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Response of key stress-related genes of the seagrass *Posidonia oceanica* in the vicinity of submarine volcanic vents

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Abstract

Submarine volcanic vents are being used as natural laboratories to assess the effects of CO_2 on marine organisms and communities, as this gas is the main component of emissions. Seagrasses should positively react to increased dissolved carbon, but in vicinity of volcanic vents there may be toxic substances, that can have indirect effects on seagrasses. Here we analysed the expression of 35 stress-related genes in the Mediterranean keystone seagrass species *Posidonia oceanica* in the vicinity of submerged volcanic vents located in the Islands of Ischia and Panarea, Italy, and compared them with those from control sites away from the influence of vents. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) was used to characterize the expression levels of genes.

Fifty one per cent of genes analysed showed significant expression changes. Metal detoxification genes were mostly down-regulated in relation to controls both in Ischia and Panarea locations, indicating that *P. oceanica* does not increase the synthesis of heavy metal detoxification proteins in response to the environmental conditions present at the two vents. The expression levels of genes involved in free radical detoxification indicate that, in contrast with Ischia, *P. oceanica* at the Panarea vent face stressors that result in the production of reactive oxygen species triggering antioxidant responses. In addition, heat shock proteins were also activated at Panarea and not at Ischia.

Overall, our study reveals that *P. oceanica* is generally under higher stress in the vicinity of the vents at Panarea than at Ischia, possibly resulting from environmental and evolutionary differences existing between the two volcanic sites. This is the first study analysing gene responses in marine plants living near natural CO_2 vents and our results call for a careful consideration of factors, other than CO_2 and acidification, that can cause stress to seagrasses and other organisms near volcanic vents.

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1 Introduction

Seagrass meadows rank amongst the most valuable ecosystems to society in terms of the flow of services and values they support (Costanza et al., 1999; Seitz et al., 2014). They form multidimensional habitats for organisms directly participating in the trophic dynamics (Mazzella et al., 1992) and are a primary food source for herbivores on coral reefs, lagoons, and other shallow habitats (Orth et al., 2006). Seagrasses reduce sediment re-suspension and their roots enhance sediment accretion, thus maintaining high water quality. Seagrass ecosystems also represent key sites for carbon storage in the biosphere and are important as CO_2 sinks (Mcleod et al., 2011; Fourqurean et al., 2012; Pendleton et al., 2012; Pergent et al., 2012).

It is consensual that increased CO_2 will not have negative effects on seagrasses, which have even been predicted to extend their distribution, locally replacing macroalgae (Harley et al., 2006). Nevertheless, associated with increased CO_2 there may be indirect effects on seagrasses, as loss of phenolic protective substances due to lowered pH (Arnold et al., 2012) and light stress effects due to the modifications in the production and biomass of epiphytic algae on seagrass leaves. Regarding epiphytes, opposing light stress effects may be expected: shading, if non-calcifying epiphytes respond positively to increased CO_2 (Martínez-Crego et al., 2014), or high light exposure, if calcifying epiphytes decline (Martin et al., 2008). Experimental evidence for increased seagrass productivity as a response to elevated CO_2 levels is also inconclusive, and a recent meta-analysis did not detect significant effects of ocean acidification on seagrass photosynthesis (Kroeker et al., 2010). In a short-term experiment, the seagrass *Zostera marina* was found to grow at increasing rates under CO_2 enrichment (Thom, 1996). Similarly, Jiang et al. (2010) found an increase in *Thalassia hemprichii* photosynthesis and leaf growth rate. Nonstructural carbohydrates increased in belowground tissues whereas in aboveground tissues the carbon content was not affected by CO_2 treatments. On the other hand, in a long-term experiment, there was no effect of increasing CO_2 levels on the aboveground productivity of *Zostera marina* (Palacios and

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Zimmerman, 2007), as opposed to belowground. Alexandre et al. (2012) showed that the net photosynthetic rate of *Zostera noltii* was positively affected by the CO_2 enrichment of the seawater, but they did not observe an increase of leaf growth rates.

A problem with the above experimental approaches is that even the longest experiments do not allow for enough time to marine plants to adapt to high CO_2 conditions thus making it difficult to forecast how they will perform in a future high CO_2 ocean. This is the main argument to use submarine volcanic vents as natural laboratories for the effects of CO_2 as this gas is the main component of emissions and the emissions have been happening for a long time (years to hundreds of years, Hall-Spencer et al., 2008). Moreover, global climatic changes are not impacting marine environments only increasing dissolved CO_2 in the water. Elevated CO_2 is increasing global mean temperature, that results in a series of physical and chemical changes in marine systems (Harley et al., 2006). Such changes, together with changes in pH, will also potentially affect global biogeochemical cycles. Volcanic vent sites, displaying high dissolved CO_2, low pH, and also toxic contaminants of volcanic origin (Vizzini et al., 2013) can be valuable systems for modelling the response of marine organisms to multiple stressors, as predicted under the future climatic scenario.

In order to study plant response to multiple stressors, and to disentangle different sources of stress, a highly promising approach is to investigate the expression of specific genes involved in the response to stress. It will give insights for understanding how marine organisms maintain or re-establish homeostatic metabolism in the face of varying physical or chemical environmental variables (Ahuja et al., 2010). Organisms react to environmental pressure by activating a series of conserved stress enzymes/proteins, that involve redox sensors (e.g. reactive oxygen species-ROS sensors, antioxidants and detoxification systems), macromolecule damage sensors (e.g. stress-inducible heat shock proteins-HSPs) and/or condition-specific proteins that help to adjust the cellular physiology and metabolism protecting against cell damage or death. Plants can also make use of their general stress-coping mechanisms to compensate the effects of elevated CO_2 (Koch et al., 2013; Tuba et al., 2007).

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Here we analyzed the expression levels of selected genes of the seagrass *Posidonia oceanica* in the vicinity of submerged volcanic vents located in the Islands of Ischia and Panarea, Italy, and compared them with those from control sites away from the influence of vents. Reverse Transcription-quantitative PCR (RT-qPCR) was used to characterize expression levels of genes involved in stress responses, antioxidant activity, metal-related responses and defense processes in adult leaves of *P. oceanica*, in order to address the stress response of plants and to understand what stress defense mechanisms are at play in the vicinity of submarine volcanic vents.

In our analysis, target genes have been selected with the attempt to include all the possible mechanisms that *P. oceanica* can activate in face of abiotic stressors (Mittler, 2006). We selected genes from the first and second line of defenses, antioxidant and stress-related enzymes. From the first line of defense we analyzed a multixenobiotic resistance transporters or ATP Binding Cassette protein (MXR/ABC), which is involved in the efflux of a large number of structurally and functionally diverse, moderately hydrophobic compounds, including anthropogenic pollutants and natural toxins (Bard, 2000). The second line of defense is characterized by detoxification reactions, such as oxidation, reduction, hydrolysis, hydration and de-halogenation of compounds to detoxify (e.g. cytochrome P450 or CYP450; Regoli and Giuliani, 2014). The second line of defense also includes Aldehyde dehydrogenases (ALDH) that detoxify a wide variety of endogenously produced and exogenous aldehydes catalyzing their oxidation to the corresponding acids (Marchitti et al., 2008). Reactive Oxygen Intermediates (ROIs), such as superoxide anion, hydrogen peroxide and hydroxyl radical, are produced by plants following many stress conditions, such as drought stress, desiccation, heat shock, heavy metals, air pollutants, nutrient deprivation, mechanical stress and high light stress (Mittler, 2002). In low quantities these intermediates may function as a signal for the activation of stress-responses and are rapidly converted to less reactive forms. They can be very damaging to DNA, RNA and proteins when present in abnormally high quantities and may activate programmed cell death (PDC). Cells possess their own free radical detoxification enzymes and we selected and analyzed catalase

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(CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX), ascorbate peroxidase (APX) and scavenger molecules such as glutathione. Even if different enzymes may have the same substrate, they differ for cell localization and/or substrate affinity (Mittler, 2002). The over-accumulation of ROIs when antioxidant/defense systems are not able to cope with stress may induce lipid peroxidation and PDC (Mittler, 2002). This is the reason why we also analyzed the expression of a lipoxygenase (LOX), involved in lipid peroxidation, and of a death specific protein (DSP5), involved in cell death (Bidle and Bender, 2008). Other enzymes analyzed here have also antioxidant/protective properties. This is the case of Peroxiredoxin Q, involved in free radical detoxification processes, and Germin-like proteins, involved in many different processes such as metal stress response, fungal attack, osmotic regulation, cell wall restructuring and superoxide dismutase activities (Carter and Thombur, 1999; Lamkemeyer et al., 2006). In addition, other proteins/enzymes have been selected for their involvement in heavy-metal responses/detoxification (Hussain et al., 2004; Ricachenevsky et al., 2013).

P. oceanica is endemic to the Mediterranean providing a fundamental engineering role and key ecological services in the coastal ecosystem (Cullen-Unsworth et al., 2014). It is therefore essential to understand which kind of stresses the species is susceptible to and how it responds to them. This is the first study analyzing key protein activation in this species representing the first step for using stress-related genes of seagrasses as indicators of environmental pressures in a changing ocean.

2 Methods

2.1 Sampling

The study has been performed in the vicinity of submarine volcanic vents at Ischia and Panarea Islands, Tyrrhenian Sea, Italy. In both cases, the hydrothermal vents are characterized by the emission into sea water of thermal waters and gases, mainly CO_2,

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inducing changes in the chemical composition of the water column and associated community (Italiano and Nuccio, 1991; Kerrison et al., 2011).

Ischia Island. The study has been performed in a very little fringe of *Posidonia oceanica* meadow involved by vent areas off the Castello Aragonese isle (Island of Ischia, 40°43.849′ N; 13°57.089′ E Naples, Italy). In this site, underwater CO_2 vents occur in the shallowest rocky bottoms, and a pH gradient is formed (Hall-Spencer et al., 2008). Archaeological evidences suggest that vent sites around the Castello Aragonese in Ischia were above sea level in the fourth century BC, but that the region underwent a tectonic lowering (bradyseism) and was flooded by about 130–150 AD (de Alteriis and Toscano, 2003; Zucco, 2003). Thus, at these sites, subsurface vent activity can be dated back to about 1800–1900 years (Lombardi et al., 2011). Three individual shoots of *P. oceanica* were randomly collected in a control site at ambient pH in Ischia (S1, about 8.14 pH) and in a site of low pH (S2, with about 7.83 pH) in dense and continuous meadows. Additional three shoots where collected in a very isolated and little spot of *P. oceanica* in the extreme low pH conditions (S3 with about 6.57 pH). The depth range varies from 3.5 to 1 m along the pH gradient.

Panarea Island. The CO_2 vents of Panarea originated from a recent volcanic activity occurred in 2002, which resulted in a series of gas bursts (Tassi et al., 2009). Sampling was conducted in two separate sites off the Island (38°38′00″ N; 15°04′00″ E): a control site with pH 8.17 (Bottaro islet) and an acidified site with pH 7.91 (Formiche shoals), both at a 12 m depth. At each sampling site, six adult shoots of *P. oceanica* were collected.

For both sites, tissues from intermediate leaves (usually the second-rank leaf in the shoot) were collected and rapidly cleaned from epiphytes with a razor blade, towel-dried and immediately stored in RNAlater©tissue collection solution (Ambion, life technologies). Samples were then transported to the laboratory, preserved one night at 4 °C and stored at −20 °C until RNA extraction.

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2.2 Sample genotyping

Samples collected for RT-qPCR analysis were also genotyped using species-specific microsatellite markers. About 50–70 milligrams of dried tissue from each individual sample were ground in a Mixer Mill MM300 (QIAGEN). Subsequent DNA extraction was carried out using the NucleoSpin® 96 Plant II kit (Macherey-Nagel) as in Tomasello et al. (2009). Individual multilocus genotypes were assessed by a total of 29 microsatellites (SSRs): 13 *P. oceanica*-specific anonymous loci putatively neutral and widely employed to assess neutral genetic variation (e.g. Procaccini and Waycott, 1998; Alberto et al., 2003; Migliaccio et al., 2005; Serra et al., 2010) and 16 loci, representing a sub-set of the new EST-linked microsatellites developed from two existing *P. oceanica* EST-libraries (Arranz et al., 2013). PCR conditions were designed based on Arranz et al. (2013). Multiplex amplification reactions were performed using multiplex PCR buffer (QIAGEN Multiplex PCR Master Mix).

PCR products were analyzed on an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). Electropherogram profiles were visualized and analyzed using the software PeakScanner (Applied Biosystems). Individual multilocus genotypes were determined using the software Gimlet (Valière, 2002).

2.3 RNA extraction and cDNA synthesis

Portions of seagrass leaf tissue were grinded into a fine powder with mortar and pestle containing liquid nitrogen. About 100 mg of powered tissue was used for the RNA extraction using Aurum™ Total RNA Mini Kit (BIO-RAD) as in Mazzuca et al. (2013). After lysis solution, samples were homogenized using the Qiagen Tissue Lyser and Tungsten Carbide Beads (3 mm) (Qiagen) for 3 min at 20.1 Hz. RNA quantity was assured by Nano-Drop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies) monitoring the absorbance at 260 nm; purity was determined by monitoring the 260/280 and 260/230 nm ratios using the same instrument. Both ratios were about 2.0. All samples were free from protein and organic solvents used during RNA extraction. RNA quality

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was evaluated by agarose gel electrophoresis that showed intact RNA, with sharp ribosomal bands. Total RNA (500 ng) was retro-transcribed into cDNA with the iScript™ cDNA Synthesis Kit (BIO-RAD) following the standard protocol, using the GeneAmp PCR System 9700 (Perkin Elmer). The reaction was carried out in 20 µL final volume with 4 µL 5× iScript reaction mix, 1 µL iScript reverse transcriptase and DNase-free H_2O. The mix was first incubated 5 min at 25 °C, followed by 30 min at 42 °C and finally heated to 85 °C for 5 min.

2.4 Oligo design and PCR (Polymerase chain reaction) optimization

Primers for genes of interest (GOI) were designed considering sequences from the seagrass EST database Dr. Zompo (Wissler et al., 2009), unpublished sequences from the transcriptome of *P. oceanica* (D'Esposito et al., 2015) or from the generic online databases GenBank (http://www.ncbi.nlm.nih.gov/genbank/; Table 2). Primers were designed using the software Primer3 v. 0.4.0 (http://frodo.wi.mit.edu/primer3/). Table 1 lists selected GOI, their functions, primers' sequences and amplicon sizes. Primers were optimized as in Serra et al. (2012). The sequences are deposited in GenBank under the Accession numbers shown in Table 1.

2.5 Best reference gene (RG) assessment

In order to analyze the expression levels of specific GOI, a panel of seven putative reference genes (RGs) was first screened to find the most stable genes in the seagrass *P. oceanica* in both natural CO_2 enriched sampling sites. The screened panel included the eukaryotic initiation factor-4A (eIF4A) and the ones already published in Serra et al. (2012): ubiquitin (UBI), ribosomal protein L23 (L23), elongation factor 1-alpha (EF1A), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal RNA 18S (18S) and ubiquitin-conjugating enzyme (NTUBC2). Three different algorithms were utilized to identify the best RGs in our experimental design: BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004).

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2.6 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Expression level analyses were then performed for specific GOIs related to antioxidant activity, stress and detoxification processes (Table 1). Primer efficiencies were calculated for each oligo pair generating standard curves with five dilution points by using the cycle threshold (Ct) value vs. the logarithm of each dilution factor and using the equation $E = 10^{-1/\text{slope}}$. RT-qPCR was performed as in Dattolo et al. (2014). Control sites (S1 pH 8.14 and 8.17, for Ischia and Panarea Islands, respectively), were used as reference conditions. Statistical analyses were performed using the GraphPad Prim statistic software, V4.00 (GraphPad Software). Statistical significant gene regulation was considered at $p < 0.05$.

3 Results

3.1 Best reference gene (RG) assessment in Ischia and Panarea Islands

According to the mathematical approach of BestKeeper, the most stable genes were L23, GAPDH, UBI for Ischia and 18S, L23 and UBI for Panarea (Figs. S1a and S2a). NormFinder indicated eIF4a, NTUBC2 and UBI for Ischia and L23, NTUBC2 and EF1A for Panarea, as best candidate reference genes (Figs. S1b and S2b). According to geNorm analysis, the two most stable genes were eIF4A and NTUBC2, in Ischia (Fig. S1c) and L23 and UBI, in Panarea (Fig. S2c). All these genes were below the threshold M value of 1.5, which indicates that a gene can be considered suitable as a RG (Figs. S1c and S2c). The approach implemented in geNorm also allowed inferring the minimum number of necessary genes to be used as RGs in given data set. Pair-wise variation values were always < 0.15 in both sampling sites (V value; Figs. S1d and S2d), indicating that only two genes were sufficient for the analysis. Nevertheless, when results were not consistent among the different approaches utilized, we also included a third RG in the analysis. The best RGs identified for each statistical

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approach and utilized for normalizing GOI expression levels in the two sampling sites were L23, eIF4a and NTUBC2 in Ischia, and L23, 18S and UBI in Panarea (Table 2).

3.2 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Adult intermediate leaves utilized for gene expression analyses were previously genotyped with microsatellites, assuring that there were at least 3 different genotypes for each gene expression replicate. Results obtained from all distinct genotypes, using the site with normal pH as control, show that different gene category or specific gene functions have different behaviour in the two sampling sites. HSP expression levels showed opposite patterns (Fig. 1). In Ischia (Fig. 1a) all HSPs were significantly down-regulated, while in Panarea (Fig. 1b) they were up-regulated. In particular, the transcription factor HSFA5 and HSP90 were significantly down-regulated at both S2 and S3 Ischia sites. DNAJ was down-regulated only at S3 site. HSP83 was the only gene that was significantly expressed at both locations showing down-regulation at Ischia and up-regulation at Panarea. In Panarea, up-regulation was statistically significant for DNAJ and DehSP (Fig. 1b).

Regarding genes involved in detoxification and antioxidant activity, the expression levels were mainly higher in Panarea compared to Ischia (Fig. 1). The Peroxiredoxin Q gene, Prx Q, and a gene for free radical detoxification, GPX, were significantly up-regulated at both locations whereas the Germin-like protein, GLP, was down-regulated. The ascorbate-related gene sAPX, the glutathione-related enzyme GR, the superoxide dismutase SODCP and the cytochrome gene CYP were down-regulated at Ischia and up-regulated at Panarea. The transporter protein ABC was significantly over-expressed in Panarea but not at Ischia. The ascorbate peroxidase gene, APX3 was down-regulated in both locations, although its expression was only significant at Ischia, The genes that were significantly expressed at Ischia showed the same regulation trend in both sites sampled even though in some cases one of them was not significant.

Most metal-related genes were down-expressed both at Ischia and Panarea (Fig. 1). Four out of 8 metal-related genes were significantly down-expressed in Ischia whereas

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only one was down-expressed at Panarea. The only metal-related gene that was significantly up-regulated was the heavy metal ATPase 5 protein gene, HMATPase5, only at the S3 site of Ischia.

4 Discussion

To our knowledge, there are no published data on the gene expression of photosynthetic organisms in the vicinity of submarine volcanic vents. Here we analysed the expression of 35 genes of the Mediterranean keystone seagrass species *Posidonia oceanica*, putatively involved in different levels of stress response to volcanic emissions in relation to natural control sites. Fifty one per cent of genes analysed in this study showed significant expression changes either in the two sites of Ischia, in Panarea or in both locations (summarized in Fig. 2). A consistent gene response at the three sites was observed for three genes, Heavy Metal Associated-domain (HMA), Glutathione Peroxidase (GPX) and Peroxiredoxin Q (PrxQ). HMA was significantly down-regulated at both Ischia and Panarea, showing that plants do not synthesize more heavy metal detoxification proteins in proximity to volcanic emissions compared to the control site. This was further supported by the consistent pattern observed both at Ischia and Panarea, of the down-regulation of most metal detoxification genes examined suggesting that the putative heavy metal emission from the vents at Ischia and Panarea do no cause stress on *P. oceanica* plants. The bioavailability of heavy metals, which depends on pH and redox potential, may be low at the sites where plants grow as Vizzini et al. (2013) pointed out for the volcanic vents of Vulcano Island, Italy.

Glutathione Peroxidase (GPX) and Peroxiredoxin Q (Prx Q), involved in free radical detoxification, were significantly up-regulated in both sites in Ischia and in Panarea in relation to control sites, suggesting that *P. oceanica* plants are activating similar antioxidant protective mechanisms. Peroxiredoxins are ubiquitous thioredoxin- or glutaredoxin-dependent peroxidases, the function of which is to destroy peroxides, while GPX is important for reducing cytotoxic hydroperoxides. In contrast, the activ-

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ity of another antioxidant gene, the Germin-like protein (GLP; Gucciardo et al., 2007)), was down-regulated in both sites in Ischia (although in one it was not significant) and also in Panarea, indicating that this antioxidant defence system was not active in plants at the vicinity of vents.

Many contrasting patterns in the expression of the studied genes were observed between Ischia and Panarea, indicating that different environmental stresses are at play. Fourteen out of the 18 genes with significant expression were different between Panarea and Ischia. Panarea plants activated antioxidant enzymes such as sAPX, GR, SODCP, and detoxification proteins such as CYP and HSP83 compared with the Ischia plants, where these enzymes were down-expressed or did not show any significant change. Most of these genes are also activated after various types biotic and abiotic stressors in different plants species (see Vranovà et al., 2002 for a review), including seagrasses (i.e. heat stress in *Z. marina*; Bergmann et al., 2010). Our results indicate that, in contrast with Ischia, *P. oceanica* at Panarea faces stressors near the vents that result in the production of reactive oxygen species (ROS) that trigger antioxidant responses. There are only few published studies on the occurrence of antioxidant responses in seagrasses, mostly based on indirect observations of photosynthetic parameters derived from chlorophyll *a* fluorescence (Ralph et al., 1998; Campbell et al., 2006) and our work is the first one to show the expression of genes associated to the antioxidant responses in *P. oceanica*. It is thus difficult to pinpoint the environmental factors that triggered the antioxidant responses of *P. oceanica* in the vicinity of Panarea volcanic vents. Multiple factors have been described to cause them in higher plants that are probably not applicable. These include excess light, drought, high salinity, extreme cold, heat shocks, excess of heavy metals, high UV levels, and pathogen infections (Pessarakli, 2011). Recent research has shown that the presence of epiphytes may cause the production of reactive oxygen species leading to oxidative stress in *P. oceanica* (Costa et al., 2015).

The activation of heat shock protein genes such as HSP83 and DehSP in Panarea plants is also worth of attention. HSPs play an essential role as molecular chaper-

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ones by assisting the correct folding of nascent and stress-accumulated misfolded proteins, and by preventing their aggregation. HSPs induction and synthesis is not only a response to high temperature, which does not occur in Panarea, but also to many different types of stress, including exposure of cells to toxins or nitrogen deficiency (Santoro, 2000). As HSPs are very sensitive to even minor hits, they are suitable as an early warning bio-indicator of cellular hazard (Gupta et al., 2010). Our observation that HSPs were down-regulated at Ischia and up-regulated at Panarea supports the overall finding that relevant environmental differences exist between the two volcanic sites. An alternative hypothesis is that *P. oceanica* gene expression responses at Panarea are still going through the initial phase of acclimation to stress whereas at Ischia the species has already adapted to existing stress. Thus, the gene expression differences revealed in this work may be a component of the species homeostatic evolutionary compensation. The volcanic vent in Ischia, in fact, can be as old as about 2000 years, as indicated by archaeological evidences (Lombardi et al., 2011), whereas the vent in Panarea is only about ten years old. It is quite possible that some of the Ischia genotypes of *P. oceanica* have been there since the onset of the volcanic vents as it has been recently revealed that the longevity of this species can be up to thousands of years (Arnaud-Haond et al., 2012).

This is the first time that the assessment of gene expression data is performed in marine plants in the vicinity of submarine volcanic vents, generally assumed to be natural laboratories to investigate the effects of increased CO_2 and acidification. In our analysis, we identified a subset of genes that were coherently expressed in both sites, and that could be further explored for suggesting their use as early-warning indicators of low pH conditions in photosynthetic marine organisms. Nevertheless, caution should be taken when using only natural volcanic vents as a proxy of future ocean acidification scenario, and experimental work in controlled laboratory conditions is necessary to unambiguously test organismal response to increased CO_2 and low pH conditions. Our results call for a careful consideration of other factors that can cause stress to seagrasses and other organisms near the vents and that may confound the effects of

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CO_2 and acidification. In order to allow that, the knowledge of general stress coping, stress avoiding, and tolerance mechanisms is needed and the analysis of more than one gene category is required.

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Table 1. List of selected genes of interest, with their abbreviations and functions.

Abbrev	Gene name	Function
HSP90	HSP90	Stress Protein
DNAJ	Chaperone protein DNAJ	Stress Protein
HSP83	HSP83	Stress Protein
OzSP	OZONE STRESS PROTEIN	Stress Protein
DEHSP	DEHYDRA STRESS PROTEIN	Stress Protein
LBP	Luminal bindibg protein LBP	Stress Protein
SHSP	SH STRESS PROTEIN	Stress Protein
HSFA5	HEAT SHOCK FACTOR A5	Heat shock protein transcrition factor
ABC	ABC_MDH	Transporter protein
CYP450	Cytochrome P450	Primary metabolism/detoxification
ALDH	Aldehyde dehydrogenase	Primary metabolism/detoxification
GST	Glutathione S-transferase	Antioxidants
GPX	Glutathione peroxidase	Antioxidants
GSH-S	Glutathione synthase	Antioxidants
GR	Glutathione reductase	Antioxidants
CAT	Catalase	Free radical detoxification
SODCP	Superoxide dismutase [Cu-Zn], chloroplastic	Free radical detoxification
CSD1	cu zn superoxide dismutase, cytosolic	Free radical detoxification
FSD	chloroplast iron superoxide dismutase	Free radical detoxification
MSD	manganese superoxide dismutase	Free radical detoxification
AR	Ascorbate Reductase	Antioxidants
APX	ascorbate peroxidase, microsomal	Antioxidants
sAPX	ascorbate peroxidase, chloroplastic (stromal)	Antioxidants
Prx Q	Peroxiredoxin Q	Antioxidants
GLP	Germin-like protein	Antioxidants
DSP5	Death specific protein 5	Apoptosis
LPX	Lipooxygenase	Lipid Metabolism
FtsH2	ATP-dependent zinc metalloprotease	Metal-related gene
HMA	Heavy metal transport detoxification domain	Heavy metal-domain
NRAMP1	Root-specific metal transporter	Heavy metal-transporter
HMATPase	Heavy metal p-type ATPase	Heavy metal-ATPase
HMATPase5	Heavy metal ATPase 5 protein gene	Heavy metal-ATPase
MT3	Metallothionein 3 (*)	Heavy metal-stress response
Fe-SP	Iron-stress related protein	Heavy metal-stress response
MTP	Metal tolerance protein	Heavy metal-related gene

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Table 2. Best reference genes as given by BestKeeper, NormFinder and Genorm analyses, for each sampling location (Ischia and Panarea). Genes are ranked from the most stable (in bold) to the less stable.

Rank	BestKeeper	NormFinder	GeNorm
ISCHIA (S2 and S3)			
1	**L23**	**eIF4a**	**eIF4a-NTUBC2**
2	GAPDH	NTUBC2	UBI
3	UBI/eIF4a/NTUBC2	EF1A	EF1A
4	EF1A	UBI	GAPDH
5	18S	L23	L23
6		GAPDH	18S
7		18S	
PANAREA			
1	**18S**	**L23**	**L23/UBI**
2	L23	NTUBC2	NTUBC2
3	UBI/NTUBC2	EF1A/UBI	18S
4	eIF4A	18S	EF1A
5	GAPDH	eIF4A	eIF4A
6	EF1A	GAPDH	GAPDH

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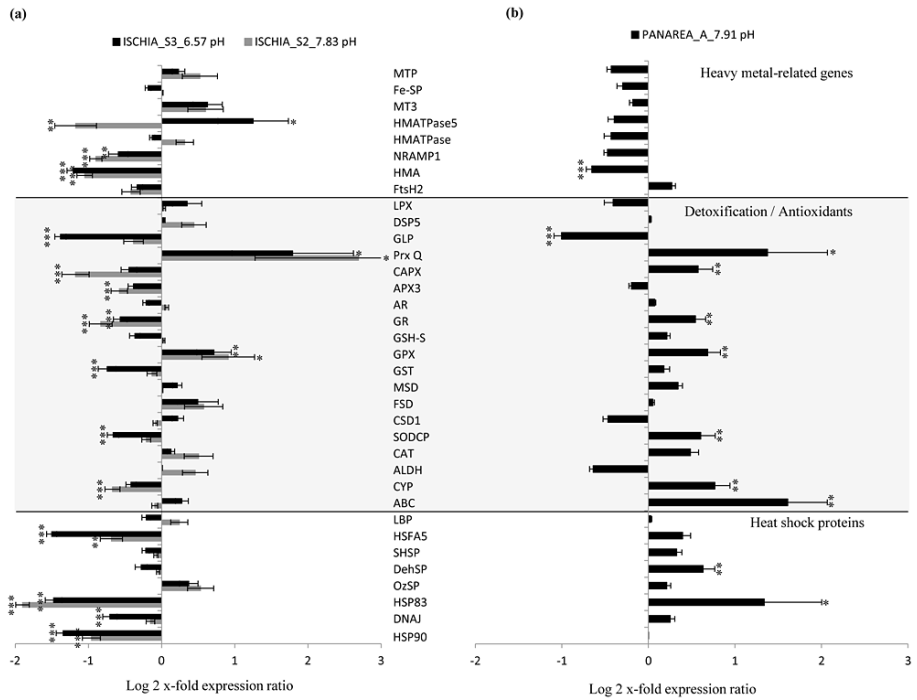

Figure 1. Expression levels of genes of interest in *P. oceanica* collected in **(a)** Ischia Island (S2 and S3 sites with pH 7.83 and 6.57), compared to the control site (pH 8.14), and in **(b)** Panarea Island (pH 7.91) compared to the control site (pH 8.17).

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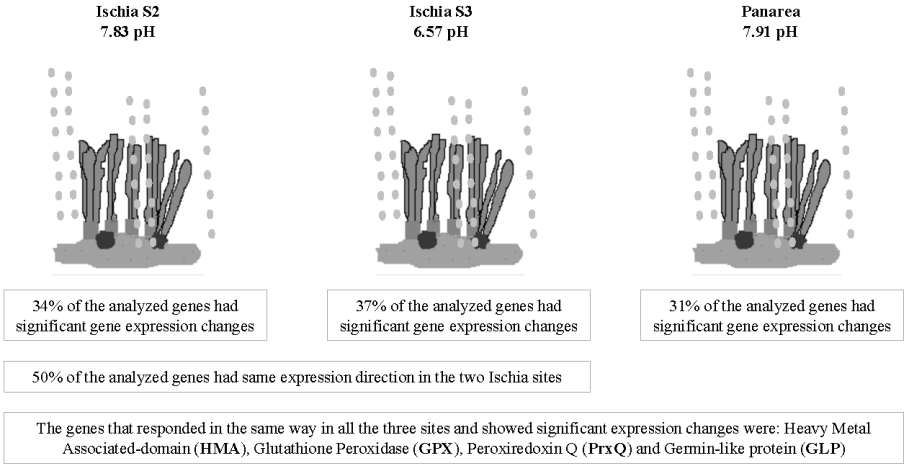

Figure 2. Summary of gene expression results obtained in Ischia (S2, pH 7.83 and S3, pH 6.57) and Panarea (pH 7.91) sites.

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