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Molecular response of *Sargassum vulgare* to acidification at volcanic CO_2 vents: insights from de novo transcriptomic analysis

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Abstract

Ocean acidification is an emerging problem that is expected to impact ocean species to varying degrees. Currently, little is known about its effect on molecular mechanisms induced in fleshy macroalgae. To elucidate genome wide responses to acidification, a transcriptome analysis was carried out on *Sargassum vulgare* populations growing under acidified conditions at volcanic CO_2 vents and compared with populations in a control site. Several transcripts involved in a wide range of cellular and metabolic processes were differentially expressed. No drastic changes were observed in the carbon acquisition processes and RuBisCO level. Moreover, relatively few stress genes, including those for antioxidant enzymes and heat-shock proteins, were affected. Instead, increased expression of transcripts involved in energy metabolism, photosynthetic processes and ion homeostasis suggested that algae increased energy production to maintain ion homeostasis and other cellular processes. Also, an increased allocation of carbon to cell wall and carbon storage was observed. A number of genes encoding proteins involved in cellular signalling, information storage and processing and transposition were differentially expressed between the two conditions. The transcriptional changes of key enzymes were largely confirmed by enzymatic activity measurements. Altogether, the changes induced by acidification indicate an adaptation of growth and development of *S. vulgare* at the volcanic CO_2 vents, suggesting that this fleshy alga exhibits a high plasticity to low pH and can adopt molecular strategies to grow also in future more acidified waters.

Keywords: brown algae, climate change, CO_2 vents, fleshy macroalgae, molecular response, mRNA-Seq, ocean acidification

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Introduction

The anthropogenic emission of carbon dioxide (CO_2) in the atmosphere has increased in the postindustrialization era and it is still accelerating. Over the last 200 years, oceans have absorbed a major portion of these emissions, acting as an efficient sink for CO_2 (Sabine *et al.* 2004). However, current CO_2 emission rates exceed the buffering capacity of the oceans and cause a shift of marine carbonate chemistry and a decrease of pH. Consequently, oceanic pH, which has already decreased 0.1 units since last century, might face a drop of extra 0.3–0.5 units by the end of this century, a phenomenon known as ocean acidification (OA; Caldeira & Wickett 2005).

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A wide range of marine organisms is affected by OA, in particular calcifiers are more sensitive than noncalcifying organisms because the reduction of the saturation state of $CaCO_3$ in seawater causes faster dissolution of their skeletons (Kroeker *et al.* 2010). Therefore, the effect of OA may widely affect ocean biology, causing a shift in the community structure and population dynamics (Porzio *et al.* 2011; Kroeker *et al.* 2013; Harvey *et al.* 2014). Besides calcification, OA can interfere with other processes such as photosynthesis. Theoretically, increased CO_2 availability should benefit the growth and primary production of marine plants (Koch *et al.* 2013). Therefore, marine autotrophs which rely solely on aqueous CO_2 or having less efficient carbon concentrating mechanisms (CCM) should benefit from OA. The macroalgae with CCM could also take advantage if they shut down energy requiring active bicarbonate transport and support passive CO_2 diffusion for carbon acquisition (Wu *et al.* 2008). Conflicting results have been reported so far, when short- and long-term effects of elevated CO_2 on macroalgae were tested either separately or in combination with other stressors in the laboratory conditions. For example, among algae belonging to the *Sargassaceae*, photosynthesis was stimulated by short-term exposure to high CO_2 levels in *Hizikia fusiformis*, while it was inhibited in the germlings of *Sargassum henslowianum*, under varying light and temperature conditions (Zou *et al.* 2011; Chen & Zou 2014). In other brown macroalgae, such as *Fucus serratus*, a downregulated CCM activity has been reported at elevated CO_2 conditions and it was hypothesized that the energy saving from CCM was used to increase their growth (Johnston & Raven 1991). However, in other *Ochrophyta* with CCM activity, such as *Laminaria digitata* and *Saccharina latissima*, no increase in growth rate was observed when grown at high-CO_2 conditions (Roleda & Hurd 2012). These results suggest that algal species may respond differently to increased CO_2 levels. Moreover, the decrease in seawater pH caused by elevated CO_2 may affect other biochemical and physiological processes, such as ion homeostasis, energy budget, respiration, nutrient uptake, extracellular enzymatic activities, as well as early life stages of marine autotrophs (Israel & Hophy 2002; Rokitta *et al.* 2012; Roleda *et al.* 2012; Hofmann *et al.* 2013).

Most of the effects of elevated CO_2 and OA have been observed in short-term laboratory experiments on a single isolated species. This hampers the scaling up of the observations to predict the impact in natural environments. Indeed, a gene expression study of the coccolithophore *Emiliania huxleyi* revealed that the results of long-term experiments significantly differed from those obtained with short-term exposure (Benner *et al.* 2013). However, studies of the long-term adaptive response of algae to lowered pH and/or high CO_2 are limited to microalgae (Collins & Bell 2006; Lohbeck *et al.* 2012; Benner *et al.* 2013). Investigations of the long-term adaptive response in macroalgae, such as large habitat-forming species, are difficult to perform in the laboratory, due to their longer life cycle, seasonality and lack of community interactions. In addition, artificial laboratory conditions may cause stress, which could affect the results (Widdicombe *et al.* 2010). Therefore, areas with natural CO_2 vents represent useful experimental systems to investigate the impact of OA on macroalgae in their natural ecosystem. Such sites have been discovered around the globe and few of them have already been used as a 'natural laboratory' to study elevated CO_2/OA (Vizzini *et al.* 2010; Lauritano *et al.* 2015; Linares *et al.* 2015).

Off the Ischia Island along Castello Aragonese, natural underwater vents of volcanic origins release gases, mainly CO_2, causing seawater acidification (Hall-Spencer *et al.* 2008). This is one of the best characterized natural CO_2 vent sites in terms of physicochemical parameters and the presence of autotrophs and associated invertebrate communities (Hall-Spencer *et al.* 2008; Martin *et al.* 2008; Kerrison *et al.* 2011; Porzio *et al.* 2011, 2013; Kroeker *et al.* 2013; Garrard *et al.* 2014; Lauritano *et al.* 2015). Volcanic CO_2 vents acidify the seawater causing a pH gradient with three distinct zones with mean pH of 8.1, 7.8 and 6.7, respectively. In the most acidified area of the vents, the biomass is dominated by fleshy brown algal species, particularly *Sargassum vulgare* C. Agardh (Porzio *et al.* 2011, 2017). *S. vulgare* with its long and complex thallus is a prevalent habitat-forming species along the Mediterranean coast. Its population provides shelter and breeding ground for a large number of associated invertebrate species, mainly molluscs and amphipods (Chemello & Milazzo 2002). This fucoid alga shows synchronous gamete release under calm sea conditions. The negatively buoyant eggs immediately settle down and mating occurs in close vicinity. Moreover, for fucoids and other organisms with direct development, spawning/fertilization and settlement/recruitment processes are tightly coupled (Pearson & Serrão 2006). The dispersal of propagules, gametes and zygotes of many species of fucoid algae is limited to short distance, sometimes within metres, thus limiting the gene flow (Kendrick & Walker 1991). Based on this, it can be assumed that specimens living for decades in our study site with elevated CO_2 and lowered pH conditions could be more isolated and has adopted different mechanisms to persist. Considering that *S. vulgare* is a benthic species, its capability to survive in acidified conditions requires fine coordination at the molecular level which is largely unknown due to the lack of genomic information.

In order to understand how this macroalga can thrive in lowered pH/high CO_2, we investigated the transcriptional pattern of *S. vulgare* under these conditions. Through a next-generation transcriptome sequencing analysis, we compared the global gene expression profile of the population from the Ischia Island with the population growing in a control area at current CO_2 and pH levels. To confirm physiological relevance of these transcriptional differences, we measured the activities of selected enzymes.

Sargassum species, as well as other fucoid algae, provide relevant ecological and economical roles in shallow coastal areas, where they are challenged by increasing anthropogenic pressure. Results from this study will help us to understand the mechanisms used in coping lowered pH environment by habitat-forming macroalgae.

Material and methods

Study site and sample collection

The algae were collected along the coast of the Ischia Island at two locations: Castello Aragonese (acidified site, 40°43.87N, 013°57.78E) and Lacco Ameno (control site, 40°45.35N, 013°53.13E; Fig. 1). Castello Aragonese has peculiar characteristic features due to CO_2 bubbles from volcanic vents, resulting in elevated CO_2 levels and lowered pH. Venting activities at this site date back almost 2000 years (Lombardi *et al.* 2011). The vents release gas comprising of 90.1–95.3% CO_2, 3.2–6.6% N_2, 0.6–0.8% O_2, 0.08–0.1% Ar, 0.2–0.8% CH_4, with no traces of harmful sulphur gases and no rise in the temperature (Hall-Spencer *et al.* 2008). The venting activities are variable at the hour scale, but on average the pH values, where *Sargassum vulgare* occurs, are constantly around 6.7. Almost 6 km northwest from Castello Aragonese, at Lacco Ameno, the unique *S. vulgare* population growing at the same hydrodynamic and physical conditions of the acidified population but at current pH value (8.1) has been found and used as control samples. In both sites, *S. vulgare* grows at the same depth (<1 m depth), in sheltered bays with the same geographical exposure (south–southwest). Salinity around Ischia is typical of Tyrrhenian coastal waters and varies generally between 37 and 38.5 psu (Ribera d'Alcalà *et al.* 2004; Lorenti *et al.* 2005; Kroeker *et al.* 2011). The salinity was 37 ± 0.5 ppt at the time of sample collection in March 2014 at both locations. Hence, depth and salinity were not confounding factors. Temperature was continuously monitored using a HOBO data logger (Onset Comp. Corporation, Bourne, MA, USA) which was positioned at Lacco Ameno and Castello Aragonese throughout the year in 2013, and in March 2014. An underwater PAR spherical sensor QSI-140 (Biospherical Instruments, San Diego, USA) was used to obtain noon PAR irradiances

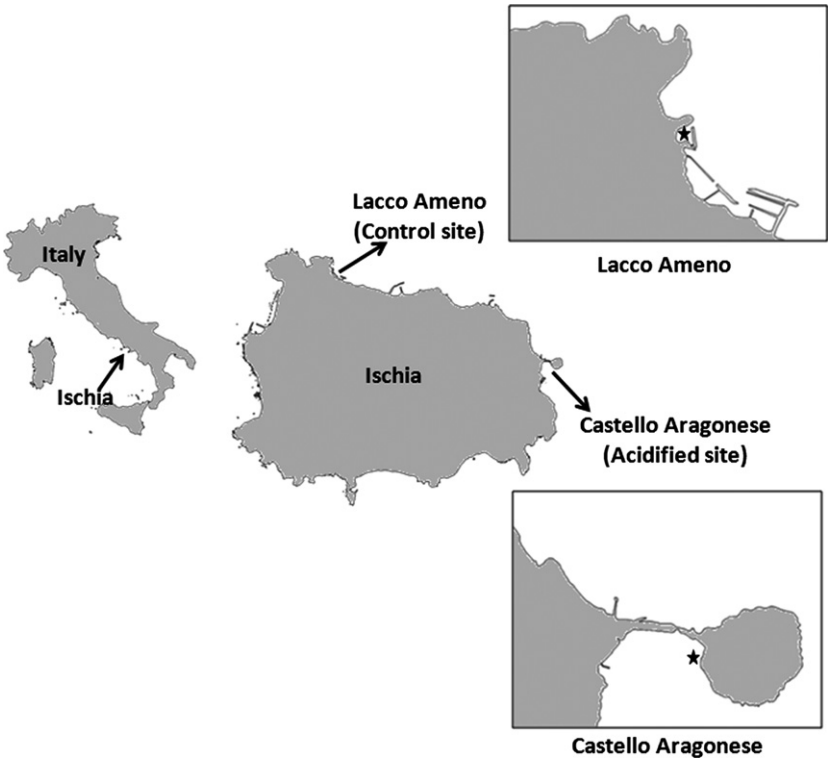

Fig. 1 Study sites around the coast of the Ischia island: Castello Aragonese (CO_2 vents; acidified site) and Lacco Ameno (control site; Image generated through QGIS version 2.12.2).

and determine *Kd* coefficient from the surface to 1 m depth (Lorenti & De Falco 2004).

In order to avoid the influence of unpredictable and difficult to measure environmental factors between the two locations, the samples were collected at same time point (6th March 2014 at midday). A total of nine thalli of similar size (8–10 cm frond length) were handpicked in three different patches in each site along a coastal stretch of 15 m to cover the natural variability of the two local populations. The samples were transported within an hour to the laboratory where apical tissues were cleaned with filtered seawater to remove the visible epiphytes. The samples from each patch were pooled together to create one biological replicate. Samples were then snap-frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction and enzymatic assays.

RNA extraction and library preparation

RNA was extracted using CTAB extraction method (Sim *et al.* 2013) with minor modifications. The frozen algal tissue was ground to powder in liquid nitrogen and cethyltrimethylammonium bromide (CTAB) extraction buffer (100 mM Tris–HCl pH 8.0, 2 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 50 mM DTT, 10% β-mercaptoethanol) was added (1:10 w/v). The mixture was vortexed vigorously and incubated for 10 min at 60 °C followed by chloroform–isopropanol extraction (24:1). The supernatant was collected and precipitated with LiCl for 2 h at −80 °C. The pellet collected after centrifugation at 10 000 ***g*** for 30 min was washed with 80% molecular grade ethanol. Total RNA was treated with DNase (Roche) for 20 min at 30 °C and cleaned using RNease plant mini kit (Qiagen). RNA quality and quantity were assessed by NanoDrop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies Inc., Wilmington, DE, US) and Plant Nano chip using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). About 2.5 μg of total RNA samples with high purity and high integrity (RIN >7.5) was used to isolate poly(A) mRNA for the preparation of Illumina RNA-Seq libraries. The libraries were prepared using TruSeq Stranded Total RNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA), and their quality was checked with high-sensitivity DNA kit (Agilent Technologies).

De novo transcriptome assembly and data analysis

All six libraries (three from acidified and three from control site) were multiplexed and sequenced in a single lane on an Illumina Hiseq 1000 sequencer (Illumina Inc.) generating 100-bp paired-end sequences. Raw reads were prefiltered by removing low-quality reads (>50 bases with quality <7 or >10% undetermined bases). Adapter sequences were clipped from raw reads using Scythe (version 0.98; https://github.com/najoshi/scythe) and were quality-trimmed using Sickle with a PHRED quality score (Q score) minimum threshold of 20 calculated in a window of 10 nucleotides (version 0.94; https://github.com/najoshi/sickle). *S. vulgare* contigs were assembled de novo using Trinity assembler (version 2.0) with default parameters (Grabherr *et al.* 2011) on the two samples separately. Reads were mapped to assembled putative transcripts using bowtie (Langmead & Salzberg 2012). Contigs represented by less than 100 reads were removed. Redundancy of common transcripts among assemblies was removed by CD-HIT-EST clustering of transcriptome sequences with 95% of sequence identity (Li & Godzik 2006). The threshold was empirically selected as the best in clustering sequences with minimal loss of sequencing data mapping and thus representativeness of the reference data set. The expression analysis was performed through RSEM (version 1.1.21) using default parameters, and expression values have been converted to FPKM (Fragments per Kilobase of exon per Million fragments mapped; Roberts *et al.* 2011). Differential expression analysis was performed using EDGER software by setting a log fold change on base 2 (LogFC) threshold ≥1, corresponding to a twofold induction or repression and a false discovery rate (FDR) <0.05 (5%). We performed automatic functional annotation with BLAST2GO (Conesa *et al.* 2005) and Annocript program (Musacchia *et al.* 2015) using NCBI nr as reference database (*E*-value $<10^{-3}$). The Gene Ontology (GO) terms were assigned based on annotation with an *E*-value 10^{-6} and 45% cut-off followed by InterProScan and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis in BLAST2GO. The regulated transcripts with known functions were also classified into pathways using MAPMAN (Thimm *et al.* 2004). The WEGO software was used to classify the transcripts according to their functions (Ye *et al.* 2006).

Quantitative polymerase chain reaction

To validate the RNA-Seq results, the expression level of four genes (two up- and two downregulated) was measured using quantitative PCR (qPCR). Based on transcriptome analysis, the target genes and three reference genes were selected. The list of genes and primers is available in electronic supplementary material (Table S1, Supporting information). Complementary DNAs were synthesized from 1 μg total RNA and qPCRs were performed with ABI ViiA™ 7 Real-Time

PCR System (Applied Biosystems, USA) using FAST START SYBR® green master mix according to the manufacturer's instructions. The following thermal profile was used: 95 °C for 10 min, 1 cycle for cDNA denaturation; 95 °C for 1 s and 60 °C for 20 s, 39 cycles for amplification, and 1 cycle for melting curve analysis (from 60 to 95 °C) to verify the presence of a single product. The qPCR was performed in triplicate for each sample. The relative expressions were measured using REST (Relative Expression Software Tool), and statistical significance was calculated using the Pair Wise Fixed Reallocation Randomization Test (Pfaffl 2001; Pfaffl *et al.* 2002).

Enzymatic activities

Proteins were extracted (Murshed *et al.* 2008) and quantified (Lowry *et al.* 1951) from frozen material. Enzyme activities were determined using existing protocols. Ascorbate peroxidase and monodehydroascorbate reductase were measured according to Murshed *et al.* (2008), peroxiredoxin according to Horling *et al.* (2003), superoxide dismutase according to Dhindsa *et al.* (1981), glutathione S-transferase according to Habig *et al.* (1974), *o*-aminophenol oxidase according to Suzuki *et al.* (2006), glutamine synthetase according to Temple *et al.* (1996), cellulose synthase according to Wood & Bhat (1988), phosphoglycolate phosphatase according to Ames (1966) and cytochrome oxidase according to Goyal & Srivastava (1995). NADH dehydrogenase activity was measured according to Galante & Hatefi (1978) with a modified reaction mixture (1 mL) containing 50 mM phosphate buffer (pH 7.4), 0.1% Triton X-100 (v/v), 1.6 mM potassium ferricyanide, 0.17 mM NADH and 30 μg mitochondrial protein in phosphate buffer. This reaction mixture had slightly lower pH value and contained lower and higher concentrations of NADH and potassium ferricyanide, respectively, as compared to the original protocol. Samples treated with L-3,4-dihydroxyphenylalanine (L-DOPA) to suppress NADH dehydrogenase activity were used as negative controls. The absorbance was measured at 410 nm, and NADH dehydrogenase activity was calculated using an extinction coefficient of 1 mM^{-1} cm^{-1}.

Statistical analysis

Student's *t*-tests were performed in order to assess differences between study sites. The enzymatic data were analysed using SPSS version 21 (SPSS Inc, Chicago, IL, USA). Condition of homogeneity of variance was examined by Levene's test. Independent sample *t*-test was performed on the data to determine the significant difference between the mean values.

Results

Abiotic variables

Temperature at both sites varied annually between 14 and 28 °C, with warmest water occurring in August and coldest in February and March. Annual means of temperature showed no significant differences between the two study sites ($t = 0.96$, $P > 0.05$) or between March monthly means ($t = 0.084$, $P > 0.05$; Fig. S1, Supporting information). Regarding PAR irradiance, the average *Kd* of the two water masses was similar (0.18 and 0.20 at the acidified and the control site, respectively). Therefore, we conclude that temperature and light were not confounding factors.

Transcriptomic coverage and annotation results

Illumina-based RNA-Seq generated a total of 163 962 462 reads with an average of 25 million of reads per sample (87 870 172 and 76 092 290 reads from acidified and control sites, respectively). 148 262 131 (90.4%) were retained after trimming of low-quality reads, and removal of adapter sequences and short reads. These reads were de novo assembled for each of the two different geographically isolated populations separately to accommodate for genotype-specific transcript sequences, generating 126 085 contigs representing putative transcripts in control samples and 262 909 in acidified samples. These putative transcripts were filtered to remove background expression noise and redundancy among samples, following which 53 421 and 73 253 transcripts remained in control and acidified samples, respectively. A nonredundant reference transcriptome was obtained by combining sequences common to the two data sets (Wachholtz *et al.* 2013; Garcia-Seco *et al.* 2015). The filtered reads were mapped back to the reference transcriptome. An average alignment rate of 77.29% was obtained and a total of 67 131 putative transcripts were generated. All raw and processed next-generation sequencing data from this study are available in the NCBI Gene Expression Omnibus under Accession no. GSE78707. The length of these transcripts ranged from 301 to 22 219 bases with an average length of 2237 bases and a median length of 1906 bases. Of the 67 131 transcripts, only those with expression values FPKM ≥0.1 in at least two replicates from either of the two conditions were considered to be expressed and used for differentially expressed genes (DEGs) analysis. This filter returned 18 464 putative transcripts for which functional annotation was generated. The average GC content of the *Sargassum vulgare* expressed transcripts were 50.31% which falls within the range of reported values for *Phaeophyceae* (Le Gall *et al.* 1993). A total of

13 986 (75.75%) was found to have significant hit (*E*-value $<10^{-3}$) with published sequences. The similarity distribution showed that almost 50% of the hits had a similarity of 40–60%, 40% of the hits had a similarity of 60–80% and nearly 10% of the hits had a similarity of 80% or higher with the known sequences in the database. For almost 80% of the transcripts, the first hit was with *E. siliculosus* proteins, the only brown alga for which genome sequence was available at the time of data analysis. At least one GO term was assigned to 9677 transcripts. These transcripts were annotated and GO level four terms, based on the principal categories of cellular component (CC), molecular function (MF) and biological process (BP), are shown in Fig. S2 (Supporting information). KEGG enzyme codes were assigned to 2841 transcripts representing 119 biological pathways. The highest numbers of transcripts were assigned to primary metabolism, in particular to carbohydrate (21%), amino acid (20%), nucleotide (15%), energy (12%), cofactors and vitamins (10%), followed by lipids (6%; Fig. S3, Supporting information). There were 111 (0.6%) long noncoding RNAs which are usually involved in transcriptional regulation. These satisfied the following conditions: (i) length 200 nucleotides, (ii) lack of similarity with any protein, domain and other short ncRNA from Rfam and rRNAs, (iii) longest open reading frame (ORF) <100 amino acids and (iv) noncoding potential score >0.95.

Global change of gene expression under acidified conditions

In lowered pH/high-CO_2 conditions, 380 transcripts of 18 464 (2.05%) were differentially expressed, of which 245 transcripts were upregulated and 135 transcripts were downregulated (FDR $\leq$ 0.05). Among all DEGs, 93 showed no similarity to known annotated proteins and 41 exhibited similarity to proteins of unknown functions. Among the transcripts similar to proteins of known functions, 153 were up- and 93 downregulated. To identify the pathways affected under elevated CO_2/acidified conditions, we performed a GO term enrichment analysis using DEGs as test set and whole transcriptome as background in BLAST2GO. Thirty-two GO terms were enriched including 21 in BP followed by seven in MF and four in CC ($P \leq 0.05$; Fig. 2). Overrepresented GO categories included generation of precursor metabolites and energy, various cellular and metabolic processes, and transport. The number of the transcripts in each enriched GO term is shown in Table S2 (Supporting information). KEGG annotation assigned 98 transcripts (25.7%) belonging to a number

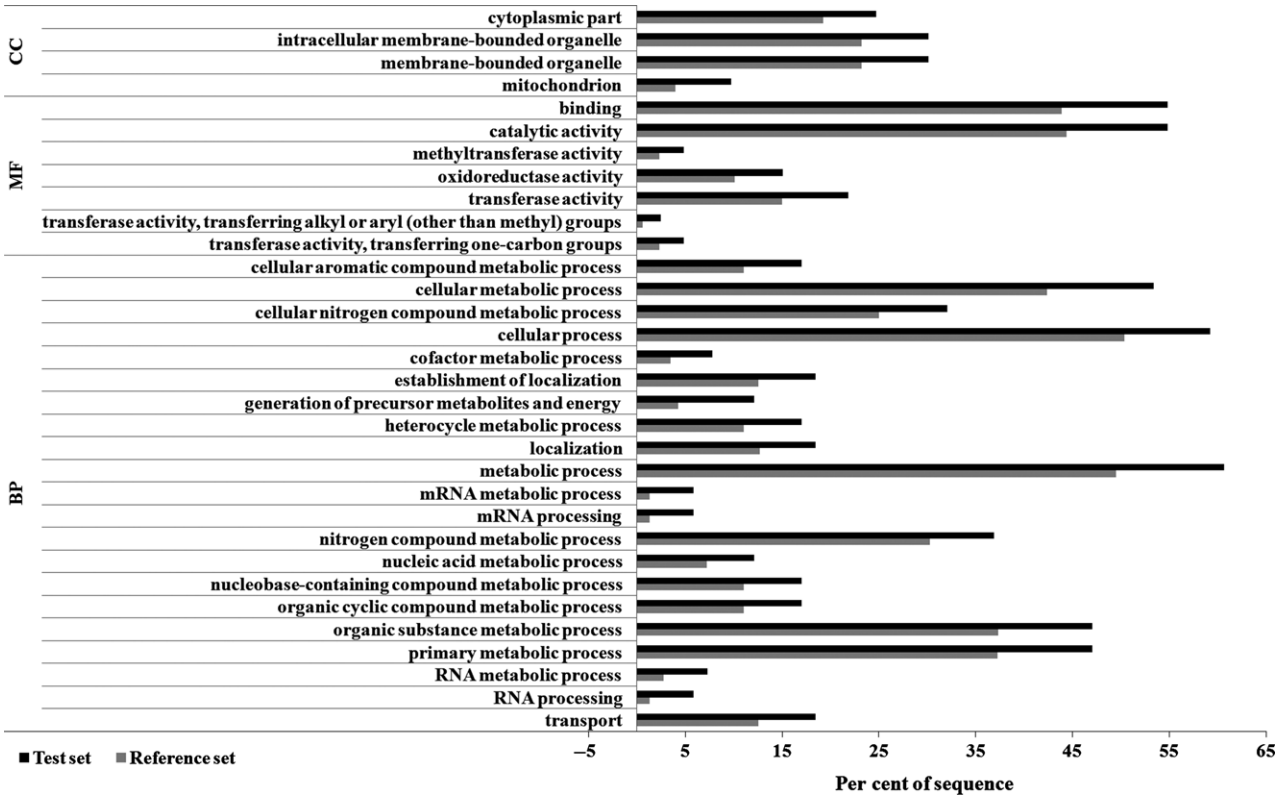

Fig. 2 Overrepresented GO terms under acidified conditions in the three major functional categories, namely biological process (BP), molecular function (MF) and cellular component (CC).

of biological pathways (Fig. 3). The most representatives are related to the metabolism of energy (19%), carbohydrates (18%), amino acids (15%), nucleotides (12%) and lipids (12%). KEGG enrichment analysis indicated that oxidative phosphorylation was overrepresented.

To identify relevant mechanisms involved in OA response, we manually classified DEGs into specific groups according to their putative functions: metabolism, ion transport, cellular stress response mechanisms, information storage and processing, membrane protein and cellular signalling (Table S3, Supporting information). An overview of the biochemical pathways affected by OA has been reported in Fig. 4.

Metabolism. Among the genes encoding enzymes of the carbohydrate metabolism, fructose-1,6-bisphosphate aldolase was upregulated, whereas phosphoglycerate mutase (PGM) and triose phosphate isomerase (TPI) were downregulated. In relation to carbon storage and metabolism, the gene encoding for the enzyme involved in mannitol biosynthesis, for example haloacid dehalogenase-like hydrolase (HAD), was upregulated. A series of transcripts related to cell wall synthesis, for example cellulose synthase (CESA), trehalose phosphate phosphatase (TPP), UDP-D-glucuronic acid epimerase (GAE), cell wall-associated hydrolase (CWAH), membrane-bound sulphotransferase (ST), mannuronan C-5-epimerase (Mc5E) 4 and mannosyl-oligosaccharide alpha mannosidase (MAN), were upregulated, whereas fucose kinase (FK) and Mc5E were downregulated.

Concerning nitrogen metabolism, the gene encoding the enzyme glutamine synthetase (GS) was downregulated under acidified conditions.

The upregulation of the transcripts encoding for mitochondrial electron transport chains and oxidative metabolism genes (FAD-linked oxidoreductase, NADH dehydrogenase, cytochrome oxidases) suggests that energy metabolism was upregulated in the *S. vulgare* population at the acidified site.

A total of 16 transcripts encoding alpha, beta and gamma forms of carbonic anhydrase (CA) were found in the transcriptome; however, only one α-CA was upregulated. On the contrary, the RuBisCO transcript was not differentially expressed between the populations living at acidified and control sites. Furthermore, several transcripts involved in light-harvesting complex and generally photosynthetic process (chlorophyll *a–b* binding proteins, photosystem II S4 domain protein, cytochrome *b6f* complex iron–sulphur subunit, cytochrome *b*) and in pigment biosynthesis (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase) were upregulated. Also phosphoglycolate phosphatase (PGPase), an important photorespiratory enzyme, was upregulated in *S. vulgare* population living at the acidified site.

Ion transport. The expressions of a number of transporters involved in ion fluxes as well as in the transport

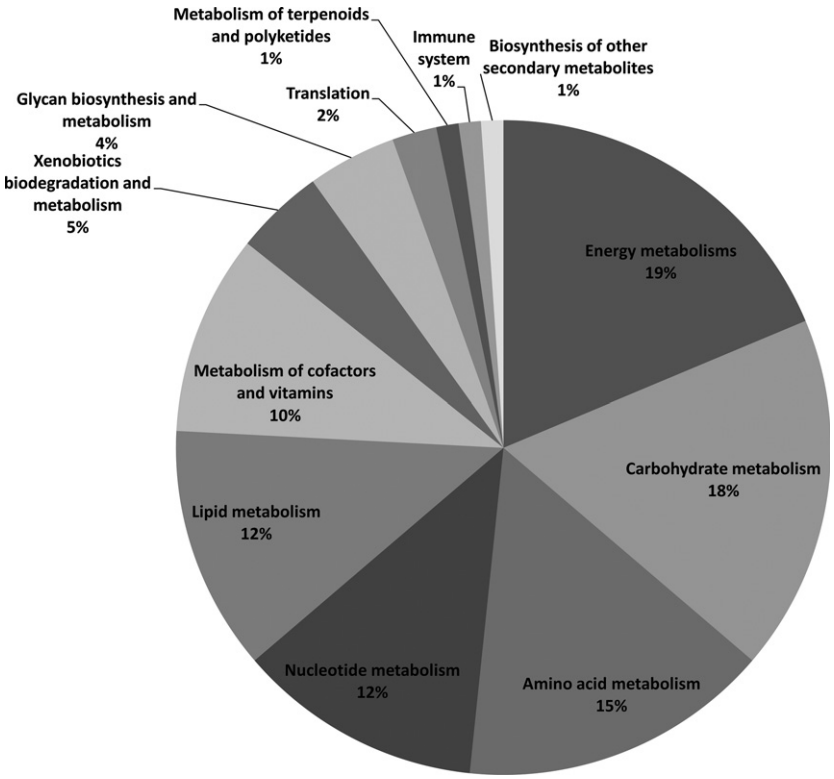

Fig. 3 KEGG biological pathways analysis of differentially expressed genes under acidified conditions.

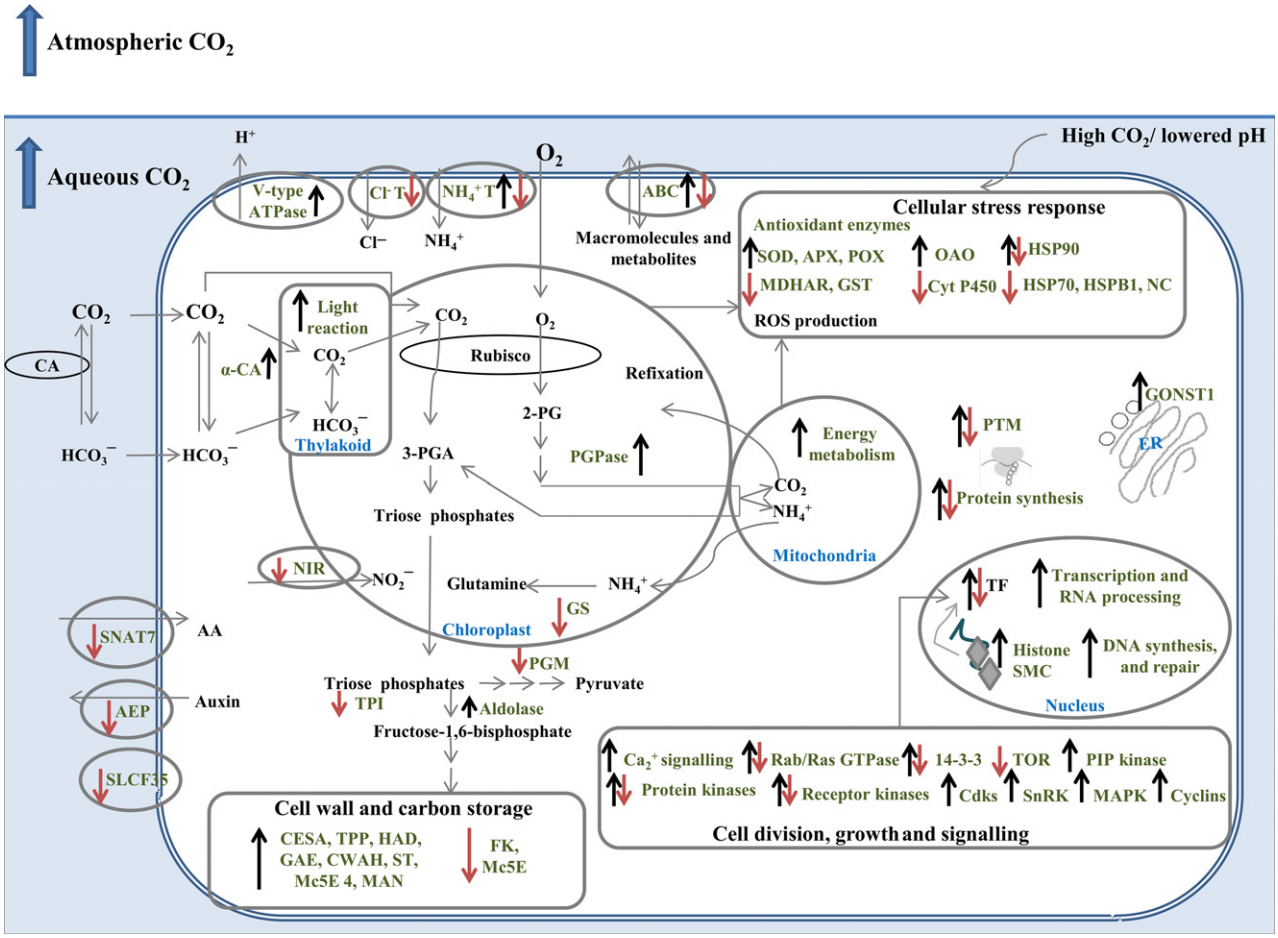

Fig. 4 An overview of the cellular events involved in the response of *Sargassum vulgare* to acidification at natural CO_2 vents. The differential process and enzymes are shown in green font. Black and red arrow represents up- and downregulation, respectively. AA, amino acid; ABC, ABC transporter; AEP, auxin efflux transporter; APX, ascorbate peroxidase; CA, carbonic anhydrase; Cdks, cyclin-dependent kinases; CESA, cellulose synthetase; Cl-T, chloride transporter; CWAH, Cell wall-associated hydrolase; Cyt P450, Cytochrome P450; ER, endoplasmic reticulum; FK, fucose kinase; GAE, UDP-D-glucuronic acid 4-epimerase; GONST1, golgi nucleotide sugar transporter 1; GS, glutamine synthetase; GST, glutathione S-transferase; HAD, haloacid dehalogenase-like hydrolase; HSP, heat-shock protein; HSPB1, protein associated with heat-shock protein 27; MAN, mannosyl-oligosaccharide alpha mannosidase; MAPK, mitogen-activated protein kinase; Mc5E, mannuronan C-5-epimerase; MDHAR, monodehydroascorbate reductase; NC, nuclear chaperones; NH_4^+T, ammonium transporter; NIR, formate/nitrite transporter; OAO, *o*-aminophenol oxidase; PGM, phosphoglycerate mutase; PGPase, phosphoglycolate phosphatase; PIP, phosphatidylinositol phosphatase; POX, peroxiredoxin; PTM, post-translational modifications; SLCF35, solute carrier protein family 35; SMC, structural maintenance of chromosomes; SNAT7, sodium-coupled neutral amino acid transporter; Snrk, SNF1-related protein kinase; SOD, superoxide dismutase; ST, sulphotransferase; TF, transcription factor; TOR, target of rapamycin; TPI, triose phosphate isomerase; TPP, trehalose phosphate phosphatase; V-type ATPase, V-type proton ATPase.

of various metals and metabolites were significantly different between the two sites. These include V-type proton ATPase (V-type ATPase), solute carrier family 35 (SLCF35) and the ABC (ATP-binding cassettes) type (ABC), ammonium (NH_4^+T), formate/nitrite (NIR), Golgi nucleotide sugar (GONST1), voltage-gated chloride channel (Cl–T), sodium-coupled neutral aminoacid (SNAT7) and auxin efflux (AEP) transporters.

Cellular stress response mechanisms. Some of the DEGs were involved in cellular stress response such as those encoding for heat-shock proteins (HSPs) and antioxidant enzymes. The detected HSPs belonged to two different families: HSP90 and HSP70. For HSP90, two transcripts were upregulated, whereas another transcript and two transcripts coding for HSP70 were downregulated. Also, the protein associated with the heat-shock protein 27 (HSPB1), and two other nuclear chaperones (NC) were downregulated. Moreover, few genes encoding proteins with antioxidant activities were differentially expressed. These included upregulated transcripts for ascorbate peroxidase (APX),

peroxiredoxin (POX) and superoxide dismutase (SOD), and downregulated transcripts for monodehydroascorbate reductase (MDHAR) and glutathione S-transferase (GST). Other transcripts likely associated with oxidoreductase activity, that is cytochrome P450 (cyt P450) and ortho-aminophenol oxidase (OAO), were down- and upregulated, respectively.

Information storage and processing. Several transcripts involved in DNA repair and RNA processing were upregulated in *S. vulgare* under acidified conditions. Two transcripts encoding histone proteins and three transcripts for structural maintenance of chromosomes (SMC) gene, likely involved in altering gene expression under high-CO_2/low-pH conditions, were upregulated. Among transcripts encoding transcription factors (TF), some were upregulated while others appeared downregulated.

The expression of several transcripts involved in protein synthesis, including ribosomal structure and biogenesis, was affected. Also some of the transcripts involved in posttranslational modifications (PTM) were differentially expressed. In total, the expression of 16 transcripts encoding elements involved in formation of transposable elements was altered, including 11 upregulated and five downregulated.

Membrane protein and cellular signalling. Among transcripts involved in cell division, growth and signal transduction, a cyclin A and cyclin B were upregulated. In addition, Rab/RasGTPase, various kinases, proteases, phosphatases, 14-3-3 proteins, TOR (the target of rapamycin) and some membrane proteins were differentially expressed.

Validation of gene expression analysis

Four genes were chosen to validate the RNA-Seq results by quantitative real-time PCR: phosphoglycolate phosphatase, sterol methyltransferase, ABC transporter and glutamine synthetase. The expression of these genes, normalized using the three reference genes, 60S ribosomal protein, α tubulin and elongation factor 1 alpha, confirmed the RNA-Seq data (Fig. 5).

Enzymatic activities

The activity of selected enzymes involved in stress response, energy metabolism, cell wall synthesis, photorespiration and nitrogen metabolism was measured to supplement the results of RNA-Seq at a functional level. Consistent with the upregulation of their transcripts, we observed increased activities of superoxide dismutase, ascorbate peroxidase, peroxiredoxin, ortho-aminophenol oxidase, cytochrome oxidase, NADH dehydrogenase, phosphoglycolate phosphatase and cellulose synthase in samples from the acidified site (Fig. 6). Similarly, decreased activity of monodehydroascorbate reductase was found consistent with downregulation at gene level. However, activity of glutathione S-transferase and glutamine synthetase differed with gene expression results.

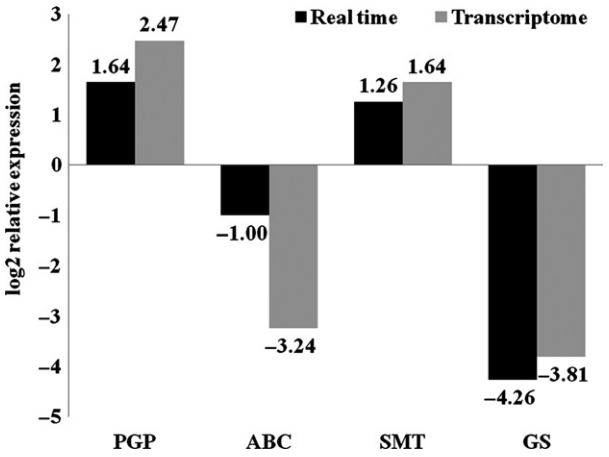

Fig. 5 Comparison of gene expression of selected genes by de novo transcriptome and real-time qPCR. PGP, Phosphoglycolate phosphatase; ABC, ATP-binding cassette transporter; SMT, Sterol methyltransferase; GS, Glutamine synthetase.

Discussion

In this study, we explored the genomewide transcriptomic response to chronic acidification of a habitat-forming fleshy macroalga, *Sargassum vulgare*, in its natural environment. We generated for the first time large-scale genomic information on this macroalga, which could be further used in eco-physiological studies. The sampling was performed once from two locations because the uniqueness of natural CO_2 vents and the lack of other control *S. vulgare* populations at similar environmental conditions did not allow to extend the analysis at a wider spatial scale. However, the number of collected samples along the coastal stretches was high enough to represent the variability of response of local populations. The similarity of the environmental factors between the two sites suggested that the observed molecular response was due to lowered pH/high CO_2.

The analysis of DEGs highlighted the molecular strategies adopted by *S. vulgare* to live at the volcanic CO_2 vents (Fig. 4).

Photosynthetic metabolism

It is commonly believed that increased CO_2 in seawater affects carbon acquisition and fixation. However, our

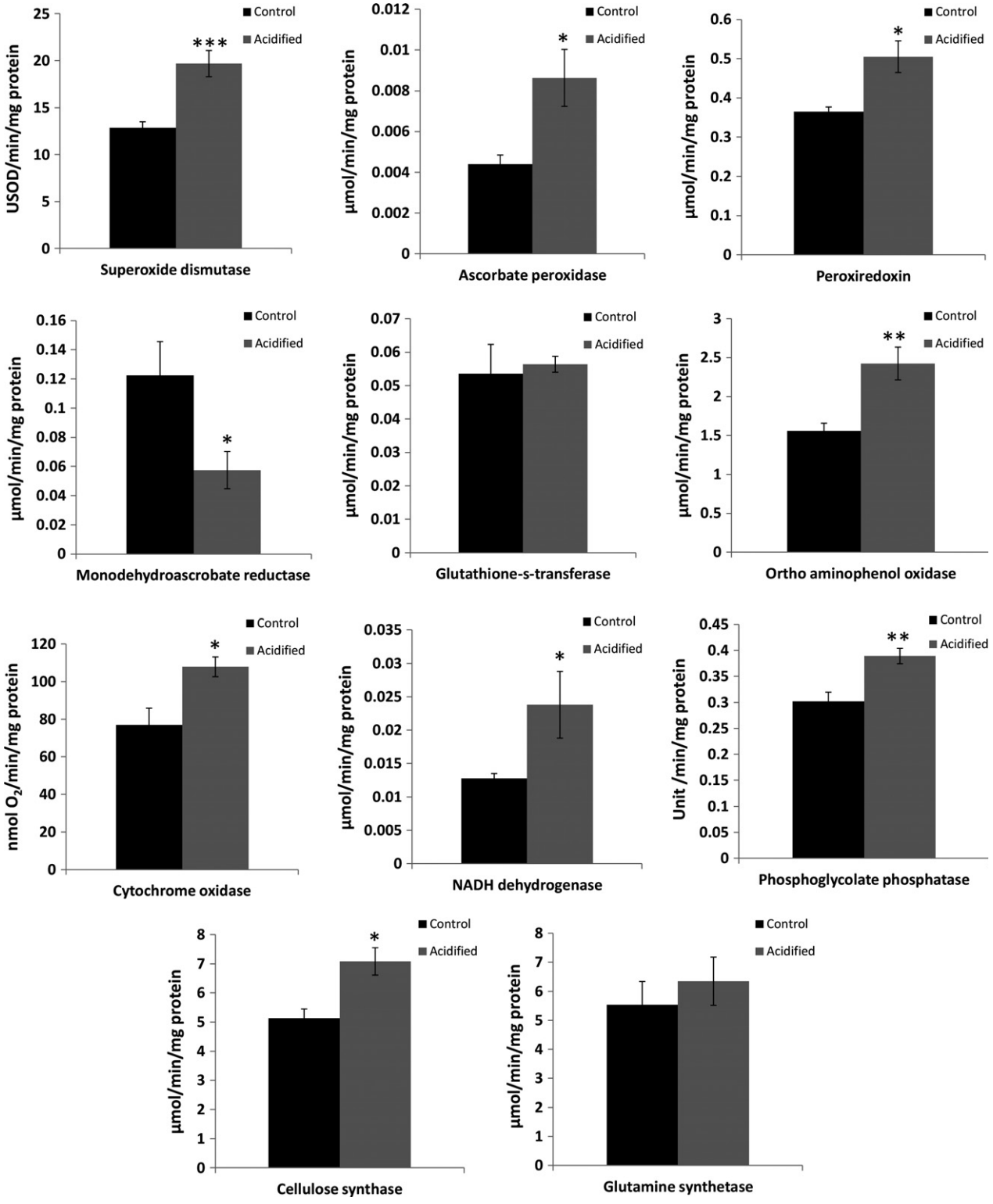

Fig. 6 Activities of selected enzymes involved in the oxidative stress, energy metabolism, photorespiration and cell wall synthesis ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

transcriptome data revealed no drastic changes of the related genes in *S. vulgare* at the acidified site. Indeed, only one transcript for α-CA was upregulated and no differences were observed in RuBisCO expression and bicarbonate ion transporters. The reason for induction of α-CA in high CO_2 compared with ambient CO_2 may

be due to a fine regulation of the gene at the higher pressure of CO_2 in acidified site. Assays of CA activity would advance our knowledge in this issue. The increase of a specific type of α-CA (CAH2) was also reported under high-CO_2 conditions in *Chlamydomonas* (Spalding 2008). Upon exposure to high CO_2 for long term (250 or 500 generations), no differences in RuBisCO and CA expressions were reported in the microalga *E. huxleyi* (Benner *et al.* 2013; Lohbeck *et al.* 2014). Similarly, expression of small subunit of RuBisCO in the diatom *Thalassiosira pseudonana* was also not altered when grown for a long time under increased CO_2 conditions (Crawfurd *et al.* 2011).

Photorespiration can act as a photoprotective mechanism by diverting the excess excitation energy that may lead to reactive oxygen species formation with consequent damage to biomolecules (Takahashi & Murata 2008). Our finding that both the transcription and the activity of the photorespiratory enzyme phosphoglycolate phosphatase were increased in *S. vulgare* at the acidified site suggests that photorespiration is still active under high-CO_2 conditions. However, during photosynthesis, phosphoglycolate phosphatase is also involved in removing inhibitors of Calvin cycle and glycolate metabolism, allowing an increase in carbohydrate production. Noteworthy, in corals a decline in the phosphoglycolate phosphatase was associated with a decrease in the productivity (Anthony *et al.* 2008; Crawley *et al.* 2010).

Considering that in the marine environment, photosynthesis is CO_2 limited, a higher number of autotrophs would lead to a better photosynthetic performance in high-CO_2 conditions. Indeed, the increase of the transcripts related to light-harvesting complex and electron chain of light reactions at the acidified site may indicate the ability to respond to acidification by increasing its energy capture and antioxidative functions (Chen *et al.* 2014).

Carbohydrate metabolism

The regulation of the genes involved in the carbohydrate metabolism (fructose-1,6-bisphosphate aldolase, phosphoglycerate mutase and triose phosphate isomerase) indicated the activation of anabolic pathways (gluconeogenesis, Calvin cycle) instead of the glycolytic catabolic ones. Moreover, the upregulation of haloacid dehalogenase-like hydrolase, which has been shown to have mannitol-1-phosphatase activity in *E. siliculosus* (Groisillier *et al.* 2014), suggested the activation of the pathway leading to the formation of mannitol, one of the main forms of carbon storage in brown algae. Also transcripts related to the formation and structures of cell wall were upregulated together with the increased transcription and enzymatic activity of cellulose synthase. Overall, these results indicate increased allocation of carbon to cell wall and storage in *S. vulgare*, similar to the response of higher plants to elevated CO_2 (Li *et al.* 2008).

Ion homeostasis

More energy may be needed to maintain the pH homeostasis inside the cells (Rokitta *et al.* 2012), which is a critical factor for a range of cellular functions in the algae (Taylor *et al.* 2012). Indeed, in *S. vulgare* living at the acidified site there is the upregulation of V-type proton ATPase which pumps protons across the plasma membrane. In the CO_2-tolerant green microalga *Chlorococcum littorale*, an increase in V-type proton ATPase expression was reported when exposed to high CO_2 levels (Sasaki *et al.* 1999). By contrast, no difference in the V-ATPase expression was found in the sensitive green alga *Stichococcus bacillaris* exposed to similar conditions (Dietz *et al.* 2001). This indicated that *S. vulgare* acquired the tolerance to live in high-CO_2 conditions. Even if evolutionarily distant from macroalgae, in marine invertebrates such as corals and sea urchins, this transporter was downregulated in response to OA in short-term experiments (Todgham & Hofmann 2009; Kaniewska *et al.* 2012). In addition to this transporter, the transcript of H^+ transporter ATP synthase, generally involved in pH homeostasis, was constitutively high in both acidified and control sites. The regulation of many transporters involved in ion fluxes, transport of macromolecules and metabolites (chloride, ammonium, ABC, formate/nitrite, sodium-coupled amino acid, auxin efflux and solute carrier protein family 35 transporters) in *S. vulgare* at the acidified site might be the consequence of the changes in the water chemistry which affects solubility, adsorption, toxicity, and metal redox processes (Millero *et al.* 2009).

Cellular stress response

The antioxidant defence machinery of *S. vulgare* is upregulated, as demonstrated by the increase at transcription and enzymatic levels of superoxide dismutase, ascorbate peroxidase, peroxiredoxin and ortho-aminophenol oxidase, likely to remove reactive oxygen species deriving from the increased oxidative metabolism. This is further confirmed by the upregulation of genes involved in oxidative phosphorylation and increased activities of cytochrome oxidase and NADH dehydrogenase, thus supporting the view that *S. vulgare* under acidified condition exhibits an increased energy demand. The finding that the majority of the transcripts encoding HSP90 were upregulated under acidified

conditions is in contrast with the downregulation of the same genes in the seagrass *Posidonia oceanica* living at Castello Aragonese CO_2 vents (Lauritano *et al.* 2015). On the other hand, the downregulation of HSP70 observed in *S. vulgare* is in accordance with laboratory experiments mimicking climate change scenarios, in which *E. huxleyi* was exposed to acidified conditions for 215 generations (Benner *et al.* 2013). The upregulation of some antioxidant enzymes and HSPs in the algae at the acidified site further indicates that algae are adopting molecular strategies to thrive in lowered pH conditions.

Information processing

A general increase in DNA repair, RNA processing, protein synthesis and posttranslational modifications suggests the relevance of these processes in determining tolerance to acidification in *S. vulgare*. Increased translation and posttranslational modifications under acidified conditions suggest that this species utilizes the metabolic energy in synthesizing proteins and modifying them to live at high-CO_2 conditions (Pan 2013; Pan *et al.* 2015). Interestingly, genes encoding proteins for RNA processing, transcription factors and posttranslational modifications were found to be elevated in *E. huxleyi* strains grown under future ocean conditions for more than 200 generations (Benner *et al.* 2013). The regulation of the transcription of histone proteins along with SMC gene and other transcription factors under acidified conditions could be related to the faster growth of *S. vulgare* at the acidified site, likely due to modification of the chromosome structure and altered gene expression (Hennon *et al.* 2015). The presence of the regulated transcripts for enzymes capable of doing transpositions such as reverse transcriptase indicated that the mobile elements may be involved in generating genetic variability at the acidified site which, in turn, increases the possibility of adaptation under varying environmental conditions (Capy *et al.* 2000).

Cell signalling

The regulation of membrane proteins and cellular signalling pathways further supports the capacity of the algae to acclimatize/adapt to varying environmental conditions. Briefly, the induction of calcium signalling in acidification response is highlighted by the upregulation of a specific transcript predicted in MAPMAN to encode for calmodulin with possible function in thigmomorphogenesis, a complex plant response to abiotic stimuli. Furthermore, the upregulation of transcripts encoding Rab/RasGTPase and MAPK suggests that MAPK signalling cascades may be also involved in mechanism of response to OA, as also demonstrated in intertidal macroalgae (Parages *et al.* 2014). The regulation of cyclic adenosine 3′,5′-monophosphate (cAMP) in *S. vulgare* may contribute to the acclimatization to elevated CO_2 areas, as also reported for the diatom *T. pseudonana* (Hennon *et al.* 2015). Altogether, the cellular signalling components and membrane receptors affected by elevated CO_2/decreased pH seem to contribute towards growth, division and development of *S. vulgare* at the acidified site. Indeed, the canopy of this macroalga looks healthy and flourishing at the CO_2 vents of Castello Aragonese.

Conclusions

The results here reported improve our knowledge about the molecular mechanisms underpinning the adaptive response of the brown alga *Sargassum vulgare* to OA. In the future, the identification of the unknown genes revealed by our RNA-Seq analysis should help to further characterize the pathways involved in the survival of *S. vulgare* at the CO_2 site and predict molecular strategies of other fucoid algae growing at the same environmental stress conditions and long-term acclimation to acidification. However, results may differ in other benthic macroalgae for their different carbon uptake strategies. Our results are relevant considering that some fucoids are endangered species for their high sensitivity to pollution. By contrast, the ability of *S. vulgare* to adapt to acidified conditions at volcanic CO_2 vents suggests that in the future, it could be a successful species in a more acidified scenario. This is ecologically relevant because *S. vulgare* is a habitat-forming species contributing to shaping macroalgal communities and trophic dynamics.

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Conflict of interest

The authors have declared that no competing interests exist.

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A.K., I.C., F.P.P., M.D., A.P. and M.C.B. conceived and designed the experiments. A.K. performed the experiments. A.K., I.C., A.P. and M.C.B. analysed the RNA-Seq data. H.A.E., G.T.S.B. and H.A. performed enzymatic assays. H.A.E. and A.K. analysed enzymatic assays. A.K., I.C., F.P.P., A.P. and M.C.B. drafted the manuscript.

Data accessibility

RNA-Seq raw sequence reads and normalized expression values for each transcript are available through the NCBI Gene Expression Omnibus under Accession no. GSE78707.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 List of genes and primers used in qPCR.

Table S2 Number of transcripts in each enriched GO term.

Table S3 List of differentially expressed genes (DEGs) in different categories.

Fig. S1 Monthly means of temperature in the two sites of Castello Aragonese (acidified site) and at Lacco Ameno (control site).

Fig. S2 GO annotation of annotated genes in GO level 4.

Fig. S3 KEGG biological pathways analysis of whole transcriptome.