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ORIGINAL ARTICLE

Hypersalinity and Hydrogen Peroxide Upregulation of Gene Expression of Antioxidant Enzymes in *Ulva fasciata* Against Oxidative Stress

Ming-Shiuan Sung · Yi-Ting Hsu · Yuan-Ting Hsu · Tzure-Meng Wu · Tse-Min Lee

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Abstract The modulation of manganese superoxide dismutase (MnSOD), FeSOD, ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) gene expression and activities and antioxidants in *Ulva fasciata* against hypersalinity (90‰)-induced oxidative stress was studied. Increases in H_2O_2 contents but no changes in lipid peroxidation and protein carbonyl group contents suggest oxidative damage did not occur in 90‰ condition. Antioxidants were consumed for reactive oxygen species (ROS) scavenging indicated by decreased ascorbate and glutathione contents by 90‰. Antioxidant enzymes were differently expressed by 90‰ for ROS removal. MnSOD activity and transcript increased 1 h after 90‰ treatment with a peak at hour 3, while FeSOD activity increased fast to the plateau after 1 h and its transcript increased after 3 h. APX activity increased 1 h after 90‰ but its transcript rose till 3 h, and GR activity increased after 1 h with a peak at hour 3 but its transcript increased till 3 h. CAT activity and transcript increased after 12 h. Enzyme activity is transcriptionally regulated by 90‰ except a fast increase in FeSOD, APX, and GR activities during 1 h. APX is responsible for early H_2O_2 decomposition while CAT scavenges H_2O_2 in the later period. The inhibition of 90‰ induced increase of H_2O_2 content and FeSOD activity and transcript by treatment of a H_2O_2 scavenger, dimethylthiourea, and the increase of FeSOD transcript of 30‰ grown thalli by H_2O_2 treatment suggest that H_2O_2 mediates the upregulation of FeSOD by hypersalinity while other enzymes is modulated by factors other than H_2O_2.

Keywords Antioxidant enzyme · Gene expression · Hydrogen peroxide · Hypersalinity · Real-time quantitative RCR · *Ulva fasciata*

Introduction

Salinity fluctuation is one of the factors restricting the growth and distribution of intertidal and estuarine macroalgae (Lobban and Harrison 1997). The physiological and biochemical responses are extensively studied to understand how algae respond and adapt to salinity changes (Kirst 1990). However, effects of salinity stress on the induction of oxidative stress are limited in algae (Jahnke and White 2003; Liu et al. 2007). The defense responses of algae to salinity stress-induced oxidative stress are also waited to be elucidated.

Reactive oxygen species (ROS) can be generated from chloroplast and mitochondrion electron transport flow due to excitation of O_2 by excess electron to form singlet oxygen (O_2^1), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$; Halliwell and Gutteridge 1989). For enzymatic scavenging of ROS, superoxide dismutase (SOD; EC 1.15.1.1) converts $O_2^{\cdot-}$ to H_2O_2 that is scavenged by ascorbate peroxidase (APX; EC 1.11.1.11) by AsA oxidization. Monodehydroascorbate and dihydroascorbate (DHA) that is oxidized AsA are reduced by GSH oxidation, and oxidized glutathione (GSSG) is then reduced to glutathione

M.-S. Sung · Y.-T. Hsu · Y.-T. Hsu · T.-M. Wu · T.-M. Lee (✉)
Institute of Marine Biology, National Sun Yat-sen University, Kaohsiung, Taiwan 80424, Republic of China
e-mail: tmlee@mail.nsysu.edu.tw

M.-S. Sung · Y.-T. Hsu · Y.-T. Hsu · T.-M. Wu · T.-M. Lee
The Kuroshio Research Group of the Asia-Pacific Ocean Research Center, National Sun Yat-sen University, Kaohsiung, Taiwan 80424, Republic of China

(GSH) by glutathione reductase (GR; EC 1.6.4.2) via utilization of reducing equivalent from NAD(P)H (Asada 1999). H_2O_2 can also be scavenged by catalase (CAT; EC 1.11.1.6; Willekens et al. 1997).

ROS is known as a signal for regulation of antioxidant defense system (Mittler 2002; Neill et al. 2002; Apel and Hirt 2004; Mittler et al. 2004). For example, H_2O_2 can upregulate the expression of genes involved in antioxidant defense (Levine et al. 1994; Karpinski et al. 1999; Morita et al. 1999; Mullineaux et al. 2000). Using cDNA microarray technology, Desikan et al. (2001) have identified 175 nonredundant expressed sequence tags (EST) that are regulated by H_2O_2 in *Arabidopsis*; 113 ESTs are induced and 62 ESTs are repressed.

The study on a marine microalga, *Dunaliella tertiolecta*, has shown that the activities of APX and monohydroascorbate reductase, but not dihydroascorbate reductsase, SOD, GR, and CAT, are induced to cope with hypersaline stress (Jahnke and White 2003). We have recently identified that long-term (4 days) exposure to hypersalinity enhances the generation of ROS in the intertidal green macroalga *Ulva fasciata* Delile, and oxidatively attacks macromolecules indicated by increased lipid peroxidation and peroxide contents (Lu et al. 2006). *U. fasciata* is able to defense the long-term hypersalinity-induced oxidative stress, but it faces daily fluctuated salinity changes instead of long-term salinity stress. Thus, changes of H_2O_2, thiobarbituric acid reacting substance (TBARS), and protein carbonyl group contents and antioxidant enzyme activities by 90‰ treatment (12 h) were examined to clarify whether oxidative stress occurs upon short-term exposure to hypersalinity and whether enzymatic defense is upregulated against oxidative stress. As we know, how expression of genes of antioxidant enzymes of macroalgae is modulated by hypersalinity is not clear. So, the full-length cDNAs of MnSOD (*UfMnsod*, GenBank no. EF437244), FeSOD (*UfFesod1*, GenBank no. EF437245, and *UfFesod2*, GenBank no. EF437246), APX (*Ufapx*, GenBank no. ABB88581), and GR (*Ufgr*, GenBank no. ABB88584) and partial α-tubulin cDNA (*Uftua*, GenBank no. EU701065) were cloned from *U. fasciata* (Wu and Lee (2008). Then, the time-course (12 h) changes in transcripts of MnSOD, FeSOD, APX, GR, and CAT in response to 30‰ and 90‰ were determined and compared to enzyme activity changes. Next, in the attempt to determine whether H_2O_2 plays a role in the regulation of gene expression of antioxidant enzymes, 30‰ grown thalli were pretreated with dimethylthiourea (DMTU), a H_2O_2 scavenger (Levine et al. 1994), for 12 h in darkness and then transferred to 90‰ under light condition for 3 h for SOD, APX, and GR assay or for 12 h for CAT assay. The role of H_2O_2 was confirmed by the application of 0.05 and 0.2 mM H_2O_2 to 30‰ grown thalli.

Materials and Methods

Algal Culture and Treatments *U. fasciata* of 15–20 cm plant height were collected from intertidal regions of Hsitzu Bay in Kaohsiung in southern Taiwan. Plants were washed with natural seawater to remove sands and rhizoidal portions were cut to avoid microbial contamination in following cultures. Thalli were preincubated at 25°C for 14 days in 30‰ nutrient-enriched artificial seawater (ASW; 403.5 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 30 mM $MgSO_4$, 10 mM Tris-HCl (pH 8.0), N- (NH_4^+ and NO_3^-) and P- (PO_4^{3-}) free Provasoli nutrient solutions (Provasoli 1968), 3 mM $NaHCO_3$, 400 μM $NaNO_3$, and 20 μM Na_2HPO_4). Light time was 12 h and photosynthetically active radiation (400–700 nm) was 120 μmol photons·m^{-2}·s^{-1}. Before salinity treatment, thallus segments (3–5 cm in length) of 30 g wet weight (w. wt.) were incubated in a Petri dish (internal diameter, 30 cm) containing 2 L of 30‰ nutrient-enriched ASW for 24 h at 25°C (12 h of 120 μmol photons·m^{-2}·s^{-1} and 12 h darkness). Thalli of 1.20 g w. wt. were transferred to a polycarbonate vessel (Magenta GA-7 vessel, Sigma, St. Louis, MO, USA) containing 300 mL of 30‰ or 90‰ nutrient-enriched ASW (Lee et al. 2005). ASW of 90‰ was prepared by adding NaCl in 30‰ nutrient-enriched ASW (Liu et al. 2000). The photoperiod was 12 h (150 μmol photons·m^{-2}·s^{-1}) and temperature was 25°C.

For DMTU treatment, thalli of 1.20 g w. wt. were cultured in 300 mL of 30‰ nutrient-enriched ASW containing DMTU for 12 h in darkness and then transferred to 30‰ or 90‰ ASW under 150 μmol photons·m^{-2}·s^{-1} for 3 and 12 h. Thalli were sampled and stored in −70°C freezer till analysis. Thalli sampled at hour 3 were used for assay of FeSOD, MnSOD, APX, and GR, while those sampled at hour 6 were used for CAT assay.

For H_2O_2 treatment, H_2O_2 of 0.05 and 0.2 mM was applied to 30‰ grown thalli (1.20 g w. wt. thalli in a polycarbonate vessel containing 300 mL of 30‰ nutrient-enriched ASW) at 150 μmol photons·m^{-2}·s^{-1} and sampled after 0.5 and 3 h, respectively.

Determination of H_2O_2, Protein Carbonyl Group, and Thiobarbituric Acid Reacting Substance Contents H_2O_2 was determined according to Shiu and Lee (2005). Thalli of 0.1 g w. wt. were ground in liquid nitrogen and 1 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 H_2O_2 contents based on decomposition of H_2O_2 by peroxidase. KOH (4 M) of 11.5 μL was added to 0.2 mL supernatant to adjust pH to 7.5 and centrifuged at 12,000×*g* for 1 min at 4°C. Then, the supernatant was loaded to a 1-mL column of Amberlite IRA-410 and then 0.8 mL of distilled water was passed

through, and the eluate was collected. H_2O_2 contents in the eluate were determined within 10 min post elution. Four hundred microliters of 12.5 mM 3-dimethylaminobenzoic acid, 0.4 mL of 10 mM 3-methyl-2-benzo-thiazoline hydrazone, and 0.02 mL of 0.25 unit/mL horseradish peroxidase (Sigma, MO, USA) were added to 0.5 mL eluate. The absorbance at 590 nm was recorded over 120 s at 30°C for estimation of H_2O_2 contents from the standard curve.

Protein carbonyl group contents were determined according to Packer and Glazer (1990). Thalli of 0.1 g w. wt. were ground in liquid nitrogen and 1 mL of 0.1 M phosphate buffer (pH 7.0) containing 1 mM Na_2 ethylenediamine tetraacetic acid (EDTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) was added. After centrifugation at 12,000×*g* for 5 min at 4°C, 450 μL supernatant was mixed with 50 μL of 10% (*w*/*v*) streptomycin and incubated at room temperature for 15 min. After centrifugation at 12,000×*g* for 10 min at 4°C, 250 μL supernatant was mixed with 500 μL 2,4-dinitrophenylhydrazine (Fluka, USA). The supernatant of 250 μL was mixed with 500 μL Milli-Q water as the blank and incubated at room temperature in darkness for 1 h and vortexed every 10 min. Then, 500 mL of 20% TCA was added and incubated in ice under darkness for 5 min. After centrifugation at 12,000×*g* for 5 min at 4°C, the supernatant was discarded and 1 mL of washing solution (absolute ethanol (Merck, Germany) to ethyl acetate (BDH, England)=1:1 (*v*/*v*)) was added. By centrifugated at 12,000×*g* for 5 min at 4°C, the pellet was washed. The washing step was repeated and the pellet was dissolved in 600 mL of 6 M guanidine-HCl (J.T. Baker, USA) and incubated at 37°C for 15 min. After centrifuged at 12,000×*g* for 10 min at 4°C, the absorbance at 370 nm was detected. The extinction coefficient is 22,000 M^{-1} cm^{-1}.

The above TCA supernatant was also used for determination of lipid peroxidation as thiobarbituric acid reacting substance contents resulting from thiobarbituric acid reaction as described by Health and Packer (1968). TBARS contents were calculated based on A_{532}–A_{600} with the extinction coefficient of 155 $mM^{-1}{\cdot}cm^{-1}$.

Determination of Ascorbate and Glutathione Contents Thalli of 0.25 g w. wt. were ground in 2.5 mL of 5% (*w*/*v*) trichloroacetic acid. After centrifugation at 12,000×*g* for 10 min at 4°C, the supernatant was collected as TCA extract. Total AsA (AsA + DHA) and AsA contents in TCA extract were measured according to Hodges et al. (1996). Total AsA contents were determined in 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 incubated 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 incubated at a 40°C water bath for 1 h and detected at 525 nm. For determination of AsA contents, the chemicals and procedure were same as above except the replacement of DTT and *N*-ethylmaleimide by distilled water. Total AsA and AsA contents were estimated from the standard curve of 0–40 nmol L-AsA. DHA contents were calculated by the subtraction of AsA from total AsA.

Total GSH contents were determined by the absorbance at 412 nm according to Griffiths (1980). K_2CO_3 (1.25 M) of 38.7 μL was added to 0.3-ml TCA extract to adjust pH to 7.0 and centrifuged at 12,000×*g* for 1 min under 4°C for collecting supernatant. For determination of total GSH, 0.1 mL of supernatant was added in 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, and 0.1 mL of 0.5 unit/mL glutathione reductase (Sigma, MO, USA)) and 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 contents were determined as described above. A standard curve was prepared with different concentrations of oxidized GSH (0–20 nmol; Sigma). The reduced GSH contents were calculated by the subtraction of oxidized GSH contents from total GSH contents.

mRNA Quantification by Real-Time Quantitative PCR Total RNA was extracted by TRIZOL Reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instruction. After DNase I digested, 5 mg of RNA sample was transcribed to complementary DNA with PowerScript Reverse Transcriptase Kit (Clontech, CA, USA) using $Oligo(dT)_{18}$ according to the manufacturer's instruction. Real-time polymerase chain reaction (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 one time Smart Quant Probe Master Mix, 0.3 μM of each primer, and cDNA corresponding to 10 ng 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 fluorescence was analyzed by ABI Prism 7000 SDS Software using auto C_T to determine threshold of each gene, and the $2^{-\Delta\Delta CT}$ method was used to calculate the C_T values. The product was analyzed by electrophoresis to

assess the presence of a unique final product. PCR product is a DNA fragment with certain length as predicted. Then, PCR products were purified from the gel and cloned for nucleotide sequencing. The sequences of PCR products agreed with predicted gene sequence. The relative change in mRNA level was normalized to a reference gene (α-tubulin) and the fold of increase was calculated relative to RNA sample from 30‰ thalli or without chemical treatment. Each replicated sample was assayed at least three times, and their average was calculated as the value of the sample. Data shown were the average of three replicated samples. 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), CAT (*Ufcat*, GenBank no. ABB88582), and the partial length of α-tubulin (*Uftua*, GenBank no. EU701065) were used for primer design. The forward and reverse primers were listed in Table 1.

Determination of Enzyme Activity The crude enzymes were extracted according Shiu and Lee (2005). 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 L-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 assay. For APX, thalli of 0.0125 g d. wt. were ground in liquid nitrogen and 0.5 mL of extraction buffer (0.1 M sodium phosphate buffer (pH 6.8) containing 1% (*w*/*v*) polyvinylpolypyrrolidone (PVPP), 0.5 mM L-AsA (freshly prepared), 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, thalli of 0.0125 g d. wt. were ground 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 CAT, lyophilized thalli of 0.0055 g d. wt. were homogenized in liquid nitrogen and 0.5 mL of 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 stand for 30 min for fully precipitation. The mixture was centrifuged at 10,000×*g* for 10 min at 4°C and the pellet was dissolved by 150 μL of extraction buffer that was used for CAT assay. Soluble protein contents were assayed by coomassie blue dye binding method (Bradford 1976) with bovine serum albumin as standard.

SOD activity was determined based on the inhibition of photochemical conversion of nitroblue tetrazolium (NBT) to NBT-diformazan (Giannopolitis and Ries 1977). Total SOD activity was detected in the reaction mixture 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. SOD isoforms were identified by adding 3 mM KCN to inhibit CuZnSOD activity and 3 mM KCN and 5 mM H_2O_2 to inhibit CuZnSOD and FeSOD activities. CuZnSOD activity was subtracted by total SOD activity with the activity of FeSOD and MnSOD. FeSOD activity was subtracted by the activity of FeSOD and MnSOD with MnSOD activity. One unit (U) of SOD is defined as the 50% inhibition of activity of the control (without extract added). To avoid the loss of enzyme activity, SOD activity was determined within 30 min after extraction. The activities of MnSOD and FeSOD were detected but CuZnSOD activity was not detected.

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). GR activity was monitored by A_{340} for β-NADPH oxidization as GSSG reduction according to 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 Kato and Shimizu (1987). One unit of enzyme activity is defined as 1 μmol·min^{-1} for APX, GR, and CAT. APX, GR, and CAT activities were determined within 60 min after extraction.

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 data were shown as the mean of three replicates with a vessel as

Table 1 Real-time quantitative PCR primers for antioxidant enzymes and α-tubulin gene in *U. fasciata*

Gene	Nucleotide sequence
UfFesod1	Forward 5′-TGCACGCCGAAGGACATA-3′
	Reverse 5′-CCAAAGCCATTGTGAATCGAG-3′
UfFesod2	Forward 5′-ATTGAGTCGGTGAGCCT-3′
	Reverse 5′-TGCACACAAGCGTTGTTAC-3′
UfMnsod	Forward 5′-TGCACGCCGAAGGACATA-3′
	Reverse 5′-CCAAAGCCATTGTGAATCGAG-3′
Ufapx	Forward 5′-GTTTCAGGCAGGCAGCA-3′
	Reverse 5′-ATTCGCATTGTTCTGGGAATC-3′
Ufgr	Forward 5′-GATTTAGGCCAGGCGGA-3′
	Reverse 5′-TCATTTCATCT GATCATATAACAGAACCC-3′
Ufcat	Forward 5′-GAA TACCTTGACCAAAGTGGTT-3′
	Reverse 5′-GTAAGTGCAGTCTACGTCG-3′
α-tubulin	Forward 5′-GTGGGCTATTAAATGGAGTATTGTT-3′
	Reverse 5′-ACAGATAGGGTATCAAAGCGAA-3′

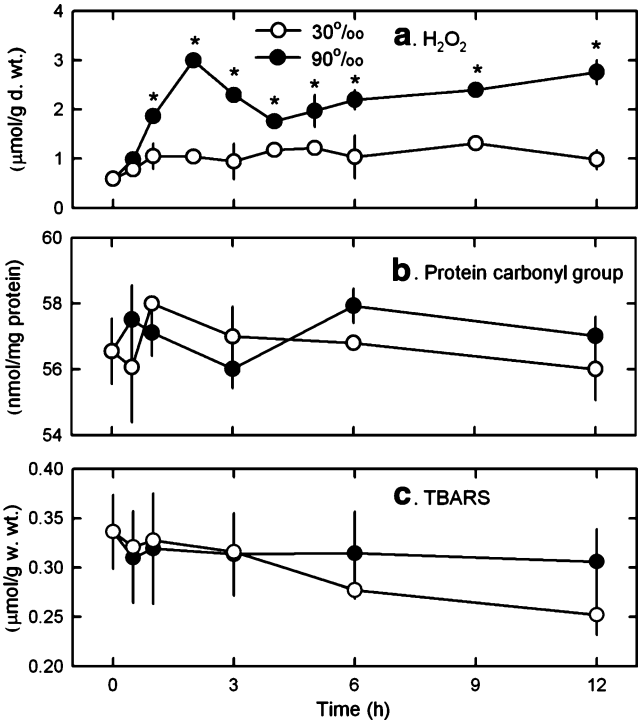

Fig. 1 H_2O_2 (**a**), protein carbonyl group (**b**), and TBARS (**c**) contents of *U. fasciata* in response to 30‰ (*open circle*) or 90‰ (*closed circle*). Data are means ± SD (n=3) and *asterisk* indicates significant difference by *t*-test

a replicate and analyzed by analysis of variance (ANOVA). The differences among means were tested by *t*-test following significant ANOVA at $P<0.05$.

Results

H_2O_2, TBARS, and Protein Carbonyl Group Contents H_2O_2 contents did not change after 0.5 h of 90‰ treatment but increased after 1 h and reached the maximum at hour 2, followed by a decrease first and then a gradual increase after 6 h ($P<0.05$; Fig. 1a). The early increase of H_2O_2 accumulation during the 0–3 h after exposure to hypersalinity reached to approximately 3 μmol H_2O_2/g d. wt. and, after a decline, there was a steady-state increase to 3 μmol H_2O_2/g d. wt. after 12 h of 90‰ treatment.

To elucidate whether oxidative stress occurs during 90‰ treatment, protein carbonyl group and TBARS contents were determined. As compared to the 30‰ control, the contents of protein carbonyl group (Fig. 1b) and TBARS (Fig. 1c) were not affected by 90‰ over 12 h ($P>0.05$). Thus, even H_2O_2 of 90‰ treated thalli was accumulated to the level significantly higher than the 30‰ controls, oxidative damage of macromolecules can be effectively prevented by *U. fasciata* upon exposure to hypersalinity.

Antioxidant Enzyme Activities and mRNA Levels Hypersalinity markedly increased the activities and transcripts of

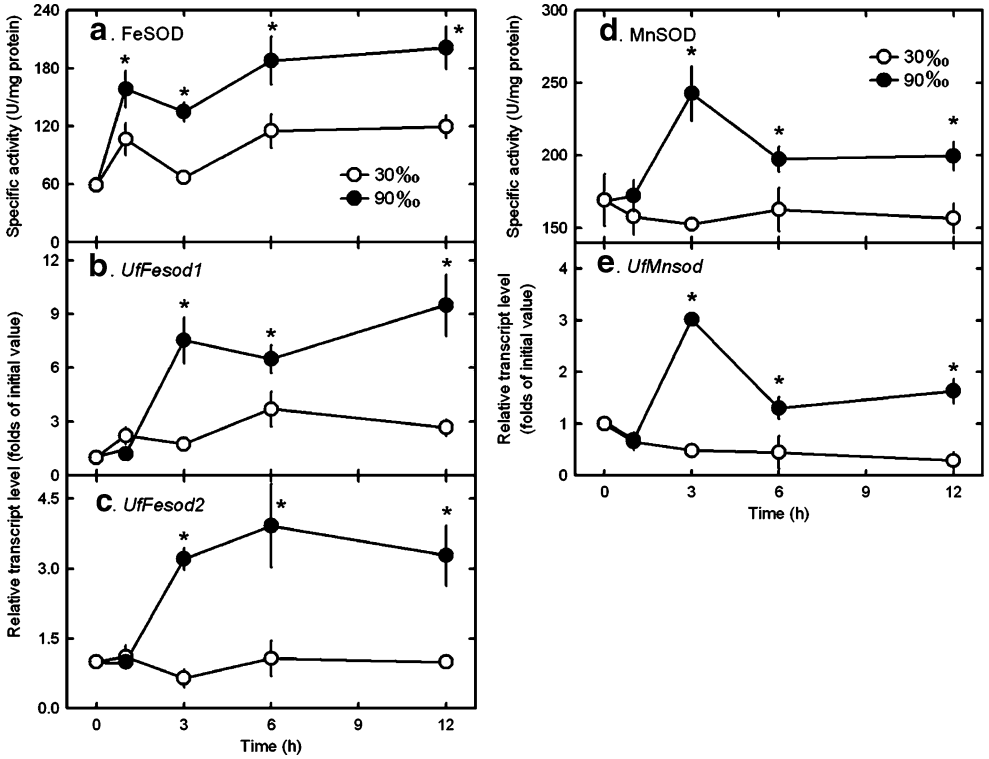

Fig. 2 Time-course changes in the activity and transcript of FeSOD (**a**–**c**) and MnSOD (**d**, **e**) of *U. fasciata* in response to 30‰ (*open circle*) or 90‰ (*closed circle*). Data are means ± SD (n=3) and *asterisk* indicates significant difference by *t*-test

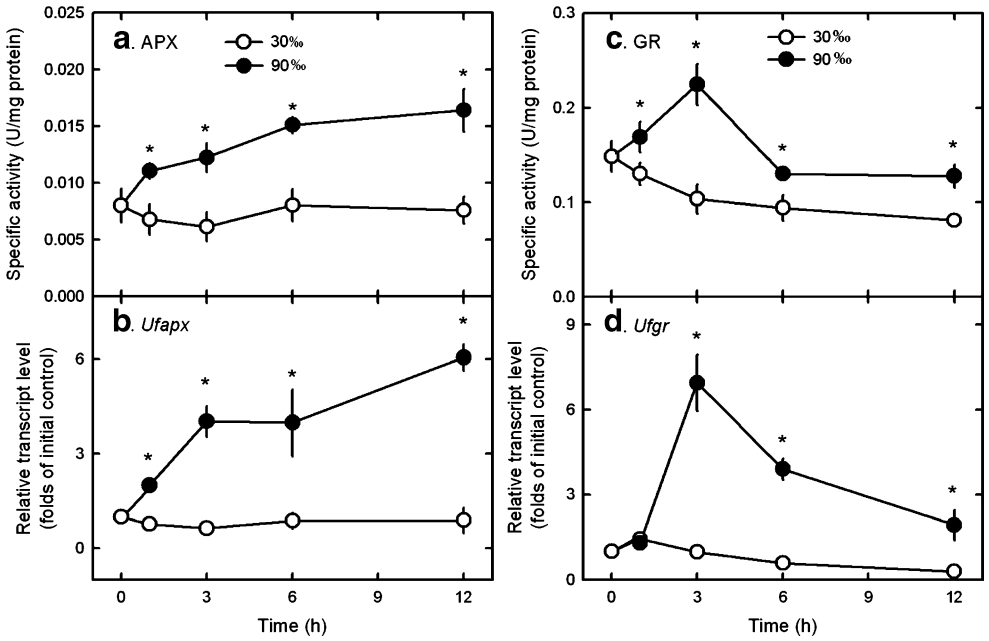

Fig. 3 Time-course changes in activities of APX (**a**) and GR (**c**) and transcripts of *Ufapx* (**b**) and *Ufgr* (**d**) in *U. fasciata* in response to 30‰ (*open circle*) or 90‰ (*closed circle*). Data are means ± SD (n=3) and *asterisk* indicates significant difference by *t*-test

SOD, APX, GR, and CAT (P<0.05), but time-course patterns were different among different enzymes. The activity of FeSOD increased immediately after 90‰ treatment and reached the plateau fast after 1 h (Fig. 2a). The transcripts of *UfFesod1* (Fig. 2b) and *UfFesod2* (Fig. 2c), two isoforms of FeSOD gene, did not change 1 h after exposure to 90‰ and then increased to a plateau after 3 h, which were eightfold (*UfFesod1*) and fourfold (*UfFesod2*) of the 30‰ control, respectively. The magnitude of the increase in the transcript of *UfFesod1* was higher than that of *UfFesod2* (Fig. 2b,c). MnSOD activity (Fig. 2e) and transcript (Fig. 2f) did not change 1 h after 90‰ treatment but rose at hour 3 followed by a decrease after 6 h, which was still higher than the 30‰ control.

APX activity (Fig. 3a) and transcript (Fig. 3b) increased as time advanced after exposure to 90‰. The activity of GR increased significantly after 1 h of 90‰ treatment and peaked at hour 3 (Fig. 3c), and its transcript increased till 3 h followed by a decrease (Fig. 3d). CAT activity and transcript increased till 12 h (Fig. 4).

Effects of DMTU Treatment on H_2O_2 Contents and Enzyme Activities and mRNA Levels DMTU of both 5 mM and 10 mM inhibited the increase of H_2O_2 contents by 3 and 12 h after 90‰ treatment (Fig. 5). The extent of inhibition increased as DMTU contents increased. Because CAT activity and transcript increased 12 h after 90‰ treatment, the effect of DMTU on CAT was assayed after 12 h of DMTU treatment. Other enzymes were assayed after 3 h of DMTU treatment. DMTU affected the activities and transcripts of FeSOD (Fig. 6a,f,g) but did not affect those of MnSOD (Fig. 6b,h), APX (Fig. 6c,i), GR (Fig. 6d,j), and CAT (Fig. 6e,k). The 90‰ induced increase in FeSOD activity (Fig. 6a) and *UfFesod1* (Fig. 6e) and *UfFesod2* (Fig. 6f) transcripts were inhibited by both 5 and 10 mM DMTU; the extent of inhibition increased as DMTU contents increased.

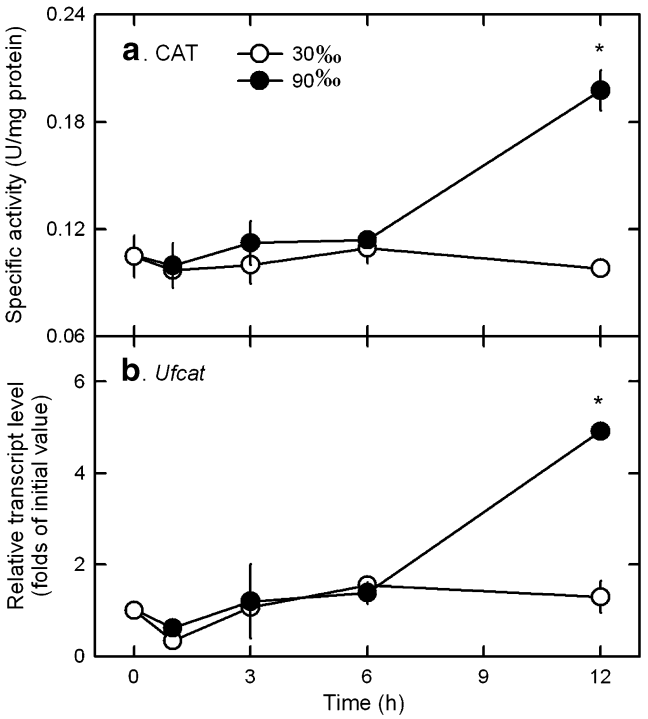

Fig. 4 Time-course changes in activities (**a**) and transcripts (**b**) of CAT in *U. fasciata* in response to 30‰ (*open circle*) or 90‰ (*closed circle*). Data are means ± SD (n=3) and *asterisk* indicates significant difference by *t*-test

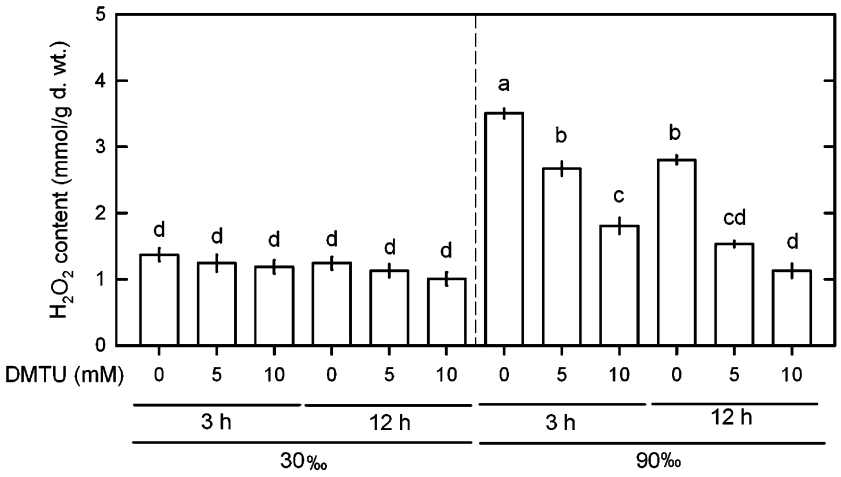

Fig. 5 Effect of DMTU on H_2O_2 contents of *U. fasciata* in response to 30‰ or 90‰ for 3 and 12 h. Data are means ± SD (n=3) and *different symbol* indicates significant difference at P<0.05

Effects of H_2O_2 Treatment on mRNA Levels of Antioxidant Enzymes In attempts to clarify the responses of gene expression of antioxidant enzymes to H_2O_2, H_2O_2 of 0.05 and 0.20 mM were applied to the 30‰ grown thalli under light condition and then thalli were sampled at 0.5 and 3 h. Chlorophyll *a* fluorescence (F_v/F_m and F_v'/F_m') and chlorophyll contents were not affected by 0.05 or 0.20 mM H_2O_2 (data not shown). It suggests that a 0.5–3 h exposure to 0.05 and 0.20 mM H_2O_2 did not influence photosynthetic electron transport.

The transcripts of *UfFesod1* and *UfFesod2* were increased by both 0.05 and 0.20 mM H_2O_2 but those of *UfMnsod*, *Ufapx*, *Ufgr*, and *Ufcat* did not change (Fig. 7). Increases of *UfFesod1* by both 0.05 and 0.20 mM H_2O_2 were relatively fast and significant than those of *UfFesod2* (Fig. 7).

Discussion

This investigation shows that H_2O_2 production in *U. fasciata* could be stimulated by 12-h exposure to hypersalinity. Enhanced ROS production by salinity stress has also been observed in a green microalga *Chlamydomonas reinhardtii* (Yoshida et al. 2004). But, increased salinity did not affect the production of ROS in a harmful algal bloom species *Chattonella marina* (Liu et al. 2007). We have found that there were two peaks for H_2O_2 production in *U. fasciata* exposed to hypersalinity: one was in the early period (0–6 h) and the other was in the late period (6–12 h). Recently, Dring (2006) documented that stresses tend to result in a gradual and continued buildup of ROS in macroalgae, while hyperosmotic shock induces a more rapid and intense production of ROS, described as an "oxidative burst". In this study, the production of H_2O_2 in *U. fasciata* showed a transient increase during the early period after 90‰ exposure followed a steady-state increase during the later period (>6 h). It is not only "oxidative burst" for *U. fasciata* in early response to hypersalinity but also accumulation of H_2O_2 by prolonged hypersalinity treatment. It is likely that hyperosmotic and ionic effects interact for H_2O_2 production in *U. fasciata* as time advanced after hypersalinity. Because we have observed that water content (around 4.10 g H_2O/g d. wt.) did not change 2 h after exposure to 90‰ but showed a significant decrease after 3 h and reached the bottom (around 2.80 g H_2O/g d. wt.) after 4 h (data not shown), it seems likely that late increase in H_2O_2 contents (6–12 h after 90‰ treatment) is resulted from enhanced generation of H_2O_2 due to water loss and ionic stress and decreased activities of some ROS scavenging enzymes, such as MnSOD and GR. The early H_2O_2 peak is possibly due to ionic effects.

Although H_2O_2 accumulated over the 12-h period, oxidative stress did not occur in *U. fasciata* exposed to hypersalinity as indicated by no changes in lipid peroxidation and protein carbonylation. Evidence shows that nonenzymatic and enzymatic defense mechanisms are used for ROS scavenging. Not only a fast decline in the contents of antioxidants, especially glutathione, but also an increase in antioxidant enzyme activities were observed in *U. fasciata* after hypersaline treatment. Our previous study has shown that a long-term (4-day) exposure to 90‰ increased H_2O_2, TBARS and peroxide contents (Lu et al. 2006). It is evident that oxidatively attack on macromolecules can be effectively prevented by *U. fasciata* when exposed to short-term hypersalinity, but this ability is greatly declined after 4 days of 90‰ treatment. It might be due to a decrease in antioxidant defense capacity. Activities of CAT and APX were increased by 4 days of hypersalinity treatment, but the activities of SOD and GR did not increase (Lu et al. 2006). However, the short-term hypersalinity treatment increased the activities of SOD,

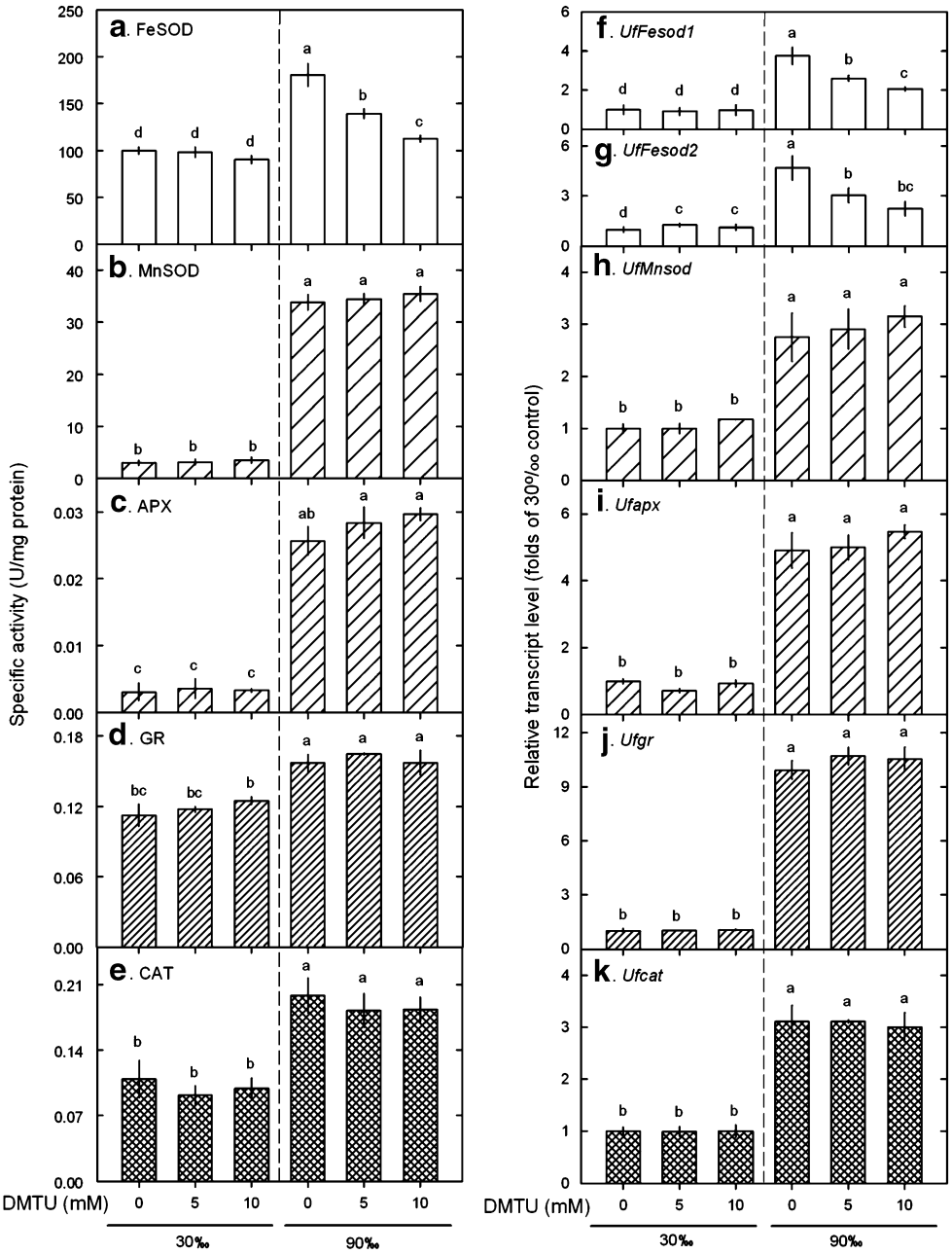

Fig. 6 Effect of DMTU on activities of FeSOD (**a**), MnSOD (**b**), APX (**c**), GR (**d**), CAT (**e**) and transcripts of *UfFesod1* (**f**), *UfFesod2* (**g**), *UfMnsod* (**h**), *Ufapx* (**i**), *Ufgr* (**j**), and *Ufcat* (**k**) of *U. fasciata* in response to 30‰ or 90‰. Data are means ± SD (n=3) and *different symbol* indicates significant difference at $P<0.05$

APX, GR, and CAT. Therefore, we hypothesize that SOD and GR are critical for *U. fasciata* against oxidative stress occurring during short-term exposure to acute hypersalinity.

Activities of antioxidant enzymes except CAT increased fast 1–3 h after hypersalinity. Since CAT activity and transcript were upregulated till 12 h, CAT is not involved in early oxidative defense. The steady-state increase of FeSOD activity and the transient increase of MnSOD activity by hypersalinity suggest that when *U. fasciata* was exposed to hypersalinity, SOD is responsible for detoxifying $O_2^{\cdot-}$ to a less toxic ROS, H_2O_2, which can be degraded by concomitantly increased APX activity. By using transgenic tobacco, it has been identified that the expression of SOD isoform along with APX leads to increased protection, the coexpression of both SOD and APX provided the highest levels of protection against membrane damage in leaf discs and visual symptoms in whole plants (Kwon et al. 2002). We thus propose that the immediate increase in activities of SOD and APX as well as GR by hypersalinity is essential for *U. fasciata* to survive in intertidal regions with regular salinity fluctuations. Meanwhile, it highlights the necessity to study the regulation of antioxidant enzyme activity in detail to elucidate the difference in defense mechanism between short-term

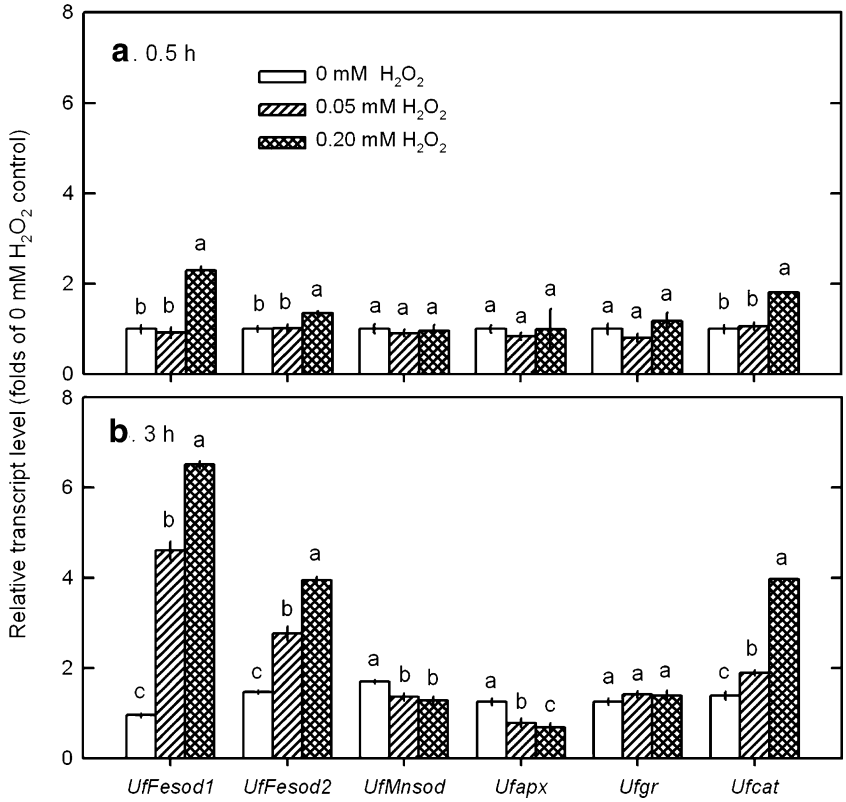

Fig. 7 Effect of 0.05 and 0.20 mM H_2O_2 on transcripts (means ± SD, n=3) of *UfFesod1*, *UfFesod2*, *UfMnsod*, *Ufapx*, *Ufgr*, and *Ufcat* of *U. fasciata* grown under 30‰ conditions. *Different symbol* indicates significant difference at P<0.05

responses and long-term survival mechanisms. To date, there is still little information at molecular level as to how regulation of antioxidant enzyme activities in algae is affected by short-term and long-term hypersaline stress.

We have previously found that the activities of SOD, APX, GR, and CAT can be increased for ROS scavenging in *U. fasciata* upon exposure to ultraviolet B radiation (Shiu and Lee 2005). In addition, our recent study demonstrates that the maintenance of antioxidant homeostasis and the induction of activities of antioxidant enzymes via enhanced gene expression are used by *U. fasciata* to cope with the Cu-induced oxidative stress, but the defense capacity cannot sufficiently alleviate oxidative damage occurring under the condition of higher Cu concentrations (Wu and Lee 2008). It seems to suggest that the activities of ROS scavenging enzymes can be effectively upregulated in this intertidal green macroalga to defense oxidative stress.

Immediate increases of FeSOD, APX, and GR activities without mRNA increase 1 h after 90‰ treatment suggest that early increase in activities of FeSOD, APX, and GR by 90‰ is not modulated at the transcriptional level. Instead, they might be under translational regulation for quick modulation of enzyme activity in order to scavenge ROS generated soon after transferring of *Ulva* to hypersalinity. However, the coincidence between antioxidant enzyme activities and transcripts as 90‰ treatment ≥3 h indicates that increased enzyme activity by 90‰ during 3–12 h is attributable to gene expression.

H_2O_2 is considered not only toxic cellular metabolites but also a signaling molecule that mediates the responses to various stimuli (Mittler 2002; Neill et al. 2002; Apel and Hirt 2004; Mittler et al. 2004). The present result showed that H_2O_2 mediated the upregulation of FeSOD gene expression by hypersalinity and the subsequent activity increase in *U. fasciata*. When H_2O_2 accumulated under 90‰ condition was scavenged by DMTU, the increases in activity of FeSOD and transcripts of *UfFesod1* and *UfFesod2* by 90‰ can be inhibited. *UfFesod1* and *UfFesod2* transcripts in 30‰ grown thalli were increased by H_2O_2 treatment. The H_2O_2 modulation of expression of genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production, was observed in *Arabidopsis* (Baxter-Burrell et al. 2002). By the modulation of cellular H_2O_2 contents via the application of APX, CAT, and SOD inhibitors, hydroxyurea, aminotriazole, and diethyldithiocarbamate, respectively, Morita et al. (1999) suggest that rice cytosolic APX is regulated by H_2O_2 generated through $O_2^{\cdot-}$ dismutation via SOD. But, this study showed that short-term effect of 90‰ (3 or 12 h) on increases of transcripts and activities of MnSOD, APX, GR, and CAT in *U. fasciata* cannot be inhibited by DMTU treatment, and their transcripts and activities did not respond to H_2O_2 treatment. It seems that other factors execute their function independently of H_2O_2 for upregulation of MnSOD, APX, GR, and CAT activities and transcription in *U. fasciata*. It is in contrast to the results

of our previous study that H_2O_2 mediates the increase of APX, GR, and CAT activities in *U. fasciata* by 1 day of 90‰ exposure (Lu et al. 2006). Regulatory systems in response to short-term hypersalinity are likely changed after the long-term hypersalinity treatment. The short-term defense mechanism and the long-term survival responses following hypersalinity are possibly related to dynamic nature of algal regulatory systems.

It can be concluded that antioxidant consumption and immediate upregulation of FeSOD, APX, and GR activities without mRNA increase within 1 h after 90‰ treatment followed by the increases of antioxidant enzyme activities via gene expression are one of defense mechanisms for *U. fasciata* to cope with oxidative stress accompanied with salinity fluctuations in intertidal regions and estuaries. The upregulation of FeSOD gene expression and activity by 90‰ is mediated by H_2O_2, but MnSOD, APX, GR, and CAT gene expression and enzyme activity are regulated by factors other than H_2O_2.

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