Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 19(2007) 1311–1317
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Reactive oxygen species metabolism during the cadmium hyperaccumulation of a new hyperaccumulator *Sedum alfredii* (Crassulaceae)

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Received 5 February 2007; revised 18 April 2007; accepted 17 May 2007

Abstract

Sedum alfredii Hance, a newly discovered hyperaccumulator, could serve as a good material for phytoremediation of Cd polluted sites. Malondialdehyde (MDA), reactive oxygen species (ROS) and antioxidases (catalase (CAT); superoxide dismutase (SOD); peroxidase (POD)) in the leaf were determined when *S. alfredii* was treated for 15 d with various $CdCl_2$ concentrations ranging from 0 to 800 μmol/L. The results showed that the production rate of 2′,7′-dichlorofluorescein (DCF), which is an indicator of ROS level, reached up to the maximum at 400 μmol/L $CdCl_2$ and then declined with the increase of $CdCl_2$ concentration, while MDA accumulation tended to increase. CAT activity was significantly inhibited at all tested $CdCl_2$ concentrations and SOD activity was sharply suppressed at 800 μmol/L $CdCl_2$. However, the enhancement of POD activity was observed when $CdCl_2$ concentration was higher than 400 μmol/L. In addition, its activity increased when treated with 600 μmol/L $CdCl_2$ for more than 5 d. When sodium benzoate, a free radical scavenger, was added, *S. alfredii* was a little more sensitive to Cd toxicity than that exposed to Cd alone, and the Cd accumulation tended to decline with the increase of sodium benzoate concentration. It came to the conclusions that POD played an important role during Cd hyperaccumulation, and the accumulation of ROS induced by Cd treatment might be involved in Cd hyperaccumulation.

Key words: cadmium; hyperaccumulation; reactive oxygen species; *Sedum alfredii*

Introduction

Cadmium (Cd) is widespread in the atmosphere, soil and water owing to various anthropogenic activities (Sanità di Toppi and Gabbrielli, 1999) and is a non-essential element for life. Cd is soluble and can be easily absorbed by many organisms, resulting in some toxicity. Cd accumulation in plants can reduce the growth of root and stem, cause leaf roll and chlorosis, change the color of roots to brown, interfere with the water-balance, damage the photosynthetic apparatus, inhibit the stomata opening, and finally lead to the death of plants (Kahle, 1993; Sanità di Toppi and Gabbrielli, 1999). Cd could also be concentrated in human body through many pathways such as food chain and resulted in several diseases (Gerhardsson *et al*., 2002; Waisberg *et al*., 2003).

Biotic and abiotic stresses such as pathogenic invasion, wounding, drought, salt, heat and cold could result in oxidative damages in many organisms. Similarly, oxidative damages may be one of the mechanisms of Cd toxicity as indicated by reactive oxygen species (ROS) accumulation and lipid peroxidation (Dixit *et al*., 2001; Sandalio *et al*., 2001; Shah *et al*., 2001; Schtzendbel and Polle, 2002; Cho and Seo, 2005). Cd could displace Fe and Cu ions in proteins and then these ions might stimulate the production of free radicals by Fenton's reaction (Rea *et al*., 2004). It has also been reported that an NADPH-oxidase like enzyme was involved in the process of ROS induction by Cd (Olmos *et al*., 2003). In addition, Cd could bind between semiubiquinone and cytochrome b_{566} of the Q_0 site of cytochrome *b* of complex III and result in the accumulation of semiubiquinones at the Q_0 site and the induction of ROS generation in mitochondria (Wang *et al*., 2004). However, the synchronous action of antioxidants prevents abundant ROS formations and alleviates oxidative damages. Superoxide dismutase (SOD; E.C.1.15.1.1) catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen, catalase (CAT; E.C.1.11.1.6) directly eliminates hydrogen peroxide predominantly in the peroxisome and peroxidase (POD; E.C.1.11.1.7) scavenges the hydrogen peroxide to water with the help of small molecules such as ascorbic acid, glutathione and phenolics in the cell.

Although high concentration of ROS could cause irreversible damage, however, under several manipulative levels, a certain concentration of ROS, more particularly H_2O_2, could play a vital role as regulatory molecules in signal transduction and gene expression (Vanderauwera *et al*., 2005). In the plant-pathogen interaction ROS is centrally involved in the induction of pathogen defense genes, such as those encoding pathogenesis-related proteins,

Project supported by the Wuhan Chenguang Project for Youth Scholar (No. 20045006071-24) and the "211 Project" of Educational Ministry of China. *Corresponding author. E-mail: bsqiu@public.wh.hb.cn.

regulating the accumulation of phenylpropanoid compounds, or encoding ROS detoxifying enzymes (Levine *et al*., 1994; Mittler *et al*., 1999; Schenk *et al*., 2000).

Phytoremediation offers an effective, environmentally nondestructive and cheap remediation method for Cd polluted sites (Prasad, 2003; Eapen and D'Souza, 2005). Its development depends on the discovery of new hyperaccumulator and elucidation of the mechanism of metal hyperaccumulation. *Sedum alfredii* Hance, a newly discovered hyperaccumulator, is highly tolerant to Cd and its growth in terms of dry weight is not influenced even at 400 μmol/L external $CdCl_2$ solution (Yang *et al*., 2004; Xiong *et al*., 2004). It can grow up to 60 cm height and propagate three to four times in a year if the environmental conditions are favorable (Zhou and Qiu, 2005). Meanwhile, it possesses many other merits such as asexual reproduction, fast growth, large biomass and extraordinarily strong ability to accumulate up to more than 10000 mg/kg Cd in the leaf (Yang *et al*., 2004; Zhou and Qiu, 2005). Thus, *S. alfredii* could serve as a good material for the phytoremediation of Cd polluted sites. However, no study has ever been reported the reactive oxygen species metabolism during Cd hyperaccumulation of *S. alfredii*. In this aricle, we investigated the mechanisms of Cd accumulation mainly concerning the roles of antioxidases and reactive oxygen species.

1 Materials and methods

1.1 Plant material, growth conditions and treatments

S. alfredii was collected from an old Pb/Zn mining area in China. Healthy and similar sized shoots were chosen and grown in hydroponics medium for 20 d for the initiation of new roots. The compositions of nutrient solution were: 2 mmol/L $Ca(NO_3)_2{\cdot}4H_2O$, 0.1 mmol/L KH_2PO_4, 0.5 mmol/L $MgSO_4{\cdot}7H_2O$, 0.1 mmol/L KCl, 0.7 mmol/L K_2SO_4, 10 μmol/L H_3BO_3, 0.5 μmol/L $MnSO_4{\cdot}H_2O$, 0.5 μmol/L $ZnSO_4{\cdot}7H_2O$, 0.2 μmol/L $CuSO_4{\cdot}5H_2O$, 0.01 μmol/L $(NH_4)_6Mo_7O_{24}$ and 100 μmol/L Fe-EDTA. The nutrient solution was renewed every four days and continuously aerated with an aquarium air pump, and the pH value was adjusted to 5.8 with 1 mol/L HCl or NaOH. Plants were grown at 25°C and 200 μmol/(m^2·s) with a 14-h light/10-h dark cycle.

S. alfredii was then cultivated for 15 d with the treatments (control, 200, 400, 600 and 800 μmol/L $CdCl_2$) to assay enzymes activities, lipid peroxidation and ROS levels in the leaf because the accumulation of Cd in the leaf was much higher than in the stem and root (Yang *et al*., 2004; Zhou and Qiu, 2005). POD activity and ROS levels in the leaf were also determined at the day 5, 10 and 15, when *S. alfredii* was exposed to 600 μmol/L $CdCl_2$. In order to study ROS effects on Cd accumulation, *S. alfredii* was respectively treated with 600 μmol/L $CdCl_2$, 600 μmol/L $CdCl_2$ + 100 μmol/L sodium benzoate (SB) or 600 μmol/L $CdCl_2$ + 200 μmol/L SB for 5 d. All nutrient solutions were renewed every two days.

1.2 Estimation of lipid peroxidation

MDA (malondialdehyde) is considered as a useful index of lipid peroxidation. Thiobarbituric acid-reactive substances assay was used to measure the level of MDA (Hodges *et al*., 1999). The top fully expanded leaf was chosen, homogenized with pestle and mortar in 3 ml 80:20 (v/v) of ethanol:water with quartz sand, then the homogenate was centrifuged at 3000×*g* for 10 min and the volume of supernatant was recorded. The sample was diluted twice with 80:20 (v/v) of ethanol:water and then 2 ml diluted sample was added to a glass tube with 2 ml of either (A): –TBA solution comprised 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene, or (B): +TBA solution containing the above plus 0.6% TBA. Sample was then mixed vigorously, heated at 95°C in water boiler for 25 min, cooled quickly, and centrifuged at 3000×*g* for 10 min. Absorbance was recorded at 440, 532 and 600 nm, respectively, with the spectrophotometer. MDA equivalents were calculated according to Hodges *et al*. (1999).

1.3 ROS analysis

2′,7′-Dichlorohydrofluorescein diacetate (DCFH-DA) has been used as a molecular probe for ROS levels in the cell (Allan and Fluhr, 1997; Collén and Davison, 1997). When DCFH-DA enters the cell, it is deacetylated by esterase and trapped in cells as the nonfluorescent compound DCFH. Then, DCFH can be oxidized by H_2O_2 with the aid of POD, producing highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Cathcart *et al*., 1983). DCFH shows selectivity for H_2O_2 over the other free radicals (Vowells *et al*., 1995). However, other radicals could be rapidly changed into H_2O_2 *in vivo* by SOD and thus could be indirectly detected by this molecular probe.

Chose the top fully expanded leaf, washed with distilled water for 5–7 min to remove the produced ROS as a consequence of incision (Rodrłguez *et al*., 2002), then dried with filter paper and weighed its fresh weight. Put the sample into a tube, added 10 ml loading buffer (Tris 10 mmol/L, KCl 50 mmol/L, pH 7.2) and 5 μl DCFH-DA (100 mmol/L, dissolved in dimethyl sulphoxide), and then kept in dark for 15 min at 25°C. The sample was washed with loading buffer quickly and homogenized with pestle and mortar in 4 ml loading buffer with quartz sand at 0°C. The resulting homogenate was centrifuged for 10 min with 15000×*g* at 4°C and then the supernatant was transferred to another tube and the volume was recorded. The result was detected through the fluorescence spectrophotometer (F-4500, Hitachi) at the excitation wavelength of 488 nm and the emission wavelength of 525 nm. The blank without leaf addition was subtracted from each sample. Finally, DCF content was valued according to the standard DCF concentration curve.

1.4 Enzymes activities

The top fully expanded leaf was selected to test the activities of antioxidative enzymes. The leaf was homogenized with pestle and mortar in 5 ml extract buffer (66.7

mmol/L phosphate buffer, pH 7.7, 100 mmol/L EDTA, 4% polyvinylpolyrrolidone) with quartz sand at 0°C, the homogenate was centrifuged at 15000×*g* and 4°C for 20 min, and then the supernatant was used for following assays of enzyme activities. Total protein was determined according to Bradford (1976) using bovine albumin for calibration.

SOD activity was determined according to the reductive degree of nitroblue tetrazolium (NBT) in the light with some modifications (Beauchamp and Fridovich, 1971; Aravind and Prasad, 2003). Added 0.2 ml crude enzyme solution to the reaction buffer (containing 66.7 mmol/L phosphate buffer, pH 7.7, 13 mmol/L *dl*-methionine, 0.1 μmol/L EDTA, 75 μmol/L NBT, 2 μmol/L riboflavin), the mixture was then transferred into a plate and incubated at 40 μmol/(m^2·s) and 25°C for 10 min. The NBT reduction was measured at 560 nm with a spectrophotometer. The control tube was added with the crude enzyme solution that had been boiled at 100°C for 20 min and was incubated under the same conditions as samples, while a tube with crude enzyme solution was kept in the dark and served as a blank. One unit of SOD activity was defined as the amount of enzyme required to inhibit the photoreduction of NBT by 50%.

POD activity was measured monitoring the formation of tetraguaiacol from guaiacol in the presence of H_2O_2 and POD (Chance and Maehly, 1955). Crude enzyme solution (0.2 ml) was added to the reaction mixture containing 1 ml of 30 mmol/L H_2O_2, 1.08 ml 20 mmol/L guaiacol and 0.72 ml 66.7 mmol/L phosphate buffer (pH 7.0). The reaction was recorded for 2 min at 470 nm with a spectrophotometer. POD activity was valued using the extinction coefficient 26.6 L/(mmol·cm) for tetraguaiacol. One unit of POD was defined as 1 nmol tetraguaiacol produced per minute per μg protein.

CAT activity was detected spectrophotometrically following the consumption of H_2O_2 at 240 nm (Aebi, 1984). Mixed 100 μl crude enzyme solution with 1.9 ml phosphate buffer (pH 7.0) at room temperature, then added 1 ml of 30 mmol/L H_2O_2 and followed the decrease in the absorbance at 240 nm every 3 s for 1.5 min. CAT activity was calculated with the extinction coefficient 39.4 L/(mmol·cm) for H_2O_2. One unit of CAT was defined as 1 μmol H_2O_2 consumption per minute per μg protein.

1.5 Cd determination

The intact plants were rinsed with tap water for several minutes and the roots were completely immersed in 20 mmol/L Na_2-EDTA for 15 min (Zhou and Qiu, 2005). Then, the intact plants were washed thoroughly with deionized water. The root, stem and leaf were separated respectively, heated at 70°C for 48 h, and their dry weights were recorded. Each sample was digested in 5 ml pure nitric acid according to the microwave technique until clear solution and diluted with deionized water. Inductively coupled plasma atomic emission spectrometry (ICP-AES) (Optima 2000 DV, Perkin-Elmer Instruments) was used for Cd analysis.

2 Results

MDA accumulation tended to increase with the increasing $CdCl_2$ concentration although it was not significantly different from each other ($P < 0.05$, Tukey multiple comparison) (Fig.1). DCF production rate, another good indicator of ROS levels, was enhanced with the increase of $CdCl_2$ concentration and reached up to the maximum at 400 μmol/L $CdCl_2$. DCF production rate was then decreased significantly at 600 and 800 μmol/L $CdCl_2$ compared with that at 400 μmol/L $CdCl_2$ ($P < 0.05$, Tukey multiple comparison) (Fig.1).

The antioxidases such as SOD, CAT and POD activities were measured after 15 d treatments with various $CdCl_2$ concentrations. SOD activity was almost not affected under Cd concentrations less than 600 μmol/L and significantly decreased when exposure to high $CdCl_2$ concentrations, about 7% of the control at 800 μmol/L $CdCl_2$ ($P < 0.05$, Tukey multiple comparison) (Fig.2). CAT, one of the two H_2O_2 scavenged enzymes, was sensitive to $CdCl_2$ exposure and its activity decreased significantly under all tested $CdCl_2$ treatments ($P < 0.05$, Tukey multiple comparison) (Fig.2). Even under the lowest $CdCl_2$ concentration (200 μmol/L), it was only about half of the control. However, the other H_2O_2 scavenged enzyme POD changed at the opposite trend. The enhancement of POD activity was observed when $CdCl_2$ concentration was higher than 400

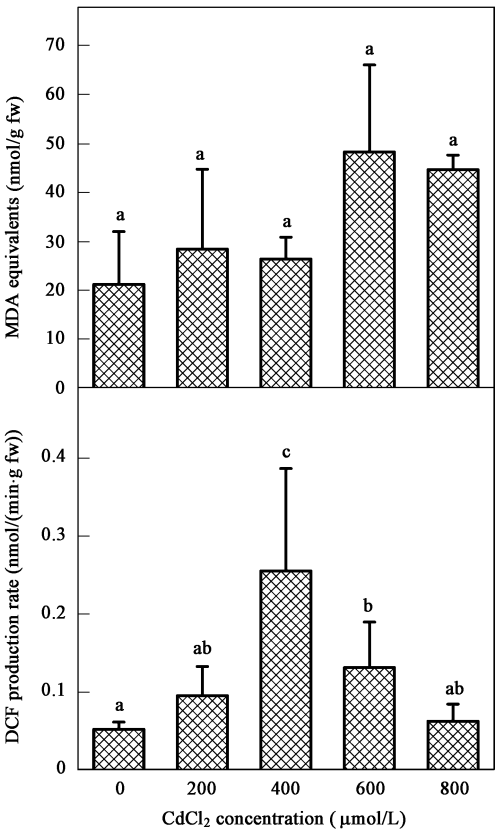

Fig. 1 DCF production rate and MDA accumulation in the leaf of *Sedum alfredii* Hance after being exposed to various $CdCl_2$ concentrations for 15 d. Those with different letters on error bar are significantly different ($P < 0.05$, Tukey multiple comparison). Data are means ± SD (*n*: 3–4).

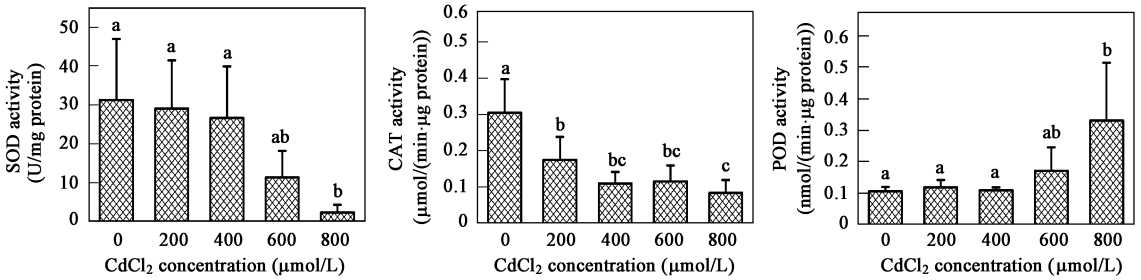

Fig. 2 Antioxidases activities in the leaf extract of *Sedum alfredii* Hance after being exposed to various $CdCl_2$ concentrations for 15 d. Those with different letters on error bar are significantly different ($P < 0.05$, Tukey multiple comparison). Data are means ± SD (n: 3–4).

μmol/L and reached the maximum at the highest $CdCl_2$ concentration ($P < 0.05$, Tukey multiple comparison) (Fig.2). Among these three ROS scavenged antioxidases, POD might play an important role when *S. alfredii* was exposed to Cd stress.

The amount of Cd accumulation was the maximum in the leaf, stem and root of *S. alfredii* when grown at 600 μmol/L $CdCl_2$ (Zhou and Qiu, 2005), so this concentration was selected to further test the role of POD activity in Cd accumulation and to assay ROS effects on Cd accumulation. POD activity was similar to that of the control after the exposure to 600 μmol/L $CdCl_2$ for 5 d. However, it increased significantly in the following 5 or 10 d ($P < 0.05$, Tukey multiple comparison) (Fig.3). After being treated with 600 μmol/L $CdCl_2$, DCF production rate increased a little at the day 5 and reached the maximum at the day 10 ($P < 0.05$, Tukey multiple comparison) (Fig.3). At the day 15, it was decreased by 31.6% compared to that at the day 10 (Fig.3).

The change of root color into red-brown is one symptom of Cd toxicity. It appeared when *S. alfredii* was exposed to 600 μmol/L $CdCl_2$ for 5 d (Figs.4a and 4b). However, this symptom had not been observed for plant subjected to 200 μmol/L SB for 5 d (Figs.4a and 4c). Compared to Cd treatment alone, the addition of 200 μmol/L SB made *S. alfredii* more sensitive to Cd toxicity and the color of its root was a little more red-brown (Figs.4b and 4d). A clear trend of decreased Cd accumulation was observed when SB was added to samples treated with 600 μmol/L $CdCl_2$, although the results were not significantly different (Fig.5). The Cd content in the leaf, stem and root was about 90% of that exposed to 600 μmol/L $CdCl_2$ alone when the plants were treated with 600 μmol/L $CdCl_2$ + 100 μmol/L SB for 5 d. It was further decreased for samples treated with 600 μmol/L $CdCl_2$ + 200 μmol/L SB. This indicated that the ROS scavenger could reduce the Cd accumulation in *S. alfredii*.

3 Discussion

In the present study, ROS was accumulated in *S. alfredii* especially at 400–600 μmol/L $CdCl_2$ (Figs.1 and 3). This is in agreement with published reports of Shah *et al*. (2001) and Olmos *et al*. (2003) that Cd enhanced superoxide anion generation and hydrogen peroxide accumulation. However, at high Cd concentrations (600–800 μmol/L), SOD activity decreased (Fig.2), so the efficiency of the

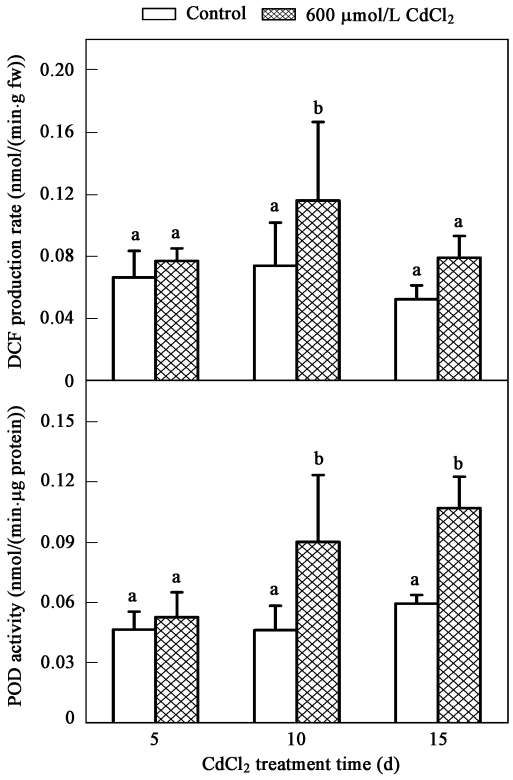

Fig. 3 Time-course dependence of POD activity and DCF production rate in the leaf of *Sedum alfredii* Hance after being exposed to 600 μmol/L $CdCl_2$. Those with different letters on error bar are significantly different ($P < 0.05$, Tukey multiple comparison). Data are means ± SD (n: 3–4).

dismutation of superoxide into H_2O_2 by SOD would be reduced. In addition, the increase of POD activity at high Cd concentrations (600–800 μmol/L) could scavenge H_2O_2 (Fig.2). Consequently, the concentration of H_2O_2 indicated by DCF production rate would decrease, although MDA (a free radical cascade reaction product of lipid peroxidation) tended to increase at high Cd concentrations (600–800 μmol/L) (Fig.1). Furthermore, DCFH shows selectivity for H_2O_2 over the other free radicals (Vowells *et al*., 1995). Thus, DCF production rate at 600–800 μmol/L $CdCl_2$ was less than that at 400 μmol/L (Fig.1).

The accumulation of Cd was increased with the increase of Cd concentration and reached to a very high level in *S. alfredii* (Zhou and Qiu, 2005). Among three tested ROS scavenging enzymes, POD in the leaf extract was the only one increased during this process (Fig.2). Thus, POD might play an important role in *S. alfredii* during the process of Cd hyperaccumulation. This was further

Fig. 4 The root growth of *Sedum alfredii* Hance after being exposed to different treatments for 5 d. (a) control; (b) 600 μmol/L $CdCl_2$; (c) 200 μmol/L sodium benzoate; (d) 600 μmol/L $CdCl_2$ + 200 μmol/L sodium benzoate.

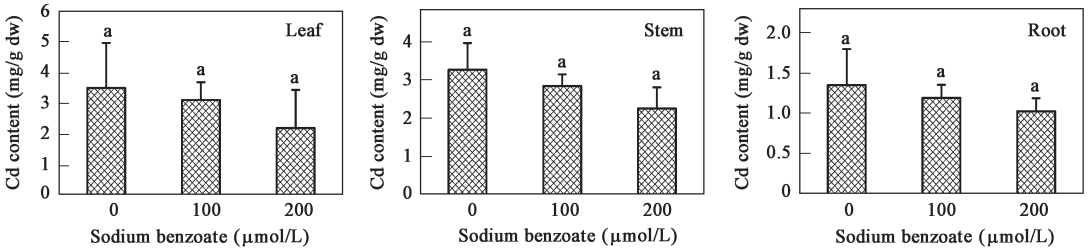

Fig. 5 Effects of the free radical scavenger sodium benzoate (SB) on Cd accumulation in the leaf, stem and root of *Sedum alfredii* Hance. Samples were treated with 600 μmol/L $CdCl_2$, 600 μmol/L $CdCl_2$ + 100 μmol/L SB or 600 μmol/L $CdCl_2$ + 200 μmol/L SB for 5 d. Those with different letters on error bar are significantly different ($P < 0.05$, Tukey multiple comparison). Data are means ± SD (n: 3–8).

supported by the time-course experiment that POD activity increased with the time of Cd exposure (Fig.3). POD had a large family of isoenzymes in higher plants and its involvement in the response against environmental stress was well known (Gara, 2004). The increase of POD activity resulted in enhanced tolerance to UV irradiation (Takahama, 2004) and its activity was also raised by Cd treatment in many plants (Shah *et al*., 2001; Metwally *et al*., 2003; Ranieri *et al*., 2005; Smeets *et al*., 2005; Mishra *et al*., 2006). ROS accumulated at a certain concentration could function as a signal mediating adaptive response to various stresses, such as enhancing the expression of antioxidant genes *CAT1*, *cAPX* and *GR1* (Zhang *et al*., 2006). The increase of POD during Cd hyperaccumulation in the leaf of *S. alfredii* may be activated by ROS induced by Cd through some unkown way. Cd-induced increase of POD activity was suggested to efficiently reduce H_2O_2 accumulation in the leaves of bread wheat (Ranieri *et al*., 2005). In contrast, POD activities in the leaves of pea were depressed after being treated with 50 μmol/L Cd for 28 d (Sandalio *et al*., 2001). In the present study, CAT and SOD activities in the leaf of *S. alfredii* were inhibited by 15 d Cd treatment (Fig.2). It had been reported that the activities of these two enzymes decreased with Cd treatment in *Arabidopsis* seedlings (Cho and Seo, 2005) and in the leaves of pea plants (Sandalio *et al*., 2001). The reduction of CAT activity by Cd was suggested to result from the decrease of protein content (Sandalio *et al*., 2001). In addition, Dixit *et al*. (2001) and Zhang *et al*. (2005) found that CAT and SOD activities were induced by Cd and these results may be due to the lower Cd concentrations and shorter time treatment. ROS accumulation increased after *S. alfredii* was exposed to 600 μmol/L $CdCl_2$ (Figs.1 and 3). SB was an effective ROS scavenger and could alleviate lipid peroxidation caused by heavy metals (Chien *et al*., 2000; Tripathi and Gaur, 2004). However, the accumulation of Cd did not increase with the increase of SB concentration but tended to decrease in *S. alfredii* (Fig.5). Furthermore, *S. alfredii* with 200 μmol/L SB addition was a little more sensitive to Cd toxicity compared to Cd treatment alone (Figs.4b and 4d). MAPKs, which could mediate ROS signal transduction in plant cell, were induced by Cd in rice and alfalfa seedlings (Jonak *et al*., 2004; Yeh *et al*., 2004; Pitzschke and Hirt, 2006). Lin *et al*. (2005) reported that 200 μmol/L SB addition was able to effectively prevent Zn-induction MAPK activation. The decline of Cd accumulation caused by SB addition may be due to the reduced activities of ROS signal transduction pathway in *S. alfredii*.

Cd hyperaccumulation in *S. alfredii* might be a complex phenomenon including tolerance and accumulation. Usually, it involves several steps such as absorption, translocation and accumulation in the shoots, and many genes may participate in these processes (Bert *et al*., 2003). Molecular researches on this new hyperaccumulator are required in the future, for example, the identification of related genes, elucidation of their expression, regulation and function, and investigation on signal transduction events

during the cellular response to Cd stress. Meanwhile, many works from different domains are required to develop the phytoremediation with *S. alfredii*.

Acknowledgements

The authors are grateful to Mr. W B Zhou for some helpful advices.

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