), according to the manufacturers' instructions. All RT-qPCR reactions were conducted in triplicate and contained a 1:5 dilution of the cDNA template. Among transcripts shown to be significantly differentially regulated by RNA-Seq, 10 were selected to have their expression data validated by RT-qPCR. These were genes involved in photosynthesis, protein folding and transport, isoprenoid biosynthesis, translation, autophagy, and ion transport. Primer design and optimization procedures were carried out as outlined in Serra *et al.* (2012) and Dattolo *et al.* (2014). Table 5 lists transcript names, primer sequences

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and percent efficiencies. Relative quantitative analysis of transcripts was obtained using the Relative Expression Software Tool (REST) 2009 ver. 2.0.13. (Pfaffl *et al.* 2002). The REST 2009 software estimates up and down gene regulation by using randomization and bootstrapping techniques. The *eukaryotic initiation factor 4A* (eIF4A), which has been previously tested in *C. nodosa* at different CO_2 condition (Olivé *et al.* 2016), was selected as reference gene for RT-qPCR assays.

Results

Transcriptome sequencing and de novo assembly

An average of 43.5 million reads were obtained for each of the nine sequenced libraries, for a total of 391,433,655 (100 bases, paired-end) reads (Table 1). After raw data were adapter- and quality-trimmed and filtered, remaining reads were used for the *de novo* assembly of *C. nodosa* transcriptome, which resulted in 171,105 contigs. For each sample, the single reads were mapped back to the assembled reference transcriptome thus generated. More than 78% of reads had at least one alignment, while 21.32% failed to align (Table 1). In order to limit sequencing and assembly artifacts we selected only the contigs showing an expression level greater than 1 cpm in at least 2 out of 9 samples, yielding a total of 59,478 unique sequences (Table 2 and S4). Total length of the transcripts was observed to be 136,813,002 bases (136.8 Mb). Distribution of contigs length is shown in Fig. S4. The minimum and maximum sequence length were respectively 202 and 18,275, with a mean sequence length of 2,300 bases, and N50 = 2,279 (Table 2), both values are comparable with the values obtained for *Z. marina* Illumina transcriptome dataset by Kong *et al.* (2014). Transcriptome G-C composition was 41.42 % (Table 2).

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Functional annotation

De novo assembled *C. nodosa* transcriptome was annotated using the Annocript pipeline. In total, 55,343 contigs (93.05 % of all unique contigs) showed at least one hit with an E-value lower than $1.0E^{-5}$ (Table S4). The percentage of transcripts annotated with a Swiss-Prot identifier was 72.68 % (43,231 contigs), while 92.68 % were annotated in UniRef90 (55,126 contigs), and 75.73 % in Conserved Domains (CDD) (45,045 contigs). We also identified 110 putative long non-coding RNAs. The high rate of annotation and classification of the assembled transcriptome represents more than what had been obtained for any other seagrass transcriptome currently available.

Table 3 and Fig. S5 summarize all the results from the functional annotation of the transcriptome. The annotation process also retrieved information on Gene Ontology (GO) terms, metabolic pathways and enzyme codes. GO terms were assigned to 57.39 % (34,133) of contigs (Table 3), including Biological Processes (59.45 %), Molecular Functions (80.82 %), and Cellular Components (60.53 %). Pathway information were obtained for 10.13 % (6,028) of the total number of contigs (Table 3), while enzyme identifiers were assigned to 23.59 % (14,032) of them.

Differential expression analysis

A biological coefficient of variation (BCV) of 0.277 was obtained for our experiment (Fig. S6; Common dispersion = 0.077). In total, 170 transcripts were found significantly differentially expressed (FC > ±2 and FDR ≤ 0.05) in *C. nodosa* under elevated pCO_2 (1,200 μatm) relative to control condition (400 μatm) after 15 days of exposure (t_1), including 153 up-regulated and 17 down-regulated. The full list of differentially expressed genes (DEGs), and their annotation can be retrieved from Table S5.

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Among the DEGs, the most highly expressed in high CO_2 (logFC > 3) were the enzyme *Bifunctional pinoresinol-lariciresinol reductase*, involved in the biosynthesis of plant lignans (logFC = 12.9), three transcripts encoding for as many *Probable LRR receptor-like serine/threonine-protein kinase* and a *Serine/threonine-protein phosphatase*. Six highly expressed transcripts (logFC > 3) were not annotated and thus the functions of their products remain unknown. Conversely, CO_2 enrichment induced a strong down-regulation (logFC < -3) of two transcripts associated to putative retro-elements, both annotated as *Retrovirus-related Pol polyprotein from transposon TNT 1-94* and of the *Zinc finger protein CONSTANS-LIKE 5*.

Gene Ontology and Pathway enrichment analysis

GO enrichment analysis of DEGs was used to identify significantly over-represented functional gene classes with respect to the whole *C. nodosa* transcriptome. We found 47 significantly enriched GO terms (adjusted *p*-value <0.05) that were clustered into "Biological Processes", "Molecular Functions" and "Cellular Components" categories. Figure 1 shows the top 15 significantly enriched GO terms in each GO class division (only the top 15 are shown in case they were more). The most significant Biological Processes were "iron ion homeostasis" (p = 8.21E-42), "ion transport" (p = 1.72E-33), "thiamine biosynthetic process" (p = 2.41E-14), "photosynthesis, light harvesting" (p = 7.03E-14) and "response to bacterium" (p = 3.92E-13) (Fig. 1a). Other relevant over-represented GO categories included "autophagy", "response to biotic stimulus", "tricarboxylic acid cycle", "protein folding", and "gluconeogenesis" (Fig. 1a). Significantly enriched terms in the Molecular Function category were related to "ferric-chelate reductase activity" (p = 1.70E-55), "farnesyltranstransferase activity" (p = 3.49E-16), "3-isopropylmalate dehydratase activity" (p = 1.16E-14), "4 iron, 4 sulfur cluster binding" (p = 1.33E-12), "nutrient reservoir activity" (p = 1.07E-08) (Fig. 1b).

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The most frequently occurring GO terms within Cellular Components included "mitochondrion", "chloroplast", "chloroplast stroma", and "membrane" (Fig. 1c). All significantly enriched GO terms are summarized in Table S6. Pathway enrichment analysis showed that most of the DEGs were enriched in the "isoprenoid biosynthesis" and "carbohydrate biosynthesis" classes (Fig. 2a). All 11 significantly enriched pathways (adjusted p-value <0.1), are summarized in Table S7. Enriched Gene Ontology terms and Pathways in high CO_2 uncovered functional gene classes relevant to fundamental plant metabolic processes, as the formation and breakdown of carbohydrates (i.e. Calvin cycle, glycolysis / gluconeogenesis, tricarboxylic acid (TCA) cycle), the production of plant isoprenoids, protein folding, and ion homeostasis. A graphical representation of major transcript levels changes occurring in *C. nodosa* in response to elevated pCO_2 compared to control conditions, is shown in Fig. 3. In the following sections, we focus on a subset of DEGs within most relevant GO and pathway enriched classes.

Carbohydrate metabolism

Up-regulated transcripts emphasized functions related to carbon metabolism and utilization (Table 4 and Table S5). Most of them encoded components involved in respiratory pathways: glycolysis, tricarboxylic acid cycle and mitochondrial electron transport chain. In particular, we observed the up-regulation of the transcript for the cytosolic *Glucose-6-phosphate isomerase 1,* an essential enzyme of glycolysis that catalyzes the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate, and of a number of genes belonging to the TCA pathway (*Aconitate hydratase*, two *Isocitrate dehydrogenases NADP, 2-oxoglutarate dehydrogenase, Succinyl-CoA ligase ADP-forming subunit beta*). Downstream from the TCA cycle, the mitochondrial *NADH dehydrogenase ubiquinone iron-sulfur proteins 1* and *3*, and *NADH dehydrogenase ubiquinone flavoprotein 1*, which encode for core subunits of the

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mitochondrial NADH:ubiquinone oxidoreductase (Complex I), together with the transcript for the *Gamma carbonic anhydrase 3*, which mediates Complex I assembly, were also significantly induced by elevated CO_2. Equally up-regulated were some transcripts for genes with a function in carbohydrate biosynthesis, associated to the Calvin Cycle (*Ribulose-phosphate 3-epimerase, Ribulose bisphosphate carboxylase small chain 2*), and to gluconeogenesis (*Phosphoenolpyruvate carboxykinase ATP 2*). Chloroplastic *Phosphoglucomutase,* involved in starch biosynthesis, and *Alpha, alpha-trehalose-phosphate synthase*, involved in trehalose biosynthetic process, were also induced under CO_2 enrichment.

Protein folding and repair

Transcripts for a number of genes with a function in protein metabolism were found up-regulated in plants exposed to high CO_2 (Table 4 and Table S5). The majority of them encode molecular chaperones, which assist in *de novo* folding or refolding of stress-denatured proteins, protein transport or proteolytic degradation. Specifically, three up-regulated transcripts (*Heat shock 70 kDa protein 6*, *20 kDa chaperonin, Chaperone protein ClpC1*) mediate the folding of newly translated polypeptides or the degradation of denatured proteins within the chloroplast. Differently, *Heat shock 70 kDa protein* and *Chaperonin CPN60-1* are implicated in mitochondrial protein import and assembly and may prevent misfolding or promote the refolding of polypeptides generated under stress conditions in the mitochondrial matrix. Three transcripts coding for as many putative peptidyl-prolyl cis-trans isomerase (PPIs) were also found to be up-regulated in high-CO_2 plants. PPIases are known to accelerate the folding of proteins, but may also regulate the activity of other proteins. Yet, two distinct subunits of the T-complex protein 1 (*T-complex protein 1 subunit beta*, *T-complex protein 1 subunit delta*), which is known to assist the folding of proteins upon ATP

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hydrolysis, were found among up-regulated genes. Finally, elevated CO_2 also induced the expression of transcripts for *Peptide methionine sulfoxide reductase A* and *26S proteasome regulatory subunit 4* involved in protein repair and degradation; and *ADP-ribosylation factor 1*, a GTP-binding protein which mediate protein trafficking.

Ion and pH homeostasis

Six transcripts for two putative different isoforms of ferric-chelate reductases (NADH) were down-regulated in elevated CO_2 (*Ferric reduction oxidase 4* and *Ferric reduction oxidase 2*) (Table 4 and Table S5). Diverse roles for ferric reductase oxidases (FROs) in both iron and copper metabolism have been highlighted in plants. FRO2 isoforms are responsible for the reduction of extra cellular ferric iron chelates to soluble ferrous iron, whereas FRO4 has been newly shown to act as a copper-chelate reductase to facilitate its uptake from the soil.

Conversely, high CO_2 / low pH induced the expression of transcripts encoding for a number of membrane proteins, such as sodium hydrogen antiporters (*Na^+/H^+ antiporter NhaD)* and proton channels (Vacuolar-type H+ -ATPases) (Table 4 and Table S5), that play an integral role in pH homeostasis of the cells.

Isoprenoid Metabolism

Elevated CO_2 caused transcript level changes of key genes related to plastidic isoprenoids biosynthetic routes (Table 4 and Table S5). Two transcripts encoding for chloroplastic *Geranylgeranyl pyrophosphate synthase* (GPPS) were induced in *C. nodosa* by high CO_2. Similarly, we observed the up-regulation of *1-deoxy-D-xylulose-5-phosphate synthase*, a limiting enzyme for plastidic isoprenoid biosynthesis and essential for chloroplast development. The transcript for a chloroplastic *Geranylgeranyl diphosphate reductase,* which

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catalyzes the reduction of geranylgeranyl diphosphate to phytyl diphosphate, was also found significantly over-expressed in high-CO_2 plants.

Other relevant differentially expressed genes categories

Autophagy-related genes appeared to be responsive to elevated CO_2. Specifically, four transcripts for three putative different isoforms of autophagy-related protein 8 resulted significantly down-regulated (*Autophagy-related protein 8g*, *Autophagy-related protein 8e*, *Autophagy-related protein 8f*) (Table 4 and Table S5). The possible involvement of certain ATG genes in plant carbon metabolism and signaling has been recently suggested, and ATG8e in particular belongs to a core carbon signaling response shared by a large number of *Arabidopsis* accessions (Ren *et al.* 2014; Sulpice *et al.* 2009).

On the contrary, high CO_2 induced the expression of transcripts involved in plant defense responses, such as major latex proteins (*MLP-like protein 423*), that have been associated with pathogen defense responses as well as environmental stresses such as drought, wounding, and oxidative stress, and transcripts related to thiamine biosynthetic process (*Thiamine thiazole synthase 2*, *Phosphomethylpyrimidine synthase*) (Table S5).

Finally, a number of transcripts encoding enzymes involved in different amino-acids biosynthetic routes (e.g. *3-isopropylmalate dehydratase*, *Ketol-acid reductoisomerase*, *Adenosylhomocysteinase*) were also up-regulated in high-CO_2 plants (Table 4 and Table S5).

Verification of RNA-Seq results by RT-qPCR

Quantitative Real-Time PCR was carried out to verify RNA-Seq results. Ten out of the 170 transcripts significantly differentially expressed in high (1,200 μatm) versus control (400 μatm) pCO_2 were chosen for validation, based on their relevance for the physiological processes exhibiting higher variation. Selected transcripts belonged to categories with a

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known role in photosynthesis (*Photosystem II D2 protein*, *Ribulose bisphosphate carboxylase small chain)*, protein folding and transport (*Heat shock 70 kDa protein 6*, *ADP-ribosylation factor 1*, *Peptidyl-prolyl cis-trans isomerase*), isoprenoid biosynthesis (*Geranylgeranyl pyrophosphate synthase*), translation (*Ubiquitin-60S ribosomal protein L40*), autophagy (*Autophagy-related protein*), ion transport (*Ferric reduction oxidase 2*) and chloroplast organization (*Chloroplast stem-loop binding protein of 41 kDa*) (Table 5). Specific primer pairs were established and tested for amplification (Table 5). Figure 4 shows a plot of the Log 2 fold expression changes estimated with RT-qPCR (n = 6), together with the corresponding results obtained from transcriptome analysis. The trend of up- or down-regulation was consistent for all but one of the selected transcripts (Fig. 4), although significant results according to REST 2009 software (Pfaffl *et al.* 2002) were found for 5 out of the 10 transcripts (see hypothesis test *P(H1)* in Table 6). The difference between the two methods is possibly due to the higher number of biological replicates used in the RT-qPCR experiment, and/or to the different sensitivity of the two techniques. However, a highly significant positive correlation was established between the two methods (R = 0.89, Pearson; $p < 0.0006$), confirming the validity of RNA-Seq data.

Discussion

Our RNA-Seq analysis unveil, for the first time, the genome-wide molecular changes that modulate the physiological responses of seagrasses to high CO_2 and low pH.

The enhanced availability of CO_2 was primarily reflected in an increased expression of transcripts associated with carbohydrate metabolism and respiratory pathways. Specifically, a significantly greater abundance of transcripts for enzymes involved in glycolysis, the tricarboxylic acid cycle, and mitochondrial electron transport chain, was found. Although the transcriptome profile by itself cannot predict how gene expression translates into metabolic

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consequences, due to the multiple levels of gene regulation, this provides important evidence for an overall stimulation of the respiratory activity by elevated CO_2 in *C. nodosa* plants.

Leaf respiration is a key determinant of growth and maintenance of plant tissues and the carbon cycle (Amthor 1995), but the underlying mechanisms and the effects of elevated CO_2 have not yet been fully elucidated (Amthor 1991; Davey *et al.* 2004; Drake *et al.* 1999; Gonzalez-Meler *et al.* 2004; Wang & Curtis 2002).

In seagrasses, the effects of CO_2 enrichment on leaf mitochondrial respiration have been rarely addressed. The few existing studies reported a general lack of response in both the short and the long-term. For example, Zimmerman *et al.* (1997) found no significant impact on leaf respiration after 45 days of exposure of the eelgrass *Zostera marina* to elevated CO_2. Similarly, respiration rate was found not to vary with DIC enrichment in the two tropical seagrass species *Cymodocea serrulata* and *Halodule uninervis,* after two weeks of exposure (Ow *et al.* 2015).

On the other hand, our observations are in agreement with molecular and physiological responses of some terrestrial plants when grown at elevated CO_2, such as soybean (Ainsworth *et al.* 2006; Leakey *et al.* 2009), *Arabidopsis thaliana (*Markelz *et al.* 2014*;* Watanabe *et al.* 2014*)*, and tomato (Li *et al.* 2013), where an increased foliar respiratory capacity was driven by a greater abundance of proteins, carbohydrates, and transcripts encoding enzymes throughout the respiratory pathway.

The up-regulation of respiratory metabolism might be supported by an augmented availability of photosynthates from enhanced photosynthesis and increased energy demand for biosynthesis and stress-related processes under elevated CO_2 and low pH. High-CO_2 effects on photosynthesis in plants are largely dependent on the duration of the exposure. In the short-term, the exposure to elevated CO_2 can stimulate photosynthesis and lead to an increased activity of the primary enzyme responsible for CO_2 fixation, Rubisco (Cheng *et al.*

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1998; Pritchard & Amthor 2005). Though, a reduction in photosynthetic capacity, termed "photosynthetic acclimation" (Ludewig & Sonnewald 2000), has also been described, and is accompanied by a reduced expression of genes involved in photosynthesis and CO_2 assimilation (Gupta *et al.* 2005; Kaplan *et al.* 2012; Li *et al.* 2008; Li *et al.* 2006; Taylor *et al.* 2005).

In seagrasses, short and medium term laboratory and mesocosm experiments showed an optimization of photosynthetic performance (e.g. maximum photosynthetic rates, photosynthetic efficiency, and pigment content) in response to CO_2 enrichment (Alexandre *et al.* 2012; Beer & Koch 1996; Campbell & Fourqurean 2013; Jiang *et al.* 2010; Ow *et al.* 2015; Zimmerman *et al.* 1997). Accordingly, here we observed a significant up-regulation of some transcripts related to photosynthesis, including light reaction functions, and CO_2 fixation. For example, the transcript for the subunit *psaK* of the Photosystem I reaction center, transcripts for light-harvesting and electron transport-related proteins (*Ferredoxin-1*), as well as the small subunit of Rubisco (*Ribulose-1,5-bisphosphate carboxylase/oxygenase small chain 2*) and the *Ribulose-phosphate 3-epimerase*, which catalyzes fundamental steps of the Calvin cycle, were all induced in high-CO_2 plants.

Apparently, our findings, contrasts with the idea that *C. nodosa* has a C_4-like mechanisms of carbon fixation (Beer *et al.* 1980). It is assumed, in fact, that C_4 plants are less affected (or not at all) than C_3 species by increasing CO_2, because their photosynthesis is saturated at current atmospheric CO_2 and photorespiration is minimized thanks to the operation of the carboxylating enzyme phosphoenolpyruvate carboxylase (PEPC) (Bowes 1993; Bowes & Ogren 1972; Bowes *et al.* 2002). However, over the last decade, many studies have shown that several C_4 weeds and crop can also significantly increase their photosynthetic rates, growth and total biomass under elevated CO_2 (Cousins *et al.* 2001; Maroco *et al.* 1999; Wand

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et al. 1999; Ziska & Bunce 1997), and also modify gene expression levels (De Souza *et al.* 2008).

The increased respiratory energy demand of *C. nodosa* under simulated CO_2-driven acidification can be attributed to enhanced protein turnover (including degradation and "re-synthesis"), carbohydrate translocation, sucrose synthesis, maintenance of ion homeostasis, and other factors (Bouma *et al.* 1994; Li *et al.* 2013; Noguchi & Yoshida 2008). Accordingly, the GO terms "protein folding" and "ion transport" have been identified as two of the most frequently occurring in our transcriptome dataset.

A considerable number of transcripts encoding molecular chaperones, which assist the folding of newly synthesized polypeptides, refolding of stress-denatured proteins, protein trafficking and proteolytic degradation (Ellis 1987; Hartl *et al.* 2011), in different cellular compartments, was found up-regulated in high-CO_2 plants.

It was of particular interest to observe the induction of a peptidyl-prolyl cis-trans isomerase of the FK506-binding protein class (*FK506-binding protein 1A*), that has recently been shown to modulate intracellular pH homeostasis in *Arabidopsis* (Bissoli *et al.* 2012).

In plants, the components of the pH homeostatic machinery, including intracellular pH sensors, signal-transducing molecules, regulators of cation transport and the most pH-sensitive cellular systems are largely unknown (Felle 2001). However, recent transcriptomic studies have indicated that intracellular acidification increases the expression of several chaperones, resembling heat stress-induced changes in gene expression patterns (Bissoli *et al.* 2012). Therefore, our results confirm that low cytosolic pH, here caused by seawater acidification, generates misfolded proteins and that damage to protein structure may be one of the most important problems induced by intracellular acid stress, as suggested elsewhere (Bissoli *et al.* 2012; Kawahata *et al.* 2006; Mira *et al.* 2010; Schüller *et al.* 2004; Timmins-Schiffman *et al.* 2014). Furthermore, the role of chaperone molecules would be restricted not

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only to assist in refolding proteins, but also in triggering H^+ extrusion, through the activation of H^+-ATPases, to restore intracellular pH (Bissoli *et al.* 2012). In accordance with this, a number of transcripts for membrane proteins, such as sodium hydrogen antiporters (*Na^+/H^+ antiporter NhaD)*, and proton channels (Vacuolar-type H^+ -ATPases) were also found significantly up-regulated. This was already observed in a range of marine organisms under ocean acidification (Evans & Watson-Wynn 2014; Harms *et al.* 2014; Hu *et al.* 2014; Li *et al.* 2016; Pan *et al.* 2015). Interestingly, Na^+/H^+ antiporters in plants have recently been shown to be regulated by distinct Ca^{2+}-dependent mechanisms, based on the Ca^{2+}-binding protein calmodulin (Ranty *et al.* 2006; Yamaguchi *et al.* 2005), whose expression levels were also find to increase in our dataset.

High CO_2 / low pH conditions induced the down-regulation of two putative different isoforms of ferric-chelate reductases (NADH), that have an important role in both iron and copper metabolism (Jain *et al.* 2014). In particular, FRO2 isoforms are involved in the transfer of electrons from the cytosol across the plasma membrane to reduce extra cellular ferric iron chelates to soluble ferrous iron, whereas FRO4 has been recently shown to act as a copper-chelate reductase to facilitate its uptake from the soil (Jain *et al.* 2014). These isoforms are strongly induced by iron and copper limitation, respectively (Connolly *et al.* 2003; Jain *et al.* 2014). Thus, the regulation of ion transport and pH homeostasis appears very likely to be a second important mechanism contributing to the increased energy demand of *C. nodosa* under seawater acidification.

A proportion of the C presumably flowing through the glycolytic pathway was diverted into *C. nodosa* secondary metabolism, and in particular to the synthesis of polyphenols and isoprenoid compounds. In fact, pathway enrichment analysis identified "isoprenoid biosynthesis" as one of the most represented in high CO_2.

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Isoprenoids represent a hugely diverse group of plant metabolites which can function either as primary metabolites, participating in essential cellular processes such as photosynthesis and respiration, or as specialized secondary metabolites (Pulido *et al.* 2012; Vranová *et al.* 2012).

Specifically, we observed the induction of the transcripts for (i) *Geranylgeranyl pyrophosphate synthase* (GPPS), a crucial branch point enzyme that catalyzes the formation of geranylgeranyl pyrophosphate, a precursor of several biochemical pathways including those leading to the biosynthesis of carotenoids, gibberellins, prenyl quinones, chlorophylls, and geranylgeranylated proteins (Okada *et al.* 2000; Takaya *et al.* 2003; Tata *et al.* 2015), (ii) *1-deoxy-D-xylulose-5-phosphate synthase,* involved in the first step of the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway, which synthesizes 1-deoxy-D-xylulose 5-phosphate (DXP) from D-glyceraldehyde 3-phosphate and pyruvate (Estévez *et al.* 2001), which is in turn utilized in plastidic isoprenoids precursors biosynthesis, as well as in the production of thiamin (vitamin B1) (Julliard & Douce 1991), and (iii) and *Geranylgeranyl diphosphate reductase*, that catalyzes the reduction of geranylgeranyl diphosphate to phytyl diphosphate, providing phytol for both chlorophyll and tocopherol synthesis (Keller *et al.* 1998).

Remarkably, the most up-regulated transcript among all DEGs was the enzyme *Bifunctional pinoresinol-lariciresinol reductase*, involved in lignans (-)-hinokinin biosynthesis (Bayindir *et al.* 2008). Lignans are plant phenolic compounds, derived biosynthetically from phenylpropanoids (Moss 2000), and widely distributed among angiosperms and gymnosperms. A variety of biological functions for plant lignans have been documented, including plant defense mechanisms (Harmatha & Dinan 2003; MacRae & Towers 1984; Zhang *et al.* 2014).

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Elevated atmospheric CO_2 is known to trigger the accumulation of plant carbon-based-secondary compounds (phenolics and terpenoids) in terrestrial plants (Bidart-Bouzat & Imeh-Nathaniel 2008; Coley *et al.* 2002; J. Mattson *et al.* 2005; Lindroth 2010; Peñuelas & Estiarte ; Penuelas *et al.* 1997; Stiling & Cornelissen 2007; Valkama *et al.* 2007; Xu *et al.* 2015). This has been frequently explained on the basis of the carbon-nutrient balance hypothesis (Gebauer *et al.* 1997; Karowe *et al.* 1997), which predicts an increase in carbon allocation to secondary metabolism due to increased resources (carbohydrates) availability (Bryant *et al.* 1983; Bryant *et al.* 1987). However, this should not be considered as a simple cause-effect relationship, since the interactions of plants with herbivores and pathogens are a major factor determining variability in plant chemical defenses (Bidart-Bouzat & Imeh-Nathaniel 2008).

Under ocean acidification an accumulation of phenolic compounds has been shown in phytoplankton (Jin *et al.* 2015) and macroalgae (Celis-Plá *et al.* 2015), and this was also already observed in *C. nodosa* (Silva *et al.,* in preparation). On the contrary, Arnold *et al.* (2012) found lower concentrations of phenolic compounds in the seagrass *C. nodosa* growing close to a CO_2 vent site in Vulcano Island. Our results, obtained in a controlled mesocosm system suggest that high CO_2 / low pH conditions trigger accumulations of transcripts involved in the biosynthetic routes of diverse secondary metabolites, leading to an improvement of *C. nodosa* carbon-based chemical defenses.

In summary, we have identified, for the first time, large-scale gene expression changes that occur when a seagrass is exposed to high pCO_2, at levels projected by the end of the century. Our transcriptomic data provide evidence that CO_2-driven OA alters plants primary and secondary metabolite pathways. The up-regulation of respiratory metabolism, supported by an augmented carbohydrates availability, appears to be related to an increased energy demand

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for biosynthesis and stress-related processes under elevated CO_2 and low pH. Changes in the expression of genes related to energy metabolism paralleled the expression of those related to ion / pH homeostasis maintenance and regulation of protein folding. Finally, high *p*CO2 seems to stimulate seagrass chemical defense, as suggested by the up-regulation of enzymes involved in the synthesis of polyphenols and isoprenoid compounds.

By demonstrating which biological processes are most sensitive to OA, this research constitutes a major advance in the mechanistic understanding of seagrass metabolism, in the context of altered seawater chemistry and global climate change. Future studies should be designed to assess the interspecies differences in gene expression responses amongst seagrasses and in the long-term, and to integrate gene expression with physiological data in order to better understand how gene transcription translates into metabolic consequences. Furthermore, other environmental conditions such as light and nutrients, could vary species response to CO_2 enrichment and low pH and this deserves further examination.

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Data Accessibility

Supporting sequence data are available in the ENA (European Nucleotide Archive) repository (http://www.ebi.ac.uk/ena) under Accession numbers: HADH01000001-HADH01059478.

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Author Contributions

J.S., G.P., R.S. (Rui Santos), and I.B., planned and designed the research, J.S., I.O., M.M.C., and M.R. performed the sampling campaign, mesocosms maintenance, sample collection and seawater chemistry measurements, M.R. carried out the molecular work and interpretation of data, R.S.(Remo Sanges) and F.M. performed all the bioinformatic analyses. M.R. wrote the manuscript with the help of all other co-authors.

Figure captions

Figure 1. GO enrichment analysis. Top 15 GO terms (y axis labels; adjusted *p*-value <0.05) associated to DEGs in high pCO_2, clustered as "Biological Process" (a), "Molecular Function" (b) and "Cellular Components" (c), and enriched respect to the whole transcriptome (grey bars). The full list of 47 enriched GO terms can be retrieved from Table S6.

Figure 2. Pathways enrichment analysis. Top 15 pathways (y axis labels; adjusted *p*-value <0.1) associated to DEGs in high pCO_2, clustered as "pathways level 1" (a) and "pathways level 2" (b), and enriched respect to the whole transcriptome (grey bars). The full list of 11 enriched pathways can be retrieved from Table S7.

Figure 3. Graphical representation of major transcript levels changes occurring in *C. nodosa* under elevated pCO_2 (1,200 µatm), compared to control conditions (400 µatm). Each colored square represents the statistically significant treatment response (FC > ±2 and FDR ≤ 0.05) of a unique transcript encoding an enzyme or a protein subunit. Detailed information on selected transcripts and their annotation are provided in Table 4. The full list of DEGs can be retrieved from Table S5. C: chloroplast; CYT: cytosol; M: mitochondrion; ER: endoplasmic reticulum; Nc: nucleus.

Figure 4. Verification of RNA-Seq results by RT-qPCR. Log 2 (high CO_2 / control CO_2) values for ten transcripts at the end of the experiment (t_1): c45675_g1_i1, c45592_g3_i6, c37061_g3_i1, c44949_g7_i1, c12412_g1_i1, c30548_g1_i1, c31115_g1_i3, c33790_g1_i1, c23065_g1_i1, c35362_g1_i1. Transcripts annotation and primer sequences are listed in Table 5. RT-qPCR data are presented as mean ± SD for n = 6. For significant values according to REST 2009 analysis (*P(H1),* see Table 6).

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Supporting information captions

Figure S1. Example of daily variation of *p*CO2 in reference and high-CO_2 experimental tanks.

Figure S2. Scheme of the experimental system.

Figure S3. Daily variation of physico-chemical parameters measured in the mesocosms during the acclimation and the experimental period for both present day pCO_2 conditions (Tank Ref) and elevated pCO_2 (Tank CO_2) conditions. (a) pH; (b) Salinity; (c) Temperature; (d) Total Dissolved Inorganic Carbon (DIC); (e) Total Alkalinity.

Figure S4. Distribution of contig lengths.

Figure S5. Transcript annotation information. (a) Histogram of e-values for UniRef and SwissProt databases, respectively; (b) Closer organisms; (c) Distribution of hit and query coverages for UniRef and SwissProt databases; (d) Histogram of longest ORFs length.

Figure S6. Plot of biological coefficient of variation (BCV), calculated in edgeR, either assuming a common value of dispersion (red), trended value (blue) or tagwise (black), as a function of the average log counts per million of mapped reads.

Table S1. Average values of physico-chemical parameters measured in the mesocosms during the acclimation and the experimental period for both present day pCO_2 conditions (Tank Ref) and elevated pCO_2 (Tank CO_2) conditions. Data are mean ± se.

Table S2. Average values of physico-chemical parameters measured in the header-tanks during the acclimation and experimental period for both present day pCO_2 conditions (Ref) and elevated pCO_2 (CO_2) conditions. Data are mean ± se.

Table S3. PCR conditions for multiplexing. All primers were assembled in the same multiplex (7-plex). Concentration, microsatellite repeat-motifs, and fluorescent dye are provided for each primer.

Table S4. Full annotation of *C. nodosa de novo* generated transcriptome. For each contig, length, first HSP result (lowest e-value), as given from the BLASTx output against Swiss-Prot and UniRef90, and related description are reported. Domain composition of putative proteins, as given by Rpstblastn against the Conserved Domains Database (CDD) is also indicated. For each sequence, the longest ORF (Open Reading Frame) and a non-coding potential score are specified. Annocript also associates the best scored putative proteins to GO terms, Enzyme Commission identifiers from the ExPASy database, and UniPathways.

Table S5. Full list of significantly DEGs (FC > ±2 and FDR ≤ 0.05) in *C. nodosa* under elevated pCO_2 (1,200 µatm) relative to control condition (400 µatm) after 15 days of exposure (t_1) and their annotation.

Table S6. Results of Gene Ontology (GO) enrichment analysis. GO terms with at least 5 transcripts and adjusted *p*-value smaller than 0.05 were considered as significant. The analysis shows the GO terms as “Biological Processes”, “Molecular Functions”, and “Cellular Components” enriched in high CO_2, respect to the whole *C. nodosa* transcriptome.

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Table S7. Results of Pathway enrichment analysis. Pathways with at least 5 transcripts and adjusted *p*-value smaller than 0.1 were considered as significant. The analysis shows the Pathways as “pathways level 1”, “pathways level 2”, “pathways level 3” enriched in high CO_2 respect to the whole *C. nodosa* transcriptome.

Table 1. Read number and alignment summary

Category	**Statistics**
Total number of reads	391,433,655
Mean number of reads per sample	43,492,628
Reads aligned	308,098,530
% Reads aligned	78.7
Reads not aligned	83,335,125
% Reads not aligned	21.3

Table 2. Assembly statistics

Category	**Statistics**
Total number of sequences	59,478
Maximum sequence length (in bases)	18,275
Minimum sequence length (in bases)	202
Mean sequence length (in bases)	2,300
N50 value	2,279
Total transcripts length (in bases)	136,813,002
Mean percentage of N	0.00
Mean percentage of GC	41.42

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Table 3. Transcript annotation information

Category	**Statistics**
Total number of annotated sequences	55,339
Number of hits against Swiss-Prot	43,231
Number of hits against UniRef90	55,126
Number of hits against Conserved Domains Database	45,045
Number of hits with Enzyme Commission identifiers	14,032
Number of hits with Gene Ontology terms	34,133
Number of hits with Pathway information	6,028
Number of non-coding sequences	110

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Table 4. Selected differentially expressed genes (FC > ±2 and FDR ≤ 0.05) in *C. nodosa* under elevated pCO_2 (1,200 µatm) relative to control condition (400 µatm) after 15 days of exposure. The full list of DEGs can be retrieved from Table S5.

Description	Transcript Name	logFC high CO_2 / control CO_2	FDR
Photosynthesis (light reactions)			
Photosystem I reaction center subunit psaK, chloroplastic	c12206_g1_i1	2.4599	0.0424
Chlorophyll *a-b* binding protein of LHCII type I, chloroplastic	c47634_g4_i3	2.6363	0.0189
Chlorophyll *a-b* binding protein of LHCII type I, chloroplastic	c47634_g4_i9	2.1861	0.0477
Photosystem II CP43 chlorophyll apoprotein	c37061_g3_i1	-1.1605	0.0222
Ferredoxin-1	c39669_g1_i1	2.8330	0.0209
Calvin cycle			
Ribulose-1,5-bisphosphate carboxylase/oxygenase small chain 2, chloroplastic	c35362_g1_i1	2.4538	0.0470
Ribulose-phosphate 3-epimerase, chloroplastic	c33844_g1_i1	2.4392	0.0125
Glycolysis / Gluconeogenesis			
Phosphoenolpyruvate carboxykinase ATP 2	c47944_g1_i3	2.6590	0.0246
Glucose-6-phosphate isomerase 1, cytosolic	c37969_g1_i1	2.6553	0.0047
TCA cycle and mitochondrial electron transport chain			
Succinyl-CoA ligase ADP-forming subunit beta, mitochondrial	c27993_g1_i1	2.6561	0.0330
Aconitate hydratase, mitochondrial	c40717_g1_i1	2.5414	0.0246
2-oxoglutarate dehydrogenase, mitochondrial	c23814_g1_i1	2.5234	0.0125
Isocitrate dehydrogenase NADP	c38293_g1_i2	2.6976	0.0096
Isocitrate dehydrogenase NADP	c38293_g1_i1	2.7262	0.0173
NADH dehydrogenase ubiquinone iron-sulfur protein 1, mitochondrial	c35994_g1_i1	2.6406	0.0084
NADH dehydrogenase ubiquinone iron-sulfur protein 3, mitochondrial	c34702_g1_i1	2.6513	0.0189
NADH dehydrogenase ubiquinone flavoprotein 1, mitochondrial	c26326_g1_i1	2.4641	0.0351
Gamma carbonic anhydrase 3, mitochondrial	c34205_g1_i1	2.4965	0.0263
Starch and trehalose biosynthesis			
Phosphoglucomutase, chloroplastic	c28179_g1_i2	2.8284	0.0059
Alpha alpha-trehalose-phosphate synthase UDP-forming 6	c32701_g1_i1	2.7679	0.0054
Cell wall			
Leucine-rich repeat extensin-like protein 6	c32830_g1_i1	2.0365	0.0173
Protein folding, repair and transport			
T-complex protein 1 subunit beta	c24188_g1_i1	2.5719	0.0172
T-complex protein 1 subunit delta	c29725_g2_i1	2.3688	0.0222

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Peptidyl-prolyl cis-trans isomerase	c46125_g5_i2	2.4815	0.0216
Peptidyl-prolyl cis-trans isomerase CYP19-4	c12412_g1_i1	2.5135	0.0405
FK506-binding protein 1A	c49444_g1_i1	2.5054	0.0381
Heat shock 70 kDa protein 6, chloroplastic	c30548_g1_i1	2.3332	0.0351
Heat shock 70 kDa protein, mitochondrial	c39554_g6_i1	2.4936	0.0351
20 kDa chaperonin, chloroplastic	c12765_g1_i2	2.8003	0.0351
Chaperone protein ClpC1, chloroplastic	c27879_g1_i1	2.3389	0.0134
Chaperonin CPN60-1, mitochondrial	c40249_g1_i1	2.3947	0.0479
Probable mediator of RNA polymerase II transcription subunit 37e	c40832_g2_i1	2.6299	0.0153
Peptide methionine sulfoxide reductase A	c12053_g1_i2	2.6702	0.0266
26S proteasome regulatory subunit 4 homolog B	c35440_g1_i1	2.6304	0.0157
ADP-ribosylation factor 1	c44949_g7_i1	2.5128	0.0351
Ion and pH homeostasis			
Ferric reduction oxidase 4	c45675_g1_i2	-1.7758	0.0100
Ferric reduction oxidase 2	c45675_g1_i1	-1.5056	0.0027
Ferric reduction oxidase 2	c45675_g1_i5	-1.4763	0.0027
Ferric reduction oxidase 2	c45675_g1_i4	-1.4898	0.0027
Ferric reduction oxidase 2	c45675_g1_i6	-1.4961	0.0027
Ferric reduction oxidase 2	c45675_g1_i7	-1.5000	0.0027
V-type proton ATPase catalytic subunit A isoform 1	c31286_g1_i1	2.7444	0.0329
Vacuolar proton ATPase a2	c32906_g1_i2	2.6164	0.0330
Vacuolar proton ATPase a3	c32906_g1_i1	2.5246	0.0351
Na^+/H^+ antiporter NhaD	c23761_g1_i1	2.8814	0.0282
Isoprenoids and polyphenols biosynthesis			
Geranylgeranyl pyrophosphate synthase, chloroplastic	c31115_g1_i2	2.4981	0.0312
Geranylgeranyl pyrophosphate synthase, chloroplastic	c31115_g1_i3	2.4820	0.0351
1-deoxy-D-xylulose-5-phosphate synthase 1, chloroplastic	c36479_g1_i1	2.8260	0.0246
Geranylgeranyl diphosphate reductase, chloroplastic	c29233_g1_i1	2.5222	0.0351
Bifunctional pinoresinol-lariciresinol reductase	c955_g1_i2	12.9033	0.0330
Autophagy			
Autophagy-related protein 8g	c45592_g3_i6	-2.2564	0.0001
Autophagy-related protein 8e	c45592_g3_i5	-1.0704	0.0134
Autophagy-related protein 8e	c45592_g3_i4	-1.0573	0.0153
Autophagy-related protein 8f	c45592_g3_i1	-1.9047	0.0000
Amino-acid biosynthesis			

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3-isopropylmalate dehydratase large subunit	c8458_g1_i1	2.7535	0.0209
3-isopropylmalate dehydratase small subunit 3	c34359_g1_i2	2.4252	0.0222
Ketol-acid reductoisomerase, chloroplastic	c37400_g1_i1	2.5911	0.0220
Adenosylhomocysteinase	c46054_g2_i1	2.4565	0.0337
Thiamine biosynthesis			
Thiamine thiazole synthase 2, chloroplastic	c44350_g2_i1	2.7317	0.0208
Phosphomethylpyrimidine synthase, chloroplastic	c37164_g1_i1	2.8190	0.0437
DNA Integration			
Retrovirus-related Pol polyprotein from transposon TNT 1-94	c48028_g1_i2	2.8940	0.0027
Retrovirus-related Pol polyprotein from transposon TNT 1-94	c45874_g1_i4	-7.5633	0.0027
Retrovirus-related Pol polyprotein from transposon TNT 1-94	c48028_g1_i4	2.7435	0.0034
Retrovirus-related Pol polyprotein from transposon TNT 1-94	c45874_g1_i15	-4.8290	0.0045
Retrovirus-related Pol polyprotein from transposon TNT 1-94	c47814_g1_i2	-1.8422	0.0125

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Table 5. List of transcripts used for transcriptome validation via RT-qPCR

Transcript name	Description	Primer (5'- 3')	S (bp)	*E* (%)	R^2
c45675_g1_i1	Ferric reduction oxidase 2	F:ATCTCCACCACTCGTCCAAC R:GGTTCGCTTTCTCGAGTGAC	167	94%	0.999
c45592_g3_i6	Autophagy-related protein	F:AGTTTCAGGCAAGAGCATGAC R:CTGCCTTCTCCACGATCACT	100	100%	0.998
c37061_g3_i1	Photosystem II D2 protein	F:CCGCTTTTGGTCACAAATCT R:CGGATTTCCTGCGAAACGAA	162	100%	0.986
c44949_g7_i1	ADP-ribosylation factor 1	F:TCAACGTCGAGACAGTCGAG R:CGCTCACGATCATTGCTATC	145	97%	0.999
c12412_g1_i1	Peptidyl-prolyl cis-trans isomerase	F:CACTTCGAAGGCTCCATGTT R:ACAAGTACCCAGGACCAACG	157	92%	0.981
c30548_g1_i1	Heat shock 70 kDa protein 6 chloroplastic	F:GAGGTTGTTGCTCTTGGTGC R:GATGTTGGGAGGGTGGTGTT	158	100%	0.979
c31115_g1_i3	Geranylgeranyl pyrophosphate synthase	F:CGAGTGCATGTGCTATGGAA R:GGGCAACATCTTCACCGTAT	129	100%	0.980
c33790_g1_i1	Chloroplast stem-loop binding protein of 41 kDa b chloroplastic	F:AAGGCATGTGCTGAGGCTAT R:AGTCCATACTCAGGCCTCCA	167	100%	0.985
c23065_g1_i1	Ubiquitin-60S ribosomal protein L40	F:GTGGAGGCCTCAGACACAAT R:TGTAGTCCGCAAGTGTCCTG	130	100%	0.985
c35362_g1_i1	Ribulose-1,5-bisphosphate carboxylase/oxygenase small chain 2 chloroplastic	F:GCCCACACAACAACAAGATG R:GGCTCTTGTCCTGGACGTAA	156	98%	0.997

S: base pair; E: percent efficiency; R^2: correlation coefficient.

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Table 6. Relative Expression Report by REST 2009

Contig Name	Expression	Std. Error	95% C.I.	*P(H1)*	Result
c45675_g1_i1	1.245	0.550 - 3.290	0.379 - 7.137	0.600	
c45592_g3_i6	0.24	0.160 - 0.378	0.115 - 0.491	**0.001**	**DOWN**
c37061_g3_i1	0.841	0.428 - 1.879	0.218 - 2.933	0.590	
c44949_g7_i1	2.989	0.856 - 10.739	0.439 - 22.019	0.072	
c12412_g1_i1	2.371	1.166 - 4.888	0.671 - 6.779	**0.032**	**UP**
c30548_g1_i1	2.197	0.997 - 4.465	0.608 - 8.241	**0.043**	**UP**
c31115_g1_i3	1.739	0.495 - 5.759	0.321 - 9.194	0.286	
c33790_g1_i1	2.472	0.757 - 6.421	0.477 - 17.181	0.087	
c23065_g1_i1	2.778	0.968 - 7.090	0.539 - 18.493	**0.049**	**UP**
c35362_g1_i1	2.61	1.014 - 6.303	0.661 - 17.026	**0.045**	**UP**

P(H1): Probability of alternate hypothesis that difference between sample and control groups is due only to chance. Significantly differentially regulated contigs (P(H1)< 0.05) are indicated in bold. P(H1) < 0.1 are underlined.

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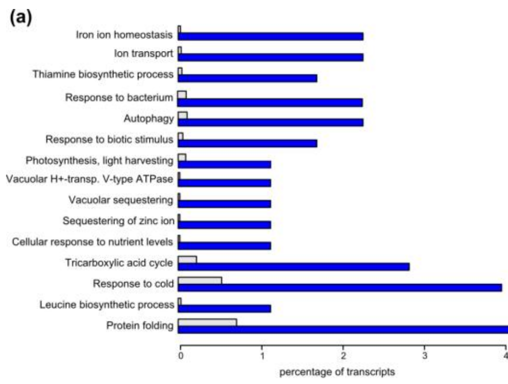

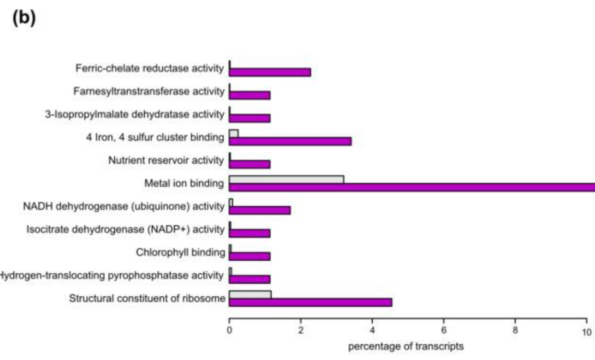

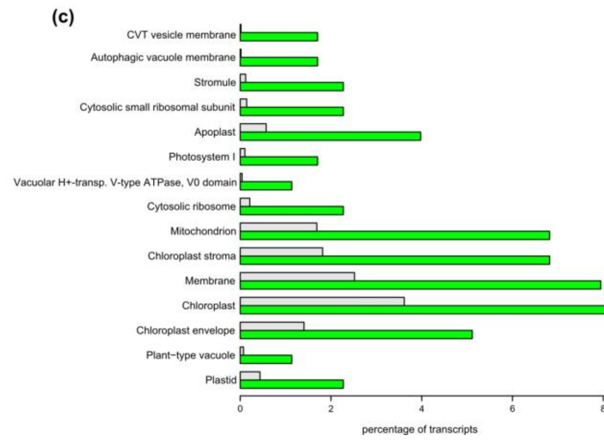

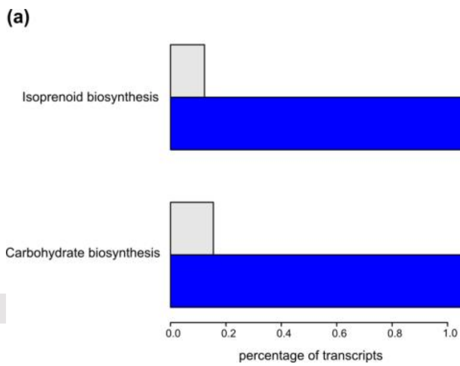

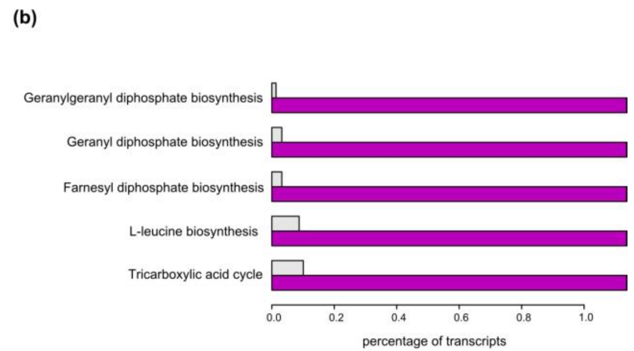

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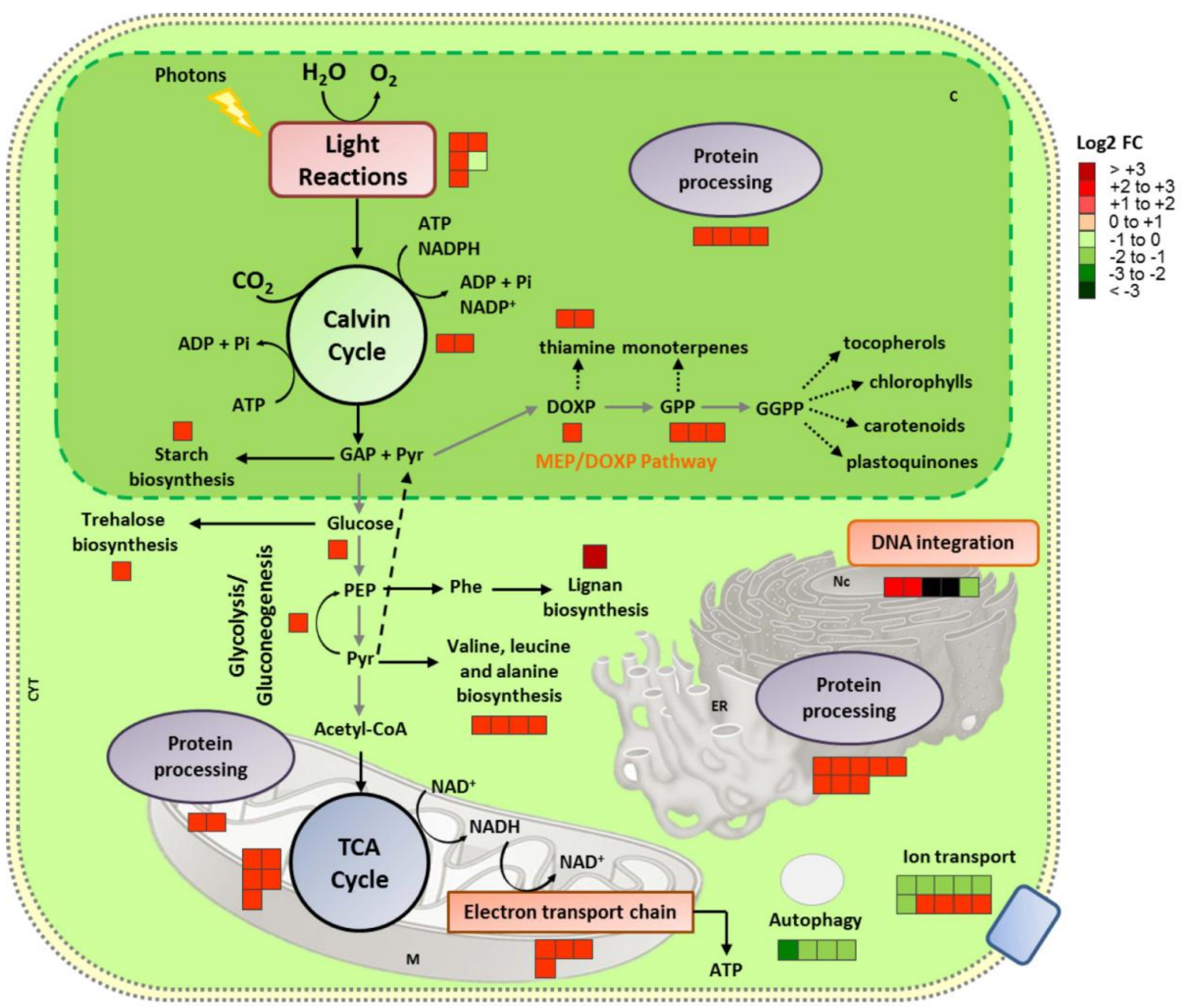

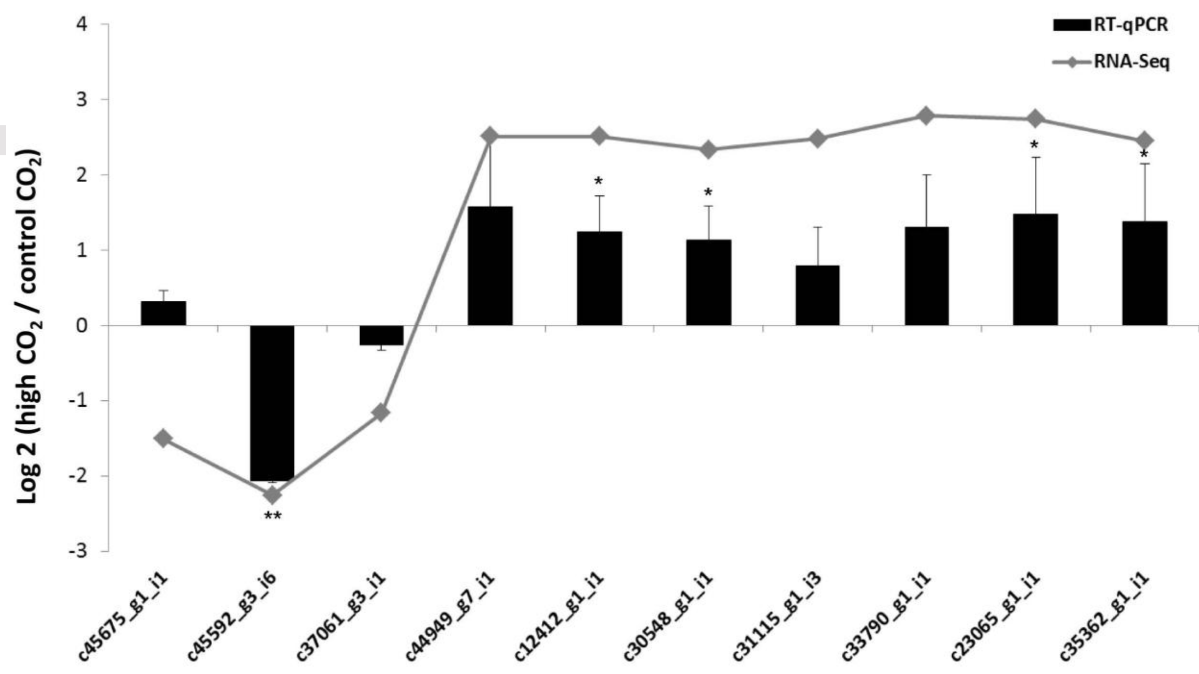

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