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Proteomic analysis and identification of copper stress-regulated proteins in the marine alga *Scytosiphon gracilis* (Phaeophyceae)

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ABSTRACT

A proteomic analysis combining peptide *de novo* sequencing and BLAST analysis was used to identify novel proteins involved in copper tolerance in the marine alga *Scytosiphon gracilis* (Phaeophyceae). Algal material was cultivated in seawater without copper (control) or supplemented with 100 μg L^{-1} for 4 days, and protein extracts were separated by two-dimensional gel electrophoresis (2-DE). From the proteins obtained in the copper treatment, 25 over-expressed, 5 under-expressed and 5 proteins with no changes as compared with the control, were selected for sequencing. Tryptic-peptides obtained from 35 spots were analyzed by capillary liquid chromatography and tandem mass spectroscopy (capLC/MS/MS), and protein identity was determined by BLASTP. We identified 19 over-expressed proteins, including a chloroplast peroxiredoxin, a cytosolic phosphomannomutase, a cytosolic glyceraldehyde-3-phosphate dehydrogenase, 3 ABC transporters, a chaperonine, a subunit of the proteasome and a tRNA synthase, among others. The possible involvement of these over-expressed proteins in buffering oxidative stress and avoiding metal uptake in *S. gracilis* exposed to copper excess is discussed taking into consideration the information available for other plant models.

Algae belonging to the genus *Scytosiphon* (Phaeophyceae) include species distributed in cold and temperate coastal regions throughout the world. In addition to their seasonal presence in the middle-lower rocky intertidal zone along the Chilean coasts (Hoffman and Santelices, 1997), they monopolize copper-polluted areas in the northern part of the country (Camus et al., 2005; Contreras et al., 2005; Medina et al., 2005). Copper tolerance was experimentally demonstrated in *Scytosiphon lomentaria* and *Scytosiphon gracilis*; plants exposed to copper excess in laboratory and in the field activate antioxidant enzymes such as catalase, glutathione peroxidase, and ascorbate peroxidase, among others and consume antioxidant compounds such as ascorbate and glutathione (Contreras et al., 2005, 2007). Furthermore, using reciprocal transplant experiments, Contreras et al. (2005) demonstrated that these antioxidant responses were rapidly expressed and reversible. Similarity in the copper-induced antioxidant responses in both *S. lomentaria* and *S. gracilis* led to the conclusion that copper tolerance is a constitutive trait in the genus *Scytosiphon* (Contreras et al., 2007). However, and considering the evidence available from vascular plant models, it seems reasonable to expect that tolerance to copper in *Scytosiphon* spp. involves more than the antioxidant responses.

The aim of this work was to determine novel proteins involved in copper tolerance in *S. gracilis* using a proteomic approach. Six replicates (40–60 pooled individuals of *S. gracilis*) of 10–20 g of fresh tissue each, were collected in central Chile (Maitencillo, 32°39.5′S, 71°26.6′W), kept in plastic bags with seawater and transported at 10 °C. In the laboratory, thalli were split in 3 replicates exposed to seawater from a pristine area in central Chile (Quintay; <0.5 μg L^{-1} copper) and 3 replicates exposed to seawater supplemented with 100 μg L^{-1} copper. All cultures were incubated for 96 h at 12 ± 2 °C, 10:14 h light:dark photoperiod and 10–30 μmol m^{-2} s^{-1} photon flux density. Protein extracts were prepared using the phenol method with an initial desalting step (Contreras et al., 2008) and 500 μg of proteins was separated by 2-DE using immobiline strips of pH 3–10 and 4–7 (Immobiline™ DryStrip; Amersham Biosciences, Uppsala, Sweden), 12.5% polyacrylamide gel for SDS-PAGE and Coomassie Blue for staining. The procedure was repeated 15 times in order to ensure reproducibility and the spots selected for analysis were identified using Z3 2-DE gel analysis software (Compugen, Tel Aviv, Israel) accord-

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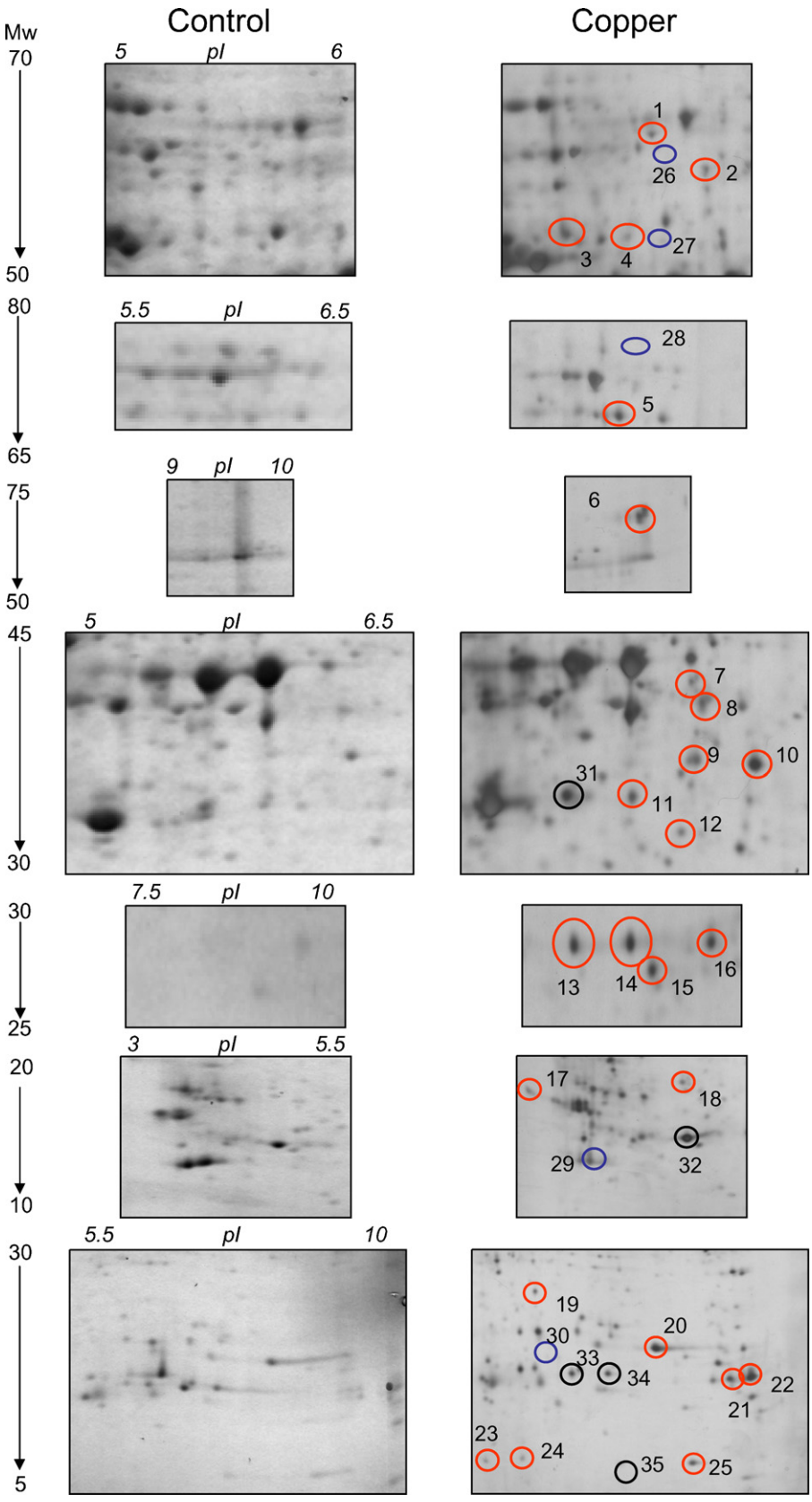

Fig. 1. Proteins separated (500 μg) by 2-DE in *S. gracilis* cultivated in control and copper-treated conditions. Molecular masses of protein standards are indicated on the left, *pI* range is shown on each section, and labelled spots indicate proteins identified by LC/MS/MS (see Table 1). Over-expressed proteins in red, under-expressed proteins in blue and proteins without changes in black.

Table 1
Proteins differentially expressed in *S. gracilis* exposed to copper excess.

Spot[a]	Protein	Expression level[b]	No. of peptides analyzed[c]	Specie, no. access[d]	*pI*, Mw (kDa)
1	Transferase	Over	13	*Dechloromonas aromatica* (Q47F82)	5.6, 65
2	tRNA synthetase	Over	24	*Helicobacter pylori* (P56126)	5.9, 60
3	Phosphomannomutase	Over	24	*Schizosaccharomyces pombe* (Q9UTJ2)	5.3, 53
4	Proteosome, subunit α	Over	24	*Oryza sativa* (Q10KF0)	5.5, 53
5	ATP synthase, subunit α	Over	13	*Syntrophus aciditrophicus* (Q2LQZ7)	6.0, 67
6	Ribulose biphosphate carboxylase large chain	Over	22	*Porphyra yezoensis* (Q760T5)	9.6, 65
7	Glyceraldehyde 3-phosphate dehydrogenase 1	Over	22	*Gracilaria verrucosa* (P30724)	6.2, 43
8	Peptidase/protease	Over	19	*Methanothermobacter* (O27355)	6.3, 42
9	tRNA binding protein	Over	23	*Anaplasma* (Q2GJX4)	6.2, 38
10	ATP binding protein	Over	24	*Methanocaldococcus jannaschi* (Q58049)	6.4, 38
14	Transcriptional regulator	Over	24	*Mesorhizobium loti* (CAD31581.1)	8.7, 29
17	Carbohydrate kinase	Over	35	*Salmonella enterica* (YP_152740.1)	3.0, 18.2
19	RNA binding protein	Over	28	*Bacillus phage* (P06953)	6.4, 25
20	ABC transporter subunit	Over	40	*Theileria parva* (XP_764551.1)	8.0, 20.6
21	RNA polymerase, subunit α	Over	19	*Francisella tularensis* (Q5NHU3)	9.2, 17.5
22	Peroxiredoxin	Over	20	*Porphyra purpurea* (P51272)	9.5, 18
23	Chaperonine	Over	10	*Caulobacter crescentus* (P48222)	5.6, 8
24	ABC transporter subunit	Over	45	*Desulfitobacterium hafniense* (ZP_01371968.1)	6.2, 8.2
25	ABC transporter subunit	Over	35	*Janibacter* sp. (ZP_00996449.1)	8.6, 7.3
27	Acetylglutamate kinase	Under	15	*Pyrococcus abyssi* (Q9V1I5)	5.7, 53
28	Phosphoenolpyruvate carboxykinase	Under	10	*Moorella thermoacetica* (Q2RH96)	6.1, 76
29	Dehydrogenase	Under	19	*Yarrowia lipolytica* (Q6CEJ6)	4.0, 13.7
31	ATP-dependent RNA helicase subunit 2	=	28	*Ajellomyces capsula* (A6R603)	5.8, 34
32	Cytochrome *c* oxidase, subunit 2	=	21	*Pisum sativum* (P08744)	4.9, 15
34	Phosphoglycerate kinase	=	22	*Triticum aestivum* (P12783)	7.3, 18
35	Photosystem I iron sulfur center	=	10	*Porphyra yezoensis* (Q1XDB3)	7.6, 6.5

(=) indicates no significant changes in expression level between copper treatment and control. Spots 11, 12, 13, 15, 16, 18, 26, 30 and 33 were not identified by BLASTP.
[a] Numbers correspond to proteins illustrated in Fig. 1.
[b] Changes in expression level compared with controls.
[c] Number of peptides analyzed by LC/MS/MS.
[d] NCBI access number of the species with the highest identity obtained by BLASP.

ing to the manufacturer's instructions. Protein spots were excised from the gels and digested with trypsin. The resulting peptides were dried, dissolved in 7 μL of 1% formic acid, and sequenced by capillary liquid chromatography followed by tandem mass spectrometry (capLC/MS/MS) using an injection volume of 0.8 μL and positive electrospray ionization (ESI) and operated using a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF II, Micromass Waters). Once confirmed, LC/MS/MS data were searched using BLASTP by partially aligning the analyzed proteins from *S. gracilis* to a NCBI database sequence from related organisms. From a total of about 1000 spots reproducibly separated on gels, 2-DE from copper treatment showed ca. 60 over-expressed, 20 under-expressed and hundreds of equally expressed proteins in comparison with the control. A total of 25 over-expressed, 5 under-expressed and 5 equally expressed proteins (Fig. 1) were selected for analysis. Peptide sequencing and BLAST analysis allowed the identification of 29 proteins, including 19 over-expressed and potentially involved in copper tolerance and oxidative stress buffering (Table 1). Among them, we identified a peroxiredoxin (PRX, spot 22). This enzyme reduces hydrogen peroxide and fatty acid hydroperoxides and its activity is normally coupled to thioredoxin and thioredoxin reductase activities (Dietz et al., 2006). PRXs are mainly located in plant mitochondria and chloroplasts and their expression is increased by salinity, drought and heavy metals (Baier and Dietz, 1997; Wood et al., 2003; Dietz et al., 2006). In algae, PRXs have been also detected in *Chlamydomonas reinhardtii* (Goyer et al., 2002), in *Porphyra purpurea* (Reith and Munholland, 1995) and in *Ulva compressa* (Contreras, personal communication), where its expression is regulated by oxidative stress and copper. Therefore, this PRX is likely involved in the control of copper-induced oxidative stress in *S. gracilis*, in synergism with other antioxidant enzymes such as ascorbate peroxidase and glutathion peroxidase.

Among the over-expressed proteins, a cytosolic phosphomannomutase (PMM, spot 3) was also detected. PMM catalyzes the conversion of mannose 6-phosphate to mannose 1-phosphate, required for the synthesis of GDP-mannose, an intermediate in the biosynthesis of the two major cell wall polysaccharides of brown algae, alginate and sulfated fucans (Mabeau and Kloareg, 1987) and also in ascorbate biosynthesis (Qian et al., 2007). An increase in the synthesis of precursors of cell wall polysaccharides, which are known to bind heavy metals (Davis et al., 2003), might provide a mechanism to increase the buffering capacity of the algal cell wall. Furthermore, an increase in ascorbate synthesis is also consistent with the need for ascorbate in copper-stressed plants, as shown in our previous studies (Contreras et al., 2005).

Another enzyme identified among those over-expressed in response to copper stress was a cytosolic glyceraldehyde-3-phosphate dehydrogenase (G3PDH, spot 7). G3PDH belong to the glycolitic pathway and, in humans, oxidative stress is known to increase its expression (Duan et al., 2008). It has been also shown that depending on its redox state, this enzyme regulates the expression of other proteins by binding to mRNAs (Rodríguez-Pascual et al., 2008). In wheat and maize seedlings the specific activity of the enzyme was found to increase after oxidative conditions imposed by methyl viologen (Bustos et al., 2008). Transformation of G3PDH into *Arabidopsis* protoplast strongly suppressed heat shock induced H_2O_2 production and cell death (Baek et al., 2008). These results suggest that G3PDH in plants remains as an active enzyme required to maintain energy and reducing power and control the generation of H_2O_2 under oxidative stress conditions. Another important function of the G3PDH is its involvement in the reactivation of a DNA repairing enzyme in human cells (Azam et al., 2008). Thus, over-expression of G3PDH likely plays an important role in helping *Scytosiphon* to attenuate the negative effects of oxidative stress caused by copper.

We also identified 3 ABC (ATP binding cassette) transporters (spots 20, 24 and 25), involved in translocation of a wide variety of compounds across cell membranes, including ions, carbohydrates, lipids, xenobiotics, drugs and heavy metals (Ehrmann et al.,

1998; Sipos and Kuchler, 2006; Gaillard et al., 2008). Translocation of metal complexes from the cytosol to the vacuole supports their role in heavy metal tolerance (Clemens, 2001). This is the case of the yeast ABC transporter YCFI, which participates in cadmium tolerance by translocating glutathione-cadmium complexes to the vacuole (Szczypka et al., 1994). In *Arabidopsis thaliana* dozens of tissue- and metal-specific ABC transporters were identified, including the AtMRP6 induced by cadmium and hydrogen peroxide (Gaillard et al., 2008) and the AtMRP3 induced by various metals (Zientara et al., 2009). In *C. reinhardtii*, an ABC transporter similar to the yeast ScYCF1 was reported to participate in cadmium sequestration and tolerance (Wang and Wu, 2006). Considering the number of ABC transporters involved in heavy metal tolerance in a wide diversity of organisms, their over-expression in copper-stressed *S. gracilis*, might correspond to equivalent roles in modulating copper homeostasis and oxidative stress in this alga.

Finally, we identified an over-expressed proteasome subunit (spot 4) known for catalyzing intracellular degradation of unfolded and oxidized proteins in eukaryotes (Hellman and Estelle, 2002). For example, cadmium increased the 20S proteasome activity in maize (Pena et al., 2007) and *A. thaliana* with mutations in the 26S proteosome regulatory zone was hypersensitive to oxidant agents (Kurepa et al., 2008). Thus, the increased expression of a proteosome subunit in *S. gracilis* may help to remove damaged proteins resulting from copper-mediated oxidative stress.

Two additional over-expressed proteins were identified, although their involvement in the responses of *S. gracilis* to copper excess remains uncertain. One was an aminoacyl-tRNA synthetase (ARS, spot 2), key enzyme involved in protein synthesis. *Nicotiana benthamiana* mutants for glutamyl- and seryl-tRNA synthetase genes developed severe leaf yellowing and cells with reduced number of chloroplast, although the expression of the chlorophyll biosynthesis genes remained unchanged (Kim et al., 2005). On the other hand, salt and cold stress triggered over-expression of methionyl- and phenylalanyl-tRNA synthetase transcripts in maize (Zheng et al., 2006). The other over-expressed protein was a chaperonine (CHAP, spot 23), a family of proteins that facilitate the maintenance of the correct configuration of structural proteins and the activation of multiple enzymes. For example, tomato plant exposed to salt excess displayed an increased expression of several chaperonine genes (Zhou et al., 2007), whereas deletion of a chaperonine gene in *A. thaliana* led to accelerated cell death compared to wild plants (Ishikawa et al., 2003).

Two ATP-user enzymes were identified among the under-expressed proteins; phosphoenolpyruvate carboxykinase (PEPCK, spot 28) involved in gluconeogenesis and an acetylglutamate kinase (AGK, spot 27) involved in arginine biosynthesis. Certainly there is much information needed regarding under-expressed enzymes in a condition of stress, particularly when those enzymes are part of primary metabolic pathways. Under-expression of these enzymes may suggest that when plants are under stress conditions, energy is diverted into fuelling those metabolic pathways directly involved in detoxification and/or defence, sacrificing some aspects of their basic metabolism. Among those whose level of expression was not affected by exposure to copper excess, a mitochondrial (cytochrome *c* oxidase, spot 32) and a chloroplastic (photosystem I iron sulfur center, spot 35) proteins were identified. It is important to mention that a number of proteins remained as non-identified, and it is likely that some of them are involved in tolerance to copper stress in *Scytosiphon*.

In summary, we report the identification of several proteins potentially involved in the control of copper-mediated oxidative stress in *S. gracilis*, which complements our previous biochemical understanding of the mechanisms involved in copper tolerance in this brown alga (i.e. rapid and reversible up-regulation of enzymatic activities, such as ascorbate peroxidase and glutathione reductase). Furthermore, this proteomic analysis provides novel target proteins which may reveal the involvement of other metabolic pathways in copper tolerance, such as the carbohydrate metabolism which can mobilize new resources to synthetize *de novo* anionic polysaccharides; the latter compounds play important chelating roles in algal cell walls and also an important component of brown algal exudates, whose production increases in copper-stressed algae (Andrade et al., in press).

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