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ORIGINAL ARTICLE

Identification of Copper-Induced Genes in the Marine Alga *Ulva compressa* (Chlorophyta)

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Abstract In order to identify genes/proteins involved in copper tolerance, the marine alga *Ulva compressa* was cultivated with 10 μM copper for 3 days. The activities of antioxidant enzymes ascorbate peroxidase (AP), peroxiredoxin (PRX), thioredoxin (TRX), and glutathione-S-transferase (GST) and the level of lipoperoxides were determined in the alga cultivated with and without copper addition. Antioxidant enzyme activities and lipoperoxides level increased in response to copper excess, indicating that the alga was under oxidative stress. A cDNA library was prepared using *U. compressa* cultivated with 10 μM copper for 3 days. A total of 3×10^4 clones were isolated and 480 clones were sequenced, resulting in 235 non-redundant ESTs, of which 104 encode proteins with known functions. Among them, we identified proteins involved in (1) antioxidant metabolism such as AP, PRX, TRX, GST, and metalothionein (MET), (2) signal transduction, such as calmodulin (CAM), (3) calcium-dependent protein kinase (CDPK) and nucleoside diphosphate kinase (NDK), (4) gene expression, (5) protein synthesis and degradation, and (6) chloroplast and mitochondria electron transport chains. Half of the identified proteins are potentially localized in organelles. The relative level of 18 genes, including those coding for AP, PRX, TRX, GST, MET, CAM, CDPK, and NDK were determined by quantitative RT-PCR in the alga cultivated with 10 μM copper for 0 to 7 days. Transcript levels increased in response to copper stress and most of them reached a maximum at days 3 and 5. Thus, the selected genes are induced by copper stress and they are probably involved in copper acclimation and tolerance.

Keywords Antioxidant enzymes · Lipoperoxides · cDNA library · Copper-induced genes · Marine alga · *Ulva compressa*

Introduction

Ulva compressa Linnaeus (Chlorophyta), formerly *Enteromorpha compressa* (Hayden et al. 2003), is a cosmopolitan metal-tolerant species that dominates copper-enriched coastal areas in northern Chile (Castilla 1996; Ratkevicius et al. 2003; Medina et al. 2005) and other parts of the world (Villares et al. 2001; Pereira et al. 2009). It was demonstrated that in copper-enriched coastal areas, *U. compressa* accumulated the metal and showed an increased activity of ascorbate peroxidase (AP), a reduced level of glutathione and water-soluble phenolic compounds and an increased level of ascorbate, which is accumulated as dehydroascorbate (Ratkevicius et al. 2003). On the other hand, these *U. compressa* showed no catalase (CAT), glutathione peroxidase (GP) and dehydroascorbate reductase (DHAR) activities (Ratkevicius et al. 2003). In addition, *U.*

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compressa cultivated with 10 μM copper for 0 to 7 days showed an increase in activities of antioxidant enzymes AP, glutathione reductase (GR) and glutathione-S-transferase (GST) and of defense enzymes phenylalanine ammonia lysase (PAL) and lipoxygenase (LOX) (González et al. 2010). It is important to highlight that *U. compressa* cultivated with copper concentrations up to 50 μM copper survived after 7 days of exposure and their normality was demonstrated by growing the plants in control seawater for seven additional days (González et al. 2010).

Linking physiological responses to stress with their genetic bases, particularly using current genomic and proteomic approaches, would certainly improve our understanding of the relationship between organisms and their environment. However, although fast development of, and more expedite access to, genomic tools have boosted research looking for the genetic bases of a broad range of biologically relevant processes in a diverse array of organisms, studies on algae are still scarce (reviewed by Peters et al. 2004 and Stanley et al. 2005). In this context, partial sequencing of cDNA libraries to generate expressed sequence tags (ESTs) provides an alternative and effective approach to gene discovery. In fact, unspecific ESTs collections are available for some red (*Porphyra yezoensis*, *Porphyra haitanensis*, and *Chondrus crispus*), brown (*Laminaria digitata* and *Sargassum binderi*), and green (*Ulva linza* and *U. fasciata*) seaweeds (Crépineau et al. 2000; Lee et al. 2000; Nikaido et al. 2000; Roeder et al. 2005; Stanley et al. 2005; Collén et al. 2006; Wong et al. 2007; Xiaolei et al. 2007). However, only few studies have focused on algae exposed to abiotic stress as models to obtain specific ESTs collections. Some examples of ESTs collections obtained from microalgae exposed to abiotic stress include *Chlamydomonas* sp. W80 cultivated at high salinity (Miyasaka et al. 2000) and *Thalassiosira pseudonana* exposed to copper excess (Davis et al. 2006). Regarding macroalgae, a cDNA library was recently prepared from *Fucus serratus* and *F. vesiculosus* exposed to desiccation and heat (Pearson et al. 2010). In addition, a subtractive hybridization library was obtained from *U. fasciata* and several copper-responsive genes coding for antioxidant enzymes were isolated (Wu and Lee 2008). From a physiological perspective however, available information indicates that different species of *Ulva* show different levels of copper tolerance and different biochemical responses to copper stress (Ratkevicius et al. 2003; Wu and Lee 2008; Han et al. 2008). In this context, the model used in this study (i.e., *U. compressa*) is particularly tolerant to copper stress since it is the only ulvophyte, when compared with the other species of the genus for which cDNA libraries are available, naturally thriving in copper-impacted sites (Ratkevicius et al. 2003).

Thus, we used *U. compressa* exposed to copper excess to prepare a cDNA library from where putative genes potentially involved in copper tolerance were identified. The activities of AP, PRX, TRX and GST as well as the level of lipoperoxides, were measured to demonstrate the occurrence of a copper-induced oxidative stress condition. The relative transcript level of 18 identified genes was analyzed by quantitative real time RT-PCR in order to detect their induction in response to copper excess.

Methods

Sampling and Culture Conditions

Individuals of *U. compressa* were collected during low tide in Cocholgüe (36° 40′ S), a non-impacted site of southern Chile (Ratkevicius et al. 2003), and transported to the laboratory in a cooler on ice. In the laboratory, algae were rinsed with filtered seawater and cleaned manually. Ultrasound was applied to remove epiphytic bacteria and debris. Seawater was obtained from Quintay (33° 12′S) in central Chile, filtered through 0.2 μm pore size membrane filters and stored in darkness at 4°C.

To prepare the cDNA library, 30 g of fresh algal tissue were cultured in 1 L of 0.2 μm-filtered seawater supplemented with 10 μM (635 μg L^{-1}) of $CuCl_2$ (Merck, Darmstadt, Germany). The material was incubated for 3 days at 12°C, with 12:12 h photoperiod and 40–50 μmol m^{-2} s^{-1} irradiance. Culture medium was aerated and changed every 24 h. For antioxidant enzyme activities and lipoperoxides level detection, 45 g of *U. compressa* were incubated in 1.5 L of 0.2 μm-filtered seawater without copper addition and with 10 μM copper for 3 days, in triplicate. For quantitative RT-PCR detection, 3 g of *U. compressa* were incubated in 100 mL of 0.2 μm-filtered seawater without copper addition and with 10 μM copper for 0, 1, 3, 5 and 7 days, in triplicate. In all cases, samples were rinsed with 100 mM Tris–HCl-10 mM EDTA, blotted dry, weighed and frozen in liquid nitrogen.

Detection of Antioxidant Enzyme Activities

Preparation of protein extracts from *U. compressa* and detection of AP activity were performed as described in Ratkevicius et al. (2003).

GSH-dependent PRX activity was determined in 1 mL reaction mixture containing 100 mM phosphate buffer pH 7.0, 5 mM hydrogen peroxide, 0.5 mM glutathione (GSH), 1U of glutathione reductase (GR), 0.15 mM NADPH and 50 μg of protein extract. The decrease in absorbance at 340 nm due to NADPH consumption was monitored for 10 min. PRX activity was calculated using the extinction coefficient of NADPH (ε=6.3 mM^{-1} cm^{-1}).

TRX activity was analyzed in 1 mL reaction mixture containing 100 mM phosphate buffer pH 7.0, 2.5 mM 5,5′ dithiobis(2- nitro-benzoic acid) (DTNB), 0.5 mM dithiothreitol (DTT) and 50 μg of protein extract. The decrease in absorbance at 412 nm due to DTNB reduction to 2-nitro 5-thiobenzoate was monitored for 10 min. TRX activity was calculated using the extinction coefficient of DTNB (ε=13.6 mM^{-1} cm^{-1}).

GST activity was detected in 1 mL of reaction mixture containing 100 mM phosphate buffer pH 7.0, 0.5 mM GSH, 1 mM 1-chloro 2, 4 dinitrobenzene (CDNB) and 50 μg of protein extract. The increase in absorbance at 340 nm due to GSH-CDNB adduct formation was monitored for 1 min. GST activity was calculated using the extinction coefficient of the GSH-CDNB adduct (ε=9.6 mM^{-1} cm^{-1}).

Detection of Lipoperoxides

Lipoperoxide levels were determined as thio-barbituric acid reactive species (T-BARS) and expressed in micromoles per gram of dry tissue as described in Ratkevicius et al. (2003).

Preparation of the cDNA Library

Total RNA was isolated from 5 g of fresh tissue using RNeasy mini Kit (Qiagen, Hilden, Germany) according to the manufacturer protocol and mRNA was obtained using DynaBeads (Invitrogen, Oregon, USA) from the total RNA. The synthesis of cDNAs, their size fractionation and cloning were obtained using SMART™ cDNA Library Construction Kit (Clontech, Mountain View, CA, USA) and the recombinant plasmids were sequenced from their 5′ and 3′ ends as described in Wellenreuther et al. (2004).

Identification and Classification of ESTs

Sequences were obtained from 480 recombinant plasmids using an automatic sequencer. In the case of clones containing an insert, sequences corresponding to 5′ and 3′ ends of the vector were eliminated. Nucleotide sequences were then translated into amino acids using the ExPASy proteomics server (http://www.expasy.org/tools/dna.html) into the six potential reading frames and analyzed for sequence similarities using BLASTX program (NCBI, USA). Reading frames with the highest sequence similarity scores were used to analyze protein similarity using BLASTP program. Threshold values were set above 50 for high-scoring segment pairs (HSP), with a minimum significance of $1\times e^{-4}$ and a percentage of identity higher than 30%. ESTs coding for known proteins were classified into functional categories using the KO (KEGG Orthology) database for ortholog grouping and hierarchical classification of genes (Kenehisa et al. 2004). The putative subcellular localization of the proteins was determined using the TARGETP (http://www.cbs.dtu.dk/services/TargetP/, Emanuelsson et al. 2000), WOLFPSORT (http://psort.ims.u-tokyo.ac.jp/, Horton et al. 2007), and ChloroP (http://www.cbs.dtu.dk/services/ChloroP/, Emanuelsson et al. 1999) servers based on *U. compressa* proteins including the N-terminal region or in proteins present in databases showing the highest similarity (Table 1).

Quantification of Gene Transcript Levels

Total RNA was extracted from 0.5 g *U. compressa* using the FavorPrep Plant Total RNA Purification kit (Favorgen, Ping-Tung, Taiwan) and quantified with Quant-iT Ribogreen RNA assay kit (Invitrogen, Oregon, USA). Transcripts of the genes coding for ascorbate peroxidase (AP), peroxiredoxin (PRX), thioredoxin (TRX), glutathione-S-transferase 1 (GST), metallothionein (MET), calmodulin (CAM), calcium-dependent protein kinase (CDPK), nucleoside diphosphate kinase (NDK), histone acetyltransferase (HAT), cytosolic ribosomal small subunit of 20 kDa (S20), cytosolic ribosomal large subunit of 31 kDa (L31), ubiquitin1 (UBI), ubiquitin-related modifier (URM), photosystem I subunit X (PSAK), photosystem II 10 kDa subunit (PSBR), photosystem II 6.1 kDa subunit (PSBW), citochrome c oxidase copper chaperone COX11, citochrome c oxidase copper chaperone COX17 and actin (ACT) were amplified by real-time RT-PCR using a thermocycler Rotor Gene 6000 (Corbett Research). PCR primers were designed based on the identified sequences using the Beacon Designer software (Premier Biosoft International, CA, USA) to amplify a DNA fragment of ca. 100 bp (see Supplementary Information). Actin primers were designed based on the actin gene sequence from *U. pertusa* (Kakinuma et al. 2004). RT-PCR reactions were done using SensiMix One-Step kit (Quantace, London, UK), 6 ng of total RNA, 400 nM of each primer and 3 mM magnesium chloride. The reverse transcription step was done at 42°C for 30 min with an inactivation step at 95°C for 10 min. The PCR amplification step used 40 cycles of 15–20 s at 95°C for denaturation, 15–20 s at a specific annealing temperature for each pair of primers (see Supplementary Information), 20 s at 72°C for elongation and 10 s at 80°C to minimize non-specific annealing of primers, except for amplification of NDK transcripts where eight initial cycles of 15 s at 95°C, 15 s at 52°C and 20 s at 72°C were added to increase amplification efficiency. Fragments amplified by RT-PCR were detected by fluorescence using SYBR Green I included in the amplification kit. RT-PCR reactions were done in triplicate with total RNA extracted from three independent replicates. Samples were averaged, normalyzed using ΔΔCT method and mean

Table 1 Functional category, identity, cellular destination of proteins, and accession number of identified genes in *U. compressa*

Functional category	Putative identity	Species and accession number	*E* value	Putative destination	*Ulva* EST accession number
1. Signal transduction	Calmodulin	*Micromonas* sp./ACO68388.1	9e-73	mit	FD387500
	Protein kinase	*Chlamydomonas reinhardtii*/BAH56709.1	3e-16	cyt	FD387532
	Histidine kinase	*Ricinus communis*/EEF30556.1	2e-07	nuc	FD387495
	Aspartyl beta-hydroxylase	*Micromonas* sp./ACO68374.1	2e-05	cyt	FD387597
	Phospholipase A	*Medicago truncatula*/ABN08447.1	2e-15	chl	FD387458
2. Transcription, splicing, and replication	Histone acetyltransferase	*Cyanothece* sp./EAZ92251.1	9e-08	nuc	FD387455
	Factor CCR4	*Ostreococcus tauri*/CAL56061.1	2e-14	nuc	FD387528
	Pirin-like protein	*Methylovorus* sp./ACT51347.1	6e-47	nuc	FD387448
	Kelch repeat protein	*Herpetosiphon aurantiacus*/ABX03583.1	4e-15	nuc	FD387649
	Splicing factor 3B subunit 1	*Arabidopsis thaliana*/BAB09858.1	1e-93	nuc	FD387562
	U2 snRNP auxiliary factor	*C. reinhardtii*/EDP08396.1	7e-57	nuc	FD387679
	U6 snRNA-associated protein	*R. communis*/EEF38593.1	2e-21	nuc	FD387533
	Replication initiation factor	*Lentisphaera araneosa*/EDM26373.1	5e-14	chl	FD387477
	Histone H4	*Gymnochlora stellata*/ACF24577.1	3e-47	nuc	FD387494
3. Basal metabolism	Ribulose 3-P epimerase	*O. tauri*/CAL53452.1	3e-13	chl	FD387667
	Prephenate dehydratase	*A. thaliana*/AAM61395.1	2e-23	chl	FD387663
	Adenylylsulphate kinase	*Ulva intestinalis*/AAC26856.1	3e-25	mit	FD387524
	Methylenetetrahydrofolate dehydrogenase	*O. tauri*/CAL54416.1	3e-46	cyt	FD387534
	Nucleoside diphosphate kinase	*Nicotiana tabacum*/AAX63738.1	8e-61	cyt	FD387504
	3-oxoacyl reductase	*Polytomella parva*/ABH11007.1	8e-38	chl	FD387509
	COQ5 methyltransferase 1	*O. tauri*/CAL57786.1	8e-57	mit	FD387555
	COQ5 methyltransferase 2	*O. tauri*/CAL51663.1	5e-27	mit	FD387623
	Hydroxyphenylpyruvate dioxygenase	*Salvia militiorrhiza*/ABO69440.1	5e-46	chl	FD387462
	Phosphoglucomutase	*Oryza sativa*/AAX95576.1	4e-33	chl	FD387605
	Nucleoside triphosphatase	*A. thaliana*/AAM15184.1	4e-59	cyt	FD387484
	Nudix hydrolase	*C. reinhardtii*/EDP07665.1	2e-35	chl	FD387507
4. Antioxidant metabolism	Ascorbate peroxidase	*U. fasciata*/ABB88581.1	9e-46	cyt	FD387604
	Peroxiredoxin	*Glossina morsitans*/AAT85821.1	1e-42	cyt	FD387607
	Thioredoxin	*C. reinhardtii*/EDP10052.1	6e-28	chl	FD387643
	Thioredoxin-like	*O. sativa*/BAG95268.1	2e-32	cyt	FD387564
	Glutathione-S-transferase 1	*Leptospira interrogans*/AAN50554.1	5e-56	cyt	FD387475
	Glutathione-S-transferase 2	*Coccidioides posadasii*/EER26370.1	1e-18	mit	FD387657
	Glutathione-S-transferase 3	*R. communis*/CAG22554.1	2e-43	cyt	FD387461
	Metallothionein	*Crassostrea virginica*/AAQ23918.1	3e-05	mit	FD387450
5. Protein synthesis and degradation	Ribosomal protein L5	*Thalassiosira pseudonana*/ACI64112.1	4e-19	cyt	FD387651
	Ribosomal protein L9	*O. lucimarinus*/ABO95768.1	2e-40	cyt	FD387652
	Ribosomal protein L10	*O. sativa*/BAD13131.1	4e-11	cyt	FD387615
	Ribosomal protein L23	*C. reinhardtii*/EDO99405.1	1e-44	cyt	FD387554
	Ribosomal protein L28	*Perikinsus marinus*/EER07009.1	8e-04	cyt	FD387680
	Ribosomal protein L31	*C. reinhardtii*/EDP05369.1	2e-38	cyt	FD387653
	Ribosomal protein L35	*A. thaliana*/EDQ66778.1	4e-28	cyt	FD387521
	Ribosomal protein S12	*C. reinhardtii*/EDO99351.1	1e-37	cyt	FD387644
	Ribosomal protein S15	*Danio rerio*/AAH81516.1	3e-33	cyt	FD387602
	Ribosomal protein S20	*C. reinhardtii*/EDO99813.1	6e-39	cyt	FD387681
	Initiation factor 2B	*C. reinhardtii*/EDP03599.1	7e-69	cyt	FD387557

Table 1 (continued)

Functional category	Putative identity	Species and accession number	*E* value	Putative destination	*Ulva* EST accession number
	Initiation factor 4A	*C. reinhardtii*/EDO98621.1	2e-40	cyt	FD387654
	Small Hsp17	*Funaria hygrometrica*/AAD09182.1	3e-70	cyt	FD387516
	Co-chaperone P23	*C. reinhardtii*/EDP04006.1	3e-18	cyt	FD387634
	Prefoldin KE2	*C. reinhardtii*/EDP00061.1	5e-06	cyt	FD387445
	Disulfide isomerase 1	*C. reinhardtii*/EDO98781.1	2e-32	cyt	FD387567
	Disulfide isomerase 2	*Triticum aestivum*/BAH20801.1	2e-13	cyt	FD387610
	Cyclophilin B	*C. reinhardtii*/EDP01732.1	5e-68	cyt	FD387571
	Ubiquitin 1	*A. thaliana*/CAB81074.1	3e-77	cyt	FD387471
	Ubiquitin 2	*A. thaliana*/CAB81074.1	1e-97	cyt	FD387577
	Ubiquitin-conjugating enzyme E2S	*Zea mays*/ACF81599.1	3e-47	cyt	FD387449
	Ubiquitin modifier URM1	*C. reinhardtii*/EDP06508.1	4e-26	cyt	FD387454
	Ring-box protein RBX1	*C. reinhardtii*/EDP03017.1	2e-50	cyt	FD387523
	Proteasome regulatory subunit	*C. reinhardtii*/EDP04786.1	4e-57	cyt	FD387493
	Autophagocytosis protein	*C. reinhardtii*/EDP07491.1	6e-30	cyt	FD387646
6. Membrane transporters	ABC transporter	*Phytophtora infestans*/EEY63309.1	2e-08	chl	FD387556
	Drug/metabolite transporter	*Dyctioglomus thermophilum*/ ACI19179.1	2e-04	ud	FD387568
7. Vesicular transport	Dynein light chain subunit 9	*C. reinhardtii*/EDP06482.1	3e-31	cyt	FD387674
	GTP-binding protein Yptc1	*Volvox carteri*/AAA34255.1	4e-76	cyt	FD387611
	GTP-binding protein Yptc6	*C. reinhardtii*/EDP04353.1	6e-42	cyt	FD387588
	GTP-binding protein ran-3	*N. sylvestris*/AAT40987.1	5e-33	cyt	FD387566
	ADP-ribosylation factor	*A. thaliana*/AAM65870.1	2e-53	cyt	FD387592
8. Cell wall synthesis	Galactosyltransferase	*Ixodes scapularis*/EEC16656.1	5e-08	sec	FD387548
	UDP-glucoronic acid decarboxylase	*C. reinhardtii*/EDP06176.1	1e-79	cyt	FD387618
9. Photosynthesis and chloroplast proteins	Photosystem I, subunit PSAD	*C. reinhardtii*/EDO99874.1	1e-62	chl	FD387469
	Photosystem I, subunit PSAG	*O. lucimarinus*/ABO95977.1	1e-26	chl	FD387573
	Photosystem I, subunit PSAH	*Micromonas sp.*/ACO62801.1	7e-20	chl	FD387632
	Photosystem I, subunit PSAK	*Z. mays*/ACG29417.1	2e-11	chl	FD387582
	Photosystem II, subunit PSBP	*C. reinhardtii*/EDP03062.1	2e-52	chl	FD387483
	Photosystem II, subunit PSBR	*C. reinhardtii*/EDP08565.1	1e-31	chl	FD387498
	Photosystem II, subunit PSBS	*Acetabularia acetabulum*/ DAA05916.1	5e-05	chl	FD387491
	Photosystem II, subunit PSBW	*C. reinhardtii*/EDO97291.1	3e-14	chl	FD387503
	ATPase, subunit delta	*O. tauri*/CAL55216.1	5e-18	chl	FD387629
	Chlorophyll -binding protein	*A. acetabulum*/AAC79711.1	4e-14	chl	FD387517
	Fe/S cluster assembly protein	*Ciona intestinalis*/XP_002127710.1	2e-20	chl	FD387598
	Heme-binding protein SOUL	*C. reinhardtii*/EDO97429.1	2e-20	chl	FD387656
	Copper transport protein	*R. communis*/EEF28512.1	9e-11	chl	FD387467
	Thylakoid protein PGR5	*C. reinhardtii*/EDP07159.1	7e-24	chl	FD387572
	RNA polymerase RpoA	*Pseudendoclonium akinetum*/ AAV80614.1	8e-18	chl	FD387590
	RNA polymerase RpoB	*C. moewusii*/ABU88331.1	5e-23	chl	FD387530
	RNA polymerase RpoN	*Strongylocentrotus purpuratus*/ XP_001176583.1	9e-23	chl	FD387633
	Ribosomal protein S10	*A. thaliana*/AAL87380.1	1e-24	chl	FD387446
	Ribosomal protein L21	*C. reinhardtii*/EDP05027.1	3e-30	chl	FD387581
	Ribosomal protein L35	*O. sativa*/BAG90148.1	9e-11	chl	FD387552
	Ribosomal protein L37	*O. tauri*/CAL54678.1	9e-33	chl	FD387617
	Ribosomal protein L47	*Z. mays*/ACG28300.1	6e-13	chl	FD387496

Table 1 (continued)

Functional category	Putative identity	Species and accession number	*E* value	Putative destination	*Ulva* EST accession number
	Metaloendopeptidase M48	*Nostoc punctiforme*/ACC84018.1	2e-25	chl	FD387589
	Peptidase S16	*Shewanella sp*/ABI39345.1	3e-07	chl	FD387662
	Phosphatase PP1	*C. reinhardtii*/EDP04484.1	9e-06	chl	FD387624
	DNA ligase	*C. reinhardtii*/EDO96848.1	3e-73	chl	FD387497
	Senescence protein DIN1	*Z. mays*/ACG42699.1	2e-20	chl	FD387474
10. Respiration and mitochondrial proteins	NADH: ubiquinone reductase subunit B16.1	*O. tauri*/CAL54798.1	1e-22	mit	FD387510
	Cytochrome c oxidase subunit II, COX2	*P. akinetum*/AAQ18779.1	1e-34	mit	FD387647
	Cytochrome c oxidase copper chaperone, COX11	*O. lucimarinus*/ABO97035.1	2e-39	mit	FD387515
	Cytochrome c oxidase copper chaperone, COX17	*T. pseudonana*/EED88856.1	2e-10	mit	FD387501
	NADH dehydrogenase	*C. reinhardtii*/EDO96701.1	3e-13	mit	FD387473
	Protein traslocase TOM22	*Z. mays*/ACG45388.1	3e-07	mit	FD387451
	Intermembrane import protein	*R. communis*/EEF49653.1	2e-09	mit	FD387478
	Ribosomal protein S6	*O. lucimarinus*/ABO93930.1	2e-07	mit	FD387506
	SHOOT1 protein	*O. tauri*/CAL52561.1	2e-32	mit	FD387512

Cellular destination

cyt cytosolic, *mit* mitochondrial, *chl* chloroplast, *nuc* nuclear, *sec* secreted, *ud* undetermined

value control was subtracted from mean treated to determine fold of change in treated samples. The relative transcript level was expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

Statistical Analysis

Significant differences were determined by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (*T*). Differences between mean values were considered to be significant at a probability of 5% ($P<0.05$) (Zar 1999).

Results and Discussion

Copper Induces an Oxidative Stress Condition in *U. compressa*

Exposure of *U. compressa* to 10 μM copper for 3 days increased the activities of antioxidant enzymes AP, PRX, TRX and GST as compared with the control (Fig. 1a–d). Similarly, lipoperoxides doubled their level in fronds exposed to copper excess (Fig. 1e). Interestingly, lipoperoxides reached a similar level in *U. compressa* collected in a copper-impacted coastal area in northern Chile (Ratkevicius et al. 2003) in which copper concentration in seawater is aprox. 30 times lower (i.e., 20 μg L^{-1}) than the concentration used in this study (635 μg L^{-1}). This indicates that *U. compressa* is able to buffer lipoperoxides even at a high concentration of copper (i.e., this study). It is important to mention that the concentration of copper used in this study did not alter viability of *U. compressa* cells (González et al. 2010). Thus, *U. compressa* cultivated with a sub-lethal concentration of copper is under an oxidative stress condition.

Genes/Proteins Isolation and Identification

The cDNA library prepared with copper-stressed algae contained 3×10^4 clones of which 480 were randomly selected and sequenced. Half of the clones did not have an insert but 238 clones contained inserts ranging from 200 to 1,000 bp, with an average size of 496 bp (42% ranged from 500 to 600 bp) and corresponding to 235 non-redundant ESTs. Isolated sequences of *U. compressa* are available at NCBI ESTs database (http://www.ncbi.nlm.nih.gov/sites/entrez) with accession numbers FD387444 to FD387681. We identified 104 ESTs (44.3%) using BLASTX and BLASTP analysis showing similarity to proteins registered in databases, 51% of which were similar to proteins of green microalgae, mainly *Chlamydomonas reinhardtii*, *Ostreococcus tauri*, *O. luciminarus*, and *Micromonas* (Table 1), 25% with proteins from terrestrial plants, mainly

Arabidopsis thaliana, Ricinus communis, *Oryza sativa*, and *Zea may* and 8.7% to bacterial proteins. Only two sequences encoding AP and adenylylsulphate kinase were similar to proteins previously reported for the genus *Ulva*. We found 131 ESTs (55.7%) without similarity with previously reported databases sequences. Analysis of the amino terminal sequences showed that 50% of the identified proteins were potentially assignable to organelles (Table 1).

Genes/Proteins Involved in Antioxidant and Detoxification Metabolism

Identified genes/proteins were classified into ten functional categories (Table 1). One of these categories corresponds to proteins involved in antioxidant and detoxification metabolism (9.4%) and includes a cytosolic ascorbate peroxidase (AP) with high similarity (86%) to AP from *U. fasciata* (Table 1). The activity of AP activity and the level of *ap* transcripts increased in *U. compressa* in response to copper stress (Figs. 1a and 2a). A similar increase in *ap* transcripts has been detected in copper-stressed *U. fasciata* (Wu and Lee 2008; Wu et al. 2009). The antioxidant enzyme AP detoxifies hydrogen peroxide in plants and algae and, in *Euglena gracilis*, this enzyme also eliminates fatty acid hydroperoxides (Shigeoka et al. 1980). An increased AP activity has also been reported in the brown macroalgae *S. lomentaria* and *L. nigrescens* cultivated with copper excess (Contreras et al. 2009). Interestingly, the copper-sensitive alga *L. nigrescens* accumulated increasing amounts of lipoperoxides when cultivated with copper excess for 4 days whereas lipoperoxides reached a constant level in the copper-tolerant specie *S. lomentaria* (Contreras et al. 2009). This suggests that the control of lipoperoxides is an important feature in copper tolerance. In addition, we detected that AP activity in *U. compressa* used ascorbate and hydrogen peroxide or ascorbate and *t*-butyl hydroperoxide as substrates (A. González, unpublished). Thus, the AP identified in *U. compressa* may participate in the detoxification of hydrogen peroxide and fatty acids hydroperoxides playing an important role in the control of copper-induced oxidative stress.

A peroxiredoxin (PRX) was also identified in *U. compressa* showing a moderate similarity (58%) with a PRX from the insect *Glossina moritans*. PRX activity and *prx* transcripts increased in *U. compressa* cultivated with copper excess (Figs. 1b and 2b). In eukaryotes, PRXs are involved in the detoxification of hydrogen peroxide and fatty acid hydroperoxides (Dietz et al. 2006). In plants, *prx* transcripts increase in response to different abiotic stresses

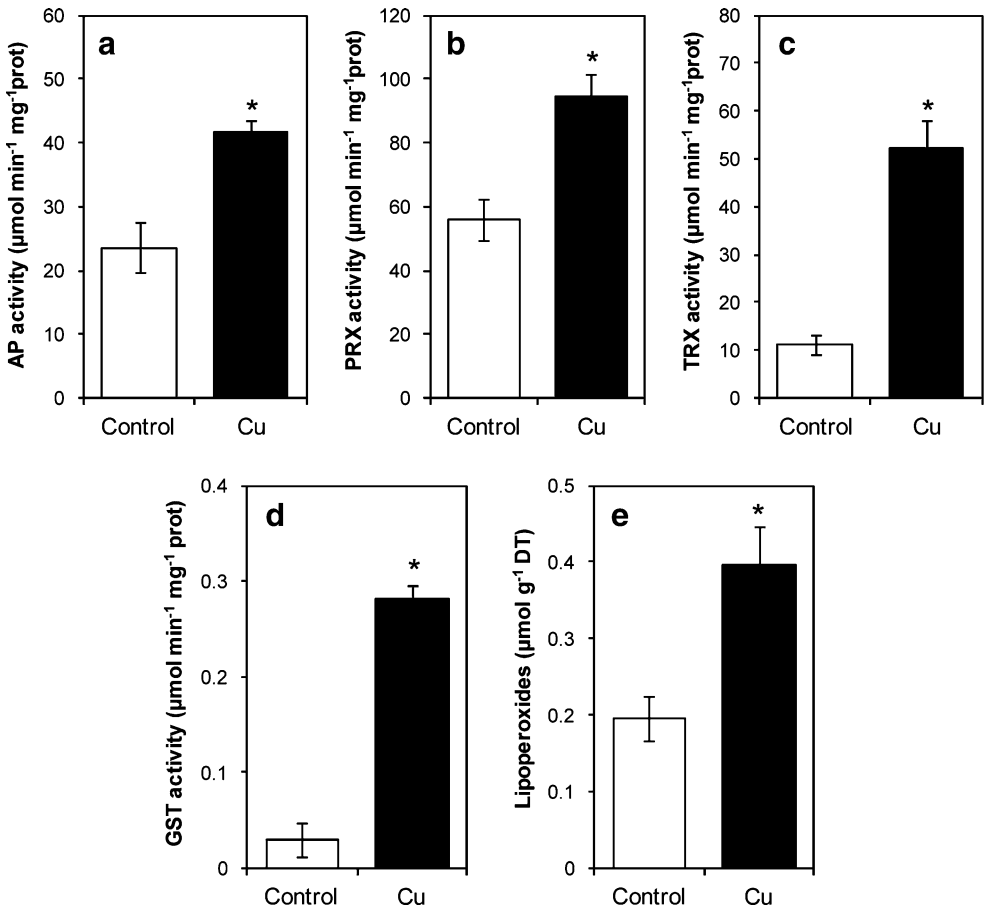

Fig. 1 Activities of antioxidant enzymes ascorbate peroxidase (*AP*), glutathione-dependent peroxiredoxin (*PRX*), thioredoxin (*TRX*), and glutathione-S-transferase (*GST*) and levels of lipoperoxides in *U. compressa* cultivated in seawater without copper (*white bars*) and with 10 μM copper for 3 days (*black bars*). *DT* dry tissue. *Bars* represent mean values of triplicates±1 SD and *asterisks* indicate significant differences ($P<0.05$)

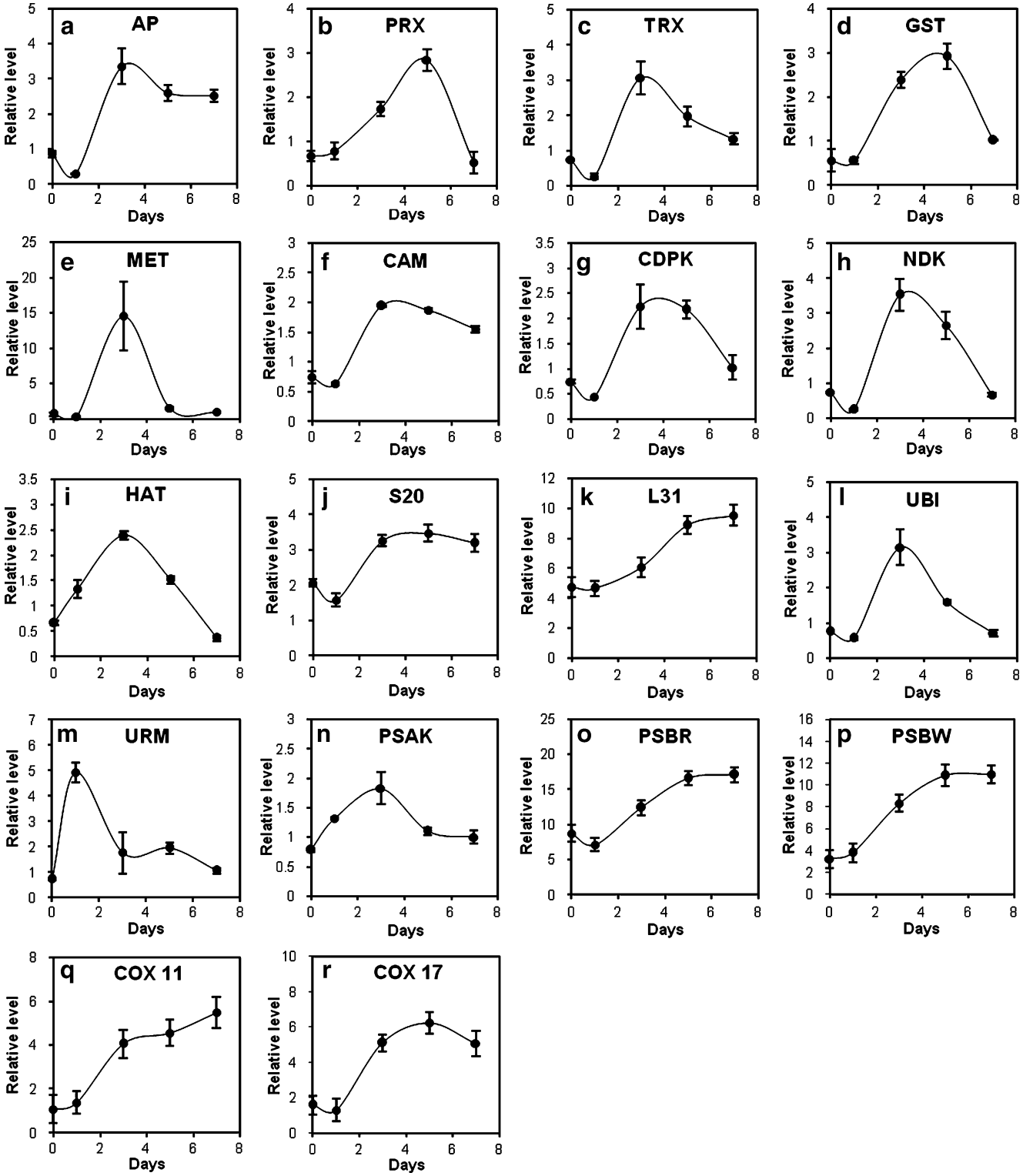

Fig. 2 Relative level of copper-induced gene transcripts in *U. compressa*. *AP* ascorbate peroxidase (**a**), *PRX* peroxiredoxin (**b**), *TRX* thioredoxin (**c**), *GST* glutathione-S-transferase 2 (**d**), *MET* metallothionein (**e**), *CAM* calmodulin (**f**), *CDPK* calcium-dependent protein kinase (**g**), *NDK* nucleoside diphosphate kinase (**h**), *HAT* histone acetyltransferase (**i**), *S20* cytosolic ribosomal small subunit of 20 kDa (**j**), *L31* cytosolic ribosomal large subunit of 31 kDa (**k**), *UBI* ubiquitin (**l**), *URM* ubiquitin-related modifier (**m**), *PSAK* photosystem I subunit X (**n**), *PSBR* photosystem II 10 kDa subunit (**o**), *PSBW* photosystem II 6.1 kDa subunit (**p**), *COX11* cytochrome c oxidase copper chaperone COX11 (**q**), *COX17* cytochrome c oxidase copper chaperone COX17 (**r**)

such as salinity, drought and heavy metals (Dietz 2003; Wood et al. 2003). Furthermore, the expression of *prx* in the green microalga *C. reinhardtii* seems regulated by light, oxygen and redox state (Goyer et al. 2002). In a previous work, and using a proteomic analysis, we detected an increased level of PRX in the brown alga *S. gracilis* cultivated with copper excess (Contreras et al. 2010). Thus, the PRX identified in *U. compressa* may play an important role in oxidative stress buffering and in lipoperoxides detoxification.

We also identified two *TRX* genes coding for a chloroplast TRX and a cytosolic TRX-like protein. TRX showed a moderate similarity (52%) with a TRX from *C. reinhardtii*. TRX activity and *trx* transcripts level increased in *U. compressa* under copper stress (Figs. 1c and 2c). TRX are small proteins catalyzing thiol-disulphide interchange and activating peroxiredoxins and organellar and cytosolic enzymes involved photosynthesis and basal metabolism (Gelhaye et al. 2005; Meyer et al. 2009). In algae, *trx*s have been isolated in *C. reinhardtii* (Decottignies et al. 1990, 1991), *G. tenuistipitata* (Hagopian et al. 2004) and *U. fasciata* (Wu et al. 2009). In *C. reinhardtii,* the expression of *trx h* and *trx m* was induced by Cd and Hg (Lemaire et al. 1999) and, consistently, the levels of TRXs and glutaredoxin increased in response to Cd (Gillet et al. 2006). In *U. fasciata*, *trx* trancripts increased in response to copper stress (Wu et al. 2009). This suggests that TRX in *U. compressa* may participate in the activation of peroxiredoxins and/or other cytosolic and organellar enzymes and as a consequence, in the control of copper-induced oxidative stress.

Three sequences coding for GSTs were isolated from *U. compressa*; GST1 showed a moderate similarity (58%) with a bacterial GST isolated from *Leptospira interrogans* while GST2 and GST3 displayed similarity with that of the fungus *Coccidioides posadasii* (36%) and the plant *R. communis* (54%), respectively. GST activity and the levels of *gst1* transcripts increased in *U. compressa* cultivated with copper excess (Figs. 1d and 2d). GSTs are involved in the detoxification toxic aldehydes derived from lipoperoxides and xenobiotics (Dixon et al. 2002; Catalá 2009). In plants, GSTs are activated in response to Al and Cu (Ezaki et al. 2004; Smith et al. 2004). In algae, GSTs activation has been reported in response to Cd stress in *C. reinhardtii* (Ding et al. 2005) and to petroleum spills in *Fucus* sp. (Cairrao et al. 2004). Therefore, it seems likely that GSTs in *U. compressa* may participate in buffering the toxic aldehydes induced by copper excess.

A sequence coding for a mitochondrial MET was also identified in *U. compressa* and the protein showed a moderate similarity (50%) with MET-IIIB from the oyster *Crassostrea virginica* (Jenny et al. 2006). Transcripts of *met* in *U. compressa* increased in response to copper stress (Fig. 2e), as those of *met* in *C. virginica*. It is important to highlight that *C. virginica* METs are encoded by a multigene family and most of the genes are heavy-metal responsive (Jenny et al. 2004; Jenny et al. 2006). There is only one previous report on a *met* gene in seaweeds (i.e., *F. vesiculosus*) and this gene was over-expressed in response to copper stress (Morris et al. 1999). These results suggest that in *U. compressa met* genes might be present as a multigene family, like in *C. virginica*, where most members could be involved copper binding and thus in buffering copper-induced oxidative stress.

Genes/Proteins Involved in Signal Transduction and Gene Expression

Another category of genes includes those involved in signal transduction (4.8%) such as a mitochondrial calmodulin (CAM), a cytosolic calcium-dependent protein kinase (CDPK), a nuclear histidine kinase, a cytosolic aspartyl beta-hydroxylase and a chloroplast phospholipase A (Table 1). A. The level of *cam* and *cdpk* transcripts increased in *U. compressa* in response to Cu excess (Fig. 2f, g). Plant CAMs and CDPKs are activated by calcium released in response to multiple environmental stimuli (Yang and Poovaiah 2003). Using W-7 and staurosporine as specific inhibitors of CAM and CDPK activities, respectively, it was established that these proteins responded to Cd excess in tobacco cells (Olmos et al. 2003; Garnier et al. 2006) and participated in the activation of NADPH oxidase in the presence of Pb in *Vicia faba* roots (Pourrut et al. 2008). CAMs and PKs are also involved in the activation of MAP kinases in response to Cu and Cd in rice (Yeh et al. 2007). Based on the above, we suggest that CAM and CDPK are probably involved in signal transduction triggered by copper stress and they may participate in the activation of a wide range of proteins and enzymes in *U. compressa*.

A gene encoding a cytosolic nucleoside diphosphate kinase (NDK), also identified in this study, showed increased transcript levels when *U. compressa* was exposed to copper excess (Fig. 2h). NDK catalyzes transfer of the gamma phosphate of nucleoside triphosphates to nucleoside diphosphates (Lascu and Gonin 2000) but also participates in signal transduction. It has been shown that NDK activates MAP kinases involved in the response to oxidative stress induced by methyl viologen, salt and cold stress in *Arabidopsis* (Moon et al. 2003). Moreover, NDK directly binds and activates the antioxidant enzyme catalase in *Arabidopsis* (Fukamatsu et al. 2003). The overexpression of *ndk* helps to overcome the oxidative stress induced by methyl viologen and cold stress in rice (Seong et al. 2007) and by methyl viologen, heat and salt stress in potato (Tang et al. 2008). Based on the above, NDK seems an additional enzyme assisting in buffering copper-induced oxidative stress in *U. compressa*.

Another category includes proteins involved in transcription, splicing and replication (8.7%) such as nuclear GCN5-related histone acetyltransferase (HAT), three regulatory transcription factors, three splicing-related proteins and a chloroplast replication factor (Table 1). The level of *hat* transcripts increased in *U. compressa* in response to copper excess (Fig. 2i). In this sense, it has been reported that GCN5-HAT is required for the activation of copper-responsive genes in the yeast *Candida glabrata* since it facilitates access of transcription factor AMT1 to the metal responsive element MRE (Koch et al. 2001). Thus, it is possible that GCN5-related HAT identified in *U. compressa* may participate in the activation of copper-responsive genes.

Genes/Proteins Involved in Basal Metabolism

Another functional category includes proteins involved in basal metabolism (11.5%) such as a chloroplast ribulose 3-phosphate epimerase which is required for carbon fixation, a prephrenate dehydratase involved in aromatic aminoacid synthesis, an adenylylsulphate kinase involved in cysteine and sulphured compounds synthesis, a metylentetrahydrofolate dehydrogenase required for purines synthesis, a nucleoside diphosphate kinase (NDK) required for nucleotide triphosphate synthesis and also involved in signal transduction (see above), a 3-oxoacyl reductase that participates in fatty acids synthesis, two methyltransferases required for ubiquinone synthesis, an hydrophenyl piruvate dioxygenase involved in tocopherol, tocotrienol and plastoquinone synthesis, a phosphoglucomutase involved in the conversion of glucose 1-P in glucose 6-P, a nucleotide triphosphatase and a nudix hydrolase involved in the degradation of oxidized nucleotides (Table 1). As mentioned before *ndk* transcripts, which encode an enzyme involved is the synthesis of nucleotides triphosphate and also in signal transduction, increased in *U. compressa* in response to copper stress (Fig. 2h). It is interesting to note that the enzyme prephrenate dehydratase participates in the synthesis of aromatic aminoacids such as phenylalanine that is required for phenolic compounds synthesis, phosphoglucomutase which is involved in ascorbate synthesis and adenylylsulphate kinase which is required for glutathione synthesis. In fact, phenolic compounds, ascorbate and glutathione are water-soluble antioxidant compounds that are synthesized in *U. compressa* in response to copper stress (Dennett et al., unpublished).

Genes/Proteins Involved in Protein Synthesis and Degradation

One of the largest functional categories of genes identified in this study comprises those involved in protein synthesis and degradation (24%) and includes 11 ribosomal proteins, two translation initiation factors, three chaperones, three disulphide isomerases, two ubiquitins (UBI), an ubiquitin-related modifier (URM), and two proteasome-associated proteins. Transcripts encoding cytosolic ribosomal proteins involved in protein synthesis such as the ribosomal small subunit S20 and ribosomal large subunit L31 increased in response to copper excess (Fig. 2j, k). A similar pattern was observed for transcripts encoding proteins UBI and URM involved in protein degradation (Fig. 2l, m). Regarding protein degradation, ubiquitin-proteasome is activated in response to several abiotic stresses and participates in mechanisms of tolerance by removing unfolded proteins and proteins damaged by oxidative stress (Dreher and Callis 2007; Pena et al. 2007; Kurepa et al. 2008) in all eukaryotes (Hellman and Estelle 2002). In addition, URM is a post-transcriptional protein modifier (Xu et al. 2006) that activates the antioxidant enzyme alkyl hydroperoxide reductase 1 in yeast, which reduces fatty acid hydroperoxides produced in response to various metals including Cu, Co, and Zn (Nguyên-nhu and Knoops 2002; Goehring et al. 2003). Thus, it seems reasonable to suggest that the increased expression of cytosolic ribosomal proteins may contribute to protein synthesis and cellular repair, that of ubiquitin in the degradation of damaged proteins and that of URM in the detoxification of lipoperoxides in copper-stressed *U. compressa*.

Organellar Genes/Proteins

Genes encoding chloroplast (25%) and mitochondrial (8.7%) proteins were also isolated. The former corresponds to four subunits of photosystem I, four subunits of photosystem II, a chlorophylla/b-binding protein, a thylakoid-binding protein, a Fe/S cluster assembly protein, a heme-binding protein, a subunit of ATP synthase, three subunits of chloroplast RNA polymerase, five chloroplast ribosomal proteins, two peptidases, a copper chaperone, a phosphatase, a DNA ligase and a senescence-related protein (Table 1). Mitochondrial proteins correspond to two subunits of mitochondrial electron transport chain, the citochrome c oxidase copper chaperones COX11 and COX17, a NADH dehydrogenase, the outer membrane protein translocase TOM22, an intermembrane import protein and a ribosomal protein (Table 1). The level of transcript encoding PSI subunit PSAK, PSII subunits PSBR and PSBW and the mitochondrial copper chaperones COX11 and COX 17 increased in response to copper excess (Fig. 2n–r). In higher plants oxidative stress causes photoinhibition mainly due to the disruption of translation of PSII subunit transcripts (Nishiyama et al. 2001; Allakhverdiev and Murata 2004; Nishiyama et al. 2006) which may also affect PSI subunits (Allakhverdiev and

Murata 2008). The increase in transcript levels of the nuclear-encoded subunits PSAK, PSBR, and PSBW in *U. compressa* exposed to copper excess might be associated to an increase in their translation in order to replace damaged proteins in photosystems. A similar phenomenon may take place in mitochondria in order to replace damaged proteins in the electron transport chain, as suggested by the increase transcripts of the copper chaperones *cox11* and *cox17* involved in cytochrome c oxidase assembly. It is important to consider that most reactive oxygen species (ROS) detected in *U. compressa* cultivated with copper excess are produced in chloroplasts and mitochondria (González et al. 2010) indicating that organellar proteins are the major targets of copper-induced oxidative stress. Thus, an efficient replacement of organellar proteins may be a key feature of copper tolerance in *U. compressa*.

Detection of Copper-Induced Genes

The relative level of transcripts encoding AP, PRX, TRX, and GST increased three to four times in response to copper excess, except *met* transcripts which increased 13 times, compare to actin transcripts which remained unchanged in algae cultivated in control conditions or with copper excess (Fig. 2a–e). The level of transcripts coding for proteins involved in signal transduction and regulation of gene expression CAM, CDPK, NDK, and HAT showed an increase of 2–3.5 times (Fig. 2f–i). The level of transcripts encoding proteins involved in protein synthesis S20, L31 and protein degradation UBI and URM increased two to five times in response to copper excess (Fig. 2j–m). The level of transcripts coding for photosystem subunits PSAK, PSBR and PSBW and mitochondrial copper chaperones COX11 and COX17 showed an increase of 1.5 to 3 times. On the other hand, transcripts coding for URM reached a maximum level at day 1 of copper exposure whereas those coding for PRX, GST, L31, PSBR, PSBW, COX11, and COX17 showed maximal levels at day 5 and the other 10 gene transcripts showed their peaks at day 3. Thus, the relative transcript level of the 18 selected genes increased in response to copper excess, suggesting that most of the identified genes in the cDNA library are copper-responsive. In addition, the relative level of most transcripts decreased after 3 days of exposure, except those of *ap*, *cam*, *s20*, *l31*, *psbr*, *psbw*, *cox11*, and *cox17*,which persisted at high levels through the end of the experiment (Fig. 2a, f, k, o–r). It is possible that genes showing a short-lasting expression are part of the acclimation mechanisms of *U. compressa* to buffer acute copper excess, whereas those that remained at high levels of expression are involved in long-term copper tolerance. In support of this view, AP activity showed a sustained increase in *U. compressa* cultivated with 10 μM copper up to 7 days (González et al. 2010), which was coupled to the sustained increase of *ap* transcripts (i.e., this study) suggesting that the increase in AP activity is due to gene transcription regulation. In addition, GST activity transiently increased in the alga cultivated with copper excess (González et al. 2010), which is in agreement with the transient increase of *gst* transcripts (i.e., this study). Furthermore, the increase in *prx* and *trx* transcript levels match with the increase of PRX and TRX activities detected after 3 days of copper exposure (i.e., this study).

Concluding Remarks

Our results showed that (1) the 18 selected genes were copper-responsive, suggesting that this feature may apply to most of the isolated genes, (2) half of the isolated genes encode putative organellar proteins, (3) the identified proteins are principally involved in antioxidant metabolism and in cellular and organellar repair, and (4) these proteins may act in a coordinated and additive or synergistic manner to ensure copper acclimation and tolerance in *U. compressa*.

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References

Allakhverdiev SI, Murata N (2004) Environmental stress inhibits the synthesis *de novo* of proteins involved in the photodamage-repair cycle of photosystem II in *Synechocystis* sp. PCC 6803. Biochim Biophys Acta 1657:23–32

Allakhverdiev SI, Murata N (2008) Salt stress inhibits photosystems II and I in cyanobacteria. Photosynth Res 98:529–539

Cairrao E, Couderchet M, Soares AM, Guilhermino L (2004) Glutathione-S-transferase activity of *Fucus* spp. as a biomarker of environmental contamination. Aquat Toxicol 70:277–286

Castilla JC (1996) Copper mine tailing disposal in Northern Chile rocky shores: *Enteromorpha compressa* as a sentinel species. Environ Monit Assess 40:41–54

Catalá A (2009) Lipid peroxidation of membrane phospholipids generates hydroxyl-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. Chem Phys Lipids 157:1–11

Collén J, Roeder V, Rousvoal S, Collin O, Kloareg B, Boyen C (2006) An expressed sequence tag analysis of thallus and regenerating protoplasts of *Chondrus crispus* (Gigartinales, Rhodophyceae). J Phycol 42:104–112

Contreras L, Mella D, Moenne A, Correa JA (2009) Differential responses to copper-induced oxidative stress in the marine macroalgae *Lessonia nigrescens* and *Scytosiphon lomentaria* (Phaeophyceae). Aquat Toxicol 94:94–102

Contreras L, Moenne A, Gaillard F, Potin P, Correa JA (2010) Proteomic analysis and identification of copper stress-regulated

proteins in the marine alga *Scytosiphon gracilis* (Phaeophyceae). Aquat Toxicol 96:85–89

Crépineau F, Roscoe T, Kaas R, Kloareg B, Boyen C (2000) Characterization of complementary DNAs from the expressed sequence tag analysis of life cycle stages of *Laminaria digitata* (Phaeophyceae). Plant Mol Biol 43:503–551

Davis AK, Hildebrand M, Palenik B (2006) Gene expression induced by copper stress in diatom *Thalassiosira pseudonana*. Eukaryot Cell 5:1157–1168

Decottignies P, Schmitter JM, Jacquot JP, Dutka S, Picaud A, Gadal P (1990) Purification, characterization, and complete amino acid sequence of a thioredoxin from a green alga, *Chlamydomonas reinhardtii*. Arch Biochem Biophys 280:112–121

Decottignies P, Schmitter JM, Dutka S, Jacquot JP, Miginiac-Maslow M (1991) Characterization and primary structure of a second thioredoxin from the green alga, *Chlamydomonas reinhardtii*. Eur J Biochem 198:505–512

Dietz KJ (2003) Plant peroxiredoxins. Annu Rev Plant Biol 54:93–107

Dietz KJ, Jacob S, Oelze ML, Laxa M, Tognetti V, Nunes de Miranda SM, Baire M, Finkenmeier I (2006) The function of peroxiredoxins in plant organelle redox metabolism. J Exp Bot 57:1697–1709

Ding Y, Miao JL, Li GY, Wang QF, Kan GF, Wang GD (2005) Effect of Cd on GSH and GSH-related enzymes of *Chlamydomonas* sp. ICE-L existing in Antarctic ice. J Environ Sci 17:667–671

Dixon DP, Lapthorn A, Edwards R (2002) Plant glutathione transferases. Genome Biol 3:1–10

Dreher K, Callis J (2007) Ubiquitin, hormones and biotic stress in plants. Ann Bot 35:1–35

Emanuelsson O, Nielsen H, Von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Plant Sci 8:978–984

Emanuelsson O, Nielsen H, Brunak S, Von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300:1005–1016

Ezaki B, Suzuki M, Motodo H, Kawamura M, Nakashima S, Matsumoto H (2004) Mechanism of gene expression of *Arabidopsis* glutathione S-transferase, *AtGST1*, and *AtGST11* in response to aluminium stress. Plant Physiol 134:1672–1682

Fukamatsu Y, Yabe N, Hasunuma K (2003) Arabidopsis NDK1 is a component of ROS signalling by interacting with three catalases. Plant Cell Physiol 44:982–989

Garnier L, Simon-Plas E, Thuleau P, Agnel JP, Blein JP, Ranjeva R, Montillet JL (2006) Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. Plant Cell Environ 29:1956–1969

Gelhaye E, Rouhier N, Navrot N, Jacquot JP (2005) The plant thioredoxin system. Cell Mol Life Sci 62:24–35

Gillet S, Decottignies, Chardonnet S, Le Maréchal P (2006) Cadmium response and redoxin targets in *Chlamydomonas reinhardtii*; a proteomic approach. Photosynth Res 89:201–211

Goehring AS, Rivers DM, Sprague GF (2003) Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. Eukaryot Cell 2:930–936

González A, Vera J, Castro J, Dennett G, Mellado M, Morales B, Correa JA, Moenne A (2010) Co-ocurring increases of calcium and organellar reactive oxygen species determine the differential activation of antioxidant and defense enzymes in *Ulva compressa* (Chlorophyta) exposed to copper excess. Plant Cell Environ 33:1627–1640

Goyer A, Haslekas C, Miginiac-Maslow M, Klein U, Le Maréchal P, Jacquot JP, Decottignies P (2002) Isolation and characterization of a thioredoxin-dependent peroxidase from *Chlamydomonas reinhardtii*. Eur J Biochem 269:272–282

Hayden HS, Blomster J, Maggs CA, Silva PC, Stanhope MJ, Waaland JR (2003) Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. Eur J Phycol 38:277–294

Hagopian JC, Reis M, Kitajima JP, Bhattacharya D, Oliveira MC (2004) Comparative analysis of the complete plastid genome sequence of the red alga *Gracilaria tenuistipitata* var. liui provides insights into the evolution of rhodoplasts and their relationship to other plastids. J Mol Evol 59:464–477

Han T, Kang SH, Park JS, Lee HK, Brown M (2008) Physiological responses of *Ulva pertusa* and *U. armoricana* to copper exposure. Aquat Toxicol 86:176–184

Hellman H, Estelle M (2002) Plant development: regulation by protein degradation. Science 297:793–797

Horton P, Park J, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K (2007) WoLF PSORT: protein localization predictor. Nucl Acids Res 259:1–3

Jenny MJ, Ringwood AH, Schey K, Warr GW, Chapman RW (2004) Diversity of metallothioneins in the American oyster, *Crassostrea virginica*, revealed by transcriptomic and proteomic approaches. Eur J Biochem 271:1702–1712

Jenny MJ, Warr GW, Ringwood AH, Baltzegar DA, Chapman RW (2006) Regulation of metallothionein genes in the American oyster (*Crassostrea virginica*): ontogeny and differential expression in response to different stressors. Gene 379:156–165

Kakinuma M, Coury DA, Inagaki E, Itoh S, Yoshiura Y, Amano H (2004) Isolation and characterization of a single-copy actin gene from a sterile mutant of *Ulva pertusa* (Ulvales, Cholorophyta). Gene 334:145–155

Kenehisa M, Goto S, Kawashima S, Okuno Y, Hattori M (2004) The KEGG resource for deciphering the genome. Nucl Acids Res 32: D277–D280

Koch KA, Allar S, Santoro N, Côté J, Thiele DJ (2001) The *Candida glabrata* Amt1 copper-sensitive transcription factor requires Swi/Snf and Gcn5 at a critical step in copper detoxification. Mol Microbiol 40:1165–1174

Kurepa J, Toh-e A, Smalle JA (2008) 26S proteosome regulatory particle mutants have increased oxidative stress tolerance. Plant J 53:102–114

Lascu I, Gonin P (2000) The catalytic mechanism of nucleoside diphosphate kinases. J Bioenerg Biomed 32:237–246

Lee EK, Seo SB, Kim TH, Sung SK, An G, Lee CH, Kim YJ (2000) Analysis of expressed sequence tags of *Porphyra yezoensis*. Mol Cell 10:338–342

Lemaire S, Keryer E, Stein M, Schepens II, Issakidis-Bourguet EG, Rard-Hirne C, Miginiac-Maslow M, Jacquot JP (1999) Heavy-metal regulation of thioredoxin gene expression in *Chlamydomonas reinhardtii*. Plant Physiol 120:773–778

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Medina M, Andrade S, Faugeron S, Lagos N, Mella D, Correa JA (2005) Biodiversity of rocky intertidal benthic communities associated with copper mine tailing discharges in northern Chile. Mar Pollut Bull 50:396–409

Meyer Y, Buchanan BB, Vignols F, Reichheld JP (2009) Thioredoxins and glutaredoxins: unifying elements in redox biology. Ann Rev Genet 43:335–367

Miyasaka H, Kanaboshi H, Ikeda K (2000) Isolation of several anti-stress genes from the halotolerant green alga *Chlamydomonas* by simple functional expression screening with *E. coli*. World J Microbiol Biotechnol 16:23–29

Moon H, Lee B, Choi G, Shin D, Prasad T, Lee O, Kwak S, Kim DH, Nam J, Bahk J, Hong JC, Lee SY, Cho MJ, Jun DJ (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in plants. Proc Natl Acad Sci USA 100:358–363

Morris CA, Nicolaus B, Sampson V, Harwood JL, Kille P (1999) Identification and characterization of a recombinant metallothionein protein from a marine alga *Fucus vesiculosus*. Biochem J 338:553–560

Nguyên-nhu NT, Knoops B (2002) Alkyl hydroperoxide reductase 1 protects *Saccharomyces cerevisiae* against metal ion toxicity and glutathione depletion. Toxicol Lett 135:219–228

Nikaido I, Asamizu E, Nakajima M, Nakamura Y, Saga N, Tabata S (2000) Generation of 10,154 expressed sequence tags from a leafy gametophyte of a marine red alga *Porphyra yezoensis*. DNA Res 7:223–227

Nishiyama Y, Yamamoto H, Allakhverdiev SI, Inaba M, Yokota A, Murata N (2001) Oxidative stress inhibits the repair of photodamage of the photosynthetic machinery. EMBO J 20:5587–5594

Nishiyama Y, Allakhverdiev SI, Murata N (2006) A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II. Biochim Biophys Acta 1757:742–749

Olmos E, Martínez-Solano JR, Piqueras A, Hellín E (2003) Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line). J Exp Bot 54:291–301

Pearson G, Hoarau G, Lago-Leston A, Coyer JA, Kube M, Reinhardt R, Henckel K, Serrao ET, Corre E, Olsen JL (2010) An expressed sequence tag analysis of the intertidal brown seaweed *Fucus serratus* (L.) and *F. vesiculosus* (L.) (Heterokontophyta, Phaeophyceae) in response to abiotic stressors. Mar Biotechnol 12:195–213

Pena LB, Pasquini LA, Tomaro ML, Gallego SM (2007) 20S proteasome and accumulation of oxidized and ubiquitinated proteins in maize leaves subjected to cadmium stress. Phytochemistry 68:1139–1146

Pereira P, De Pablo H, Rosa-Santos F, Pacheco M, Vale C (2009) Metal accumulation and oxidative stress in *Ulva* sp. substanciated by response integration into a general stress index. Aquat Toxicol 91:336–345

Peters AF, Marie D, Scornet D, Kloareg B, Cock M (2004) Proposal of *Ectocarpus siliculosus* (Ectocarpales, Phaeophyceae) as a model organism for brown algal genetics and genomics. J Phycol 40:1079–1088

Pourrut B, Perchet G, Silvestre J, Cecchi M, Guiresse M, Pinelli E (2008) Potential role of NADPH oxidase of early steps of lead-induced oxidative burst in *Vicia faba* roots. J Plant Physiol 165:571–579

Ratkevicius N, Correa JA, Moenne A (2003) Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in *Enteromorpha compressa* (L.) Grev. (Chlorophyta) from heavy-metal enriched environments in northern Chile. Plant Cell Environ 26:1599–1608

Roeder V, Collén J, Rousvoal S, Corre E, Leblanc C, Boyen C (2005) Identification of stress gene transcripts in *Laminaria digitata* (Phaeophyceae) protoplast cultures by expressed sequence tag analysis. J Phycol 41:1227–1235

Seong ES, Go J, Kim YH, Cho JH, Lim CK, Hyun H, Wang MH (2007) Regulation of marker genes involved in biotic and abiotic stress by overexpression of the *AtNDPK2* gene in rice. Biochem Biophys Res Commun 363:126–132

Shigeoka S, Nakano Y, Kitaoka S (1980) Characterization and some properties of L-ascorbic acid-specific peroxidase in *Euglena gracilis*. Arch Biochem Biophys 201:121–127

Smith AP, De Ridder B, Guo WJ, Seeley EH, Regnier FE, Goldsbrough PB (2004) Proteomic analysis of *Arabidopsis* glutathione S-transferases from benoxacor- and copper-treated seedlings. J Biol Chem 279:26098–26104

Stanley MS, Perry RM, Callow JA (2005) Analysis of expressed sequence tags from the green algal *Ulva linza* (Chlorophyta). J Phycol 41:1219–1226

Tang L, Kim MD, Yang KS, Kwon SY, Kim SH, Kim JS, Yun DJ, Kwak SS, Lee HS (2008) Enhanced tolerance of transgenic potato plants overexpressing nucleoside diphosphate kinase 2 against multiple environmental stresses. Transg Res 17:705–715

Villares R, Puentes X, Carballeira A (2001) *Ulva* and *Enteromorpha* as indicators of heavy metal pollution. Hydrobiologia 462:221–232

Wellenreuther R, Schupp I, Poustka A, Wiemann S, The German cDNA Consortium (2004) SMART amplification combined with cDNA size fractionation in order to obtain large full-length clones. BMC Genomics 5:36–43

Wong TKM, Ho CL, Lee W, Rahim RA, Phang S (2007) Analyses of expressed sequence tags from *Sargassum binderi* (Phaeophyta). J Phycol 43:528–534

Wood ZA, Poole LB, Karplus BA (2003) Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science 300:650–653

Wu TM, Lee TM (2008) Regulation of activity and gene expression of antioxidant enzymes in *Ulva fasciata* Delile (Ulvales, Chlorophyta) in response to copper stress. Phycologia 47:346–360

Wu TM, Hsu YT, Sung MS, Hsu YT, Lee TM (2009) Expression of genes involved in redox homeostasis and antioxidant defense in the marine macroalga *Ulva fasciata* by excess copper. Aquat Toxicol 94:275–285

Xiaolei F, Yongjun F, Songnian H, Guangce W (2007) Generation and analysis of 5318 expressed sequence tags from the filamentous sporophyte of *Porphyra haitanensis* (Rhodophyta). J Phycol 43:1287–1294

Xu J, Zhang J, Wang L, Zhou J, Huang H, Wu J, Zhong Y, Shi Y (2006) Solution structure of Urm1 and its implications for the origin of protein modifiers. Proc Natl Acad Sci USA 103:11625–11630

Yang T, Poovaiah BW (2003) Calcium/calmodulin-mediated signals network in plants. Trends Plant Sci 8:505–512

Yeh CM, Chien PS, Huang HJ (2007) Distinct signalling pathways for induction of MAP kinase activities by cadmium and copper in rice roots. J Exp Bot 58:659–671

Zar J (1999) Biostatistical analysis. Prentice-Hall, Englewood Cliffs