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Full Length Research Paper

Different response of phytochelatins in two aquatic macrophytes exposed to cadmium at environmentally relevant concentrations

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Phytochelatins (PCs) have been proposed as potential biomarkers for an evaluation of metal toxicity. However, most studies have been generally limited to high concentrations of metals. In this study, two submerged macrophytes *Ceratophyllum demersum* L. and *Elodea canadensis* Michx. were adopted to investigate the response of phytochelatins (PCs) and its relationship with cadmium (Cd) toxicity upon exposure to low concentrations of Cd (0.01 to 0.64 µM) usually present in low or moderately polluted environments. It was observed that, 0.01 to 0.04 µM Cd had no obvious toxic effects on the growth of two plants compared with the control plants, whereas Cd toxicity is significantly seen in *C. demersum* at 0.08 to 0.64 µM and in *E. canadensis* at 0.16 to 0.64 µM, as indicated by the significant decreases of fresh weights. Two plants showed strong capacity to accumulate Cd present in low concentrations. PCs were produced in a dependent-species and-concentration manner. Response of PCs was strong in *C. demersum* exposed to Cd concentrations studied (0.02 to 0.64 µM) and dramatically increased with the increase of Cd concentrations in solutions. The maximum amount of PCs in *C. demersum* was found at 7 days and obviously decreased with the prolonged exposure to Cd. PC concentrations in *C. demersum* were positively correlated with Cd toxicity, as measured by the growth inhibition rate of fresh weight. By contrast, Cd concentrations studied slightly or mildly induced the production of PCs in *E. canadensis*. The results suggested that, positive responses of PCs in *C. demersum* can serve as early biomarkers for reflecting Cd toxicity in low or moderately polluted water environments.

Key words: Biomarker, cadmium, *Ceratophyllum demersum* L., *Elodea canadensis* Michx., metal toxicity, phytochelatins.

INTRODUCTION

Cadmium (Cd) is a non-essential and toxic heavy metal, mainly released into environments by a variety of natural, agricultural and industrial activities. Cd is believed to cause damage even at very low concentrations (Järup et al., 1998). The toxic effects of Cd on plant growth have been extensively demonstrated. Cd inhibits plant growth and also disturbs photosynthesis, sugar metabolism, sulphate assimilation and several enzymes (Sanità di Toppi and Gabbrielli, 1999; Šottníková et al., 2003; Kevrešan et al., 2003).

Numerous methods have been made to predict toxic effects of metals in plants. Traditional chemical monitoring of metal concentrations in environments and plants can not reflect the actual metal bioavailability and their biological effects (Sneller et al., 1999). The analysis of metal concentrations in environments and plants may overestimate the toxic effects of metals. Consequently, considerable interest has been paid to the use of biomarkers for environmental quality evaluation and risk

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Abbreviations: PCs, Phytochelatins; **GSH,** glutathione; **ICP AES,** inductively coupled plasma-atomic emission spectrometer; **RIR,** relative inhibition rate; **HPLC,** high-performance liquid chromatography.

assessment. Biomarkers have been defined as "biochemical, physiological and morphological changes in plants reflecting their exposure to chemicals" (Ernst and Peterson, 1994). Biomarkers based on responses at the cellular and molecular levels represent the earliest signals of environmental disturbance and commonly used for biomonitoring (Depledge, 1994; Lowe et al., 1995). The principal biomarkers tested in plants are related to photosynthetic activity, enzyme activities, secondary metabolite synthesis, oxidative stress and detoxification mechanisms (Ferrat et al., 2003).

Phytochelatins (PCs), a family of metal-induced peptides, have the general structure (γ-Glu-Cys) $_n$-Gly (n = 2-11) (Grill et al., 1985). Among metals, Cd is the best activator known so far (Clemens, 2006). Furthermore, it has been shown that PC production occurs almost instantaneously upon exposure to metals (Grill et al., 1989) and is positively dependent on external metal concentrations and intracellular metal ion concentrations and is not correlated with metal accumulation (Keltjens and van Beusichem, 1998; Ahner et al., 1994; Sun et al., 2005a). The induction of PCs in plants under metal stress is generally considered to play an important role in metal detoxification. However, numerous studies showed that, PCs are not responsible for metal tolerance and detoxification in metal-tolerant plants and metal-hyperaccumulators (Sun et al., 2005d, 2007). More recently, considerable interest has been attracted to PCs as earlier potential biomarkers for metal toxicity due to its unique responsive properties of PCs. Direct relationships between metal exposure and PC synthesis have been examined in some terrestrial plants and algae, which largely strengthens the potentiality of PCs as biomarkers. However, few studies on the response of PCs and its relationship with metal exposure and metal toxicity have been carried out in aquatic macrophytes.

Recently, use of aquatic macrophytes to remediate the contaminated aquatic system is of considerable interest as a branch of the exciting low-cost and eco-friendly technology, phytoremediation. Among aquatic macrophytes, the submerged species are particularly useful in the abatement and monitoring of heavy metals (Gupta and Chandra, 1998). In terms of this, it was thought to be worthwhile to study their physiological response under metal stress. The induction of PCs under Cd stress has been demonstrated in some aquatic macrophytes. However, the majority of existing PC studies has been confined to high concentrations of Cd (2.5 to 200 μM) usually absent in natural Cd-contaminated environment, which were 278 to 22222 times higher than the recommended permissible limits (0.009 to 0.09 μM) in surface water of China (Yin et al., 2002; Sanità di Toppi et al., 2007). Wagner (1993) estimated that, non-polluted soil solutions contained Cd concentrations ranging from 0.04 to 0.32 μM. Soil solutions which have a Cd concentration varying from 0.32 to about 1 μM can be regarded as polluted to a moderate level. Thus, it was necessary to further demonstrate the production of PCs in closely realistic Cd-polluted environments.

Ceratophyllum demersum L. and *Elodea canadensis* Michx. are two completely submerged aquatic macrophytes and commonly seen in ponds, lakes, ditches and quiet streams. They have been demonstrated to be efficient in the Cd uptake and accumulation (Ornes and Sajwan, 1993; Gupta and Chandra, 1996; Kumar and Prasad, 2004; Fritioff et al., 2005; Fritioff and Greger, 2003, 2007). In the present work, they were typically selected to investigate PC response and its relationship with Cd toxicity under the conditions of low concentrations of Cd (0.01 to 0.64 μM), which are close to environmental realistic levels of Cd pollution. The concentration range of Cd designed was based on GB3838-2002 environmental quality standard for surface water of China (Environmental Protection Administration of the People's Republic of China, 2002). Glutathione (GSH) may be involved in metal stress in higher plants with a variety of ways, possibly including a well-known antioxidant for removal of radicals caused by metal stress in plants (Foyer et al., 2001), a potential chelator of metals reducing metal toxicity (Vögeli-Lange and Wagner, 1996; Sun et al., 2005d, 2007), a precursor of PC synthesis accompanied by a higher demand of total GSH in intact plant (Sun et al., 2005b,c) and a potential bio-marker for an evaluation of metal toxicity (Sun et al., 2005a,b,c). In this experiment, GSH in two plants was also examined.

MATERIALS AND METHODS

Plant preculture and Cd treatments

C. demersum L. and *E. canadensis* Michx. were collected from the local unpolluted fishponds and further cultivated with tap water in a large tank for 1 month in a greenhouse under the natural day-night cycle. After grown for more than one month, two plants (12 g) were transferred to 3 L plastic containers and acclimatized for a week in 10% Hoagland solutions. The solution pH value was adjusted to 6.0 with 0.1 M HCl or NaOH once every 3 days. The nutrient solutions were aerated continuously. After a week, all plants were exposed to a series of Cd concentrations (introduced as $CdCl_2$•$2.5H_2O$): 0.01, 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64μM. All Cd concentrations studied in solutions were accurately detected by inductively coupled plasma-atomic emission spectrometer (ICP-AES). Plants cultured in 10% Hoagland solutions without Cd served as control. Three replicates were used for each Cd concentration. The treated solution was renewed once every 3 days. After Cd exposure for 7, 14 and 21 days, plants were harvested, respectively. At harvesting, plants were washed thoroughly with distilled water, blotted and used for the determination of various parameters. For each harvestry, fresh plants were subdivided into two portions, one was immediately weighed, frozen in liquid nitrogen (-196°C) and stored at - 80°C for analysis of PCs and other low molecular weight thiols, the other was left for the determination of Cd.

Analytical methods of parameters

Cadmium toxicity was estimated by the relative inhibition rate (RIR) of fresh weight after Cd exposure for 21 days. The RIR of fresh weight is defined as ((average fresh weight of plant in control –

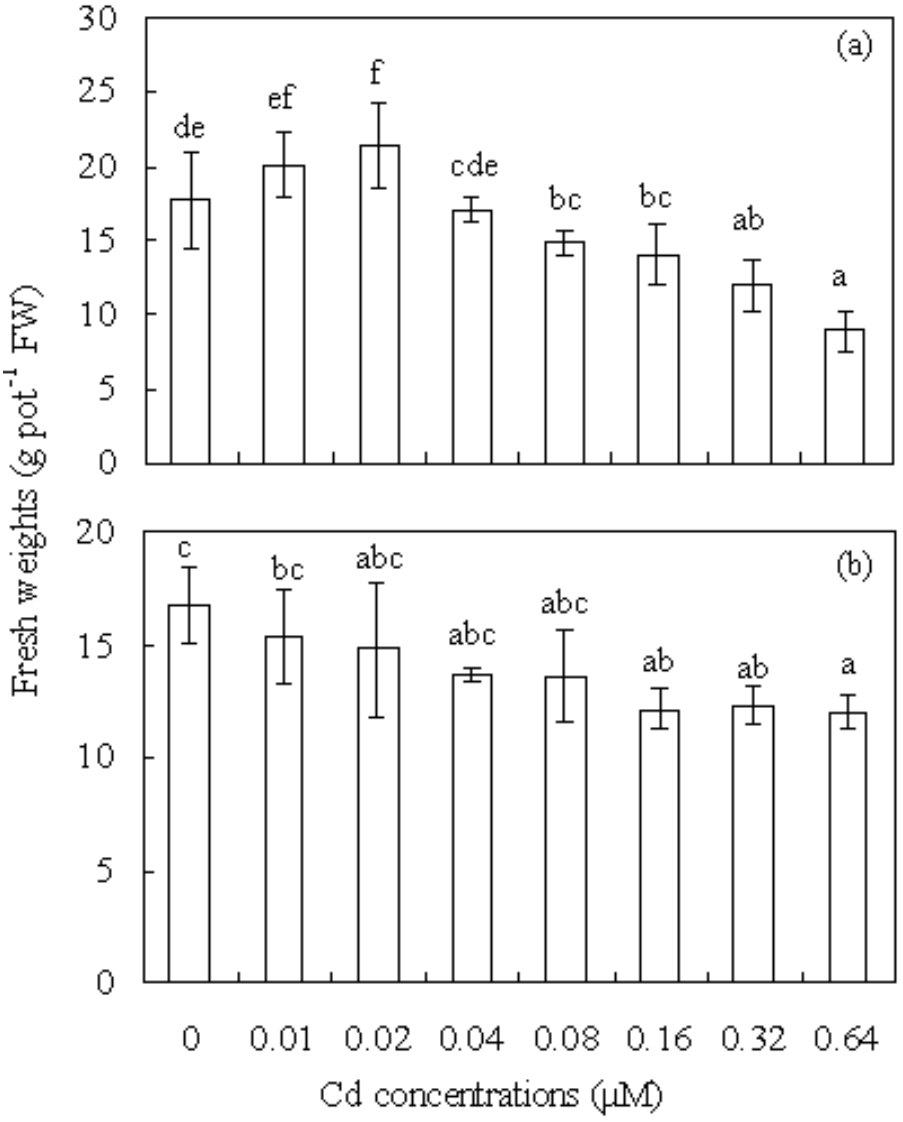

Figure 1. Fresh weights of *C. demersum* L. (a) and *E. canadensis* Michx.;(b) exposed to low concentrations of Cd for 21 days. Each value is means ± SD (standard deviation, n = 3). Different letters at the same figure pattern denote significant differences at the level of $P < 0.05$.

average fresh weight of plant in Cd-treated solutions) / average fresh weight of plant in control) × 100%). According to the method described by Sun et al. (2005d, 2007), Cd concentrations in Cd-treated solutions and two plants were tested by ICP-AES (Leeman-LABS, Prodigy, USA) and analysis of PCs and other low molecular weight thiols were determined by high-performance liquid chromatography (HPLC) by using pre-column derivatization with a fluorescent probe monobromobimane (mBBr).

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical package (version 10.0 for windows). Data were tested at significance levels of $P < 0.05$ or $P < 0.01$ by one-way ANOVA. The subsequent multiple comparisons among means were examined based on a Tukey test.

RESULTS

Plant growth

After Cd exposure for 21 days, experimental Cd concentrations (0.01 to 0.04 μM) in solutions had no toxic effects on the growth of two plants, as shown by no significant decrease of the fresh weights compared with the control plants without Cd (Figure 1a, b). Cd toxicity in *C. demersum* is significantly seen at 0.08 μM, while in *E. canadensis* at 0.16 μM and the degree increased with further increase of Cd concentrations in solutions, accompanied by significant ($P < 0.05$) decreases in the fresh weights of *C. demersum* (0.08 to 0.64 μM) and *E. canadensis* (0.16 to 0.64 μM) compared with the control

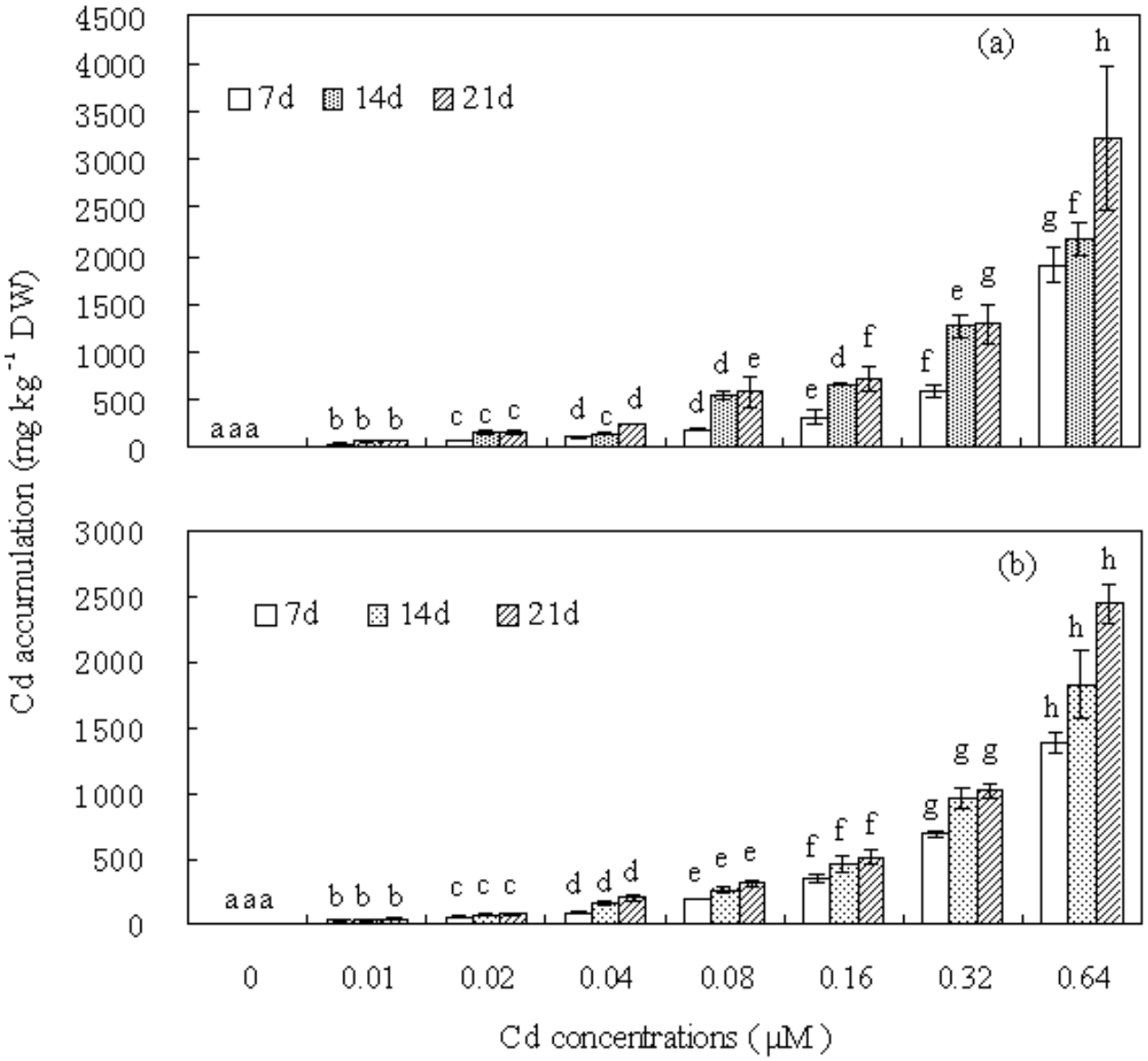

Figure 2. Cd accumulation (mg kg^{-1} DW) in *C. demersum* L. (a) and *E. canadensis* Michx.; (b) exposed to low concentrations of Cd with various exposure time. Each value is means ± SD (standard deviation, n = 3). Different letters at the same figure pattern denote significant differences at the level of $P < 0.01$.

plants without Cd addition (Figure 1a, b).

Cadmium accumulation

Cadmium accumulation in two plants increased with the increase of Cd concentrations in solutions and Cd exposure time (Figure 2a, b). Two plants showed strong ability to absorb Cd from external Cd-treated solutions. At 0.64 μM, Cd contents in *C. demersum* and *E. canadensis* were 1900.71 and 1387.01 mg ·kg^{-1} DW at 7 days, 2172.69 and 1830.33 mg ·kg^{-1} at 14 days, 3228.46 and 2453.37 mg ·kg^{-1} at 21 days.

Phytochelatin production

Phytochelatins were detectable in both control plants without Cd addition. During the period of Cd exposure (7 to 21 days) (Table 1), PCs in *C. demersum* were strongly induced at 0.02 to 0.64 μM Cd, increased with the increase of Cd concentrations in solutions. The concentrations of PCs in *C. demersum* were positively correlated with the concentrations of Cd in solutions (R^2= 0.9034 for 7 days, R^2= 0.8515 for 14 days, R^2= 0.932 for 21 days). Different from Cd accumulation in *C. demersum*, the levels of PCs obviously decreased with the prolonged exposure of Cd. In contrast to *C.*

Table 1. Concentrations of phytochelatins[a] (nmol –SH g^{-1} FW) in two aquatic macrophytes exposed to low concentrations of Cd with various exposure time.

Cd treatments (μM)	*Ceratophyllum demersum* L.			*Elodea canadensis* Michx.		
	7 d	14 days	21 days	7 days	14 days	21 days
0	7.38±0.51[b] a	6.33±1.18a	6.43±0.86ab	2.76±0.09a	3.76±0.29a	2.16±0.44a
0.01	8.76±0.87a	7.03±1.61a	5.47±1.25a	2.60±0.14a	3.43±0.51a	2.08±0.04a
0.02	20.82±1.09b	12.77±1.97b	8.09±1.28b	2.51±0.41a	3.36±0.59a	2.30±0.36a
0.04	120.51±17.09c	39.92±6.43c	32.70±7.66c	2.62±0.47a	3.48±0.38a	2.77±0.54ab
0.08	181.62±16.72d	87.07±7.31d	42.13±8.04c	3.00±0.44ab	4.55±0.96ab	3.53±0.22bc
0.16	169.80±15.03d	133.13±27.27e	66.62±9.51d	3.48±0.03b	3.84±0.49a	2.74±0.35ab
0.32	257.75±51.19e	244.07±36.79f	175.69±32.1e	5.61±0.74c	4.18±0.98ab	3.99±0.88c
0.64	441.23±35.27f	267.81±52.63f	222.67±5.96f	10.05±2.02d	4.72±0.31b	3.73±0.39c

[a] Phytochelatin data are reported as a molar concentration (normalized to FW) of the sum of PC_2, PC_3, and unidentified iso-phytochelatins being eluted after PC_4; [b]each value is means ± SD (standard deviation, n = 3). Different letters in the same column denote significant differences at the level of $P < 0.05$.

Table 2. Concentrations of glutathione (nmol g^{-1} FW) in two aquatic macrophytes exposed to low concentrations of Cd with various exposure time.

Cd treatments (μM)	*Ceratophyllum demersum* L.			*Elodea canadensis* Michx.		
	7 days	14 days	21 days	7 days	14 days	21 days
0	10.04±1.77[a]a	13.16±1.86a	22.18±3.53a	8.68±0.22a	8.96±0.58a	8.65±0.76 a
0.01	10.61±1.88a	14.13±0.94a	34.54±2.28b	7.44±1.18a	8.14±0.99a	9.22±1.43 ab
0.02	11.24±1.61a	19.17±1.00b	35.78±6.46b	9.54±1.43b	8.37±1.07a	8.81±1.06 a
0.04	18.28±1.23b	24.85±1.54c	39.92±8.69b	8.92±1.33ab	9.56±1.16ab	8.90±0.98 a
0.08	12.67±1.35a	40.85±4.04d	40.31±2.84b	9.15±0.32ab	8.61±0.93a	10.59±0.48b
0.16	16.36±1.23b	25.50±1.51c	45.65±5.70b	11.16±2.10c	10.05±2.06b	11.89±0.14cd
0.32	25.89±3.54c	42.49±8.07d	119.24±9.40c	14.65±2.70e	12.36±1.33c	12.81±0.09d
0.64	26.20±2.51c	77.70±8.74e	188.74±44.37d	13.56±1.90d	10.69±7.43b	11.77±0.08cd

[a]Values are means of three replicates ± SD (standard deviation, n = 3). Different letters in the same column denote significant differences at the level of $P < 0.05$.

demersum, Cd concentrations studied slightly or mildly stimulated the response of PCs in *E. canadensis* within 7 to 21 days. At equivalent concentrations of Cd in solutions, the levels of PCs were significantly higher in *C. demersum* than in *E. canadensis*.

Cadmium toxicity was estimated by the relative inhibition rate (RIR) of fresh weight. There existed a linearly positive relationship between PC concentrations and Cd toxicity ($R^2 = 0.9085$) in *C. demersum* after Cd exposure for 21 days. But this relationship was absent in *E. canadensis*.

Glutathione production

The levels of GSH in *C. demersum* remarkably increased with the increase of Cd concentrations in solutions and Cd exposure time (Table 2) and yet the extent was obviously lower than that of PCs. However, the slight increase of GSH was observed in *E. canadensis*. Moreover, GSH accumulation was higher in *C. demersum* than in *E. canadensis*.

DISCUSSION

C. demersum (Ornes and Sajwan, 1993; Gupta and Chandra, 1996; Kumar and Prasad, 2004; Mishra et al., 2008) and *E. canadensis* (Fritioff et al., 2005; Fritioff and Greger, 2007) has been demonstrated to accumulate Cd in significant amounts. The present study further demonstrated that two plants presented high Cd-accumulating characteristic (Figure 2a, b). The amount of Cd accumulated in two aquatic macrophytes was in a concentration- and time- dependent manner. Concomitant with Cd accumulation, Cd toxicity was obviously shown at 0.08 to 0.64 μM in *C. demersum* and at 0.16 to 0.64 μM in *E. canadensis*. From an application viewpoint,

our study proposed that *C. demersum* and *E. canadensis* may be suitable for use in remediation of lower levels of Cd pollution. Similarly, Ornes and Sajwan (1993) reported that, *C. demersum* is a powerful scavenger of Cd at low concentrations (0.1 to 0.5 ppm), but does not survive at higher concentrations (1 ppm to 10 µM).

The production of PCs at low concentrations of Cd usually present in realistic metal-polluted environments has scarcely been investigated in aquatic plants so far. In our experimental studies, low concentrations of Cd varying from 0.01 to 0.64 µM were simulated to investigate the response of PCs in two submerged macrophytes, *C. demersum* and *E. canadensis*. PCs were strongly induced at 0.02 to 0.64 µM Cd in *C. demersum* with the drastic increase of Cd concentrations in solutions, but with the slight induction of PCs in *E. canadensis*. It was clearly found that, PCs were highly sensitive and strongly induced by low concentrations of Cd in a dependent-species manner. Similar to our results, it has been shown that various natural populations of seaweeds (marine macroalgae) collected from natural environments with different levels of contamination by trace meals have different capacities for the production of PCs (Pawlik-Skowrońska et al., 2007). Such difference in PC production has also been found in various species of phytoplankton and freshwater algae (Ahner et al., 1995; Rijstenbil et al., 1998; Skowroński et al., 1998; Malea et al., 2006).

In our study, PC concentrations in *C. demersum* were positively correlated with Cd concentrations in solutions and Cd toxicity in *C. demersum*. A similar relationship has also been found in the seaweeds (Pawlik-Skowrońska et al., 2007), some algae (Ahner et al., 1994, 1997; Pawlik-Skowrońska, 2002; Pawlik-Skowrońska, 2001, 2003) and terrestrial plants (Schat and Kalff, 1992; De Knecht et al., 1995; Keltjens and van Beusichem, 1998; Snellller et al., 1999; Sun et al., 2005a,b,c). Undoubtedly, numerous direct quantitative relationships between metal concentrations in experimental solutions and fields/metal toxicity and the levels of expression of PCs provided convincing evidence that PCs can serve as earlier potential biomarkers for monitoring of environmental metal levels and biotoxicity. From an application viewpoint, due to its wide distribution in water environments, large biomass, easier collection and stronger physiological responses such as high production of PCs upon low concentrations of Cd as shown by the present experiment, it was suggested that, a submerged macrophyte *C. demersum* can be served as a useful test organism for further PC analysis in order to monitor environmental Cd-polluted levels.

Intracellular PC concentrations in the diatom are related to free Cd ion concentrations, rather than its total concentrations (Ahner et al., 1994). It was further confirmed in this study that, this dependant relation was also present in aquatic plant cells. Concomitant with the increase of Cd accumulation with the prolonged exposure of Cd (Figure 2a), PCs in *C. demersum* was largely induced after exposure to low concentrations of Cd (0.02 to 0.64 µM) for 7 days and obviously decreased after more than 7 days (Table 1). It was indicated that, the response of PCs was more sensitive in the early duration of Cd exposure and not dependent on Cd accumulation. Such phenomenon have been shown in our previous study (Sun et al., 2005a,b,c). Typically, increasing supply of citric acid in solutions increased Cd accumulation by wheat roots and yet led to a lower synthesis of PCs and lower Cd toxicity (Sun et al., 2005a). Based on present and previous results, it was warned that the time course of PC production as earlier biomarkers in response to metal stress should be emphasized in order to clearly evaluate metal toxicity in plants.

In this research, similar to the difference of PC production in these two aquatic macrophytes, *C. demersum* had a higher production of GSH than *E. canadensis*. Moreover, concentrations of GSH significantly increased with the increase of Cd concentrations in solutions and Cd exposure time in *C. demersum* (Table 2). Such difference in GSH concentrations has also been reported in three various seaweeds (Pawlik-Skowrońska et al., 2007) and three other *Fucus* species (Collen and Davison, 1999) in response to environmental stress. The difference in GSH concentrations in different aquatic macrophytes may indicate the presence of various physiological response mechanisms for handling Cd accumulation and toxicity. In accordance with our results, an increase of total GSH under Cd stress was found in *Phragmites australis* (Iannelli et al., 2002), *Lactuca sativa* (Maier et al. 2003), *Phragmites stratiotes* (Sanità di Tappi et al., 2007) and *Sedum alfredii* (Sun et al., 2005d, 2007). This increase may be attributed to the various function of GSH in organisms. Based on these backgrounds, further experimentation will be necessary to elucidate the role of the increase of GSH levels in *C. demersum*.

In conclusion, despite strong capacity to accumulate Cd, two submerged macrophytes showed different sensitiveness in PC response when exposed to low concentrations of Cd 0.01 to 0.64 µM usually present in low or moderately polluted environments. In contrast to *E. canadensis*, low concentrations of Cd studied induced the dramatic production of PCs in *C. demersum*, which was positively correlated with Cd concentrations in solutions and Cd toxicity. In terms of its wide distribution, large biomass, easier collection, strong Cd accumulation and the dramatic production of PCs, it was suggested that, *C. demersum* can be served as a useful biomonitor in water body for earlier PC analysis to predict Cd toxicity at environmentally relevant levels.

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