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# Regulation of phytochelatin synthesis by zinc and cadmium in marine green alga, *Dunaliella tertiolecta*

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday.

**Abstract**

Although $Cd^{2+}$ is a more effective inducer of phytochelatin (PC) synthesis than $Zn^{2+}$ in higher plants, we have observed greater induction of PC synthesis by $Zn^{2+}$ than $Cd^{2+}$ in the marine green alga, *Dunaliella tertiolecta*. To elucidate this unique regulation of PC synthesis by $Zn^{2+}$, we investigated the effects of $Zn^{2+}$ and $Cd^{2+}$ on the activities of both phytochelatin synthase (PC synthase) and enzymes in the GSH biosynthetic pathway. PC synthase was more strongly activated by $Cd^{2+}$ than by $Zn^{2+}$, but the difference was not very big. On the other hand, γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS) were activated by both heavy metals, but their activities were higher in Zn-treated cells than in Cd-treated cells. Dose-dependent stimulation of intracellular reactive oxygen species (ROS) production was observed with $Zn^{2+}$, but not $Cd^{2+}$ treatment. These results suggest that $Zn^{2+}$ strongly promotes the synthesis of GSH through indirect activation of γ-ECS and GS by stimulating ROS generation. This acceleration of the flux rate for GSH synthesis might mainly contribute to high level PC synthesis.


*Keywords:* *Dunaliella tertiolecta*; Phytochelatins; Glutathione; Zinc; Cadmium; Phytochelatin synthase; γ-Glutamylcysteine synthetase; Glutathione synthetase; Reactive oxygen species

## 1. Introduction

Throughout the entire plant kingdom from higher plants to eucaryotic microalgae, heavy metal-binding peptides, phytochelatins [PCs, $(\gamma\text{-Glu-Cys})_n\text{-Gly}$], are well known to play an important role in detoxification of several toxic heavy metals (Grill et al., 1985; Gekeler et al., 1988, 1989). In the presence of heavy metals, phytochelatin synthase (PC synthase, dipeptidyltranspeptidase, EC 2.3.2.15) catalyzes PC synthesis from the substrate, glutathione (GSH) (Grill et al., 1989). $Cd^{2+}$ is recognized as the strongest inducer of PC synthesis in a lot of plant materials. It has also been shown that $Cd^{2+}$ is the most effective activator of PC synthase (Zenk, 1996). On the other hand, the strength of induction of PC synthesis and activation of PC synthase by $Zn^{2+}$ was much less than that of $Cd^{2+}$ (Zenk, 1996).

In a previous study, we unexpectedly found that the levels of PCs synthesized in a marine green alga, *Dunaliella tertiolecta* ATCC 30929, treated with $Zn^{2+}$ were significantly higher than those in Cd-treated cells (Hirata et al., 2001). In both Zn- and Cd-treated cells, the level of GSH was constant while PCs increased linearly, indicating that GSH biosynthesis was also promoted by treatment with $Zn^{2+}$ and $Cd^{2+}$.

Besides activation of PC synthase by heavy metals, PC biosynthesis is thought to be regulated by the intracellular level of GSH. The formation of γ-glutamylcysteine (γ-EC) from glutamate and cysteine, a reaction catalyzed by γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2), is generally accepted as the rate-limiting step in

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the biosynthetic pathway of GSH because this enzyme is feedback-inhibited by GSH (May et al., 1998; Noctor et al., 1998). In addition to γ-ECS, glutathione synthetase (GS, EC 6.3.2.3) and *O*-acetylserine(thiol)lyase (OASTL, EC 4.2.99.8) are also thought to be involved in the regulation of GSH and PC synthesis.

GSH synthesis is also regulated by oxidative stress. Exogeneously applied and endogeneously generated $H_2O_2$ increases GSH levels in plants and cultured plant cells (Smith et al., 1984, 1985; May and Leaver, 1993). Xiang and Oliver (1998) have proposed the idea that PC synthesis is regulated at multiple levels in the presence of $Cd^{2+}$. In this model, $Cd^{2+}$ increases PC synthesis from GSH by activation of PC synthase and promotes the synthesis of GSH not only through transcriptional activation of the GSH biosynthetic pathway, but also through stimulation of endogeneous generation of ROS such as $H_2O_2$.

Similar regulatory mechanisms for heavy metal induction of PC synthesis are assumed to exist in eukaryotic algae, although the literature is limited. Furthermore, we have observed an unexpectedly strong induction by $Zn^{2+}$ in *D. tertiolecta*, suggesting that there may be some unique features in this regulatory pathway in this alga. Therefore, we have investigated the effects of both $Zn^{2+}$ and $Cd^{2+}$ on the activities of PC synthase and three enzymes of the GSH biosynthetic pathway in *D. tertiolecta*.

## 2. Results

### 2.1. Phytochelatin synthesis induced by $Zn^{2+}$ and $Cd^{2+}$ in D. tertiolecta

When *D. tertiolecta* cells were exposed to various concentrations of $Zn^{2+}$ or $Cd^{2+}$ (0–600 μM), PC levels in Zn-treated cells were significantly higher than those in Cd-treated cells, with the highest levels observed at 200 μM $Zn^{2+}$ and 400 μM $Cd^{2+}$, respectively. Typical HPLC profiles indicating specific detection of thiol-compounds in crude extracts from the cells non-treated, treated with 200 μM $Zn^{2+}$ or treated with 400 μM $Cd^{2+}$ for 24 h are shown in Fig. 1, panels A, B, and C, respectively. Analysis of time course profiles showed that the levels of GSH, the substrate for PC synthesis, did not appreciably change in either Zn- or Cd-treated cells, while PCs increased almost linearly (Hirata et al., 2001). Cysteine and γ-EC levels were much lower than those of GSH and PCs in the cells under basal cultivation conditions and were also not changed by heavy metal treatment (data not shown).

### 2.2. Activation of phytochelatin synthase by heavy metals

In higher plants and cultured plant cells in the literatures, PC synthesis is strongly induced by $Cd^{2+}$ and this heavy metal acts as the most effective activator of PC synthase (Zenk, 1996). Therefore, one possible explanation for Zn-induced high level PC synthesis is that PC synthase in *D. tertiolecta* is activated by $Zn^{2+}$ more strongly than by $Cd^{2+}$. To test this possibility, PC synthase activity was assayed by using $Zn^{2+}$ and $Cd^{2+}$ as activators in crude enzyme fractions obtained from cells treated with 200 μM $Zn^{2+}$ or treated with 400 μM $Cd^{2+}$. By treatment with these concentrations for 24 h, the highest levels of PC synthesis were observed, respectively. In all cells, the activity increased with increasing the concentration of added $Zn^{2+}$ and $Cd^{2+}$ as activators in the range from 0.1 to 10 mM (data not shown). Therefore, we compared the effect of $Zn^{2+}$ and $Cd^{2+}$ on PC synthase activity in the presence of 500 μM of both metals as activators. $Cd^{2+}$ showed higher activation efficacy than $Zn^{2+}$ in both treatments, and the efficacy of $Zn^{2+}$ was 86 and 73% of $Cd^{2+}$ in Zn- and Cd-treated cells, respectively (Table 1). These results indicate that the efficacy of $Zn^{2+}$ in activating PC synthase is less than that of $Cd^{2+}$ even though in PC synthesis is more strongly induced by $Zn^{2+}$ than by $Cd^{2+}$ in *D. teriolecta*.

### 2.3. Regulation of GSH synthesis enzymes by heavy metals

The levels of PCs synthesized in the presence of heavy metals are thought to be dependent not only on the activity of PC synthase but also on flux of the substrate, GSH. In *D. tertiolecta*, GSH levels did not decrease while PC levels increased linearly in both cells treated with $Zn^{2+}$ and $Cd^{2+}$ (Hirata et al., 2001), indicating that these ions promote GSH synthesis as well as activation of PC synthase as described above. Therefore, we investigated the effect of $Zn^{2+}$ and $Cd^{2+}$ on activities of three enzymes, γ-ECS, GS, and OASTL, in the GSH biosynthesis pathway. When cells were treated with 200 μM $Zn^{2+}$ and 400 μM $Cd^{2+}$ for 24 h, activities of both γ-ECS and GS greatly increased, although OASTL activities decreased to 64% and 84% in comparison with those in non-treated controls (Table 2). In particular in Zn-treated cells, the activities of γ-ECS and GS were 5-fold and 3-fold of those in the control, respectively, and distinctly higher than those in Cd-treated cells. These results strongly suggest that Zn-induced high level PC synthesis is due to large GSH flux achieved by increases in the activities of γ-ECS and GS in combination with relatively stronger activation of PC synthase.

### 2.4. Oxidative stress induced by heavy metals

GSH plays an important role in protecting plants from oxidative stresses caused by the generation of reactive oxygen species (ROS) and heavy metals. It has been reported that oxidative stress due to exogeneously applied $H_2O_2$ and some heavy metals promotes GSH

synthesis (Smith et al., 1984, 1985; May and Leaver, 1993). To investigate the relationship between Zn-induced increases in γ-ECS and GS activities and oxidative stress in *D. tertiolecta*, intracellular ROS levels were analyzed by flow cytometry with the fluorescent probe, 2′,7′-dichlorofluoresein diacetate (DCFH-DA). Using this probe, ROS such as $H_2O_2$ can be specifically detected and quantified (Bass et al., 1983). As seen in Fig. 2A, intracellular ROS, shown as fluorescent intensity per 10,000 cells, shifted to a higher level in cells treated with 200 μM $Zn^{2+}$. The dose-dependent increase in ROS generation observed with Zn-treatment was similar to that observed in cells treated with $Cu^{2+}$, well known as a redox-reactive metal (data not shown). On the other hand, no shift in fluorescence was observed in cells treated with 400 μM $Cd^{2+}$ (Fig. 2B) indicating that $Cd^{2+}$ has no stimulatory effect on ROS generation in *D. tertiolecta*.

## 3. Discussion

Induction of high level PC synthesis by $Zn^{2+}$ in this marine green alga is a unique phenomenon. PC synthesis is catalyzed by PC synthase, which is thought to be constitutively expressed and activated by heavy metals (Grill et al., 1989). It is also generally observed that PC synthase is strongly activated by $Cd^{2+}$ and that the activation efficacy of other heavy metals is much less than that of $Cd^{2+}$. For instance, the efficacy of $Zn^{2+}$ relative to $Cd^{2+}$ is 33, 26, and 33% in crude enzyme fractions from *Silene cucubalus* suspension culture (Grill

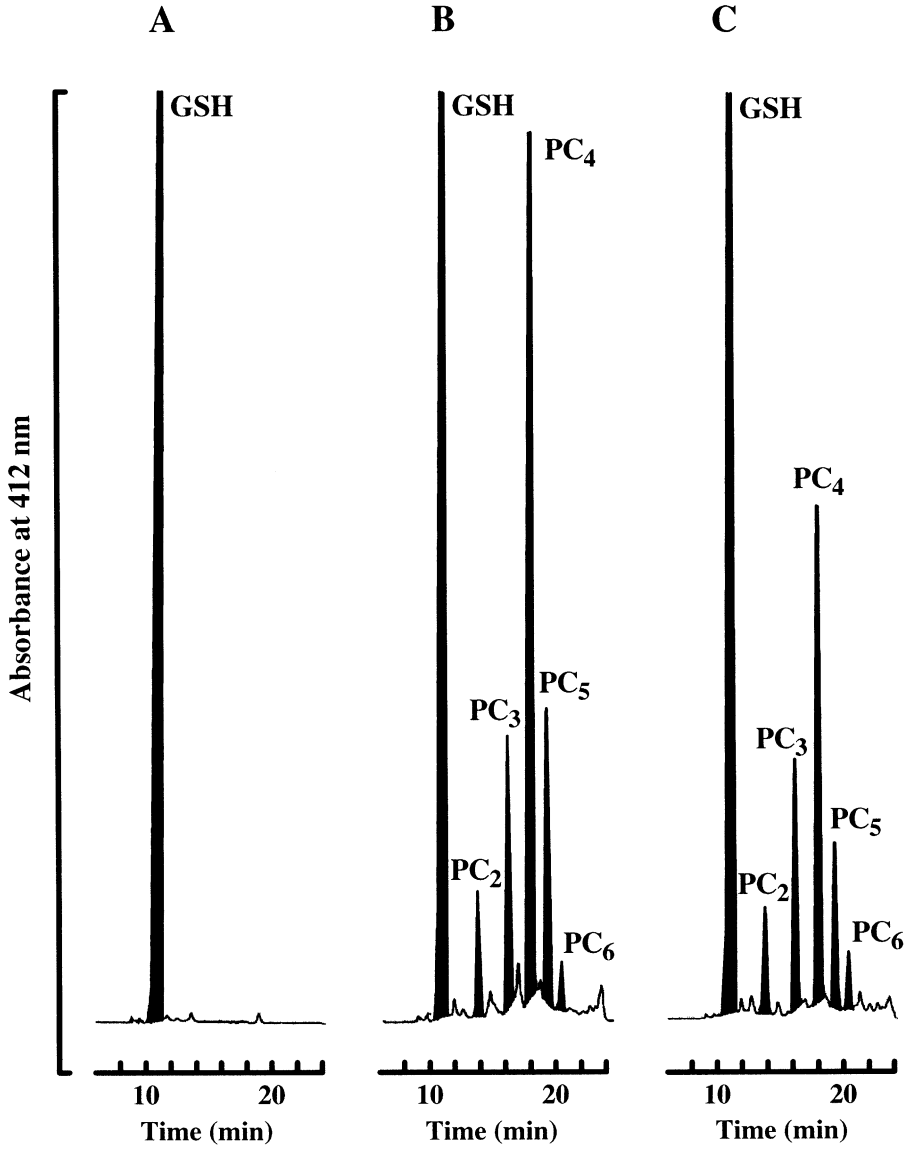


Fig. 1. HPLC profiles of detection of specific thiol-compounds in crude extracts from *Dunaliella tertiolecta* treated with heavy metals. Cells were not treated (A, control), or treated with 200 μM $Zn^{2+}$ (B) or 400 μM $Cd^{2+}$ (C) for 24 h. Phytochelatins and glutathione were extracted and analyzed by HPLC as described in Experimental.

et al., 1989), and recombinant PC synthases from *Glycine max* (GmhPCS1, homo-PC synthase) and *Arabidopsis thaliana* (AtPCS1) (Oven et al., 2002), respectively. In *D. tertiolecta*, as shown in Table 1, $Cd^{2+}$ also more strongly activated PC synthase than $Zn^{2+}$, but the efficacy of $Zn^{2+}$ relative to $Cd^{2+}$ for PC synthase activation was higher than observed in the studies of higher plants and cultured plant cells described above. Therefore, PC synthase seems to be activated more strongly by $Zn^{2+}$ in *D. tertiolecta* than in higher plants. However, to elucidate the difference of PC synthase between *D. tertiolecta* and higher plant, further study using isolated enzymes is required.

γ-ECS is commonly known as the rate-limiting enzyme of the GSH biosynthesis pathway in various organisms. Overexpression of *gsh 1* encoding γ-ECS results in increasing PC levels and enhanced tolerance to $Cd^{2+}$ in *B. juncea* (Yong et al., 1999). In the case of GS, overexpression of *gsh 2* encoding this enzyme also increases the levels of PCs in the same plant in the presence of $Cd^{2+}$ (Zhu et al., 1999). Since transcription of both *gsh 1* and *gsh 2* is induced by exposure to $Cd^{2+}$ and $Cu^{2+}$ (Schafer et al., 1998; Xiang and Oliver, 1998), the increases in activities of γ-ECS and GS observed in Cd-treated *D. tertiolecta* are thought to be due to induction of transcription by $Cd^{2+}$. However, Xiang and Oliver (1998) have also demonstrated that transcription of *gsh 1* and *gsh 2* is not induced by $Zn^{2+}$. Therefore, increases in the activities of these two enzymes by Zn-treatment cannot be explained by regulation at the level of transcription. In our study, OASTL activities in Zn- and Cd-treated cells decreased slightly (Table 3), but were still much higher than γ-ECS and GS activities. Therefore, OASTL seems not to be related to the regulation of GSH synthesis in *D. tertiolecta*.

It is well known that autooxidation of redox-reactive metals such as $Cu^{2+}$ and $Fe^{2+}$ results in formation of $O_2^-$ and subsequently in $H_2O_2$ and hydroxyl radical via Fenton-type reactions (Stohs and Bagchi, 1995). It also has been demonstrated that exposure of plants to non-redox-reactive heavy metal, $Cd^{2+}$, results in oxidative

Table 1
Activity of PC synthase of *D. tertiolecta* treated with $Zn^{2+}$ and $Cd^{2+}$

| Heavy metal treatment | Activity (nmol SH equivalent/min/mg protein) | |
|---|---|---|
| | $Zn^{2+}$ | $Cd^{2+}$ |
| 200 μM $Zn^{2+}$ | 0.32±0.09 | 0.43±0.03 |
| 400 μM $Cd^{2+}$ | 0.35±0.00 | 0.41±0.01 |

PC synthase activity was assayed in crude extracts from *D. tertiolecta* cells treated with 200 μM $Zn^{2+}$ or 400 μM $Cd^{2+}$ for 24 h by using 500 μM of $Zn^{2+}$ or $Cd^{2+}$ as an activator. Values are the means of three experiments±SD.

Table 2
Activities of enzymes in GSH biosynthesis pathway of *D. tertiolecta* treated with $Zn^{2+}$ and $Cd^{2+}$

| Heavy metal treatment | OASTL[a] | r-ECS[a] | GS[a] |
|---|---|---|---|
| Non-treated | 14.5±1.0 | 0.07±0.02 | 0.10±0.01 |
| 200μM $Zn^{2+}$ | 9.3±0.8* | 0.36±0.13* | 0.30±0.11* |
| 400μM$Cd^{2+}$ | 12.1±3.4 | 0.15±0.02* | 0.20±0.02* |

[a] nmol product/min/mg protein. Enzyme activities were assayed in crude extracts from *D. tertiolecta* cells treated with 200 μM $Zn^{2+}$ or 400 μM $Cd^{2+}$ for 24 h. Values are the means of three experiments±SD. * Significantly different ($P<0.05$) from the value of non-treated cells.

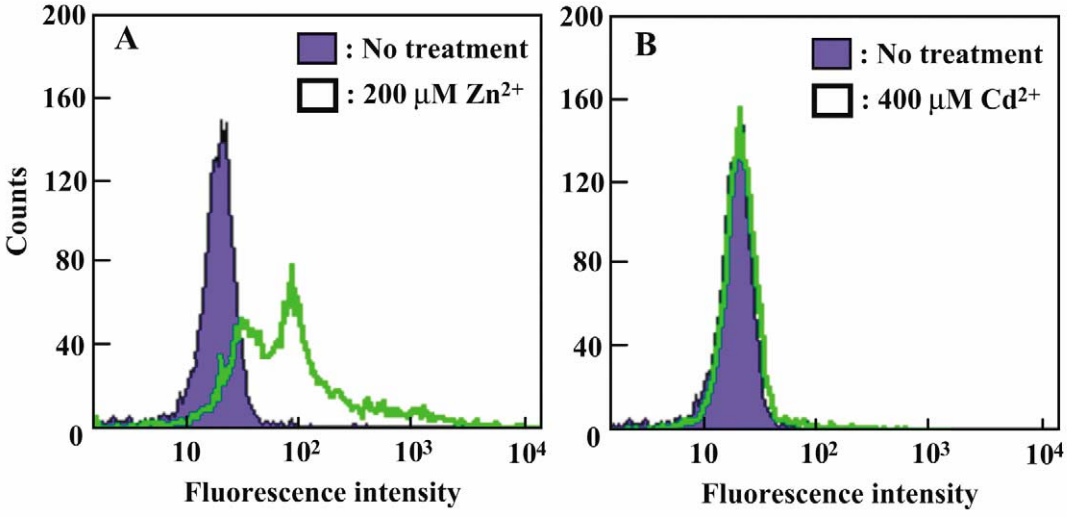


Fig. 2. Generation of reactive oxygen species in *D. tertiolecta* treated with heavy metals analyzed by flow cytometry using DCFH-DA. Cells were incubated with 5 μM DCFH-DA for 1 h in the dark and subsequently exposed to 200 μM $Zn^{2+}$ (A) or 400 μM $Cd^{2+}$ (B) for 2 h.

stress, as indicated by $H_2O_2$ accumulation (Schützendübel and Polle, 2002). Weckx and Clijsters (1997) reported that oxidative stress was also caused by treatment with $Zn^{2+}$. Therefore, at least a part of the ROS generation induced by Zn-treatment in *D. tertiolecta* may be $H_2O_2$ and it is assumed that $Zn^{2+}$ accelerates GSH flux via stimulation of $H_2O_2$ generation, which in turn promotes GSH synthesis. Xiang and Oliver (1998) have demonstrated that $H_2O_2$ does not induce accumulation of GSH metabolic gene transcripts such as *gsh 1* and *gsh 2*, although this ROS did increase GSH levels. In addition, $Zn^{2+}$ also does not induce transcription of these genes. Therefore, increases in γ-ECS and GS activities caused by this ion in *D. tertiolecta*, are more likely to be due to post-transcriptional regulation via stimulation of $H_2O_2$ generation. A novel transcriptional regulation by $Zn^{2+}$ or $H_2O_2$ that has not been found in plants may also explain the observed increases in enzyme activities. To fully address the mechanisms of GSH synthesis regulation in *D. tertiolecta*, further investigation is required, including identification and analysis of GSH metabolic genes in *D. tertiolecta*, such as the homologs of *gsh 1* and *gsh 2*.

Fig. 3 shows a schematic representation of the proposed events for PC synthesis in *D. tertiolecta*. Strong induction of PC synthesis by $Zn^{2+}$ is due to both stronger activation of PC synthase by $Zn^{2+}$ in comparison with that previously reported in higher plants, and

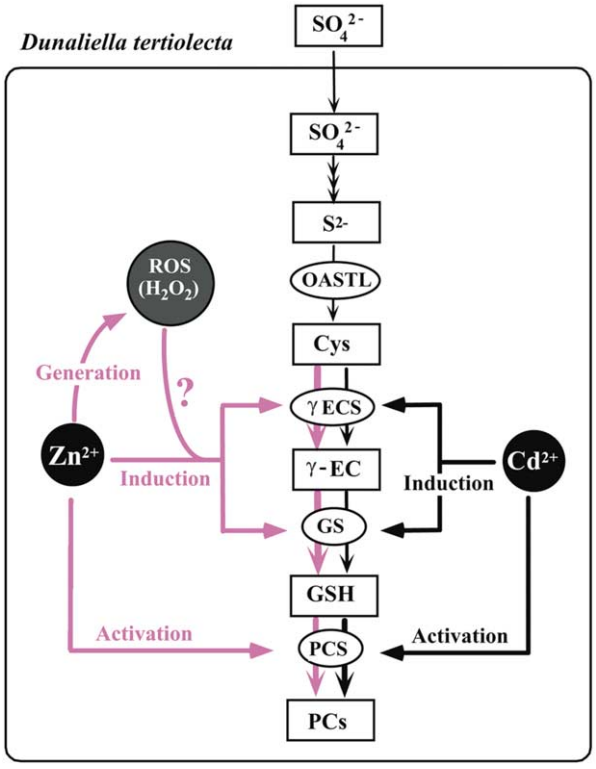


Fig. 3. Schematic representation of proposed events for PC synthesis in *D. tertiolecta* in the presence of $Zn^{2+}$ and $Cd^{2+}$.

a large flux of GSH achieved by increased activities of the GSH metabolic enzymes, γ-ECS and GS. It is possible that the enzyme activation is not a direct effect of $Zn^{2+}$, but due to $H_2O_2$ produced as a result of Zn-treatment.

$Zn^{2+}$ is an essential heavy metal and generally accepted as being far less toxic than $Cd^{2+}$. Using $Zn^{2+}$ as an effective inducer for PC accumulation in *D. tertiolecta*, we have succeeded in enhancing tolerance to toxic heavy metals (Cd, Hg, As, Pb, and Cu) and oxidative stress caused by paraquat and $H_2O_2$ (Tsuji et al., 2002). Recently, Takagi et al. (2002) have reported that detoxification of $Cd^{2+}$ in mammalian cells was achieved by expression of *AtPCS1*. Therefore, PC accumulation would be applicable not only to phytoremediation of toxic heavy metals (Mejáre and Bülow, 2001) but also to increasing tolerances in plants and animals to toxic heavy metals and oxidative stress. Therefore, the Zn-induced high level PC synthesis that we have observed may have broad practical applications.

## 4. Experimental

### 4.1. Materials

*D. tertiolecta* ATCC 30929 was cultivated in 300 ml test tubes containing 200 ml of modified f/2 seawater medium (Yoshihara et al., 1996) at 30 °C under illumination with white fluorescent light (10 W/m$^2$) with 1% $CO_2$ aeration (30 ml/min). For heavy metal treatment, $CdCl_2$ or $ZnCl_2$ were added to cells grown to a density of 525 mg dry wt/l (optical density of 1.5 at 680 nm), and the treated cells were incubated for 24 h.

### 4.2. Chemicals

DCFH-DA was obtained from Molecular Probes, OR, USA. All other reagents were purchased from Nakarai Tesque Chemical Co., Kyoto, Japan.

### 4.3. Determination of PCs and their biosynthetic intermediates

Cells exposed to heavy metals were harvested by centrifugation (1,500 *g* for 10 min at 4 °C) and resuspended in 30 mM Tris–HCl buffer (pH 8.0). The cell suspension was lyophilized and 40 mg of cells were disrupted by sonication in 1.6 ml of 0.5 N NaOH containing 0.5 mg/ml of $NaBH_4$. After centrifugation (12,000 *g* for 5 min at 4 °C), 1.25 ml of the supernatant were mixed with 0.25 ml of 3.5 N HCl. After cooling in ice bath for 15 min, 100 μl of the supernatant obtained by centrifugation (15,000 *g* for 15 min at 4 °C) were analyzed by a modified HPLC post-column system described previously (Hirata et al., 2001).

### 4.4. Enzyme extraction

Algal cells incubated for 24 h in the presence or absence of heavy metals were harvested by centrifugation (1,500 *g* for 10 min at 4 °C) and resuspended in 100 mM Tris–HCl buffer (pH 8.0) containing 10 mM $MgCl_2$ and 5 mM EDTA. The cells were disrupted by sonication and ultracentrifuged at 80,000 *g* for 45 min at 4 °C. For PCS assay, the supernatant was precipitated by 70% (w/v) saturation of $(NH_4)_2SO_4$. The precipitate obtained was dissolved in the same buffer and desalted by dialysis against the buffer. The supernatant obtained by ultracentrifugation was applied to a Sephadex G-25 column for desalinization and eluted fractions were used for OASTL, γ-ECS and GS assays. Protein content of these crude enzyme fractions was measured according to the method of Bradford (1976).

### 4.5. Assay for enzyme activity

PC synthase activity was determined in a reaction mixture (200 μl) containing 100 mM Tris–HCl buffer (pH 8.6), 5 mM mercaptoethanol, 10 mM glutathione, 0.5 mM $ZnCl_2$ or 0.5 mM $CdCl_2$ and enzyme solution including 1 mg protein. The incubation was performed at 37 °C for 120 min and terminated by addition of 40 μl of 3.6 N HCl. The amount of $PC_2$ synthesized was determined by HPLC analysis as described by Hirata et al. (2001). Since synthesis of $PC_3$ and longer PCs was not observed under experimental condition, PC synthase activity was indexed by $PC_2$ production rate.

GS activity was determined in a reaction mixture (200 μl) containing 50 mM Tris–HCl buffer (pH 8.0), 10 mM $MgCl_2$, 30 mM glycine, 5 mM γ-EC, 10 mM ATP, and enzyme solution including 1 mg protein. The incubation was performed at 37 °C for 60 min and terminated by addition of 40 μl of 3.6 N HCl. PC synthesis was not observed in this reaction. The amount of GSH synthesized was determined by HPLC analysis as described above.

γ-ECS activity was determined in a reaction mixture (200 μl) containing 100 mM HEPES buffer (pH 8.0), 40 mM $MgCl_2$, 30 mM glutamate, 1 mM cysteine, 5 mM ATP, 0.5 mM phosphoenolpyruvate, 0.5 unit pyruvate kinase, 0.5 mM dithioerythrito, and enzyme solution including 1 mg protein. The incubation was performed at 37 °C for 45 min and terminated by addition of 40 μl of 3.6 N HCl. The amount of γ-EC synthesized was determined by HPLC analysis as described above.

OASTL activity was determined according to the methods described by Saito et al. (1994). The reaction mixture (200 μl) was incubated at 30 °C for 10 min and terminated by addition of 30 μl of 6 N HCl. The amount of cysteine synthesized was determined by the acid-ninhydrin method at 560 nm as described by Gaitonde (1967).

### 4.6. Assay for generation of reactive oxygen species

The generation of intracellular ROS was analyzed by measuring the oxidation of 2′,7′-dichlorofluoresein diacetate (DCFH-DA). Algal cells were incubated for 1 h in the presence of 5 μM DCFH-DA in the dark. The cells were subsequently incubated for 2 h in the presence of $ZnCl_2$ or $CdCl_2$. The accumulation of oxidized DCFH-DA (2′,7′-dichlorofluoresein, DCF) in the cells was analyzed using flow cytometry (FACS Calibur; Becton Dickinson, USA) at FL1 parameter on a log scale for 10,000 events.

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