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Comparative analysis of peroxiredoxin activation in the brown macroalgae *Scytosiphon gracilis* and *Lessonia nigrescens* (Phaeophyceae) under copper stress

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Among thiol-dependent peroxidases (TDPs) peroxiredoxins (PRXs) standout, since they are enzymes capable of reducing hydrogen peroxide, alkylhydroperoxides and peroxynitrite, and have been detected in a proteomic study of the copper-tolerant species *Scytosiphon gracilis*. In order to determine the importance of these enzymes in copper-stress tolerance, TDP activity and type II peroxiredoxin (II PRX) protein expression were compared between the opportunistic *S. gracilis* and the brown kelp *Lessonia nigrescens*, a species absent from copper-impacted sites due to insufficient copper-tolerance mechanisms. Individuals of both species were cultured with increasing copper concentrations (0–300 μg l^{-1} Cu) for 96 h and TDP activity and lipoperoxides (LPXs) were determined together with II PRX expression by immunofluorescence and Western blot analysis. The results showed that TDP activity was higher in *S. gracilis* than *L. nigrescens* in all copper concentrations, independent of the reducing agent used (dithiothreitol, thioredoxin or glutaredoxin). This activity was copper inhibited in *L. nigrescens* at lower copper concentrations (20 μg l^{-1} Cu) compared to *S. gracilis* (100 μg l^{-1} Cu). The loss of activity coincided in both species with an increase in LPX, which suggests that TDP may control LPX production. Moreover, II PRX protein levels increased under copper stress only in *S. gracilis*. These results suggest that in *S. gracilis* TDP, particularly type II peroxiredoxin (II PRX), acts as an active antioxidant barrier attenuating the LPX levels generated by copper, which is not the case in *L. nigrescens*. Thus, from an ecological point of view these results help explaining the inability of *L. nigrescens* to flourish in copper-enriched environments.

Introduction

Copper (Cu) is an essential micronutrient, but in excess it may become harmful due to its involvement in redox reactions that generate reactive oxygen species (ROS). In the presence of superoxide or reducing agents such as glutathione (GSH) or ascorbic acid, Cu^{2+} is reduced to Cu^{+} which catalyzes the formation of hydroxyl radicals from hydrogen peroxide (H_2O_2) through the Haber–Weiss reaction (Bremner 1998).

Abbreviations – 1-Cys PRX, 1-cysteine peroxiredoxin; 2-Cys PRX, 2-cysteine peroxiredoxin; CAT, catalase; DT, dry tissue; DTT, dithiothreitol; GR, glutathione reductase; GRX, glutaredoxin; GSH, glutathione; H_2O_2, hydrogen peroxide; II PRX, type II peroxiredoxin; LPX, lipoperoxides; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate saline buffer; PRX, peroxiredoxin; PRX Q, type Q peroxiredoxin; Px, peroxidase; ROS, reactive oxygen species; *t*-BOOH, *t*-butyl peroxide; TDP, thiol-dependent peroxidase; TRX, thioredoxin.

ROS are known to participate in signaling pathways and activate non-specific defense mechanisms against stress (Mittler 2002). However, when the increase of ROS is not neutralized by cellular antioxidant mechanisms, a physiological condition known as oxidative stress (Sies 1991) develops, which leads to oxidative damage of various cell components (Halliwell and Gutteridge 1984, Buettner 1993, Powell 2000). Thus, excessive copper in the environment has potentially severe consequences for all living organisms.

One indisputable source of environmental copper pollution is mining. In Chile, as the result of this activity, coastal zones – in some cases distant from the copper mines – are directly affected by the discharge of solid and liquid wastes (Castilla and Nealler 1978, Vásquez et al. 1999, Fariña and Castilla 2001). One of the impacted areas is the coastline around Chañaral (26°16′S, 70°41′W), that started receiving copper-rich effluents from the El Salvador mine in the early 1940s. The most important effects associated with the discharge of the copper-rich effluent included high and fluctuating copper levels in coastal waters, ranging from 10 to 300 μg l^{-1} (Castilla and Correa 1997, Correa et al. 1999), beach progradation, and the disappearance of most species of algae and invertebrates (Castilla 1983, Correa et al. 2000, Medina et al. 2005). Two copper-tolerant algal species, *Scytosiphon lomentaria* (Ochrophyta, Scytosiphonales) and *Ulva compressa* (Chlorophyta, Ulvales) (Castilla and Correa 1997, Camus et al. 2005, Medina et al. 2005) are observed as the dominant organisms along the copper-enriched coast. These species are capable of accumulating high levels of copper (Andrade et al. 2006) and attenuate the copper-induced oxidative stress through various physiological mechanisms (Ratkevicius et al. 2003, Contreras et al. 2005). However, the brown kelp *Lessonia nigrescens* (Ochrophyta, Laminariales), a key stone species dominating the lower intertidal rocky shores from 16°S to 41°S, remains absent from the copper-enriched area (Medina et al. 2005). There is clear evidence that tolerance to copper stress in *S. lomentaria* is a constitutive trait, i.e. inducible and reversible in the presence or absence of copper excess (Contreras et al. 2005), a condition that extends to its sister species *S. gracilis* (Contreras et al. 2007). In comparison, the antioxidant responses recorded in *L. nigrescens* seem insufficient to attenuate the toxicity generated by the levels of copper found in the environment (Contreras et al. 2009). The absence of *L. nigrescens* from this impacted area affects negatively the local biodiversity, due to its role in regulating community structure (Cancino and Santelices 1984).

Recently, by using a proteomic analysis, a peroxiredoxin (PRX) was identified in *S. gracilis* exposed to copper excess (Contreras et al. 2010). PRXs belong to the thiol-dependent peroxidase (TDP) family, and are known to attenuate oxidative stress, reduce H_2O_2, alkylhydroperoxides and peroxynitrite and modulate redox-dependent signaling cascades (Dayer et al. 2008, Foyer and Noctor 2009, Tripathi et al. 2009, Dietz 2011). On the basis of their catalytic mechanisms and subcellular localization in vascular plants, four groups of PRXs have been distinguished: 1-cysteine peroxiredoxin (1-Cys PRX), 2-cysteine peroxiredoxin (2-Cys PRX), type Q peroxiredoxin (PRX Q) and type II peroxiredoxin (II PRX) (Dietz et al. 2002, Tripathi et al. 2009). In mammals, the six isoforms belong to three different PRX types, namely II PRX, 1-Cys PRX and 2-Cys PRX. Thus, PRX complexity is higher in plants. PRXs contain, in their active site, one or two cysteines whose thiol group is oxidized by peroxides to sulfenic acid, and subsequently reduced to its original form by two enzymatic reducing agents: thioredoxins and glutaredoxins (Rouhier and Jacquot 2002), which are reduced by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent thioredoxin reductase and GSH, respectively (Meyer 2008).

PRXs, as well as other TDP (i.e. GSH peroxidase), play an important role in buffering ROS generated by different environmental stresses. For example, the 1-Cys PRX transcript of the dehydration-tolerant *Xerophyta viscosa* Baker is induced under desiccation, heat, high light intensity and salinity stress (Mowla et al. 2002). An overexpression of 2-Cys PRX in transgenic *Festuca arundinacea* confers high tolerance to heat stress and methylviologen. This tolerance was the result of a low lipoperoxidation, probably due to peroxide detoxification and PRX chaperone activity (Kim et al. 2010). In *Arabidopsis thaliana*, PRX Q transcript levels were upregulated during light stress (Horling et al. 2003), and higher II PRX F protein and transcript levels were detected in cadmium-treated plants (Finkemeier et al. 2005). Finally, PRX Q and II PRX protein increased in poplar during the hypersensitive response associated with infection by the fungal pathogen *Melampsora larici-populina* (Rouhier et al. 2004).

In algae, the protective function of PRXs is scarcely documented. For example, the *prx* gene, with homology with the 2-Cys PRX of higher plants (Baier and Dietz 1997), was first identified in *Porphyra purpurea* (Bangiophyceae, Rhodophyta) (Reith and Munholland 1993). In the green microalga *Chlamydomonas reinhardtii* (Chlorophyta, Volvocales), seven genes have been identified as PRXs (Dayer et al. 2008). Studying the responses to desiccation stress in *Pyropia columbina* (Montagne) Nelson [formerly *Porphyra columbina* (Bangiophyceae, Rhodophyta)], Contreras-Porcia et al. (2011a, and unpublished data) found that PRX activity and its gene

expression increased significantly. Similarly, in the green alga *U. compressa* when exposed to copper excess for 7 days, genes encoding for PRX and its reducing agent, thioredoxin, increased significantly (Contreras-Porcia et al. 2011). Even though these observations suggest the involvement of PRXs and other TDP as part of the mechanism of tolerance to oxidative stress in seaweeds, functional analyses are lacking. In this context, and taking into consideration the inter-species differences in tolerance to copper-mediated oxidative stress displayed by some algae, we hypothesized that in copper-tolerant species, such as *S. gracilis*, PRXs are a key component in regulating copper-induced oxidative stress, making it possible for the species to flourish in metal-impacted sites. This hypothesis was tested by comparing TDP activity, protein II PRX expression and lipoperoxides (LPX) levels in tolerant (*S. gracilis*) and non-tolerant (*L. nigrescens*) algae exposed to copper excess.

Materials and methods

Algae and seawater sampling

Individuals of *S. gracilis* and *L. nigrescens* were collected manually during low tide in Las Cruces (33°30′S, 71°37′W) and Maitencillo (32°39.5′S, 71°26.6′W), sites with no history of heavy metal pollution (Ratkevicius et al. 2003, Contreras et al. 2005). Seawater used in all the experiments came from Las Cruces, was filtered through 0.22 μm polycarbonate membranes MF-Millipore™ (EMD Millipore, Billerica, MA) and kept in the dark at 11°C.

After collection, algae were transported to the laboratory in a cooler at 5–7°C. In the laboratory, fronds were rinsed with 0.22-μm-filtered seawater and cleaned for 10 s using an ultrasonic bath 575T (CREST, Trenton, NJ). Prior to their use, fronds were acclimated during 12 h in 0.22-μm-filtered fresh seawater at 14 ± 2°C, under 30–50 μmol m^{-2} s^{-1} photon flux density.

Copper treatments

Individuals of *S. gracilis* and *L. nigrescens* (10–20 g FW) were placed in Erlenmeyer flasks with 1 l of filtered seawater supplemented with $CuCl_2$ (Titrisol® MERCK, Darmstadt, Germany) for 96 h. Two series of copper concentrations were used: (1) 5, 10 and 20 μg Cu l^{-1} to cover the current concentrations at copper-polluted sites (Stauber et al. 2005, Andrade et al. 2006), and (2) 40, 100 and 300 μg l^{-1}, representing historic concentrations at the same sites (Castilla and Correa 1997, Correa et al. 1999). Controls without copper addition were included. Each treatment was run in triplicate at 14 ± 2°C, 30–50 μmol m^{-2} s^{-1} photonic flux density and 12:12 light : dark photoperiod. The culture medium was changed daily and, after 96 h, samples were stored at −80°C to determine enzymatic activities, LPXs content and II PRX analysis. Post incubation fresh samples were used immediately for immunofluorescence analysis. The kinetics of TDP activation was determined in both species, using samples collected after incubation for 4, 12, 24, 48, 72 and 96 h with the different copper concentrations.

Extraction and quantification of protein extracts

Algal samples were frozen in liquid nitrogen and homogenized in a mortar with a pestle. Proteins were precipitated with ammonium sulfate, stabilized in 2-mercaptoethanol 2 m*M* (Contreras et al. 2005), and quantified using the bicinchoninic acid assay (Smith et al. 1985), using Thermo Scientific Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

TDP activity assay

TDP activity, using dithiothreitol (TDP/DTT), was determined by preincubating 50–100 μg of protein extract with dithiothreitol (DTT) 0.2 m*M* in phosphate buffer 0.1 *M* pH 7.0 for 30 min at 37°C. DTT was used as reducing agent (Rhee et al. 2005) and also as an inhibitor of catalase (CAT) activity (Kunce et al. 1988). The reaction was initiated by adding 50 μ*M* H_2O_2 or *t*-butyl peroxide (*t*-BOOH) to the protein extract, and incubated for 30 min at 37°C. Reaction was stopped by adding trichloroacetic acid (10% final concentration) and centrifuged at 18 700 *g* for 10 min to precipitate the proteins. A 700 μl aliquot of the supernatant was mixed with 200 μl of $(NH_4)_2$ $Fe(SO_4)$ 10 m*M* and 100 μl of KSCN 2.5 *M*; these compounds react with the remaining peroxide forming a red-colored complex (Thurman et al. 1972). Peroxide concentrations were spectrophotometrically determined at 480 nm, using a spectrophotometer UV/Visible SmartSpec 3000 (BioRad, Hercules, CA). It is important to highlight that specificity of the TDP/DTT assay was checked by measuring the inhibitory effect of DTT on CAT activity, as this enzyme also consumes H_2O_2. This activity was determined as previously described by Contreras et al. (2005, 2009; see Appendix S1).

Determination of LPXs

Lipid peroxidation levels were determined as the amount of thio-barbituric acid reactive species (i.e. LPXs) according to Ratkevicius et al. (2003), using 3–5 g of dried tissue.

Peroxidase activity coupled to thioredoxin assay

The Px/TRX (thioredoxin) activity was determined by adding 50–100 μg of protein extract, 100 μ*M* H_2O_2, 2U TRX, 2U TRX reductase and 150 μ*M* NADPH to a final volume of 1 ml, in phosphate buffer 0.1 *M* pH 7.0. The decrease in absorbance at 340 nm, due to NADPH consumption, was monitored for 1 min and the enzymatic activity was calculated using the NADPH molar extinction coefficient (6.3 mM^{-1} cm^{-1}).

Px activity coupled to glutaredoxin (Px/GRX) assay

The Px/GRX activity was determined by adding 50–100 μg of protein extract, 100 μ*M* H_2O_2, 500 μ*M* of GSH, 1 U GSH reductase, 1 U glutaredoxin and 150 μ*M* NADPH to a final volume of 1 ml, in phosphate buffer 0.1 *M* pH 7.0. The decrease in absorbance at 340 nm due to NADPH consumption was then monitored for 1 min and the activity was calculated using the NADPH molar extinction coefficient.

Immunofluorescence detection of II PRX protein

Because of the lack of information on immunolocalization in algae, a protocol described for vascular plants was adapted for our algal models (Greenwood and Chrispeels 1985). Cross-sections of 0.5 cm of *S. gracilis* and *L. nigrescens* were treated with 4% paraformaldehyde, 0.3% glutaraldehyde and 0.1% caffeine in 0.1 *M* phosphate buffer pH 7.0 for 4 h at 4°C. Fixed material was then rinsed with phosphate buffer 0.1 *M* pH 7.0 for 12 h and dehydrated through 50, 60, 70, 80, 90 and 100% (dried) ethanol, 30 min per step at 4°C. Samples were infiltrated in a 1:1 LR-White resin (London Resin Company, Reading, Berkshire, England):pure ethanol mixture for 2 h and then embedded in pure LR-White resin for 4 h. Sections of 1–2 μm thick were obtained using a Sorvall MT-5000 ultramicrotome (Sorvall, Wilmington, DE) and mounted on glass slides pretreated with (3-aminopropyl) triethoxysilane.

For immunodetection, slides were treated for 2 h in blocking buffer, consisting of commercial 3% skim milk in phosphate saline buffer (PBS) pH 7.2, and incubated for 12 h with polyclonal primary anti-II PRX antibody diluted in blocking buffer (1:400). The slides were rinsed with blocking buffer to remove excess primary antibody and incubated for 2 h with secondary antibody (FITC-conjugated Goat anti-Rabbit IgG; Abcam, Cambridge, UK) in blocking buffer (1:200) in total darkness. The excess of secondary antibody was removed with blocking buffer and sections were observed in an Nikon Eclipse E400 fluorescence microscope (Nikon Instruments Inc., Melville, NY). The primary polyclonal antibody was prepared in rabbit (GenScript, Piscataway, NJ) using antigenic peptide sequence DAEGALGNKRC from previously described peroxiredoxin gene (NCBI accession number: FD387607) in *U. compressa* (Contreras-Porcia et al. 2011), identified as II PRX using BLAST (NCBI, Bethesda, MD) analysis.

To check the specificity of the secondary antibody, the same protocol for immunodetection was applied to cross-sections of *U. compressa*, *S. gracilis* and *L. nigrescens* exposed to 635, 40 and 10 μg l^{-1} of copper respectively, but excluding incubation with the primary antibody (see Fig. S2). Finally, data were analyzed using the IMAGEJ software for fluorescence intensity quantification (Abramoff et al. 2004).

Western blot for II PRX detection

Protein extracts from *S. gracilis* and *L. nigrescens* exposed to 0, 10, 20, 40 and 100 μg l^{-1} cooper additions were analyzed by Western blot to verify the presence and expression levels of II PRX. Proteins from *S. gracilis* exposed to 300 μg l^{-1} of copper were also analyzed. As in the II PRX immunodetection, protein extracts from *U. compressa* exposed to 635 μg l^{-1} copper were used as positive control. Proteins were extracted using the phenol method as previously described (Contreras et al. 2008). The protein pellet was re-suspended in PBS at pH 7.2 and separated according to their molecular weight using one-dimension electrophoresis (75 min at 150 V), charging a volume with 40 μg of proteins in a 15% acrylamide gel. The proteins were then transferred to a 0.45 μm nitrocellulose membrane (BioRad), for 2 h at 70 mA and 4°C. The non-specific binding sites were blocked during 12 h at 4°C in PBS pH 7.2 containing 3% BSA and 0.05% Tween-20. The membrane was first incubated with the primary anti-II PRX antibody diluted 1:2000 for 1 h and the antibody excess was removed by rinsing with PBS pH 7.2 and 0.05% Tween-20 for 10 min. The membrane was then incubated for 1 h with the secondary antibody diluted 1:15 000 (anti-rabbit-conjugated horseradish Px; Jackson ImmunoResearch, West Grove, PA) and the excess of antibody was removed as above. Finally, blots were visualized using the ECL Western Blotting Substrate (Pierce, Thermo Fisher Scientific) according to manufacturer instructions.

Statistical analyses of the results

Enzymatic activities and LPXs levels were analyzed using MINITAB software (Minitab Inc., State College, PA), with various statistical approaches including analyses of variance followed by Tukey multiple comparisons tests (T). Prior to the analyses, data were checked for normal distribution using Kolmogorov–Smirnov test and for variance homogeneity using Levene test (Zar 2010).

Results

TDP/DTT activity in *S. gracilis* and *L. nigrescens* under copper stress

TDP/DTT activity with H_2O_2 or *t*-BOOH as substrates was detected in both species (Fig. 1). The inhibitory effect of DTT on CAT activity demonstrates the specificity of the TDP/DTT assay (Fig. S1). TDP/DTT activity was higher in *S. gracilis* (Fig. 1A) than in *L. nigrescens* (Fig. 1B) at basal level as well as in all copper concentrations (species-copper concentrations interaction: $F = 27.53$, $P < 0.001$). *Scytosiphon gracilis* exposed to low concentrations of copper (i.e. 5 µg l^{-1}) showed a significant increase in TDP/DTT activity using H_2O_2 as substrate ($T = 4.12$, $P = 0.015$) but not with *t*-BOOH ($T = 0.69$, $P = 0.527$; Fig. 1A). However, the peak of TDP/DTT activity, 2.7 times higher than the controls for H_2O_2 ($T = 8.33$, $P = 0.001$) and three times higher than *t*-BOOH ($T = 6.31$, $P = 0.003$), was recorded in cultures with 40 µg Cu l^{-1}. At higher copper concentrations (i.e. 100–300 µg Cu l^{-1}) the TDP/DTT activity dropped to basal levels (i.e. exposure to 300 µg Cu l^{-1} compared to control condition; $T = 0.19$, $P = 0.855$).

In *L. nigrescens*, a significant increase in the TDP/DTT activity at 5 µg Cu l^{-1} using both H_2O_2 ($T = 5.50$, $P = 0.05$) and *t*-BOOH ($T = 4.99$, $P = 0.008$; Fig. 1B) was observed. The peak in TDP/DTT activity was 3.8 times higher than the control ($T = 15.98$, $P < 0.001$) for H_2O_2, and five times higher ($T = 16.49$, $P < 0.001$) for *t*-BOOH (Fig. 1B), a response detected only at 10 µg l^{-1} Cu. However, it is important to highlight that this peak of activity was similar to the basal level recorded in *S. gracilis* cultured without copper addition ($T = 1.10$, $P = 0.332$). Finally, there was a significant decrease of TDP/DTT activity to basal levels in *L. nigrescens* cultured with 20 µg Cu l^{-1} ($T = 1.69$, $P = 0.166$) or higher concentrations.

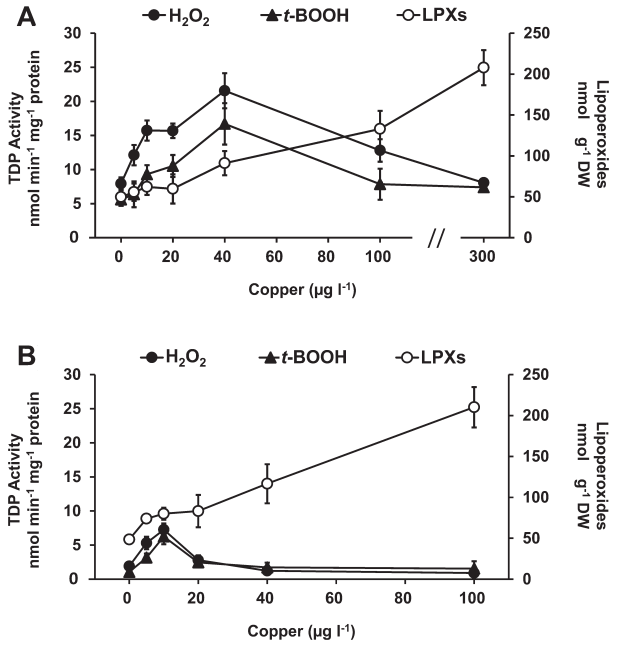

Fig. 1. TDP/DTT activity using H_2O_2 (●) and *t*-BOOH (▲) as substrates, and LPXs (○) content in (A) *Scytosiphon gracilis* and (B) *Lessonia nigrescens* cultured under different copper concentrations during 96 h. Mean values of three replicates ±SD. DW, dry weight.

Lipoperoxides

Both species showed similar LPX levels in individuals cultured without copper addition for 96 h ($T = 0.16$, $P = 0.879$) (Fig. 1B). The over-production of LPX was clearly observed in both species exposed to copper excess (Fig. 1). At 40 µg Cu l^{-1}, *S. gracilis* LPX levels almost doubled the values recorded under the control condition ($T = 3.92$, $P = 0.017$; Fig. 1A), whereas in *L. nigrescens* this increment occurred at an even lower copper concentration (i.e. 5 µg Cu l^{-1}). Maximum LPX levels in *L. nigrescens* were recorded at 100 µg l^{-1} Cu (4.3 times higher than the control).

Kinetics of Px activity under copper stress in *S. gracilis* and *L. nigrescens*

In order to understand the behavior of Pxs under copper stress, a kinetics study was conducted with both species during 96 h of culture. The results show that control *S. gracilis* displayed a stable activity, without significant differences throughout the experiment ($F = 0.18$, $P = 0.965$; Fig. 2). However, a significant increase of 1.6 times was detected after 48 h of culture in 20 µg Cu l^{-1} ($T = 3.77$, $P = 0.020$) and 2 times in 40 µg Cu l^{-1} ($T = 11.10$, $P < 0.001$; Fig. 2). The maximum TDP/DTT activity was recorded after 96 h of culture in 40 µg Cu l^{-1} (three times higher than the controls).

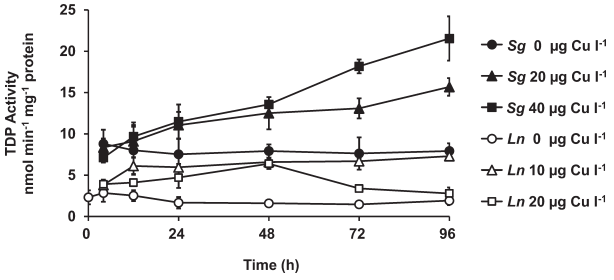

Fig. 2. Kinetics of TDP/DTT activation under copper stress in *Scytosiphon gracilis* (Sg) and *Lessonia nigrescens* (Ln) using H_2O_2 as substrate. Mean values of three replicates ±SD.

As in *S. gracilis*, TDP/DTT activity in *L. nigrescens* was influenced by the amount of copper added to the medium and it varied with the time of exposure. At 10 and 20 μg Cu l^{-1} a significant increase in the TDP/DTT activity occurred after 12 h [2.4 times higher for 10 μg Cu l^{-1} ($T = 4.96$, $P = 0.008$) and 1.6 times higher for 20 μg Cu l^{-1} ($T = 3.56$, $P = 0.024$)] than the control (Fig. 2). The peak of TDP/DTT activity occurred after 48 h in 20 μg Cu l^{-1} (4.7 times higher than the control). However, after 72 h the activity declined, reaching levels similar to the control condition ($T = 1.69$, $P = 0.166$). Thus, inactivation was only observed in *L. nigrescens* at higher copper concentrations. When the two species are compared at 20 μg Cu l^{-1}, i.e. similar to the current copper concentration at the impacted sites, TDP/DTT activity was higher in *S. gracilis* than *L. nigrescens* in all comparisons (interaction time-species: $F = 11.01$, $P < 0.001$).

TRX-coupled Px activity in *S. gracilis* and *L. nigrescens*

TRX-coupled peroxidase activity (Px/TRX) was determined in both species cultured without copper addition and with the copper addition where the maximum TDP/DTT activity was previously recorded (40 μg Cu l^{-1} for *S. gracilis* and 10 μg Cu l^{-1} for *L. nigrescens*; Table 1). The Px/TRX activity in the two species was similar under control conditions ($T = 1.56$, $P = 0.639$). In *S. gracilis*, Px/TRX activity was copper-dependent and in individuals exposed to 10 μg Cu l^{-1} was 2.5 times higher than the control ($T = 3.91$, $P = 0.020$), whereas plants exposed to 40 μg Cu l^{-1} displayed an activity 3.1 times higher than the control ($T = 5.47$, $P = 0.002$). In *L. nigrescens*, however, copper additions did not trigger increases in Px/TRX activity (10 μg Cu l^{-1}, $T = 2.04$, $P = 0.374$; 40 μg Cu l^{-1}, $T = 0.71$, $P = 0.977$; Table 1).

Table 1. Px activity (nmol min^{-1} mg^{-1} protein) using TRX and GRX as reducing agent in *Scytosiphon gracilis* and *Lessonia nigrescens* exposed to copper stress during 96 h. The copper concentrations used were those in which the maximum TDP/DTT activity was registered for each species. Each value represents the mean of three independent replicates ±SD. Superscript letters represent significant differences ($P < 0.05$) between treatments for each species.

Species	Copper (μg l^{-1})	Px/TRX	Px/GRX
S. gracilis	0	62.37 ± 32.63^{a}	84.95 ± 22.66^{a}
	10	156.99 ± 44.03^{b}	86.02 ± 16.55^{a}
	40	194.62 ± 36.26^{b}	225.81 ± 50.70^{b}
L. nigrescens	0	24.73 ± 14.90^{a}	36.56 ± 13.43^{a}
	10	34.19 ± 7.96^{a}	67.74 ± 27.56^{a}
	40	41.94 ± 20.15^{a}	31.18 ± 13.04^{a}

GRX-coupled Px activity in *S. gracilis* and *L. nigrescens*

Px/GRX activities were determined at the same copper concentrations used to determine Px/TRX activity. Both species displayed a similar Px/GRX activity ($T = 2.17$, $P = 0.317$) under control conditions (Table 1). In *S. gracilis*, Px/GRX activity increased significantly only when fronds were exposed to 40 μg Cu l^{-1} (approximately three times higher than control: $T = 6.32$, $P < 0.001$). *Lessonia nigrescens*, on the other hand, did not respond with changes in Px/GRX activity regardless the amount of added copper (Table 1).

Detection of II PRX by immunofluorescence and Western blot

An increased fluorescence intensity of II PRX, 2.6 times higher ($T = 7.31$, $P < 0.001$) than the control (Fig. 3B) and involving mainly cortical cells, was recorded in *S. gracilis* exposed to the copper concentration where the peak of Px activity had been previously recorded (Fig. 3C). Contrarily, individuals exposed to 300 μg Cu l^{-1} (which causes Px inactivation) displayed a significantly lowered fluorescence compared to 40 μg Cu l^{-1} ($T = 6.41$, $P = 0.002$) and similar to the control ($T = 0.90$, $P = 0.659$; Fig. 3D). Moreover, none of the copper additions (Fig. 3G, H) triggered a change in fluorescence intensity in *L. nigrescens* (Fig. 3F). The analysis by Western blot was consistent with observations by immunofluorescence and enzymatic activity. The II PRX expression was observed in *U. compressa* (17.4 kDa), and in *S. gracilis* (18 kDa) exposed to 10, 20 and 40 μg Cu l^{-1} (Fig. 3I). Contrary, in *S. gracilis* exposed to 100 and 300 μg Cu l^{-1}, and in *L. nigrescens* at any copper concentration (Fig. 3I), such expression was not observed.

Discussion

This study demonstrated that TDP activity was higher in *S. gracilis* than in the brown kelp *L. nigrescens* at all copper concentrations tested, using H_2O_2 and *t*-BOOH as substrates. These results are consistent with those described previously for other antioxidant enzymes in both species exposed to copper enrichment (Contreras et al. 2008, Contreras et al. 2009). In addition, *S. lomentaria* from natural populations growing at copper-impacted sites displayed a TDP activation similar in magnitude to those described for *S. gracilis* in the in vitro treatments (Appendix S1). Thus, our results demonstrate (1) an active involvement of these antioxidant enzymes in species of *Scytosiphon* responding to copper excess and (2) that differences in TDP activation between *S. gracilis*

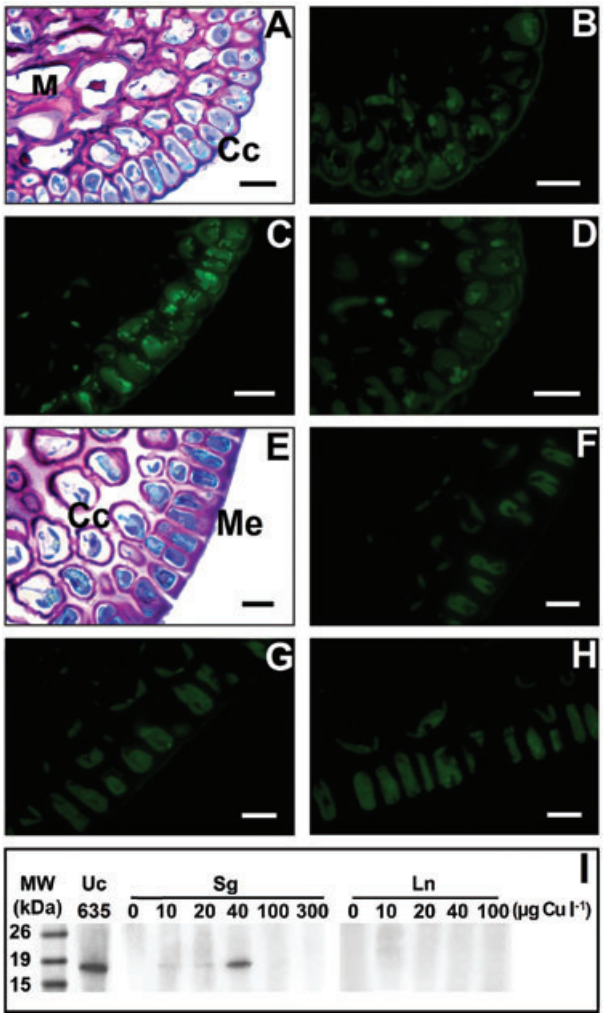

Fig. 3. II PRX detection by immunofluorescence in *Scytosiphon gracilis* (A–D) and *Lessonia nigrescens* (E–H) under copper treatment for 96 h. (A, E) Cross-sections stained with toluidine blue: CC, cortical cells; M, medulla; Me, meristoderm. (B, F) Control tissues and (C, G) tissues exposed to the concentration of copper where maximum PRX activity was recorded: (C) 40 and (G) 10 µg l^{-1} of copper. (D, H) copper concentrations where PRX inactivation was detected: (D) 300 and (H) 100 µg l^{-1} of copper. (I) Western blot to validate the specificity of the primary antibody and evaluate the II PRX expression under different copper concentrations. Uc, *U. compressa*; Sg, *S. gracilis*; Ln, *L. nigrescens*; MW, molecular weight (see Fig. S2).

and *L. nigrescens* help to understand the absence of the latter from copper-impacted coastal environments.

Our results also showed that, in *L. nigrescens*, TDP decreased at lower copper concentration, whereas in *S. gracilis* the decline occurred at higher copper concentrations (i.e.100 µg Cu l^{-1}). Loss of TDP activity could be attributed by to PRX inactivation. PRX inactivation might be due to an over-oxidation of the catalytic cysteine under high (1–5 m*M*) peroxide concentrations (Chae et al. 1994). At such high peroxide concentrations other Pxs, such as ascorbate Px and GSH Px, remain active in *S. gracilis* (Contreras et al. 2007) and in other tolerant macroalgae (Collén and Davison 1999, Ratkevicius et al. 2003, Contreras et al. 2009). Moreover, a direct action of copper on the enzyme cannot be ruled out, as has been observed in other antioxidant enzymes where the cysteine residues were oxidized by the metal (e.g. GSH reductase; Shigeoka et al. 1987, Schützendübel and Polle 2002). PRX inactivation could trigger a redox imbalance given that PRX has an important role in the control of oxidative stress induced by diverse abiotic factors. For example, in the yeast *Schizosaccharomyces pombe* 2-Cys PRX there is a redox sensor that controls the transcriptional response to H_2O_2, attenuating the oxidative stress triggered by high H_2O_2 concentrations (Vivancos et al. 2005). Therefore, we suggest that loss of PRX activity in *L. nigrescens* could be due to a high oxidative stress condition, since this state is buffered at a much lower rate compared to *S. gracilis*. This inactivation may then lead to a lower copper tolerance, expressed as important cellular alterations (i.e. protoplast retraction and indistinct thylakoidal membranes; Contreras et al. 2009). In contrast, the high activation of other antioxidant enzymes, such as those involved in Halliwell–Asada cycle, lead to an effective redox control in *S. gracilis* exposed to copper excess (Contreras et al. 2007).

This work demonstrated that under copper concentrations where TDP activity was inhibited, there was an accumulation of LPXs, which were higher in *L. nigrescens* than in *S. gracilis*. LPXs are caused by oxidative damage to membranes or other cellular structures that contain lipids, and can exacerbate an oxidative stress condition (Girotti 1998). It has been suggested that PRXs may have a protective role against lipoperoxidation by controlling its cellular harmful effects. For example, a correlation was observed between *prx* silencing and LPX over-production (Wang et al. 2006), and recently was experimentally determined a direct reduction of LPXs by specific PRXs (Cha et al. 2007, Cordray et al. 2007, Manevich et al. 2009). Thus, the high levels of LPXs in *L. nigrescens* could be related to the inhibition of PRX and other tolerance pathways, thereby, indicating a high susceptibility to copper-induced oxidative damage. On the other hand, low cellular concentrations of LPXs are involved in metabolic pathways related to the regulation of antioxidant compounds and enzymes or apoptotic pathways (Girotti and Kriska 2004, Niki 2009). Thus, the control of intracellular LPX levels by means of PRXs in *S. gracilis* to cope with oxidative stress may be an important way of modulating signaling tolerance pathways that involve these molecules.

With regards to TRX-coupled Px activity, only *S. gracilis* showed a significant response to copper excess. In vascular plants, nuclear 1-Cys PRXs and the chloroplastic 2-Cys PRX and PRX Q are susceptible to reduction only by TRX (Mowla et al. 2002, Tripathi et al. 2009). On the basis of this, we suggest that in *S. gracilis* the Px/TRX system, predominantly PRX/TRX, has a protective role in attenuating copper-induced oxidative stress in the chloroplast and/or nucleus. In *L. nigrescens*, however, insufficient PRX activity could explain the over-production and/or the poor regulation of LPXs in the chloroplast. This is consistent with previous reports showing a severe disruption of thylakoidal membranes in *L. nigrescens* exposed to copper excess (Contreras et al. 2009). The interaction between PRXs and TRXs affects not only the antioxidant responses in organisms facing oxidative stress, but also other cellular processes such as nitrogen metabolism, fatty acids biosynthesis and protein kinases that regulate stress signaling pathways (Buchanan and Balmer 2005, Montrichard et al. 2009). Therefore, activation of the PRX/TRX system in algae could be responsible for regulating the interaction of TRXs with other tolerance proteins in specific organelles.

Similar to the observed Px/TRX activity, Px/GRX activity increase in response to copper was detected in *S. gracilis*, but not in *L. nigrescens*. This enzymatic system uses GSH, an important antioxidant compound present in all organisms, as final electron donor (Anderson 1998). However, under oxidative stress Px/GRX activity, mainly PRX/GRX, may drastically decay due to low intracellular levels of GSH which, instead, may result from glutathione reductase (GR) inhibition and the subsequent failure to regenerate reduced GSH from oxidized GSH (Asada 1999). This is a reasonable explanation, as inhibition of GR activity was described in *L. nigrescens* exposed to copper excess (Contreras et al. 2009). Availability of GSH can also be affected when it is used as a precursor in the synthesis of phytochelatins, a GSH oligomer capable to bind heavy metals (Cobbett 2000). Thus, it is likely that low activity of the PRX/GRX system in *L. nigrescens* could be caused by depletion of intracellular levels of GSH. In fact, *L. nigrescens* plants exposed to the copper-impacted environment for 96 h show a reduction in these levels by up to 80% from 0.17 μmol g^{-1} dry tissue (DT) of GSH in control plants to 0.034 μmol g^{-1} DT in copper-exposed plants (Contreras-Porcia, unpublished data).

The increment in II PRX immunofluorescence in *S. gracilis* exposed at 40 μg Cu l^{-1} was consistent with the high Px activity observed using DTT or GRX as a reducing agent. Other stress factors, including desiccation, heat, xenobiotics, pathogens or photo-oxidation are also known to cause enhanced PRX levels (e.g. II PRX) in vascular plants (Mowla et al. 2002, Horling et al. 2003, Rouhier et al. 2004, Finkemeier et al. 2005, Kim et al. 2010). However, *S. gracilis* treated with 300 μg Cu l^{-1} displayed decreased II PRX-fluorescence levels coincident with a low Px activity. These results suggest that under high copper concentrations, a decline of Px activity could be due to a decrease in the II PRX protein synthesis, among other factors. Oxidation of the antigenic sequence (i.e. the II PRX protein) under high copper stress could prevent recognition by the anti-II PRX antibody, resulting in lower fluorescence intensity. Thus, this latter possibility should not be overlooked when explaining the results of this study. Finally, no changes in fluorescence were recorded in *L. nigrescens* at any copper concentration, which could be explained as in *S. gracilis* (i.e. oxidation of the antigenic sequence and a decreased in the II PRX protein synthesis).

According to the Western blot analysis, the protein determined in *S. gracilis* and *U. compressa* has a molecular weight of 18 kDa, close to II PRXs that in *A. thaliana* are located mainly in the chloroplast and mitochondria, and are susceptible to reduction by GRX (Tripathi et al. 2009). It has been also reported that expression of PRXs is tissue-specific. For example, II PRX levels increase in photosynthetically active young leaves stressed by salinity, and decrease in tissues photosynthetically inactive like senescent leaves and roots (Horling et al. 2002). This is in agreement with our observations in *S. gracilis* which, when treated with 40 μg Cu l^{-1} displayed an increase in II PRX levels, specifically in the metabolically active cortical cells, where most of the photosynthetic activity occurs. This result also suggests that PRX in *Scytosiphon*, like II PRX (PRX/GRX), could cooperate in attenuating ROS over-production during copper-disrupted metabolic processes, such as photosynthesis in chloroplasts (Asada 1999) or respiration in mitochondria (Kowaltowski et al. 2009). In the case of *L. nigrescens*, the low GRX-coupled Px activity, in addition to the lack of recognition of II PRX by immunofluorescence and Western blot, suggests a low expression of this type of PRX. Additionally, this could explain, for example, the mitochondrial alterations associated with copper stress (Contreras et al. 2009), which leads to a dysfunction of the organelle and cell death. Certainly, much more research is needed in this context. Finally, the results obtained in this study indicate that in *S. gracilis* under copper stress the TDP activities are over-induced (both Px/TRX and Px/GRX), thereby reducing both H_2O_2 and *t*-BOOH. The II PRX induction at protein level is observed mainly in the cortical cells of the fronds. These results are in contrast with those observed in *L. nigrescens*, where an insufficient

Px activity was recorded, concomitant with an overproduction of LPXs. These differences in tolerance and those previously observed in other tolerance pathways (Contreras et al. 2009), helps to explain the persistent absence of *L. nigrescens* in copper-polluted sites, where the current copper concentration does not exceed 20 μg Cu l^{-1}. In contrast, the high copper tolerance of *S. gracilis* by means of activation antioxidant responses represents a crucial ecological advantage to this species.

Conclusion

This study describes the active participation of II PRX, a thiol-dependent peroxidase (TDP) which attenuates peroxide excess generated during oxidative stress, and suggests this is another enzymatic mechanism for copper tolerance in brown algae. Px activity in the brown kelp *L. nigrescens* is lower than in *S. gracilis* regardless the copper levels at which are exposed and the reducing agent used (DTT, TRX and GRX). In addition, in *L. nigrescens* these activities are inhibited at low copper concentrations, associated with a rapid increase in LPX levels. In *S. gracilis*, this phenomenon was only detected at higher copper concentration. This observation suggests that LPXs might be regulated by TDP, probably II PRX, and in *S. gracilis* it relates to the ability of this species to tolerate copper-polluted environments. Conversely, the loss of PRX activities in *L. nigrescens* under copper stress helps to explain the serious cellular damage displayed by this species when exposed to copper excess and its failure in colonizing copper-polluted environments.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Control of CAT activity to check the TDP specificity assay in *Scytosiphon gracilis* exposed to 0 and 40 μg l^{-1} Cu, and *Lessonia nigrescens* exposed to 0 and 10 μg l^{-1} Cu for 96 h.

Figure S2. Immunofluorescence control to check the specificity of secondary antibody in cross-sections of *U. compressa*, *Scytosiphon gracilis* and *Lessonia nigrescens*.

Appendix S1. Supporting information of catalase activity assay, control for secondary antibody specificity in immunodetection, and TDP/DTT activity in Scytosiphon lomentaria from copper-polluted site.

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