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Enhancement of tolerance to heavy metals and oxidative stress in *Dunaliella tertiolecta* by Zn-induced phytochelatin synthesis

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Abstract

The synthesis of phytochelatins (PCs) in a marine alga, *Dunalliela tertiolecta*, is strongly induced by Zn. Pretreatment of the cells with Zn enhances the tolerance toward toxic heavy metals such as Cd, Hg, Cu, Pb, and arsenate. Moreover, the pretreatment also increases the tolerance toward oxidative stress caused by hydrogen peroxide or paraquat. In vitro analysis shows that PC is a stronger scavenger of hydrogen peroxide and superoxide radical than glutathione. These results suggest that PCs inducibly synthesized by Zn treatment could play a role not only in detoxification of heavy metals but also in mitigation of oxidative stress.

Keywords: Phytochelatins; Zinc; Heavy metals; Oxidative stress

In recent years, bioremediation of polluted environments has been receiving increasing attention because it is generally recognized as being both highly effective and of relatively low cost in comparison to conventional physicochemical methods. Bioremediation usually involves bioextraction by accumulation or adsorption using either microorganisms or higher plants and is useful in the removal of toxic heavy metals from contaminated soils or sediments [1,2].

Higher plants have acquired various systems to tolerate heavy metals [3]. Among them, the systems for detoxification and accumulation of heavy metals using heavy metal-binding peptides such as metallothioneins (MTs) and phytochelatins (PCs) have been intensively studied. Information obtained from these investigations is useful in developing methods for removing heavy metals from the environment.

PCs are heavy metal-binding peptides found in higher plants, microalgae, yeasts, and nematodes. These peptides conjugate specifically and strongly to several heavy metals with the and of cysteinyl thiols, rendering them non-toxic and thereby allowing them to be sequestered in the cells. For the purpose of development of heavy metal-removing techniques, an enhancement of the heavy metal tolerance and heavy metal-accumulating ability can be achieved by introduction and/or overexpression of genes concerned with the biosynthesis of PCs [4].

The biosynthesis of PCs is induced by heavy metals such as Cd, Hg, Pb, Zn, Cu, and Ag. Among these, Cd generally has the highest induction ability and is strongly conjugated by PCs [5,6]. Clearly, it is not appropriate to induce PC biosynthesis with the toxic heavy metal Cd. Moreover, Cd will conjugate strongly with PCs and interfere with further conjugation of the target heavy metals for remediation. Therefore, the biomaterial in which high levels of PCs are inducibly synthesized by Cd cannot be available for the remediation of heavy metals.

The toxicity of heavy metals is generally thought to be due to inactivation of enzymes and/or functional proteins by directly binding to them. However, recent studies show that the toxicity may be due, in part at least, to oxidative damage by generation of reactive

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oxygen species (ROS) due to the presence of heavy metals [7]. PCs have a $(\gamma\text{-Glu–Cys})_n$ Gly structure and therefore contain cysteine in abundance. It has been reported that both glutathione (GSH), a substrate of PC synthesis, and MTs display antioxidant activities [8,9]. This implies that PCs may also act as antioxidants in the same manner as GSH and MTs in the cells.

We have previously reported that Zn can induce PC synthesis much more efficiently than Cd in a marine alga, *Dunaliella tertiolecta* [10]. Because Zn is an essential metal and distributed in soil and seawater at relatively high concentrations, it is generally accepted as being far less toxic than Cd. It was also confirmed that Zn was conjugated by PCs less strongly than other toxic heavy metals such as Cd, Hg, and Cu [6]. Moreover, it has been reported that Zn used for the induction of PC synthesis was not able to form complexes with PCs in tomato and *Silene vulgaris* [11]. Therefore, it is expected that the PCs having free thiols are accumulated at high levels in *D. tertiolecta* cells following exposure to Zn and thereby the cells acquire tolerance to toxic metals both due to direct chelation and mitigation of oxidative damage. To confirm this possibility, we investigated the effect of Zn pretreatment on *D. tertiolecta* in relation to its tolerance toward toxic heavy metals and oxidative stress.

Materials and methods

Culture conditions and Zn treatment. D. tertiolecta ATCC 30929 was cultivated in a 12 well plate (Falcon 3043, USA) with modified f/2 seawater medium [12] at 25 °C under white fluorescent illumination of light ($12\,W/m^2$) with 1% CO_2 aeration (30 mL/min). Algal cells were inoculated into 2 mL medium (dry weight was adjusted to 0.14 mg/mL). To induce PC synthesis, algal cells were incubated for 12 h in the presence of 100 μM $ZnCl_2$. Then heavy metals, $CdCl_2$, $HgCl_2$, $CuCl_2$, $PbCl_2$, and Na_2HAsO_4 or oxidative stress-inducing reagents, hydrogen peroxide (H_2O_2), paraquat, were added and incubated under the same condition as described above.

Assay for cell growth and photosynthetic activity. Algal chlorophyll autofluorescence was used as indicator of cell growth. The fluorescence was measured by a fluorescence multi-plate reader (Perseptive biosystems, USA) at an excitation wavelength of 485 nm and an emission wavelength of 645 nm. The results of the cell growth was shown by percentage of the cells not treated with Zn and not incubated with heavy metals or oxidative stress-inducing reagents. Photosynthetic activity was indicated by the O_2 production rate. The cell suspension incubated for 12 h in the presence or absence of oxidative-inducing reagents was transferred to a vial (working volume, 2 mL) for the measurement of O_2 production. O_2 production in the medium was measured by an electrochemical analyzer (Bioanalysis systems, USA) and equipped with a sensor for the dissolved oxygen (WPI OXEL-1) under white fluorescent illumination of light ($30\,W/m^2$).

Assay for active oxygen generation. The generation of intracellular ROS was analyzed by measuring the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) by the modified method of Kobayashi [13]. Algal cells were incubated for 2 h in 2 mL f/2 medium in the presence of 5 μM DCFH-DA and 100 μM paraquat. Cells were harvested by centrifugation (1500*g* for 10 min 4 °C) and washed once with 2 mL medium. The cell suspension was disrupted by sonication and centrifuged (10,000*g* for 10 min at 4 °C). The supernatant was measured in a fluorescence multi-plate reader (Perseptive biosystems, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Esterase activity was measured using fluorescein diacetate (FDA). The algal cells were incubated for 10 min under dark conditions in the presence of 20 μg/mL FDA and the esterase activity was quantified by measuring the fluorescein formation using a fluorescence multi-plate reader (Perseptive biosystems, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The fluorescent intensities of DCF and FDA in the Zn-treated cells were indicated by the ratio to those in the non-Zn-treated cells.

Determination of PCs and GSH. PCs and GSH were measured according to the method described by Hirarta et al. [10]. The cells were harvested by centrifugation (1500*g* for 10 min 4 °C) and resuspended in 30 mM Tris–HCl buffer (pH 8). The cell suspension was lyophilized and disrupted by sonication in 0.5 mg/mL $NaBH_4$. The supernatant obtained by centrifugation (10,000*g* for 10 min at 4 °C was analyzed for GSH and PCs using a modified HPLC post-column system with Ellman's reagent (5,5′-dithio-bis(2-nitrobenzoic acid)).

In vitro examination. Assay for H_2O_2-scavenging activity was followed using the modified method of Lee and Lee [14]. PC_3 was reacted with H_2O_2 for 2 min in 50 mM potassium phosphate buffer (pH 7). H_2O_2 concentration was measured according to the method of Bernt and Bergmeyer [15]. The procedure of ESR assay was done according to Kumari et al. [9]. PC_3 was mixed with 0.1 mM phosphate reaction buffer (pH 8) containing 1.92 mM diethylenetriaminepentaacetic acid, 0.5 mM hypoxanthine, 50 mU/mL xanthine oxidase, and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) for 1 min. Superoxide radical (O_2^-) was measured by an ESR spectrometer (JEOL JES-TE200, Tokyo, Japan).

Assay for enzyme activity. To examine the antioxidant enzyme activities, algal cells were sonicated in 100 mM potassium phosphate buffer (pH 7.8) and ultracentrifuged at 80,000*g* for 30 min at 4 °C. The supernatant was used for the determination of antioxidant enzyme activities. Protein content was measured according to the method of Bradford [16]. Enzyme assays of catalase and APX followed the method of Lee and Lee [14]. SOD activity was performed using an SOD assay kit WST (Dojinnkagaku, Japan).

Chemicals. DCFH-DA was obtained from Molecular probes, Oregon, USA and DMPO was obtained from Labotech, Tokyo, Japan. All other reagents were purchased from Nakarai tesque, Kyoto, Japan.

Results

Tolerance to heavy metals of the Zn-treated cells

The effect of Zn-pretreatment on the tolerance to several toxic heavy metals was investigated. After induction of PC synthesis by incubation with 100 μM Zn^{2+} for 12 h, the cells were subsequently incubated with Cd, Hg, Cu, Pb, or As for 48 h. The pretreatment with Zn had no effect on the cell growth (data not shown). When the non-Zn-treated cells (non-treated cells) were incubated with 400 μM Cd^{2+} or 25 μM Hg^{2+}, the growth was completely inhibited. On the other hand, the growth of Zn-treated cells was not affected under these conditions (Fig. 1). At 10 μM As^{5+}, the growth of Zn-treated cells was suppressed by 20% compared with that of the As free cells, whereas the non-treated cells scarcely grew. It was also observed in the case of Cu and Pb that the Zn-treated cells showed better growth than the non-treated cells. These results clearly indicated that Zn-pretreatment could enhance the tolerance of *D. tertiolecta* to at least some toxic heavy metals.

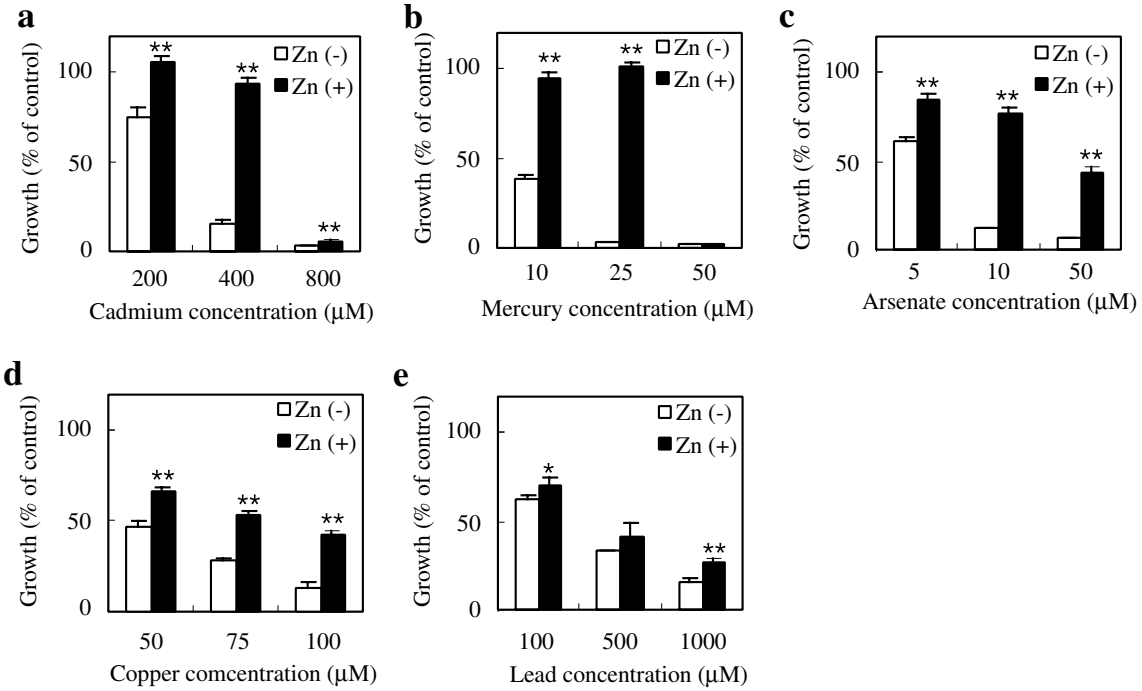

Fig. 1. Enhancement of the tolerance to toxic heavy metals by pretreatment with Zn. Algal cells were pretreated with 100 μM Zn^{2+} [Zn (+)] or non-treated [Zn (−)] for 12 h and incubated with toxic heavy metals and arsenate (a) Cd; (b) Hg; (c) As; (d) Cu; (e) Pb for 48 h. The differences in the growth were statistically significant by Student's t test ($n = 3$; *, $P < 0.05$; **, $P < 0.005$).

Tolerance to oxidative stress of the Zn-treated cells

PCs are considered to show antioxidant activity because they have an abundance of cysteine residues. If PCs play a role as antioxidants, the tolerance of Zn-treated cells to oxidative stress should be enhanced compared with the non-treated cells since they accumulate high levels of PCs. As shown in Fig. 2, in the presence of either H_2O_2 or paraquat which is known to generate O_2^-, Zn-treated cells showed better growth than the non-treated cells (Fig. 2). The Zn-treated cells also maintained a significantly higher photosynthetic activity than the non-treated cells after incubation for 12 h in the presence of 1 mM H_2O_2 or 50 μM paraquat. These results indicate that pretreatment with Zn could enhance the tolerance to oxidative stress caused by either H_2O_2 or paraquat.

Changes in intracellular reactive oxygen levels

ROS level of the cells treated with 100 μM paraquat was measured using DCFH-DA. DCFH-DA diffuses into the cells and is hydrolyzed by esterase to the water-soluble non-fluorescent substance, 2′, 7′-dichlorohydrofluorescein (DCFH), which is retained inside the cell. DCFH is oxidized by ROS, such as H_2O_2, to the fluorescent compound, 2′, 7′-dichlorofluorescein (DCF) [17]. Therefore, the intensity of fluorescence derived from DCFH-DA is directly proportional to the level of ROS in the cells, assuming that the esterase activity is not limiting. Esterase activity was measured using FDA which is hydrolyzed by the enzyme to fluorescein, a water-soluble fluorescent compound. By the addition of 100 μM paraquat, DCF formation in the *D. tertiolecta* cells under the basal cultivation conditions increased five times in comparison with the non-paraquat-added cells (data not shown). As shown in Fig. 3, the intracellular ROS level for Zn-treated cells, measured by the fluorescent intensity of DCF, was lower than that of the non-treated cells. However, the esterase activity of the Zn-treated cells, indicated by the fluorescent intensity of fluorescein, was significantly higher than that of cells incubated in the absence of Zn. This indicates that the esterase activity in the Zn-treated cells did not restrict DCF formation. Therefore, the intracellular ROS level in the Zn-treated cells was significantly lower compared with that in the non-treated cells.

Changes in intracellular GSH and PCs levels

To examine the role of PCs in response to oxidative stress, intracellular levels of GSH and PC in cells treated with oxidative reagents were measured. The cells pretreated with Zn for 12 h were incubated in the presence of 1 mM H_2O_2 or 50 μM paraquat for a further 12 h. The levels of PCs in the cells increased and reached 3–4 nmol SH equivalent/mg dw after 24 h, while PCs were unde-

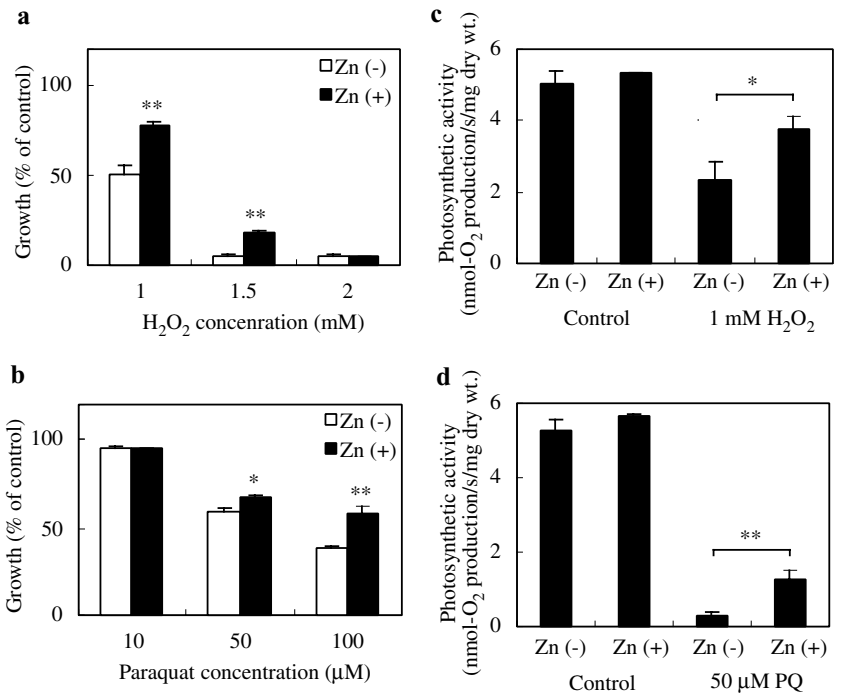

Fig. 2. Enhancement of the tolerance to oxidative stress by pretreatment with Zn. Algal cells were pretreated with 100 μM Zn^{2+} [Zn (+)] or non-treated [Zn (−)] for 12 h and incubated in the presence of (a) H_2O_2; (b) paraquat (PQ) for 48 h. Photosynthetic activities were measured after incubation for 12 h in the presence of (c) H_2O_2; (d) PQ. The differences were statistically significant by Student's t test ($n = 3$; *, $P < 0.05$; **, $P < 0.005$).

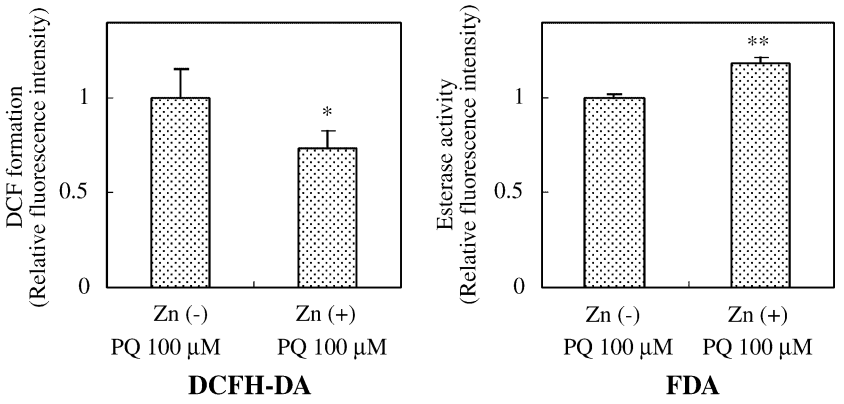

Fig. 3. Intracellular ROS level and esterase activities of Zn-treated and non-treated cells. Algal cells were pretreated with 100 μM Zn^{2+} [Zn (+)] or non-treated [Zn (−)] for 12 h and incubated in the presence of 100 μM PQ and 5 μM DCFH-DA for 2 h. DCF formation was measured as described in Materials and methods. Esterase activities were measured using FDA as described in Materials and methods. The differences in the fluorescent intensities were statistically significant by Student's t test ($n = 5$; *, $P < 0.05$; **, $P < 0.005$).

tectable in the non-treated cells (Fig. 4). In the Zn-treated cells, the PC level was significantly decreased by the H_2O_2 treatment. Likewise, a decrease in the level of PC for the Zn-treated cells was observed by incubation with paraquat.

ROS-scavenging activity of PCs

Antioxidant activity of PCs was examined by the scavenging activity for H_2O_2 and O_2^- using a PC_3 molecule. PC_3 was obtained from the culture cells *Silene cucubalus* in which PC_2 and PC_3 were predominantly synthesized. As shown in Fig. 5, PC3 showed a greater H_2O_2-scavenging activity than either GSH or ascorbic acid (AsA). ESR analysis established that the signal intensity indicating O_2^- decreased following the addition of PC_3 and the O_2^--scavenging activity was higher than that of GSH (Fig. 6). These results indicate that PCs possess a scavenging activity for H_2O_2 and O_2^- which is greater than that of GSH.

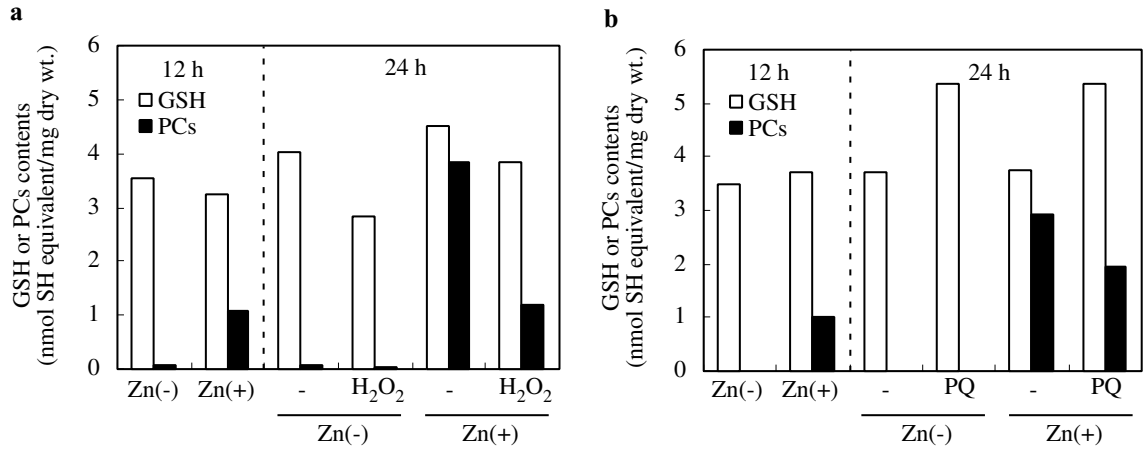

Fig. 4. Intracellular PCs and GSH levels of Zn-treated cells and non-treated cells. Algal cells were pretreated with 100 μM Zn^{2+} [Zn (+)] or non-treated [Zn (−)] for 12 h and cultivated after incubation in the presence of (a) H_2O_2 or (b) PQ for a further 12 h.

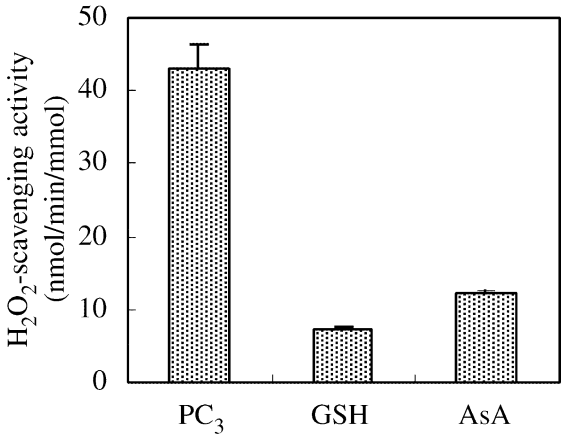

Fig. 5. The H_2O_2-scavenging activities of PC_3, GSH, AsA. H_2O_2 (10 mM) was reacted with antioxidant substances for 2 min and measured as described in Materials and methods ($n = 3$).

Discussion

Phytoremediation technology for the removal of toxic heavy metals from the environment requires higher plants which have the ability to sequester the metals efficiently and also to display a high tolerance to them. Recently, for the purpose of enhancement of the accumulating ability and tolerance, efforts have been focused on introducing mammalian MTs gene into higher plants. However, little or no effective accumulation of toxic heavy metals was attained in transgenic plants [18]. PCs play a role in detoxification and accumulation of heavy metals in higher plants and those capable of synthesizing elevated levels of PCs may prove useful for the heavy metal removal [4].

In this paper, we demonstrate that pretreatment with Zn was effective in enhancing the tolerance to Cd, Hg, Cu, Pb, and As (Fig. 1). Moreover, the pretreatment was also effective in increasing the tolerance to oxidative stress caused by H_2O_2 or paraquat (Fig. 2). Among the heavy metals detoxified, it has been previously shown that PCs conjugated to Cd, Hg, and Cu more strongly compared with Zn [6]. Thus, the detoxification of these metals in the Zn-treated cells is thought to be accomplished mainly by direct chelation to free PC or by displacement of Zn from a PC–Zn complex. It is known that the generation of ROS is stimulated by the presence of heavy metals [7] and As [19] and therefore the cells are subject to oxidative damage. It is also assumed that the detoxification of heavy metals and As by Zn-pretreatment may be due, at least in part, to the elimination of oxidative damage caused by ROS. In particular, the detoxification of Pb and As might be mainly due to such an oxidative damage elimination, because PCs cannot complex with Pb and As in vitro [6] and the formation of PC–Pb complex has not been found in vivo [11].

MTs and GSH are well known as antioxidant substances capable of scavenging ROS such as O_2^- and H_2O_2 [8,9]. However, there has been no report concerning the antioxidant activity of PCs, although a similar antioxidative function can be expected for PCs given their cysteine-rich structure. To examine the mechanism of enhanced tolerance to oxidative stress by the Zn-pretreatment, we carried out further in vivo and in vitro experiments. As shown in Fig. 3, the ROS level in the presence of paraquat in the Zn-treated cells was significantly lower than that in cells which had not been exposed to Zn. This suggests that the generation of ROS caused by paraquat was either suppressed or that the ROS was eliminated more effectively in the Zn-treated cells compared with the non-treated cells.

Zn-treated cells exhibited a lowering in the level of PC by about one-third following treatment with H_2O_2, whereas the level of GSH was not markedly affected (Fig. 4a). The reduction in the level of GSH was more

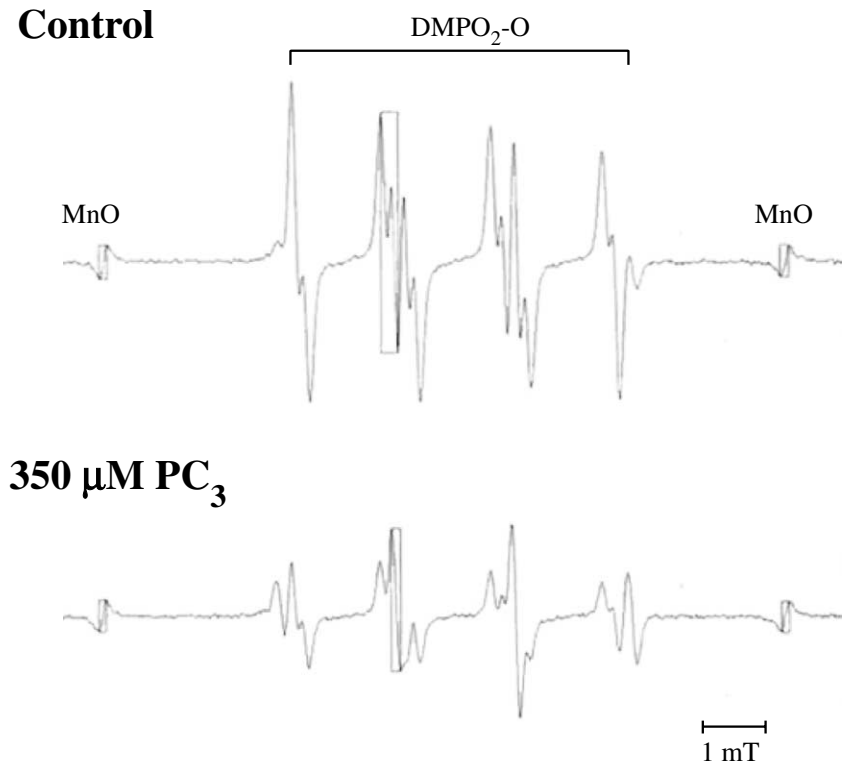

Fig. 6. The O_2^--scavenging activities of PC_3. PC_3 was mixed with the reaction buffer for 1 min. O_2^- was measured by ESR spectrometer.

pronounced in the non-treated cells following exposure to H_2O_2. Moreover, we demonstrated that PC showed a scavenging activity for H_2O_2, which was stronger than that of GSH (Fig. 5). The effect of Zn-pretreatment on the levels of carotenoid in the cells and the activities of antioxidant enzymes, catalase and APX which mainly work to scavenge H_2O_2, were examined. As a result, Zn-pretreatment did not affect the levels of carotenoids (ca. 30 nmol/mg dw) and decreased the activities of catalase and APX (data not shown). These results strongly suggested that PCs play a role in eliminating oxidative stress by scavenging ROS such as H_2O_2 directly. In that case, cysteinyl thiols in PCs might be oxidized irreversibly by H_2O_2.

Upon exposure to paraquat, the GSH level increased both in the Zn-treated cells and non-treated cells (Fig. 4b). In the Zn-treated cells, the levels of PCs went down following treatment with paraquat in comparison with the cells without paraquat, but the effect was less pronounced than in the cells exposed to H_2O_2. The total amount of GSH and PCs was consequently almost the same for Zn-treated cells whether exposed to paraquat or not, even though PCs would be consumed by scavenging ROS generated by paraquat. It is apparent from these results that paraquat stimulated the synthesis of GSH (Fig. 4). The effect of Zn-pretreatment on SOD which eliminate O_2^- was examined. As a result, the activity of SOD in the Zn-treated cells was higher than in the non-treated cells (data not shown). Then, the amount of H_2O_2 obtained by dismutation of O_2^- in the former cells must be higher than that in the latter cells. Therefore, the tolerance to paraquat might be enhanced mainly as a result of scavenging H_2O_2 by PCs in the Zn-treated cells where the activities of catalase and APX were lower compared with those of the non-treated cells. However, the scavenging ability of PCs for O_2^- was observed (Fig. 5) and this direct elimination of O_2^- might partly contribute to detoxification of paraquat.

In *D. tertiolecta* treated with Zn, the level of GSH, a substrate for PC biosynthesis was not different during induction of PC synthesis [10]. However, in other plants, *S. cucubalus* and *Senedesmus bijugatus*, the tolerance to oxidative stress was reduced by the treatment with Cu [20,21]. In these cases, the PC concentration was elevated but the level of GSH, a substrate for PC synthesis, was consequently decreased following Cu treatment. In *D. tertiolecta* treated with Zn, the GSH level was not different during PC synthesis, resulting in the enhancement of tolerance to oxidative stress. Therefore, both the levels of GSH and PCs might affect the tolerance not only to heavy metals but also to oxidative stress.

Cd is the strongest inducer for PC biosynthesis in most higher plants, which is a disadvantageous feature for the practical utilization of these plants for heavy metal removal. In a microalga, *D. tertiolecta*, we had succeeded in enhancing the tolerance to heavy metals by

Zn-induced PC synthesis. Moreover, Zn could enhance the tolerance to oxidative stress which is generally acknowledged as being caused by heavy metals resulting in damage to the cells. PC biosynthesis in *D. tertiolecta* is strongly induced by Zn, a metal of low toxicity, and this has obvious advantages in practical applications.

PC synthase (PCS: γ-glutamylcysteine dipeptidyltranspeptidase) is activated either by GSH-heavy metal chelate [22] or by heavy metals alone [23]. Generally, Cd is by far the strongest inducer [5]. Since Zn is the strongest inducer of PC synthesis in *D. tertiolecta*, the Zn-activated PCS in this alga might have different properties from the PCS found in higher plants. Therefore, cloning of PCS from *D. tertiolecta* and introduction into higher plants would be useful for development of new methodologies for phytoextraction for toxic heavy metals.

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