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Effects of cadmium on the regulation of antioxidant enzyme activity, gene expression, and antioxidant defenses in the marine macroalga *Ulva fasciata*

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ABSTRACT. This study examined the antioxidative responses of the marine macroalga *Ulva fasciata* Delile to cadmium (Cd) stress. Exposure to 0, 5, 10, 20 and 50 μM $CdCl_2$ for 4 days did not affect growth, 2,3,5-tri phenyltetrazolium chloride reduction ability, H_2O_2 production, or lipid peroxidation. The Cd contents in thalli increased linearly as $CdCl_2$ concentrations increased from 0-20 μM $CdCl_2$ and declined slightly at 50 μM $CdCl_2$. This means that long-term exposure to Cd did not produce oxidative damage to the macroalga although Cd accumulated. Ascorbate (AsA) and dehydroascorbate (DHA) concentrations increased as Cd concentrations increased while AsA/DHA ratios increased with a peak at 10 μM. Glutathione (GSH) and oxidized GSH concentrations and GSH/oxidized GSH ratios decreased as Cd concentrations increased. Cd did not affect Mn superoxide dismutase (MnSOD; EC 1.15.1.1) activities or transcripts. Cd at 50 μM increased FeSOD activities and *UfFesod1* (a gene of FeSOD isoform) transcripts but did not affect *UfFesod2* transcripts. Among isoforms of the SOD gene, only *UfFesod1* was responsible for the increase of SOD activity by Cd. The activities of ascorbate peroxidase (APX; EC 1.11.1.11) and catalase (CAT; EC 1.11.1.6) increased as Cd concentrations increased, but their transcripts were not affected by Cd, suggesting that the induction of APX and CAT activities by Cd was not under transcriptional control. Glutathione reductase (GR; EC 1.6.4.2) activities and transcripts increased as Cd concentrations increased. The present results indicate that the increase in the AsA pool, the consumption of GSH, and the induction in the activities of FeSOD, APX, GR and CAT are used by *U. fasciata* to prevent the occurrence of oxidative damage under Cd stress. The increases in the activities of FeSOD and GR by Cd can be attributed to enhanced gene expression.

Keywords: Antioxidant enzyme; Antioxidant; Cd; Gene expression; *Ulva*.

Abbreviations: APX, ascorbate peroxidase; **AsA,** ascorbate; **CAT,** catalase; **GR,** glutathione reductase; **GSH,** glutathione; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase.

INTRODUCTION

Heavy metals constitute an environmental pollutant with toxicity to biota. Since the late 19th century, heavy metals have accumulated in the environment as a result of mining and industrial activities. Because cadmium (Cd)—largely used for plastics manufacturing, Ni-Cd batteries, and the electroplating of steel—is persistent and bio-accumulated through the food chain, Cd contamination and toxicity have become of particular concern in recent years. The effects of Cd toxicity on plants are well studied (Sanitádi Toppi and Gabbrielli, 1999). Cd was found to produce oxidative damage to lipids and nucleic acids (Sandalio et al., 2001; Romero-Puertas et al., 2002, 2003; Lee and Shin, 2003; Watanabe et al., 2003). The damage caused by reactive oxygen species (ROS) is known as oxidative stress. In response, plants have developed defense systems via non-enzymatic and enzymatic scavenging of cellular ROS to cope with oxidative stress (Noctor and Foyer, 1998; Asada, 1999; Okamoto et al., 2001a, b; Pinto et al., 2003). The water-soluble ascorbate (AsA) and glutathione (GSH) and the water-insoluble α-tocopherol and carotenoids are the non-enzymatic agents that scavenge ROS (Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000; Munné-Bosch and Alegre, 2002). To scavenge ROS enzymatically, $O_2^{\bullet-}$, which can first be converted to H_2O_2 by the action of superoxide dismutase (SOD; EC 1.15.1.1), and then ascorbate peroxidase

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(APX; EC 1.11. 1.11) and glutathione reductase (GR; EC 1.6.4.2) in the ascorbate-glutathione cycle are responsible for the removal of H_2O_2 (Asaka, 1999). APX utilizes AsA to reduce H_2O_2, and AsA is in turn oxidized. Then, the oxidized AsA is regenerated back to AsA via GSH oxidation, and GSSG as the product of GSH oxidation is reduced to GSH by GR via the utilization of reducing equivalents from NAD(P)H. H_2O_2 can be degraded to H_2O by catalase (CAT; EC 1.11.1.6) (Willenkens et al., 1997) or peroxidase (POX; EC 1.11.1.7) (Asaka and Takahashi, 1987).

The antioxidative responses to Cd stress are different between algal species. Cd increased H_2O_2 concentrations, lipid peroxidation, and the activities of APX, POX and CAT in the marine microalga *Nannochloropsis oculata*, but decreased the activities of SOD and GR (Lee and Shin, 2003). In the marine red macroalga *Gracilaria tenuistipitata*, Cd increased CAT activity but did not affect SOD or APX activities (Collén et al., 2003). In the marine dinoflagellate *Gonyaulax polyedra*, acute exposure to Cd generated oxidative stress in chloroplasts while under chronic exposure, the antioxidant system was able to provide protection (Okamoto et al., 2001a, b).

Few studies on the regulation of genes and antioxidant enzymes in macroalgae exposed to heavy metals are available. *Ulva fasciata* Delile is a marine chlorophyte abundant in the intertidal regions of Taiwan, which are often subjected to heavy metal pollution from river runoff and sewage outlets. This study was planned to determine whether oxidative stress occurs in *U. fasciata* in response to Cd and to examine how the antioxidant defense system is regulated under Cd stress. After 4 days of exposure to 0, 5, 10, 20, or 50 μM $CdCl_2$, the growth rate and 2,3,5-triphenyltetrazolium chloride (TTC) reduction ability were determined, and the concentrations of H_2O_2 and the contents of thiobarbituric acid reacting substance (TBARS) as indicators of oxidative stress were examined. The concentrations of ascorbate and glutathione and the activities of SOD, APX, GR, and CAT were determined. In the attempts to investigate whether Cd can induce the gene expression of antioxidant enzymes, the genes of SOD, APX, GR, and CAT were first cloned, and transcript abundance in response to Cd stress was examined.

MATERIALS AND METHODS

Algal culture and heavy metal treatment

Ulva fasciata Delile (15-25 cm high) were collected from Hsitzu Bay, Kaohsiung, Taiwan. Following collection, whole algae were extensively washed with natural seawater to remove attached sands, and rhizoidal portions were removed to avoid microbial contamination in the following culture. Thalli were pre-incubated at 25°C for 14 days in the 35‰ artificial seawater (ASW; 403.5 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 30 mM $MgSO_4$ and 10 mM Tris-HCl, pH 8.0) containing N (NH_4^+ and NO_3^-)- and P (PO_4^{3-})-free Provasoli nutrient solutions (Provasoli, 1968). $NaHCO_3$, $NaNO_3$ and Na_2HPO_4 were then added in the final concentrations of 3 mM, 400 μM and 20 μM, respectively. The photoperiod was 12 h : 12 h, and the photon irradiance (400-700 nm) was 100-150 μmol photon·m^{-2}·s^{-1} in the absence of algae, achieved by cool-fluorescent lamps (FL40D, China Electric Apparatus Ltd., Taiwan).

After 14 days of pre-incubation, thalli of 1 g wet weight (w. wt.) were cultured in a 500-ml beaker containing 300 ml of 35‰ ASW. Carbon and N and P nutrients were provided by adding $NaHCO_3$, $NaNO_3$, and Na_2HPO_4 in the final concentrations of 3 mM, 400 μM and 20 μM, respectively, and other nutrient elements were provided by adding N- (NH_4^+ and NO_3^-) and P- (PO_4^{3-}) free Provasoli nutrient solution (Provasoli, 1968) in ASW. $CdCl_2$ was added in ASW to the final concentrations of 0, 5, 10, 20 and 50 μM. The incubation temperature was 25°C, and the photoperiod was 12 h. The photosynthetically active radiation (400-700 nm) was 150 μmol photon·m^{-2}·s^{-1} in the absence of algae. ASW was changed everyday, and after 4 days thalli were sampled for wet weight determination. Milli-Q water was used for the preparation of chemicals and ASW.

Thallus segments were divided into two parts: the first part was immediately used for TTC reduction ability assay, and the second part was fixed in liquid nitrogen and stored in -70°C for analyses of lipid peroxidation, antioxidant contents, enzyme activity, and RNA extraction.

Determination of cadmium contents

The measurement of Cd contents was modified from the method of Fuhrer (1982). The organic compounds of thallus segments were obtained by incubating 0.03 g d. wt. thalli at 550°C for 24 h. Then, ashes were digested with 200 μl of 65% HNO_3 and 200 μl of 35% H_2O_2 at 72°C for at least 10 h. After nitric acid digestion of dry ashes, the crucibles were washed with 4 ml of Milli-Q water. The solutions were used for determination of Cd contents by the Z-2000 Series atomic absorption spectrophotometer (Hitachi, Tokyo, Japan) at 228.8 nm. Cd concentrations were estimated from the standard curve of 0 - 1.5 μg l^{-1} $CdCl_2$. Cd contents (μg g^{-1} d. wt.) were calculated as follows: $A_{228.8}$ (μg l^{-1}) × 4 (ml) ÷ 0.03 (g d. wt.).

Determination of daily specific growth rate

The initial wet weight (w. wt.) was determined as WW_0. After growing for 4 days, the thalli in the beaker were weighed as a value of WW_4. The daily specific growth rate expressed as a percentage wet weight increase per day was calculated using the equation %·d^{-1} = 100 × (WW_4-WW_0)/WW_0)/4. To determine the percentage dry weight increase per day, thallus dry weight (d. wt.) was estimated from lyophilized samples (DW_4). The initial dry weights (DW_0) were estimated from the wet weight/dry weight ratio of initial thalli. The daily specific growth rate expressed as a percentage of dry weight increase per day was calculated using the equation %·d^{-1} = 100 × (DW_4-DW_0)/DW_0)/4.

Determination of TTC reduction ability

In the attempt to determine the cellular activity, thallus segments of approximately 0.05 g w. wt. were freshly sampled and incubated at 25°C in 1.5 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 0.8% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) and 35‰ ASW under darkness for 16 h (Chang et al., 1999). After triple washing with 10 ml of 35‰ ASW, intracellular insoluble formanzan was extracted with 5 ml of 95% ethanol at 80°C for 20 min. Ethanol extract was collected, and the thallus segments were extracted again with 5 ml of 95% ethanol. Ethanol extracts were combined. After making up to 10 ml with 95% ethanol, its absorbance was determined at 530 nm. The A_{530} values of Cd-treated thallus segments were calculated as a percentage of the Cd-free control.

Determination of TBARS contents and H_2O_2 concentrations

Thallus segments of approximately 0.1 g w. wt. were ground to powder in liquid nitrogen before 1 ml of 5% (w/v) trichloroacetic acid (TCA) was added. The mixture was centrifuged at 12,000 *g* for 10 min at 4°C, and the supernatant was collected for the determination of lipid peroxidation and H_2O_2 concentrations. The extent of lipid peroxidation was estimated from thiobarbituric acid reacting substance (TBARS) contents as determined according to Health and Packer (1968). TBARS contents were calculated based on A_{532}-A_{600} with the extinction coefficient of 155 $mM^{-1}\cdot cm^{-1}$.

Thallus H_2O_2 contents were determined based on the decomposition of H_2O_2 by peroxidase as described by Okuda et al. (1991). KOH (4 M) of 11.5 µl was added to 0.2 ml supernatant to adjust the pH to 7.5, and the mixture was centrifuged at 12,000 *g* for 1 min under 4°C. The supernatant was applied to a 1-ml column of Amberlite IRA-410, and residual H_2O_2 was washed out by 0.8 ml of distilled water. The contents of H_2O_2 in the eluate were determined within 10 min post column elution. For the determination of H_2O_2 in the eluate, 0.4 ml of 12.5 mM 3-dimethylaminobenzoic acid (DMAB), 0.4 ml of 10 mM 3-methyl-2-benzothiazoline hydrazone (MBTH), and finally 0.02 ml of 0.25 unit ml^{-1} horseradish peroxidase (Sigma, MO, USA) were added for the detection of absorbance at 590 nm for 3 min. A series of H_2O_2 concentrations was prepared for the determination of the H_2O_2 standard curve as in the above method. The concentrations of thallus H_2O_2 were estimated from the H_2O_2 standard curve.

Determination of ascorbate and glutathione concentrations

Thallus segments of approximately 0.25 g w. wt. were ground in liquid nitrogen, and then 2.5 ml of 5% (w/v) trichloroacetic acid (TCA) was added. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was collected for determination of ascorbate and glutathione concentrations.

The measurement of total AsA (AsA + DHA) and AsA concentrations was modified from the method of Hodges et al. (1996). Total AsA concentrations were determined in a 1-ml mixture containing 200 µl TCA extract, 50 mM potassium phosphate buffer (pH 7.4), 3 mM EDTA, and 1 mM dithiothreitol (DTT). After incubating the mixture at 25°C for 10 min, 100 µl of N-ethylmaleimide, 400 µl of 0.61 M TCA, 400 µl of 0.8 M orthophosphoric acid, and 400 µl of α, α'-bipyridyl were added. Finally, 200 µl of $FeCl_3$ was added, and the mixture was incubated at a 40°C water bath for 1 h, and the absorbance was detected at 525 nm. To determine AsA concentrations, the chemicals and procedure were the same as above, except that DTT and N-ethylmaleimide were replaced by distilled water. Total AsA and AsA concentrations were estimated from the standard curve of 0-40 nmole L-AsA. DHA concentrations were calculated by the subtraction of AsA from total AsA.

Total GSH concentrations were determined by the absorbance at 412 nm according to the method of Griffiths (1980). K_2CO_3 (1.25 M) of 38.7 µl was added to 0.3-ml TCA extract to adjust the pH to 7.0, and the mixture was centrifuged at 12,000 *g* for 1 min under 4°C, and the supernatant was collected. For the determination of total GSH, 0.1 ml of supernatant was added to the reaction mixture (0.5 ml of 200 mM sodium phosphate buffer [pH 7.5], 0.1 ml of 50 mM Na_2EDTA, 0.1 ml of 2 mM β-NADPH, 0.1 ml of 6 mM dithionitrobenzoic acid [DTNB] in 0.2 M sodium phosphate buffer (pH 7.5), and 0.1 ml of 0.5 unit ml^{-1} glutathione reductase [Sigma, MO, USA]), and then the reaction was measured at 412 nm for 3 min under 30°C. After the removal of reduced GSH by adding 2 µl of 1 M 2-vinylpyridine in 0.1 ml of supernatant and incubation at 25°C for 1 h, the oxidized GSH concentrations were determined as described above. A standard curve was prepared based on 0-20 nmole oxidized GSH (Sigma, MO, USA). The GSH concentrations were calculated by the subtraction of oxidized GSH concentrations from total GSH concentrations.

Determination of enzyme activity

The enzyme extraction was modified according our previous study (Shiu and Lee, 2005). For APX, lyophilized thalli of 0.0125 g d. wt. were homogenized in liquid nitrogen, and 0.5 ml of extraction buffer (0.1 M sodium phosphate buffer [pH 6.8] containing 1% [pH 6.8] PVPP, 1 mM L-AsA, and 0.25% [v/v] Triton X-100) was added. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was used for APX assay. For GR, lyophilized thalli of 0.0125 g d. wt. were homogenized in liquid nitrogen, and 0.5 ml of extraction buffer (0.1 M sodium phosphate buffer [pH 6.8] containing 1% [w/v] PVPP, 1 mM Na_2EDTA, and 1 mM PMSF) was added. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was used for GR assay. For SOD, lyophilized thalli of 0.0125 g d. wt. were homogenized in liquid

nitrogen and 0.5 ml of extraction buffer (0.1 M sodium phosphate buffer [pH 6.8) containing 80 μM AsA and 1 mM PMSF) was added. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was used for SOD activity assay. For CAT, lyophilized thalli of 0.0055 g d. wt. were homogenized in liquid nitrogen, and 0.5 ml of extraction buffer (0.1 M sodium phosphate buffer [pH 6.8] containing 5% [w/v] PVPP, 1 mM Na_2EDTA, 10 mM DTT and 1 mM PMSF) was added. After centrifugation at 12,000 *g* for 10 min under 4°C, the supernatant was subjected to 30% ammonia sulfate precipitation of protein and standing for 30 min for full precipitation. The mixture was centrifuged at 10,000 *g* for 10 min at 4°C, and the pellet was then dissolved in 150 μl of extraction buffer, and this was then used for the CAT assay. The soluble protein contents were determined by the coomassie blue dye binding method (Bradford, 1976) with bovine serum albumin as a standard curve. As in our our preliminary experiments, enzyme activity was determined within 1 h after extraction to avoid activity loss.

The SOD activity was determined by the inhibition of photochemical inhibition of nitro blue tetrazolium (NBT) according to a method of Giannopolitis and Ries (1977). Total SOD activity was determined in the reaction mixture that consisted of enzyme extract, 50 mM Na-phosphate buffer (pH 7.8), 13 mM L-methionine, 0.1 mM Na_2EDTA, 63 μM NBT, and 1.5 μM riboflavin. The SOD isoforms were identified by adding 3 mM KCN to inhibit CuZnSOD activity (that is, the MnSOD and FeSOD that could be detected), and 3 mM KCN and 5 mM H_2O_2 to inhibit CuZnSOD and FeSOD activities (that is, the MnSOD that could be detected). CuZnSOD activity was subtracted from total SOD activity with the activity of MnSOD and FeSOD. FeSOD activity was subtracted from the activity of both MnSOD and FeSOD with MnSOD activity. The APX activity was determined at A_{290} for DHA according to the extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano and Asada, 1981). The GR activity was monitored by A_{340} for β-NADPH oxidization as GSSG reduction according to a method of Schaedle and Bassham (1977). The CAT activity was measured at A_{420} for H_2O_2 decomposition rate using the extinction coefficient of 40 mM^{-1} cm^{-1} according to a method of Kato and Shimizu (1987). In this study, one unit (U) of enzyme activity is defined as 1 $\mu mol \cdot min^{-1}$ for APX, GR, and CAT while one unit of SOD is defined as a 50% inhibition of activity of the control (without extract added).

Cloning of MnSOD, FeSOD, APX, GR, and CAT genes

The libraries of suppressive subtractive hybridization (SSH) and rapid amplification of cDNA ends (RACE) were created for cloning the genes of antioxidant enzymes from Cu-treated thalli. Total ribonucleic acid (RNA) of thalli treated with or without extra 50 μM $CuSO_4$ (3, 6 and 9 h) was extracted using TRIZOL Reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions. Poly(A^+) mRNA was isolated from total RNA using Dynabeads® mRNA Purification Kit (Dynal Biotech ASA, Oslo, Norway) following the manufacturer' s instructions. The RNA purity was determined at 260 and 280 nm, and its integrity was checked on 1.2% (w/v) agarose/formaldehyde gel.

Because *U. fasciata* exhibited significant oxidative stress upon exposure to Cu (see Figures 1 and 2 in the Result section), the Cu (50 μM)-treated thalli were used to clone genes related to an antioxidant defense system. A cDNA library was created by SSH (Diatchenko et al., 1999) of the RNAs of Cu treatments between 0 and 50 μM using the PCR-Select™ cDNA Subtraction Kit (Clontech, CA, USA). The SSH library was done with 1 μg poly(A^+) mRNA and the TA cloning technique using the pGEM®-T Easy Vector System II (Promega, WI, USA) following the manufacturer's instructions. For screening differential expression gene fragments, probes were generated by random priming using a DIG High Prime DNA Labeling and Detection Starter Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Sequencing was done on 198 clones from forward subtractions and 57 clones from reverse subtractions. Homology searches for sequences of selected clones were performed using a Blastx search from the Basic Local Alignment Sequence Tool (BLAST) (Altschul et al., 1997) at http://www.ncbi.nlm.nih.gov/BLAST applying default parameters and non-redundant databases. From the SSH cDNA library, 300 and 360 randomly selected expressed sequence tags (ESTs) from forward and reverse clones, respectively, were analyzed. Thirty-nine clones were obtained for up-regulation by Cu^{2+} and two clones for down-regulation (Table 1 in supplement data). The results of a BLASTX search performed on the obtained total set of ESTs showed that one of the up-regulated DNA fragments could be assigned to gene coding for a protein known to be APX (SSH9). For cloning the full-length cDNA of genes from RACE library, the SSH9 fragment was used to design the reverse primer. FeSOD, MnSOD, CAT, and GR genes, which were not obtained from SSH library, were cloned directly from the RACE library using the degenerated primers.

The SMART™ RACE cDNA Amplification Kit (Clontech, CA, USA) was used to generate the 5′ and 3′ fragments of cDNAs as templates for the cloning of the full-length cDNAs of FeSOD, MnSOD, CAT, APX, and GR genes, and the BD Advantage™ 2 Kit (Clontech, CA, USA) was used for all PCR procedures. For the GR and CAT genes, we obtained the fragments by using the following degenerate primers: GR-dF (5′-GAA TTCGGNTGYGTNCCNAARAAR-3′), GR-dR (5′-AAGCTTCCWRYKGCDATYAR DAT-3′), CAT-dF (5′-GAATTCGARMGNGTNGTNCAYGC-3′), and CAT-dR (5′-AAG CTTGTRTARAAYTTNACNGC RAANCC-3′). Each reaction, which was performed in a total volume of 25 μl, contained 1× BD advantage 2 mix (Clontech, CA, USA), 0.6 μM of each primer, and 5′ fragments of

cDNAs as templates. The following degenerated PCR amplification program was used: 94°C for 5 min, 40 amplification cycles of 94°C for 30 s, then 53°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. The fragments were used to design primers (GR-R: 5′-CGC CGTCTGGGCTCGAGTATGTCATGT-3′ and CAT-R: 5′-TGACGGTAGA GAATCTGACAGCCACAGG-3′) for 5′-RACE. The gene specific primer (APX-R: 5′-TGA ACGGGGTGCATGAATCCAATC-3′) of the APX gene was designed from an SSH fragment. According to these three gene specific primers (GSPs), we made progress toward amplifying the 5′-RACE product of each gene. The conditions for the 5′-RACE of GR, CAT, and APX genes were those specified in the manufacturer's instructions. For the MnSOD and FeSOD genes, we used the degenerated reverse primers (MnSOD-dR: 5′-AAGCTTRTGYTCCCA NACRTCDATNCC-3′ and FeSOD-dR: 5′-CTGRAARTC MAGRTAGTARGCATGCTCCCA-3′) proceeding to the 5′-RACE. The amplification program was designed as follows: MnSOD gene (94°C for 5 min, 5 cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min, 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 3 min, and then finally 72°C for 5 min) and FeSOD gene (94°C for 5 min, 5 cycles of 94°C for 30 s , 65°C for 30 s , and 72°C for 3 min, 30 cycles of 94°C for 30 s , 60°C for 30 s , and 72°C for 3 min, and then 72°C for 5 min). Based on the sequence of extreme 5′-end of the 5′-RACE product, one specific primer was designed for direct amplification of the full-length cDNAs in 3′-RACE. All GSPs were chosen using the PrimerSelect program of Lasergene (DNASTAR, WI, USA). PCR products were analyzed on 2% (w/v) agarose gels, and the bands were purified by Gel-M® Gel Extraction System (Viogene, Taipei, Taiwan) and cloned with the pGEM®-T Easy Vector System II (Promega, WI, USA). After sequencing, the full-length cDNAs of MnSOD (*UfMnsod*, GenBank no. EF437244), FeSOD1 (*UfFesod1*, GenBank no. EF437245), FeSOD2 (*UfFesod2*, GenBank no. EF437246), APX (*Ufapx*, GenBank no. ABB88581), GR (*Ufgr*, GenBank no. ABB88584), and CAT (*Ufcat*, GenBank no. ABB88582) genes were obtained.

Quantitative real-time PCR detection of gene expression

Total RNA extracted using TRIZOL Reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions was DNase I digested. Five micrograms of each RNA sample was reverse transcribed to complementary DNA with PowerScript Reverse Transcriptase Kit (Clontech, CA, USA) using Oligo$(dT)_{18}$ according to the manufacturer's instructions. Real-time PCR using SYBR Green I technology on ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, USA) was performed. A master mix for each PCR run was prepared with Smart Quant Green Master Mix with dUTP & ROX Kit (Protech, Taipei, Taiwan). Each reaction, which was performed in a total volume of 25 µl, contained 1X Smart Quant Probe Master Mix, 0.3 µM of each primer, and cDNA corresponding to 10 ng input RNA in the reverse transcriptase reaction. The following amplification program was used: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s followed by 60°C for 1 min. The dissociation curves were performed after the PCR reaction, and the product was analyzed by gel electrophoresis to assess the presence of a unique final product. The fluorescence was analyzed by ABI Prism 7000 SDS Software using auto C_T to determine threshold of each gene, and the $2^{-\triangle\triangle CT}$ method was used to calculate the C_T values. The PCR product is a DNA fragment with certain length as predicted. After sequencing, the sequences of PCR products were in agreement with predicted gene fragment. The data were presented as the fold change in mRNA abundance, normalized to an endogenous reference gene (α-tublin), relative to the RNA sample from *U. fasciata* grown without extra $CuSO_4$ or $CdCl_2$ addition. The results presented were the averages of biological triplicates. The forward and reverse primers for real-time PCR were designed 5′-TGCACGCCGAAGGACATA-3′ and 5′-CCAAAGCCATTGTGAATCGAG-3′ for *UfMnsod*, 5′-TGCACGCCGAAGGACATA-3′ and 5′-CCAAAGCCATTGTGAATCGAG-3′ for *UfFesod1*, 5′-ATTGAGTCGGTGAG CCT-3′ and 5′-TGCACACAAGCGTTGTTAC-3′ for *UfFesod2*, 5′-GTTTCAGGCAGGCA GCA-3′ and 5′- ATTCG CATTGTTCTGGGAATC-3′ for *Ufapx*, 5′-GATTTA GGCCAGGCGGA-3′ and 5′-TCATTTCATCTGA TCATATAACAGAACCC-3′ for *Ufgr*, 5′-GAATA CCTTGACCAAAGTGGTT-3′ and 5′-GTAAGTGC AGTCTACGTCG-3′ for *Ufcat*, and 5′-GTGGGCTA TTAAATGGAGTATTGTT-3′ and 5′-ACAGATAGGGTA TCAAAGCGAA-3′ for the α-tubulin gene

Chemicals and statistical analyses

Chemicals were purchased from Merck (Germany) or Sigma (USA). Statistics were analyzed by SAS (SAS v 9.01, NC, USA). The present results were the mean of three replicates with a beaker as a replicate. The effects of Cd on daily specific growth rate, TTC reduction ability, TBARS contents, H_2O_2 contents, water-soluble antioxidant contents and enzyme activities, and the relative transcript abundance was analyzed by one-way analysis of variance (ANOVA). The difference among means was analyzed by Tukey's test following significant ANOVA at $p < 0.05$.

RESULTS

Growth rate, TTC reduction ability, H_2O_2 concentrations, and TBARS contents

The tolerance of *Ulva* to Cd was evaluated by daily specific growth rate and TTC reduction ability. Cd at 50 µM decreased wet weight growth rate ($F = 8.33$, $p = 0.0032$) (Figure 1A), but Cd ranging from 5-50 µM did not affect dry weight growth rate ($F = 3.92$, $p = 0.0364$) (Figure 1B) or TTC reduction ability ($F = 0.38$, $p = 0.8167$)

(Figure 1C). Neither H_2O_2 concentrations (Figure 1D) nor TBARS contents (Figure 1E) were affected by Cd (p > 0.05).

Cadmium contents

Cd contents increased significantly following $CdCl_2$ addition (F= 18.35, p= 0.0001) (Figure 2). They increased linearly from 5 to 20 μM Cd Cl_2, but exhibited a decline above 50 μM.

Ascorbate and glutathione concentrations

The concentrations of total AsA (Figure 3A) and DHA (Figure 3C) decreased by 5-10 μM Cd and increased by Cd ≥ 20 μM. Similarly, the concentrations of AsA first decreased by 5 μM Cd and then increased as Cd ≥ 10 μM, reaching a maximum at 20 μM Cd and a slight decline at 50 μM Cd (Figure 3B). The AsA/DHA ratios increased by 10 μM Cd, followed by a linear decrease (Figure 3D). The concentrations of total GSH (GSH + oxidized GSH) were not affected by 5-20 μM Cd, but decreased by 50 μM Cd (Figure 3E). The concentrations of oxidized GSH were not affected by 5 μM Cd, but increased by 10 and 20 μM Cd and decreased by 50 μM Cd (Figure 3G). The concentrations of GSH (Figure 3F) and the ratios of GSH/oxidized GSH (Figure 3H) decreased linearly as Cd concentrations increased; a drop to the bottom near zero occurred as Cd ≥ 20 μM.

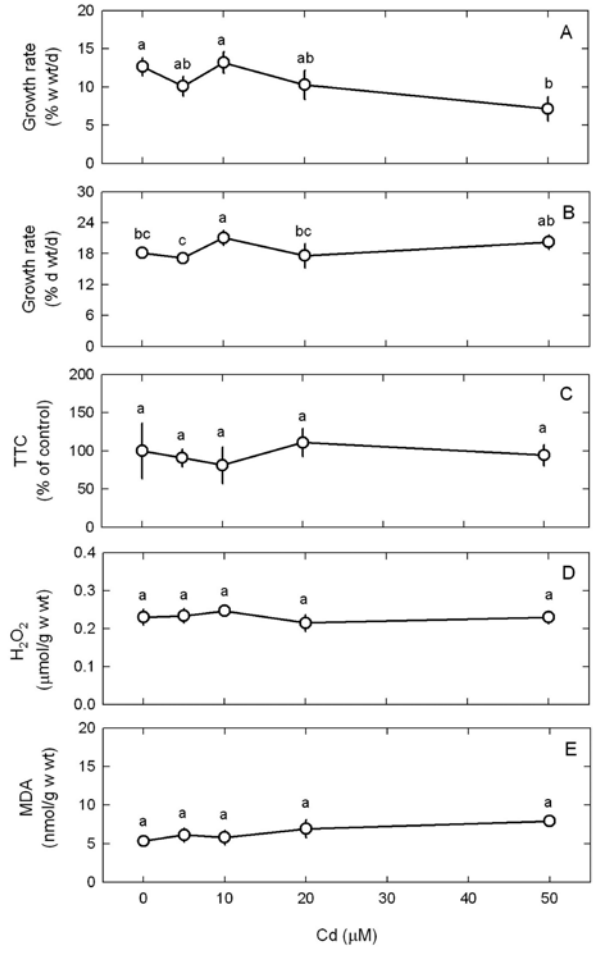

Figure 1. The specific growth rate (A: dry weight; B: wet weight), TTC reduction ability (C), H_2O_2 concentrations (D), and TBARS contents (E) (means ± SD, n = 3) of *Ulva fasciata* exposed to $CdCl_2$ for 4 days. Different letters indicate significant difference at p < 0.05 by Tukey's test.

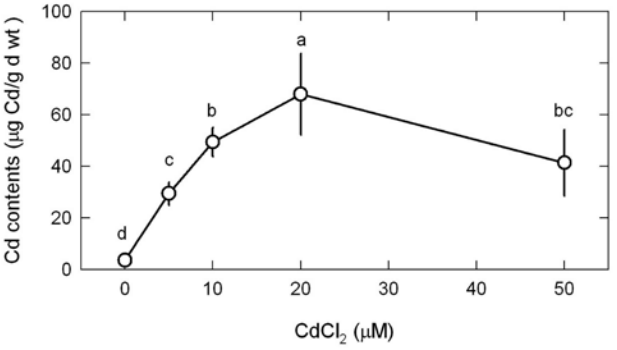

Figure 2. The contents of Cd (means ± SD, n = 3) in *Ulva fasciata* exposed to $CdCl_2$ (A, B, C, D) for 4 days. Different letters indicate significant difference at p < 0.05 by Tukey's test.

Activities of MnSOD, FeSOD, APX, GR and CAT and Transcript of *UfMnsod, UfFesod1, UfFesod2, Ufapx, Ufgr* and *Ufcat*

MnSOD activities were not affected by Cd (F = 1.66, p = 0.2434) (Figure 4A) while FeSOD activities increased by 50 (F = 25.10, p < 0.0001) (Figure 4B). APX activities (F = 7.22, p = 0.0125) underwent a slight decline by 5-20 μM Cd and increased by 50 μM Cd (Figure 4C). GR activities increased linearly as Cd concentrations increased (F = 11.33, p = 0.0010) (Figure 4D). CAT activity also increased linearly as Cd concentrations increased (F = 12.22, p = 0.0011) (Figure 4E).

The transcripts of *UfMnsod* (Figure 4F), *UfFesod2* (Figure 4H), *Ufapx* (Figure 4I), *Ufgr* (Figure 4J), and *Ufcat* (Figure 4K) were not altered by Cd while the transcript of *UfFesod1* increased linearly as Cd concentrations increased (Figure 4G).

DISCUSSION

The marine macroalga *Ulva fasciata* is tolerant to Cd ranging from 5-50 μM. Heavy metals are implicated in oxidative injury involved in the formation of ROS and their subsequent attack on proteins, lipids, and nucleic acids, leading to loss of enzyme functions, altered membrane fluidity, and genomic damage (Dietz et al., 1999). Because H_2O_2 production and TBARS contents in *U. fasciata* were not increased by Cd, Cd ranging from 5-50 μM did not produce oxidative damage to *U. fasciata*.

The present results showed that Cd uptake by *U. fasciata* occurs in a concentration dependent manner. Thallus Cd contents are proportional to the concentrations of $CdCl_2$ added to the seawater in the range from 5-20

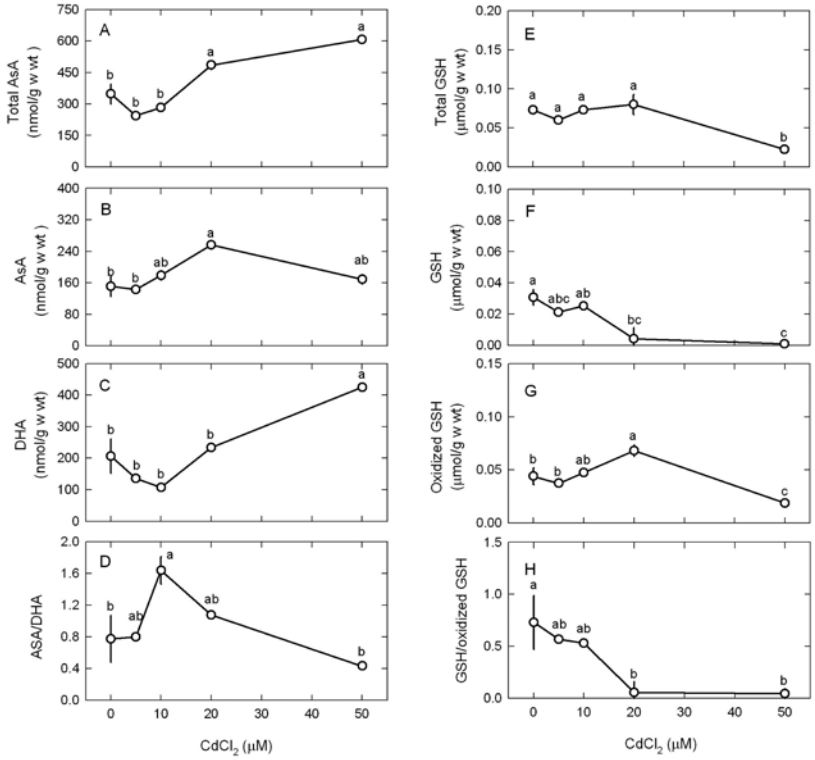

Figure 3. The concentrations of total AsA (A), AsA (B), DHA (C), AsA/DHA ratio, total GSH (E), GSH (F), oxidized GSH (G), and GSH/oxidized GSH ratio (H) (means ± SD, n = 3) in *Ulva fasciata* exposed to $CdCl_2$ (A, B, C, D) for 4 days. Different letters indicate significant difference at $p < 0.05$ by Tukey's test.

μM. However, the Cd contents in thalli were not further increased by 50 μM $CdCl_2$. This might be related to the significant loss of water from thalli that occurs upon exposure to 50 μM $CdCl_2$. The results from Figure 1A and 1B indicated marked water loss after 4 days of such exposure. We speculate that water was lost during the early period after exposure to 50 μM $CdCl_2$ and that the Cd influx decreased. As a result, the contents of Cd contents were less than in treatments with $CdCl_2$ concentrations ≤ 20 μM.

The homeostasis of an antioxidant is significantly affected by Cd. However, glutathione and ascorbate are differentially regulated. The ascorbate pool was significantly enlarged by Cd. In contrast, the concentrations of glutathione and its regeneration were significantly decreased by Cd, reflecting the fact that glutathione is consumed by *U. fasciata* to cope with Cd stress. The decrease in glutathione concentrations has been observed in the marine red macroalga *Gracilaria tenuistipitata* (Collén et al., 2003) and also in the marine green macroalga *Enteromorpha linza* (Malea et al., 2006). It has been documented that glutathione functions as an antioxidant, reacting non-enzymatically with $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ to prevent macromolecule oxidation (Noctor and Foyer, 1998; Noctor et al., 2002).Glutathione is used for synthesis of phytochelatins, which perform the intracellular sequestration of heavy metal ions in algae (De Vos et al., 1992).

Present evidence shows that the activities and gene expression of SOD isoforms are selectively regulated by Cd. The NBT-dependent activity assay and the activity staining on the native gel reveal that CuZnSOD does not exist in *U. fasciata*. A previous study comparing the CuZnSOD proteins of land plants and green algae has documented that CuZnSOD proteins appear in both charophycean algae and land plants, but other green algal groups lack them (De Jesus et al., 1989). Our results agree with their view. MnSOD and FeSOD were the main SOD in this green macroalga. MnSOD, mainly occurring in the mitochondria (Asada, 1999), were not affected by Cd, but FeSOD, mainly occurring in the chloroplasts (Asada, 1999), were increased by Cd at higher concentrations (50 μM), reflecting the fact that FeSOD is the metalloform responsible for converting $O_2^{\bullet-}$ to H_2O_2 under Cd stress. The Cd induction of SOD activity has also been observed in the marine microalga *Tetraselmis gracilis* (Okamoto et al., 1996). In the marine dinoflagellate *Gonyaulax polyedra*, the activities of FeSOD and MnSOD, but not CuZnSOD, were induced by exposure to acute Cd (Okamoto and Colepicolo, 1998).

The coincidence between FeSOD activity and the *UfFesod1* transcript, rather than *UfFesod2*, indicates that the induction of SOD activity by Cd is attributable to enhanced expression of *UfFesod1*. Okamoto et al. performed a study using the marine dinoflagellate *Lingulodinium polyedrum* which showed that gene expression of a chloroplastic FeSOD was up-regulated by Cd, but its protein amounts were not increased under Cd stress (Okamoto et al., 2001b). They suggested the translation of dinoflagellate FeSOD was controlled by other factors (Okamoto et al., 2001b). Because SOD proteins were not determined in this study, we do not know whether the translation of SOD in *U. fasciata* is up-regulated by Cd.

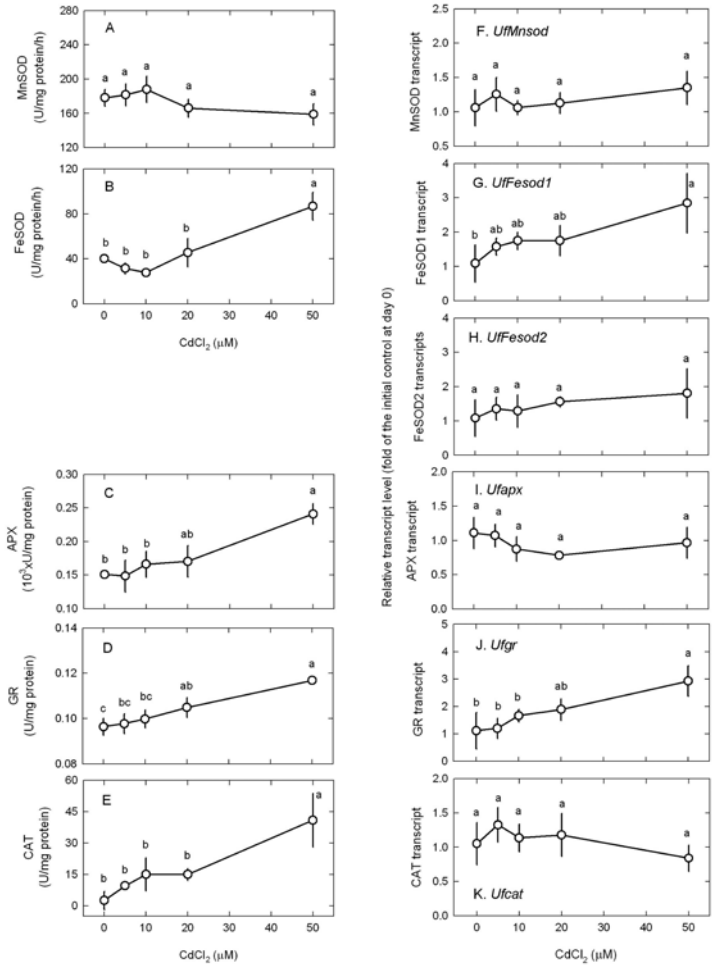

Figure 4. The activities of MnSOD (A), FeSOD (B), APX (C), GR (D), and CAT (E) and the transcripts of *UfMnsod* (F), *UfFesod1* (G), *UfFesod2* (H), *Ufapx* (I), *Ufgr* (J), and *Ufcat* (K) (means ± SD, n = 3) in *Ulva fasciata* in response to $CdCl_2$ for 4 days. Different letters indicate significant difference at $p < 0.05$ by Tukey's test.

The increases in the activities of both APX and CAT are responsible for H_2O_2 scavenging in *U. fasciata* in response to Cd stress. This explains why H_2O_2 did not accumulate in *U. fasciata* under Cd stress. However, APX and CAT activities are transcriptionally up-regulated by Cd. Possibly, the modulation of protein and/or substrate binding and turnover kinetics instead of transcriptional modulation is involved in the induction of APX and CAT activity by Cd. The induction of CAT activity by Cd has been observed in the red macroalga *Gracilaria tenuistipitata* (Collén et al., 2003). In higher plants, Cd induced oxidative stress in pea leaves, characterized by an accumulation of lipid peroxides and oxidized proteins, and a reduction of catalase and SOD activity (Sandalio et al., 2001; Romero-Puertas et al., 2002, 2003). The activities of antioxidant enzymes are up-regulated in response to Cd in rice (*Oryza sativa* L.) seedlings of the Cd-sensitive cultivar (cv. Taichung Native 1, TN1) but not in the Cd-tolerant cultivar (cv. Tainung 67, TNG67) (Kuo and Kao, 2004). The mechanisms by which Cd induces antioxidant responses and to what extent different plant species may share a common defense mechanism are not yet fully understood.

Because of the loss of water in the 50 mM $CdCl_2$-treated thalli, the involvement of Cd-induced water loss in the up-regulation of antioxidant enzyme activity and/or transcription cannot be ignored.

In conclusion, the antioxidant homeostasis in *Ulva fasciata* is markedly altered by Cd in gaining Cd tolerance, the enlargement of the AsA pool and the consumption of GSH. FeSOD, APX, GR, and CAT activities were also induced to prevent oxidative damage. Enhanced gene expression contributed to the increases in the activities of FeSOD and GR by Cd, but APX and CAT were not under transcriptional regulation.

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鎘調節大型綠藻石蓴 (*Ulva fasciata*) 的抗氧化基因、酵素活性及抗氧化能力

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本研究測定鎘逆境對大型綠藻（*Ulva fasciata*）抗氧化反應的影響。處理 0 、5 、10 、20 、50 μM 氯化鎘四天不影響石蓴生長、2,3,5-triphenyltetrazolium chloride 還原能力、H_2O_2 產生量、或脂質過氧化，但是石蓴鎘含量隨外加處理鎘濃度由 0-20 μM 增加而增加，僅在 50 μM 沒有再增高，所以石蓴在 0-50 mM 鎘之長時間處理沒有明顯的氧化逆境發生。Ascorbate (AsA) and dehydroascorbate (DHA) 含量隨外加處理鎘濃度增加而增加，AsA/DHA 比率也隨之增加，在 10 mM 達到最大值。Glutathione (GSH) 及氧化的 GSH 含量與 GSH/ 氧化 GSH 比率隨外加處理鎘濃度增加而減少。鎘外加處理不影響 Mn superoxide dismutase (MnSOD; EC 1.15.1.1) 活性及 mRNA 量，50 μM 鎘外加處理造成 FeSOD 活性增加及 *UfFesod1* (FeSOD 基因的 isoform) mRNA 量增加，但不影響 *UfFesod2* mRNA 量。所以，FeSOD 基因的表現只有 UfFesod1 受鎘外加處理影響。Ascorbate peroxidase (APX; EC 1.11.1.11) 與 catalase (CAT; EC 1.11.1.6) 活性隨外加處理鎘濃度增加而增加，但 mRNA 量不受影響。Glutathione reductase (GR; EC 1.6.4.2) 活性及 mRNA 量隨外加處理鎘濃度增加而增加。綜言之，AsA 總量增加、GSH 的消耗及 FeSOD、APX、GR 與 CAT 活性增高是石蓴去除鎘處理的氧化逆境機轉，所以沒有明顯氧化傷害產生。本研究也暗示 FeSOD 及 GR 活性增高可能是因為基因表現之關係。

關鍵詞 : 抗氧化酵素 ; 抗氧化 ; 鎘 ; 基因表現 ; 石蓴。