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Copper stress proteomics highlights local adaptation of two strains of the model brown alga *Ectocarpus siliculosus*

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*Ectocarpus siliculosus* is a cosmopolitan brown alga with capacity to thrive in copper enriched environments. Analysis of copper toxicity was conducted in two strains of *E. siliculosus* isolated from (i) an uncontaminated coast in southern Peru (Es32) and (ii) a copper polluted rocky beach in northern Chile (Es524). Es32 was more sensitive than Es524, with toxicity detected at 50 mg/L Cu, whereas Es524 displayed negative effects only when exposed to 250 mg/L Cu. Differential soluble proteome profiling for each strain exposed to sub-lethal copper levels allowed to identify the induction of proteins related to processes such as energy production, glutathione metabolism as well as accumulation of HSPs. In addition, the inter-strain comparison of stress-related proteomes led to identify features related to copper tolerance in Es524, such as striking expression of a PSII Mn-stabilizing protein and a Fucoxanthine chlorophyll a–c binding protein. Es524 also expressed specific stress-related enzymes such as RNA helicases from the DEAD box families and a vanadium-dependent bromoperoxidase. These observations were supported by RT-qPCR for some of the identified genes and an enzyme activity assay for vanadium-dependent bromoperoxidase. Therefore, the occurrence of two different phenotypes within two distinct *E. siliculosus* strains studied at the physiological and proteomic levels strongly suggest that persistent copper stress may represent a selective force leading to the development of strains genetically adapted to copper contaminated sites.

**Keywords:**
2-DE / Algae / Bromoperoxidase / Copper / Plant proteomics / Stress

1 Introduction

The release of heavy metals, including copper, by a wide range of human activities, remains a major threat for marine ecosystems, impacting benthic flora and fauna assemblages [2–4]. Copper is a micronutrient essential for all forms of life, acting as cofactor for many enzymatic systems and participating in crucial physiological processes including photosynthesis and respiration. However, copper is extremely toxic at high concentrations, particularly affecting photosynthetic organisms that suffer effects on chlorophyll structure, synthesis and function, on fatty acid metabolism, and on carbohydrate synthesis [5–7]. In addition, copper can catalyze the synthesis of the highly reactive hydroxyl radical in presence of H$_2$O$_2$ via...

*Publication of this paper was delayed after final acceptance due to awaiting publication of the *Ectocarpus* genome paper by Cock et al. [1] and the public release of the genome data.*
the Fenton reaction, a process that causes oxidative stress [8, 9]. Some local geographic areas that have been chronic recipients of wastes containing heavy metals (i.e. mine wastes) constitute a persistent stressful environment that acts as a selective force leading, in some cases, to the generation of tolerant strains genetically adapted [10–13]. In northern Chile, copper mine wastes discharges were, for almost 70 years, a major and only source of coastal pollution at Chañaral bay and surroundings, where local disturbances included the creation of large tailing beaches and the disappearance of most of the seaweeds and invertebrates [2, 14]. The process of biological recovery, expected to follow after tighter legislation, is far from being complete and [2, 14]. The pertinence of using *E. siliculosus* as a facultative metallophyte, its easy handling in the laboratory and the current efforts to develop this species as a model organism for Phaeophyceae [37] make it an attractive candidate for studying metal tolerance through global approaches such as proteomics.

At the proteomic level, whereas several studies on plant responses to heavy metal stress exist, they are mostly restricted to the exposure to arsenic, cadmium, and zinc [38, 39]. Fewer reports on this domain concern copper stress. Some proteomic studies have focused on the glutathione-S-transferase family regulation caused by copper excess [40], and the copper-binding proteins of *Arabidopsis* roots and seedlings have been explored by IMAC-MS [41].

Recently, the molecular effects of copper on the root proteome of *Cannabis sativa* was investigated by 2-DE-based analysis [42]. At the present time, no meaningful proteomic study does exist in marine algae. This is probably due to the fact that until recently no adequate techniques for obtaining high-quality protein extracts was available for these organisms [43]. In this work, copper tolerance of two *E. siliculosus* strains, originating from habitats with contrasting histories of copper levels, was analyzed by a comparative 2-DE proteomic approach. Patterns of differentially expressed proteins were compared within and between the two strains to better identify several cellular processes potentially involved in copper stress responses and tolerance.

2 Materials and methods

2.1 Plant material and cultivation treatments

*E. siliculosus* (Ectocarpales, Phaeophyceae) unialgal strains 32 (Culture Collection of Algae and Protozoa accession 1310/4, origin San Juan de Marcona, Peru 15°22′S, 75°10′W) and 524 (Culture Collection of Algae and Protozoa accession 1310/333, origin Caleta Palito, Chile 26°15′S, 70°40′W) were cultivated in 10-L plastic flasks in a culture room at 14°C using 0.22 μm filtered seawater enriched with Provasoli nutrients [44]. Light was provided by Philips daylight fluorescence tubes at a photon flux density of 40 μmol photon/m²/s for 14 h per day. Cultures were aerated with filtered (0.22 μm) compressed air to avoid CO₂ depletion.

Two days before exposing the algae to copper excess, tissues were transferred into 1 L of seawater free of organic matter prepared with 0.22 μm filtered natural SW treated overnight with 0.2 g/L activated charcoal (Merck, Germany). Treated water was then filtered at 0.45 μm to remove charcoal.
excess. Culture flasks used during the experiments were washed overnight with 1% HCl to limit copper adsorption to glass. Copper stress was triggered by changing the algae to fresh seawater free of organic matter and enriched with CuCl₂ (Merck, Germany) at nominal final concentrations of 50, 150, 250 and 500 µg/L. The term “acute stress” was employed for conditions of exposure to high copper concentrations (250–500 µg/L) during short periods of time (4–8 h). On the other hand, “chronic stress” defines the exposure to lower sub-lethal copper concentrations (50–150 µg/L) during longer periods of time (6–10 days). No nutrients were added during the experiments. Culture media were renewed every 2 days. For each kinetic point, three replicate algal samples were harvested. At the end of the experiment, thalli were frozen in liquid nitrogen and kept at −80 °C for analysis.

2.2 In vivo fluorescence measurements and microscopic observations

To monitor the intensity of the stress in the algal tissues caused by the copper treatments we measured the maximum quantum yield of PSII (dark adapted) as Fm/Fv, using a Walz Phytoco-PAM (Waltz, Germany). After 5 min of dark adaptation, samples were illuminated with modulated excitation light, provided by a combination of photodiodes emitting at 470, 520, 645 and 665 nm, at a frequency of 25 Hz, in order to define the basal fluorescence level F0. After signal stabilization, a light pulse was triggered by a red light emitting photodiode array (3500 µmol photon/m²/s intensity for 200 ms, 655 nm), which allowed measuring maximal fluorescence level Fm. The emitted fluorescence is detected at a wavelength range above 710 nm. The PSII quantum yield was then calculated as Fm/Fv = (Fm−F0)/F0. Since photosynthesis is stress sensitive, the quantum yield decreased under sub-optimal (stressful) conditions. Simultaneously to PAM measurements, loss of chlorophyll autofluorescence, accounting for cell damage, was monitored using an Olympus BX60 (Olympus, Japan) epifluorescence microscope. Thalli were observed with a 460–500 nm excitation filter and a band pass emission filter centered at 585 nm. Images were obtained using a digital camera (DIAGNOSTIC instruments model 2.1.1). The number of fluorescent cells was estimated and represented as percentage of fluorescent cells.

2.3 Protein extraction and 2-DE separation

Global soluble proteome extraction followed the protocol developed by Contreras et al. [43]. A total of 300 mg of tissue (fresh weight) was rinsed with Milli-Q water, blotted dry and fast frozen in liquid nitrogen. After tissue grinding in liquid nitrogen, powder was re-suspended in 5 mL of extraction buffer (1.5% w/v PVP, 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, 250 mM EDTA, complete protease inhibitor cocktail (Roche, Switzerland), 2% v/v β-mercaptoethanol and 0.5% w/v CHAPS) and homogenized at 4 °C for 20 min. Then, 10 mL of Tris-HCl pH 7.5 saturated phenol was added and the mixture was re-homogenized for 20 min at 4 °C. The mixture was centrifuged at 10 000 × g for 20 min and the upper phenol phase was removed. Proteins in the phenol phase were precipitated by addition of five volumes of 0.1 M ammonium acetate dissolved in methanol and incubated at −20 °C for 3 h. The extract was centrifuged at 10 000 × g for 20 min, the supernatant was discarded and the protein pellet was rinsed in ammonium acetate (0.1 M in methanol) for 20 min at −20 °C. Subsequently, the protein pellet was rinsed four times in four volumes of 80% ice-cold acetone. The pellet was then re-suspended in 2-DE compatible buffer (7 M Urea, 2 M Thiourea, 4% w/v CHAPS, 60 mM DTT, 20 mM Tris-HCl, pH 8.8, and 1 × Biolytes, pH 3–10, from BioRad, USA). A final cleaning step was incorporated using the 2-D Clean-up Kit (BioRad). The extracts were stored at −80 °C. The total protein concentration was assayed by using the 2-D quant kit (GE Healthcare, USA). For each replicate, 500 µg of total protein extract was loaded into a 17-cm non-linear ReadyStrip pH range 4–7 (BioRad). The 2-DE proteome separation and gel staining was carried out as described elsewhere [43]. Further information about the gel electrophoresis procedure is available at the MIAPE-GE table : http://www.sb-roscoff.fr/UMR7139/ectocarpus/proteomics/001/MIAPE/MIAPEGE sheet.htm.

2.4 Image analysis

Gel images obtained using an Image scanner UMAX Powerlook III (UMAX Technologies, USA) at 300 dpi resolution were analyzed with the Melanie version 5.0 software (Swiss Institute of Bioinformatics, Switzerland). Manual editing and normalization were applied after automated spot detection and matching. Spot quantification was based on spot volume (integration of spot density over spot area) as percentage of the total spot volumes of the gel to normalize for possible staining differences between gels. Gel annotations and matching fidelity were checked manually to eliminate matching errors caused by the software. Three gels representing independent biological samples were analyzed for each condition. All spots selected for MS analyses presented statistically significant variation (p < 0.05), ratio < 1.5 and an average fold change < 2 (at least in one of the stressed conditions). Further information regarding gel analyses is available at the MIAPE-GI information sheet: http://www.sb-roscoff.fr/UMR7139/ectocarpus/proteomics/001/MIAPE/MIAPEGI sheet.htm.

2.5 Protein mass fingerprints (PMF)

Coomassie blue-stained protein-bearing gel slices were cut into small pieces, washed with distilled water and de-stained with ACN. The cysteine residues were reduced by 100 µL of
10 mM DTT at 56 °C and alkylated by 150 μL of 55 mM iodoacetamide at room temperature. The iodoacetamide solution was replaced by 100 μL of 100 mM NH₄HCO₃ and gel dehydration was achieved with ACN. After evaporation in Speed-Vac (Thermo, USA), proteins were digested overnight at 37 °C in a solution containing 0.9 μg of a modified sequencing grade bovine trypsin (Roche, Germany) prepared in 25 mM NH₄HCO₃. Finally, a double extraction followed, first with 5% v/v formic acid and subsequently with 100% v/v ACN. The resulting peptide mixture was extracted and vacuum-dried, re-suspended in 1% formic acid and desalted using a ZipTip (Millipore, USA) C-18 reverse phase microcolumn. After evaporation, the desalting peptide mixture was re-suspended in 10 μL of 1% formic acid. PMF by MALDI-TOF-MS was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, USA). One microliter of tryptic digest was mixed with an equal volume of CHCA matrix (Sigma; 10 mg/L in 50% ACN, 0.1% TFA) and spotted onto the MALDI target. Spectra were acquired in positive ion reflector mode under 20 kV accelerating voltage and a mass range of 800–4000 Da. Internal calibration was performed using trypsin autolysis fragments at m/z 1433.70, 2163.05 and 2289.1. The mono-isotopic mass of tryptic peptides were used to query the specific E. siliculosus protein database deduced from the version 2 of the genome annotation (genome accessible at http://bioinformaticsystems.psb.ugent.be/webtools/bogas) using Mascot version 2.1. Search conditions included an initially permissive mass window of 200 ppm for external calibration, followed by 100-50 ppm applied for internal calibration with trypsin autodigestion fragments (when trysin peptides were detected), allowing double missing cleavage, modification of cysteines by iodoacetamide, methionine oxidation and N-terminal pyroglutamylation as variable modifications. Only significant scores were selected for publication. Further information regarding the MS procedures is accessible at the MIAPE-MS information sheet:

2.6 RNA extraction and RT-qPCR analysis

The RNA extraction, quantification, cDNA synthesis and RT-qPCR reactions followed the protocol described by Le Bail et al. [45]. For each gene, a specific pair of oligonucleotide primers in the 3’ coding sequence (Supporting Information Table S1) was designed using Beacon designer (Premier Biosoft International, USA). The qPCR reactions were done in a Chromo4 apparatus (Biorad) with a SYBR Green reaction mix AB-1162/8 from ABBgene (UK). A dilution series ranging from 91 to 121 312 copies of the E. siliculosus genome was prepared. The Dynein gene was used as internal control as previously described [45]. Relative variations in gene expression were calculated as x-fold changes compared with the appropriate control treatment.

2.7 Bromoperoxidase activity assay

Total protein extracts were obtained from of 0.2–0.5 g of fresh weight tissue, grounded in liquid nitrogen and mixed with 0.4 mL of extraction buffer containing 25 mM MOPS, pH 7.2, 15 mM MgCl₂, 15 mM EGTA, 1 mM DTT, 0.5% PVP and anti-proteases cocktail at a concentration specified by the manufacturer (Roche, Germany). Extracts were homogenized for 1 h at 4 °C, centrifuged at 10 000 x g for 30 min and the supernatant collected. Vanadate was added to the extracts at 1 mM final concentration, after which they were incubated at 4 °C for at least 1 h prior to the analysis. The total amount of proteins was quantified using the Bradford assay [46], and 20 μg were loaded on the gel. Bromoperoxidase enzymatic assay was conducted on non-denaturing gels as described elsewhere [47]. The activity levels using band density in the gels were assessed by the ImageQuant v5.2 software (Molecular Dynamics, GE Healthcare, USA). The relative changes among conditions were calculated as Volume Intensity and quantified in arbitrary units (AU).

2.8 Statistical analysis

The statistical significances of the differences among treatments were determined by the non-parametric Mann–Whitney U test run on Statistica version 5.1 software (StatSoft, USA). All conclusions are based on at least 5% level of significance (p<0.05).

3 Results

3.1 Copper toxicity tests in strains Es32 and Es524

The toxicity of copper was assessed in the two strains by in vivo measurements of chlorophyll fluorescence and by epifluorescence microscopy observations. The dose–response relationships between Cu concentrations and cell-mortality differed between both strains after 10 days of exposure to copper excess (Fig. 1A and B). In Es32, a significant drop to 70% of chlorophyll autofluorescence was observed under 10 days of incubation with 50 μg/L Cu, indicating the occurrence of cell death. On the other side, Es524 displayed a lower decay of cell-autofluorescence accounting for the deleterious effects only at 250 μg/L Cu or higher. In agreement with these observations, the F₀/F∞ ratio was negatively affected by Cu in both strains, but at different thresholds (Fig. 1C). After 10 days, a significant decline in the yield was observed in strain Es32 when copper concentration was higher than 50 μg/L, with F₀/F∞ decreasing in a dose-dependent manner. After the same time of incubation, Es524 displayed significant decrease in F₀/F∞ only for Cu concentrations of 500 μg/L. These results allowed to select the best concentrations to expose each isolate to non-lethal conditions of copper stress, treatments required for differential soluble proteome analyses.
Figure 1. Physiological effects of copper toxicity on E. siliculosus strains Es32 and Es524. (A) Comparison of epifluorescence and transmission microscopic photographs after 10 days of copper treatment. Top images represent the green cut-off filter fluorescence images with their corresponding transmission images at the bottom. For each strain, the images are organized as follows: control condition (left) and 250 μg/L of copper (right). Bars represent 0.1 mm. (B) Effect of different copper concentrations on the percentage of remaining auto-fluorescent cells during 10-day trials. Control seawater (black), and different concentrations of copper: 50 μg/L (blue), 150 μg/L (green), 250 μg/L (red) and 500 μg/L (orange). Values represent means of three independent replicates and bars represent the SE. Black-filled symbols represent a significantly different condition from the control group (p < 0.05). (C) Changes in the photosynthetic yield during 10-day trials. Control seawater (black), and different concentrations of copper: 50 μg/L (blue), 150 μg/L (green), 250 μg/L (red), 500 μg/L (orange). Values represent means of three independent replicates and bars represent the SE. Black-filled symbols represent a significantly different condition from the control group (p < 0.05).
3.2 Analyses of the proteins induced by chronic copper stress in Es32

To identify proteins specifically regulated under chronic copper stress conditions, the most sensitive strain (Es32) was exposed to 10 days at 50 µg/L nominal Cu. The 2-DE profiles of soluble proteome fractions were compared between control and treated thalli. Coomassie blue staining provided an average yield of 790 spots per gel (Supporting Information Fig. S1). Image analysis of the gels produced over 560 reproducible spot groups considering the two experimental conditions (Fig. 2A), with 58 statistically variable spots (ratio > 1.5). Among these spots, two downregulated and eight upregulated proteins were selected for MS identification because they presented enough intensity to be located and excised manually (Fig. 2A). PMF spot identifications are described in Table 1. For most of the identified spots, the 2-DE observed molecular mass and pI values are in agreement with the theoretical values obtained from the corresponding protein hits in the genomic database. Among the identified enzymes, there was a transketolase belonging to the pentose phosphate metabolism. Several enzymes involved in amino acid metabolism were also identified. For instance, copper excess enhanced the accumulation of serine hydroxymethyl transferase (SHMT) and cysteine synthase involved in the biosynthesis of glycine and cysteine, respectively. Related to this, the nucleotide metabolism enzyme S-adenosyl methionine synthetase (SAM) also appeared increased. Protein folding was involved as well since a HSP10 and the HSP cochaperone Immunophilin FKBP 11 were more abundant in stressed individuals. Finally, the chaperone calreticulin was downregulated by copper excess.

3.3 Analyses of the proteins induced by chronic copper stress in Es524

The tolerant strain Es524 was exposed during 10 days to 50 and 150 µg/L Cu. Protein extracts from Es524 were analyzed with identical criteria as for Es32. The analysis of gel images obtained under the control condition and after treatment with 50 µg/L resulted in over 423 matched spot groups. Spot intensity analysis showed 32 differentially expressed spots (ratio > 1.5). Among these spots, 20 were up-regulated and 12 down-regulated. On the other hand, comparison of control thalli with those exposed to 150 µg/L of copper produced 494 reproducible groups, from which 58 presented significant changes (Fig. 2B). For PMF, a first group of six spots corresponding to proteins accumulated under both treatments with copper excess was considered (638, 734, 745, 941, 1174 and 1176). Then, a second group was selected with nine spots only regulated by exposure to 150 µg/L, corresponding to seven up-regulated (814, 897, 584, 1089, 768 and 1184) and one down-regulated (1252) proteins. PMF results are presented in Table 2. One enzyme involved in the pentose phosphate pathway, the G6PD, was identified. As for Es32, copper excess caused
Table 1. Proteins identified in Es32 for which the level of expression changed significantly (p<0.05) under exposure to copper excess (50 μg/L for 10 days)

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Ectocarpus genomic database accession code</th>
<th>MW Theo/Obs (kDa)</th>
<th>pH Theo/Obs</th>
<th>Control (mean vol% ± MSD)</th>
<th>50 μg/L Cu (mean vol% ± MSD)</th>
<th>Average fold-change (Stress/Control)</th>
<th>Ratio (Stress/Control)</th>
<th>Peptides (matched/searched)</th>
<th>% Coverage</th>
<th>Mascot Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1612</td>
<td>Carbohydrate metabolism/Pentose phosphate pathway</td>
<td>Esi0002_0327</td>
<td>5.3/5.3</td>
<td>0.164 ± 0.055</td>
<td>0.456 ± 0.011</td>
<td>-2.5</td>
<td>-2.0</td>
<td>2/51</td>
<td>28</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>1842</td>
<td>Amino acid metabolism</td>
<td>Esi0148_0037</td>
<td>6.3/6.7</td>
<td>0.069 ± 0.013</td>
<td>0.137 ± 0.011</td>
<td>2.0</td>
<td>1.5</td>
<td>2/10</td>
<td>27</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>2023</td>
<td>Nucleotide metabolism</td>
<td>Esi0085_0190</td>
<td>5.5/5.6</td>
<td>0.038 ± 0.013</td>
<td>0.137 ± 0.013</td>
<td>3.6</td>
<td>2.4</td>
<td>1/30</td>
<td>46</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>1836</td>
<td>S-adenosyl methionine synthetase (SAM)</td>
<td>Esi0175_0026</td>
<td>5.2/5.6</td>
<td>0.080 ± 0.022</td>
<td>0.227 ± 0.013</td>
<td>2.8</td>
<td>2.1</td>
<td>15/23</td>
<td>36</td>
<td>95</td>
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<tr>
<td>1758</td>
<td>Protein folding and stabilisation</td>
<td>Esi0363_0006</td>
<td>4.2/4.8</td>
<td>0.135 ± 0.014</td>
<td>0.057 ± 0.008</td>
<td>-2.4</td>
<td>-1.9</td>
<td>12/19</td>
<td>22</td>
<td>90</td>
<td></td>
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<tr>
<td>2248</td>
<td>Immunophilin FKBP-11</td>
<td>Esi0148_0072</td>
<td>5.7/5.7</td>
<td>-</td>
<td>0.055 ± 0.001</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>6/19</td>
<td>40</td>
<td>56</td>
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<td>2279</td>
<td>Heat shock protein 10 (HSP10)</td>
<td>Esi0000_0403</td>
<td>6.3/5.8</td>
<td>-</td>
<td>0.038 ± 0.007</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>6/15</td>
<td>41</td>
<td>74</td>
<td></td>
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<td>1592</td>
<td>Unknown/hypothetical proteins</td>
<td>Esi0173_0035</td>
<td>4.6/4.9</td>
<td>0.087 ± 0.005</td>
<td>0.046 ± 0.002</td>
<td>-2.5</td>
<td>-1.9</td>
<td>11/25</td>
<td>16</td>
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<td>1961</td>
<td>Conserved hypothetical protein</td>
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<td>0.100 ± 0.012</td>
<td>0.221 ± 0.005</td>
<td>2.2</td>
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<td>26</td>
<td>102</td>
<td></td>
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<tr>
<td>1718</td>
<td>Conserved hypothetical protein</td>
<td>Esi0174_0047</td>
<td>6.0/5.5</td>
<td>0.092 ± 0.023</td>
<td>0.235 ± 0.005</td>
<td>4.0</td>
<td>2.3</td>
<td>14/40</td>
<td>20</td>
<td>63</td>
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For each spot, the classes histograms with the mean volume% and mean squared deviation (MSD) are presented in Fig. S1.

Table 2. Proteins identified in Es524 for which the level of expression changed significantly (p<0.05) under exposure to copper excess (50 and 150 μg/L for 10 days)

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Ectocarpus genomic database accession code</th>
<th>MW Theo/Obs (kDa)</th>
<th>pI Theo/ Obs</th>
<th>Control (mean vol% ± SD)</th>
<th>150 μg/L Cu (mean vol% ± SD)</th>
<th>Average fold-change (Stress/ Control)</th>
<th>Ratio (Stress/ Control)</th>
<th>Peptides (matched/ searched)</th>
<th>% Coverage</th>
<th>Mascot Score</th>
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<tr>
<td>814</td>
<td>Glucose-6-phosphate 1-dehydrogenase (G6PD)</td>
<td>Esi0159_0021</td>
<td>60/66</td>
<td>6.35.7</td>
<td>0.028 ± 0.004</td>
<td>0.066 ± 0.011</td>
<td>2.4</td>
<td>1.7</td>
<td>13/31</td>
<td>26</td>
<td>74</td>
</tr>
<tr>
<td>584</td>
<td>Glutamate synthase (GOGAT)</td>
<td>Esi0103_0010</td>
<td>140/140</td>
<td>5.25.7</td>
<td>–</td>
<td>0.035 ± 0.006</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>14/22</td>
<td>11</td>
<td>59</td>
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<td>941</td>
<td>S-adenosylmethionine synthetase (SAM)</td>
<td>Esi0175_0026</td>
<td>44/55</td>
<td>5.5/5.7</td>
<td>0.044 ± 0.008</td>
<td>0.092 ± 0.011</td>
<td>2.0</td>
<td>1.5</td>
<td>10/17</td>
<td>24</td>
<td>90</td>
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<td>733</td>
<td>Stress-induced protein STI1-like</td>
<td>Esi0172_0055</td>
<td>65/72</td>
<td>5.5/5.8</td>
<td>0.031 ± 0.004</td>
<td>0.114 ± 0.026</td>
<td>3.7</td>
<td>2.5</td>
<td>20/44</td>
<td>36</td>
<td>70</td>
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<td>734</td>
<td>Heat Shock Protein 70 (HSP70)</td>
<td>Esi0379_0027</td>
<td>72/75</td>
<td>5.0/5.0</td>
<td>0.062 ± 0.004</td>
<td>0.160 ± 0.024</td>
<td>2.6</td>
<td>2.0</td>
<td>14/19</td>
<td>23</td>
<td>75</td>
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<tr>
<td>1252</td>
<td>38 kDa ribosome-associated protein precursor</td>
<td>Esi0012_0045</td>
<td>13/55</td>
<td>6.4/5.7</td>
<td>0.146 ± 0.025</td>
<td>0.022 ± 0.031</td>
<td>–6.6</td>
<td>–2.2</td>
<td>9/15</td>
<td>18</td>
<td>72</td>
</tr>
<tr>
<td>768</td>
<td>Vanadium-dependent Bromoperoxidase (vBPO)</td>
<td>Esi0009_0080</td>
<td>70/70</td>
<td>5.9/5.8</td>
<td>0.024 ± 0.007</td>
<td>0.120 ± 0.031</td>
<td>5.0</td>
<td>2.8</td>
<td>24/43</td>
<td>30</td>
<td>155</td>
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<tr>
<td>745</td>
<td>DEAD box helicase 1</td>
<td>Esi0033_0067</td>
<td>85/71</td>
<td>9.9/5.5</td>
<td>0.130 ± 0.013</td>
<td>0.243 ± 0.009</td>
<td>2.0</td>
<td>1.6</td>
<td>19/42</td>
<td>29</td>
<td>70</td>
</tr>
<tr>
<td>897</td>
<td>DEAD box helicase 2</td>
<td>Esi0028_0100</td>
<td>99/59</td>
<td>6.1/6.0</td>
<td>0.084 ± 0.020</td>
<td>0.203 ± 0.030</td>
<td>2.4</td>
<td>1.7</td>
<td>12/20</td>
<td>12</td>
<td>56</td>
</tr>
<tr>
<td>638</td>
<td>CDC-ATPase (CDC48)</td>
<td>Esi0010_0010</td>
<td>92/103</td>
<td>4.8/4.9</td>
<td>0.100 ± 0.016</td>
<td>0.206 ± 0.025</td>
<td>2.0</td>
<td>1.6</td>
<td>20/25</td>
<td>31</td>
<td>123</td>
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<tr>
<td>1184</td>
<td>Hypersensitive-induced reaction protein (HIR)</td>
<td>Esi0126_0019</td>
<td>40/33</td>
<td>8.5/5.4</td>
<td>0.125 ± 0.013</td>
<td>0.328 ± 0.012</td>
<td>2.6</td>
<td>2.3</td>
<td>19/40</td>
<td>34</td>
<td>78</td>
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<tr>
<td>1089</td>
<td>Conserved hypothetical protein</td>
<td>Esi0175_0006</td>
<td>51/44</td>
<td>7.6/5.7</td>
<td>0.114 ± 0.017</td>
<td>0.244 ± 0.038</td>
<td>2.1</td>
<td>1.6</td>
<td>14/42</td>
<td>28</td>
<td>68</td>
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<tr>
<td>1127</td>
<td>Hypothetical protein</td>
<td>Esi0024_0144</td>
<td>32/40</td>
<td>4.6/4.6</td>
<td>–</td>
<td>0.415 ± 0.069</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>15/42</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>1174</td>
<td>Hypothetical protein</td>
<td>Esi0357_0021</td>
<td>37/37</td>
<td>5.4/5.7</td>
<td>0.111 ± 0.019</td>
<td>0.217 ± 0.016</td>
<td>2.0</td>
<td>1.5</td>
<td>16/39</td>
<td>30</td>
<td>111</td>
</tr>
</tbody>
</table>

For each spot, the classes histograms with the mean volume% and mean squared deviation (MSD) are presented in Fig S2.

Table 3. Proteins identified in Es524 proteins whose expression levels under copper excess (50 μg/L for 10 days) changed significantly compared to Es32.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Ectocarpus genomic database accession code</th>
<th>MW Theo/Obs (kDa)</th>
<th>pI Theo/Obs</th>
<th>Es32 50 μg/L Cu (mean vol% ± SD)</th>
<th>Es524 50 μg/L Cu (mean vol% ± SD)</th>
<th>Average fold-change (Es524/Es32)</th>
<th>Group ratio (Es524/Es32)</th>
<th>Peptides matched/searched</th>
<th>% Coverage</th>
<th>Mascot Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate metabolism/Pentose phosphate pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>211</td>
<td>Transketolase (TKT)</td>
<td>Esi0002_0327</td>
<td>54/41</td>
<td>5.9/5.5</td>
<td>0.652 ± 0.087</td>
<td>0.362 ± 0.013</td>
<td>−1.8</td>
<td>−1.5</td>
<td>27/49</td>
<td>39</td>
<td>134</td>
</tr>
<tr>
<td>212</td>
<td>Transketolase (TKT)</td>
<td>Esi0002_0327</td>
<td>54/41</td>
<td>5.9/5.6</td>
<td>0.057 ± 0.001</td>
<td>0.275 ± 0.016</td>
<td>4.7</td>
<td>4.4</td>
<td>16/35</td>
<td>19</td>
<td>69</td>
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<tr>
<td>534</td>
<td>Fructose-1,6-bisphosphate aldolase (SMALDO)</td>
<td>Esi0183_0064</td>
<td>42/41</td>
<td>5.4/5.5</td>
<td>0.110 ± 0.150</td>
<td>2.488 ± 0.110</td>
<td>22.3</td>
<td>8.9</td>
<td>15/61</td>
<td>27</td>
<td>73</td>
</tr>
<tr>
<td>537</td>
<td>Fructose-1,6-bisphosphate aldolase (SMALDO)</td>
<td>Esi0183_0064</td>
<td>42/41</td>
<td>5.4/5.5</td>
<td>2.445 ± 0.238</td>
<td>0.970 ± 0.010</td>
<td>−2.5</td>
<td>−2.3</td>
<td>14/48</td>
<td>25</td>
<td>79</td>
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<tr>
<td>490</td>
<td>Phosphoribulokinase (PKK)</td>
<td>Esi0000_0466</td>
<td>48/50</td>
<td>5.6/5.0</td>
<td>0.033 ± 0.074</td>
<td>0.601 ± 0.102</td>
<td>17.7</td>
<td>6.1</td>
<td>18/53</td>
<td>37</td>
<td>109</td>
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<tr>
<td>529</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase (GAPD)</td>
<td>Esi0240_0024</td>
<td>41/41</td>
<td>5.8/5.8</td>
<td>0.066 ± 0.008</td>
<td>1.171 ± 0.020</td>
<td>17.6</td>
<td>7.8</td>
<td>16/42</td>
<td>55</td>
<td>94</td>
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<td>Amino acid metabolism</td>
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<td></td>
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<tr>
<td>463</td>
<td>Glutamate ammonia ligase (GS)</td>
<td>Esi0188_0004</td>
<td>42/51</td>
<td>5.5/5.6</td>
<td>0.015 ± 0.02</td>
<td>0.588 ± 0.078</td>
<td>38.6</td>
<td>13.9</td>
<td>17/48</td>
<td>39</td>
<td>115</td>
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<td>Cell rescue and detoxification</td>
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<td></td>
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<tr>
<td>256</td>
<td>DEAD box helicase 1</td>
<td>Esi0033_0067</td>
<td>85/77</td>
<td>9.9/5.5</td>
<td>0.007 ± 0.010</td>
<td>0.091 ± 0.006</td>
<td>11.9</td>
<td>&gt; 10</td>
<td>19/42</td>
<td>29</td>
<td>70</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>656</td>
<td>Manganese stabilising protein (OEC33)</td>
<td>Esi0155_0030</td>
<td>34/35</td>
<td>5.3/5.7</td>
<td>−</td>
<td>1.727 ± 0.082</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>21/85</td>
<td>42</td>
<td>88</td>
</tr>
<tr>
<td>737</td>
<td>Fucoxanthin chlorophyll a/c binding protein (Fcp)</td>
<td>Esi0492_0002</td>
<td>21/21</td>
<td>5.0/4.2</td>
<td>−</td>
<td>0.249 ± 0.035</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>10/40</td>
<td>34</td>
<td>65</td>
</tr>
<tr>
<td>Unknown/hypothetical proteins</td>
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</tr>
<tr>
<td>615</td>
<td>Hypothetical protein</td>
<td>Esi0367_0021</td>
<td>13/32</td>
<td>5.4/5.7</td>
<td>0.046 ± 0.011</td>
<td>0.177 ± 0.033</td>
<td>3.8</td>
<td>2.5</td>
<td>13/32</td>
<td>32</td>
<td>88</td>
</tr>
</tbody>
</table>

Classes histograms with the mean volume% and mean squared deviation (MSD) are presented in Fig. S3.

differential expression of enzymes involved in amino acid biosynthesis and nucleotide metabolism related enzymes, such as GOGAT and SAM. Copper excess clearly enhanced protein folding processes through the accumulation of the chaperone HSP70, and the HSP cochaperone stress-inducible protein (STI 1). Cell cycle may also be regulated by copper, as a hypersensitive-induced reaction protein and a CDC-ATPase accumulated under stress. Interestingly, among up-regulated spots in Es524, we found two RNA helicases from the DEAD box family, both related to the cell rescue process. In addition, one specific brown algal antioxidant-related enzyme, the vanadium-dependent bromoperoxidase (vBPO), was also identified in this set of proteins.

3.4 Inter-strain differences in protein expression caused by copper excess

Finally, patterns of proteome expression were compared between Es32 and Es524 exposed to 50 µg/L of copper after 10 days. Over 406 spots matched on reproducible groups between both strains, whereas 93 proteins were differentially expressed with a ratio higher than 2 (Fig. 2C). From these, in Es524 we identified nine clearly increased and two decreased spots, which were subsequently identified (PMF identification in Table 3). Two spots, corresponding to the same locus Esi0002_0327 and encoding for a potential pentose phosphate related transketolase, showed contrasting regulation in the two strains: increased in Es524 and decreased in Es32. Several other glycolysis and pentose phosphate metabolism-related enzymes were also markedly increased in Es524: a phosphoribulokinase, a glyceraldehyde-3-phosphate dehydrogenase and a fructose 1,6-bisphosphate aldolase (SMALDO). Concerning the amino acid metabolism, a GS appeared highly increased in Es524. Photosynthesis-related processes were also stimulated in this strain. For instance, the PSI Mn-stabilizing protein of the oxygen evolving complex (OEC33) was markedly over-expressed in Es524, while it was not detectable in Es32. Similar results were obtained for a light harvesting complex (LHC) protein Fucoxanthine chlorophyll a–c binding protein (Fcp).

3.5 Expression analysis of selected key genes by RT-qPCR under acute and chronic stress conditions

Four genes, encoding for proteins identified through proteomic analyses, vBPO, HSP70, HSP10 and SAM, were chosen for RT-qPCR experiments in order to compare changes in their level of expression during acute and chronic stress (Fig. 3A and B). Acute stress was induced by exposing algal cultures to 250 and 500 µg/L of copper for 4 and 8 h. For chronic stress, final concentrations of 50 and 150 µg/L of Cu were applied for 6 and 10 days. Interestingly, vBPO is the gene whose expression level was the most altered under stress. Its

Figure 3. Changes in gene expression in E. siliculosus strains Es32 and Es524 exposed to acute and chronic stresses monitored by RT-qPCR. (A) Expression profile of vanadium bromoperoxidase (vBPO), heat shock protein 70 (HSP70), heat shock protein 10 (HSP10) and S-adenosyl methionine synthase (SAM) genes in Es32 submitted to acute Cu stresses (250 and 500 µg/L for 4 and 8 h) at the left and chronic Cu stresses (50 µg/L for 6 and 10 days) at the right. (B) Expression profile of vBPO, HSP70, HSP10 and SAM genes from Es524 exposed to acute Cu stresses (250 µg/L and 500 µg/L for 4 and 8 h) at the left and chronic Cu stresses (50 and 150 µg/L for 6 and 10 days) at the right. Relative gene expression ratios were calculated as 2n-fold variation as described in Section 2. Results are means±SE for two determinations from three independent biological samples. * Significantly different from the control group (p<0.05).
expression was induced under all the tested conditions in both strains, and more specifically, this gene was the most up-regulated under chronic stress. Accumulation of HSP70 transcripts was observed under all acute stress conditions in Es32 (Fig. 3A) and Es524 (Fig. 3B). However, during chronic stress, the transcription of this gene was up-regulated only in Es524 exposed to 150 μg/L for 10 days. Significant changes in the expression of the HSP10 gene were recorded only under acute stress, mainly in Es32 (Fig. 3A). Slight induction of this gene was observed after incubation of Es524 for 4 h in presence of 250 μg/L copper. SAM gene was down-regulated in most of the conditions tested (Fig. 3A and B).

3.6 Bromoperoxidase activity assay

To assess the importance of vBPO in the regulation of the oxidative stress created by Cu in Es32 and Es524, changes in enzymatic activity were followed on native PAGE gels. No visible activity was detected in Es32 under the control condition (Fig. 4A), while an intense band appeared under stress conditions, with an estimated molecular mass of ~140 kDa. Quantification of the band intensity showed an increase of 4E+6 AU in stressed Es32 (Fig. 4B). Protein extract from Es524 incubated in absence of copper showed only a slight coloration, indicating a low basal bromoperoxidase activity (Fig. 4C and D). After treatment with 50 or 150 μg/L, a band similar to that observed in extracts from stressed Es32 was identified, demonstrating an increase in bromoperoxidase activity. The quantification of this band was estimated at 3E+6 AU on all individuals and for both copper treatments. Similar results were obtained when replacing potassium bromide by potassium iodide during gel development, indicating that the protein identified in E. siliculosus exhibits a true vBPO activity (data not shown).

4 Discussion

Studies of differential protein expression mapping are still uncommon in marine algal models. Proteomics allowed us to analyze responses to chronic stress in two strains in the marine brown alga E. siliculosus isolated from habitats contrasting in their history of copper enrichment. Exposure of these isolates to chronic conditions of copper stress allowed the identification of proteins and genes that help to support the hypothesis that isolate Es524 from Caleta Palito is a copper tolerant ecotype, which likely evolved after more than 60 years of exceedingly high levels of copper in its normal habitat. Protein accumulation analysis in both strains shows regulation of a high number of proteins directly implicated in various mechanisms of stress control, which validates the pertinence of this approach.

4.1 Common protein expression features of the two isolates exposed to copper

Surprisingly, the marked differences in tolerance to copper between the two isolates were not accompanied by major
differences in protein expression patterns. Exposure to sublethal copper concentrations induced the expression of proteins common to both strains, underlining the crucial importance of certain metabolic pathways during the response to stress. Copper induced the accumulation of proteins involved in pathways related to energy production such as the pentose phosphate pathway. These observations are in agreement with the correlation established between alteration of the photosynthesis and the ATP pool depletion in the diatom *Phaeodactylum tricornutum* [48]. Also, as in vascular plants [39], heavy metal stress induced the onset of repairing and detoxification systems, which represent an additional energetic cost for the cells, and therefore, the accumulation of enzymes involved in energy production in both isolates should not come as a surprise. In addition, the NADPH coming from the pentose phosphate shunt is one of the main cellular sources of reducing power, which is very important for detoxification, i.e., restoration of the reduced glutathione pool. In relation to this, copper stress activates enzymes involved in amino acid metabolism. These enzymes encompassed SHMT and cysteine synthase, linked to the biosynthesis of glycine and cysteine respectively. These two enzymes, plus the GOGAT/GS, are essential for the production of amino acid precursors used during the biosynthesis of glutathione (GSH). In this context, GSH plays a pivotal role in protecting living organisms from environmental stresses [49, 50] by detoxifying ROS and toxins [9, 50, 51]. The GSH polymers PCs are fundamental for chelating metal excess in vascular plants [22]. Recently, concentrations of PCs and GSH in the brown algae *Fucus vesiculosus* and *F. serratus* have been correlated with the history of copper contamination of the sampling sites [25]. The enhanced GSH biosynthesis could also be related to the activity of the detoxification enzymes glutathione-S-transferases. Theses enzymes are particularly abundant in *E. siliculosus*, and some of them have been recently shown to be induced by copper stress (strain Es32) [52]. Theses findings, together with our results, highlight the involvement of thiol peptides in maintaining the homeostasis in brown algae under metal stress, as well as in detoxification processes. In addition, SAM, which synthesizes S-adenosyl methionine, was also up-regulated in both strains. Previous proteomic work in vascular plants and animals demonstrated that this enzyme was up-regulated under a condition of heavy metal stress [38, 53]. This enzyme belongs to the methyl cycle that interacts with the GSH biosynthetic pathway, specifically at the level of the SHMT enzyme. In vascular plants, SAM is the direct precursor for nicotianamine, involved in copper complexation [54].

Both strains showed also up-regulation of enzymes involved in protein folding and stabilization, which are important players in recycling stress-damaged proteins. HSPs, known to be induced by a variety of stress conditions, and whose role is to protect cells against protein degradation [55, 56], appeared increased in both strains. Indeed, both strains up-regulated stress-related HSPs co-chaperones such as the stress-induced protein STI 1 and the Immunophilin-FKBP 11. These co-chaperones are needed to create functionally active protein complexes by establishing interactions between HSPs [57]. Additional data obtained through gene expression analysis in Es32 showed a strong upregulation of *HSP70* and *HSP10* in response to acute copper stress. In contrast, level of expression of *HSP10* was not regulated in Es524, which is in agreement with the proteomic results. In both strains, *HSP 70* was mainly induced under acute exposures to copper excess, with a higher expression in Es32 than in Es524, suggesting an involvement in rapid stress responses rather than in adaptation.

The protein chaperone calreticulin was downregulated in Es32, whereas Es524 upregulates hypersensitive-induced reaction and CDC-ATPase proteins. These proteins participate in cell cycle control and apoptosis in several organisms, under a variety of stress, including metal ions [58–60]. Further studies should now be carried out to fully characterize the stress responses of *Ectocarpus*.

### 4.2 Strain-specific responses

Several individual proteins exhibited inter-strain qualitative or quantitative differences in expression. An increased expression of the LHC component Fcp was observed in Es524. In brown algae, and as in other photosynthetic organisms, LHC is primarily damaged by a wide range of abiotic stresses [20, 61, 62]. Therefore, the enhanced turnover of Fcp could contribute to the maintenance of the LHC integrity in this strain. Copper excess can also damage directly the PSII, generating oxidative stress as a result of the PSII dysfunction [6, 9, 63–65]. In agreement with this, a marked increase in OEC33 was observed in Es524 when compared with Es32. This could account for an enhanced turnover of OEC33, which constitutes a major copper toxicity target in PSII [64, 66]. From these observations, it seems that differences in the expression of proteins related with the photosynthetic apparatus can explain, at least partly, the different response to Cu stress displayed by the two strains used in this study. However, further research is necessary to fully address the issue of differential copper tolerance.

The enzymes involved in cell detoxification, DEAD box helicases and vBPO, appeared increased under copper stress in Es524 but not in Es32. RNA helicases from the DEAD box family are found in almost all organisms and have important roles in RNA metabolism [67]. Moreover, these enzymes have been reported to play important roles in resistance to abiotic stress in vascular plants [68, 69]. Two DEAD box helicases accumulated in copper-stressed Es524, which suggests their involvement in copper tolerance. On the other hand, vBPOs are peroxidases unique to brown and red algae, and are absent in vascular plants or metazoans [70]. They catalyze the oxidation of bromide, as well as iodide, in the presence of hydrogen peroxide, to generate oxidized halogens which are thought to be related to iodine uptake [71]. Recent studies in the kelp *L. digitata* showed
that iodine was mainly stored in the apoplastic region as iodide [36, 72]. Kürper et al. presented iodide as a powerful scavenger for a wide variety of ROS because of its high reducing capacity [36]. Furthermore, these authors suggested that vBPO could catalyze this process. Gene expression analysis showed a strong induction of vBPO in both strains under all conditions of copper excess, which highlights the importance of vBPO in stress responses. In agreement with these results, the in-gel activity assay clearly showed the increase of vBPO activity during chronic stress. Iodide metabolism is not well characterized in *E. siliculosus* and therefore this is, to our knowledge, the first study describing vBPO and its regulation under stress in this species. Based on our results and on previous work in brown algae, it is likely that vBPOs play a major role in the processes leading to ROS detoxification [36, 73]. In addition, if significant amounts of $I^-$ are released upon stress, it could be possible that complexation with Cu$^{2+}$ occurs, leading to a non-bioavailable form of copper. However, additional research is required to test this hypothesis.

4.3 Conclusions

In this work, we have characterized two strains of *E. siliculosus* presenting different copper tolerance (Es32 and EsS24). The availability of these different strains paves the way for future studies on the genetic bases of adaptive traits in seaweeds. Global proteome profiling led to identification of several pathways involved in copper tolerance and helped to better understand the differential tolerance to the metal observed between strains. In this context, photosynthesis-related proteins seem to be crucial for copper tolerance. In addition, we suggest the occurrence of an original antioxidant response of brown algae, based on halide metabolism and involving a vBPO.

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5 References


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