Chinese Science Bulletin

Synergistic defensive mechanism of phytochelatins and antioxidative enzymes in *Brassica chinensis* L. against Cd stress

CHEN LiQin[1], GUO YiFei[1], YANG LiMin[1] & WANG QiuQuan[1,2†]

[1] Department of Chemistry and the MOE Key Laboratory of Modern Analytical Science, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China;
[2] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China

***Brassica chinensis* L. was chosen and exposed to different concentrations of Cd exposure to evaluate its Cd-accumulating capacity and its potential cellular defensive mechanisms. Cd accumulation in the shoots and roots of *B. chinensis* was up to 1348.3±461.8 and 3761.0±795.0 mg per killogram of dry weight, respectively, under 200 μmol/L of Cd exposure. Increasing Cd accumulation in the plant was accompanied by rapid accumulation of phytochelatins (PCs), and the sequestration of Cd by PCs provided a primary cellular mechanism for Cd detoxification and tolerance of *B. chinensis.* Furthermore, malondialdehyde formation, hydrogen peroxide content and antioxidative enzyme activities such as superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase were observed in the shoots of Cd-stressed *B. chinensis.* Increasing enzyme activities in response to concentrations of 5 to 50 μmol/L Cd showed an efficient defense against oxidative stress, suggesting that the antioxidative system was a secondary defensive mechanism. These resulted in reduced free Cd damage and enhanced Cd accumulation and tolerance. Glutathione plays a pivotal role in these two detoxification pathways. In general, these results suggested that PCs and the antioxidative system are synergistic in combatting Cd-induced oxidative stress and that they play important roles in Cd detoxification of *B. chinensis*, and also give a deep understanding of the natural defensive mechanisms in plants under heavy metal stress.**

cadmium, *Brassica chinensis* L., phytochelatin, antioxidative enzymes

Some transitional metals such as copper (Cu) and zinc (Zn) are essential to normal processes of life at suitable concentrations, taking part in redox reactions, structural configuration and in nucleic acid metabolism as well as a multitude of enzyme-catalyzed reactions. By contrast, cadmium (Cd), another example of a transitional metal, is toxic to almost all organisms[1]. It can be absorbed by plants, animals and human beings through soil, water, air and the food chain[2]. However, any form of life has its own protection systems or mechanisms against Cd toxicity. It has been shown that Cd induces the synthesis of phytochelatins (PCs) in plants and MTs in animals during which Cd is transformed and/or sequestered into physiologically tolerable and metabolically inactivated forms[3−5]. For plants, PCs are a family of peptides that consist of repetitions of γ-Glu-Cys dipeptide followed by a terminal Gly. The basic structure is $(\gamma\text{-Glu-Cys})_n$-Gly, where n is from 2 to 11[4]. In addition, a number of structural variants of PCs such as $(\gamma\text{-Glu-Cys})_n$-β-Ala, $(\gamma\text{-Glu-Cys})_n$-Glu, $(\gamma\text{-GluCys})_n$-Ser and $(\gamma\text{-Glu-Cys})_n$,

Received August 20, 2007; accepted November 5, 2007
doi: 10.1007/s11434-008-0062-6
†Corresponding author (email: qqwang@xmu.edu.cn)
Supported by the National Natural Science Foundation of China (Grant Nos. 20535020, 20475046 and 20775062), the National "863" Hi-Tech Project of China (Grant No. 2006AA06Z404) and the National Basic Research Program of China (Grant No. 2003CD415001)

have been identified in some species of plants[6]. The synthesis of PCs starts with activation of phytochelatin synthase (also known as γ-glutamylcysteine dipeptidyl transpeptidase) by a variety of metal ions, among which Cd is the most effective one[7]. A study with Cd-sensitive mutants of *Arabidopsis thaliana* provided compelling evidence for the participation of PCs in the cellular mechanism for protection against Cd[8]. In particular, two Cd-sensitive mutants were impaired in terms of the synthesis of glutathione (GSH) and PCs synthesis, respectively[9,10]. Overexpression of both GSH synthetase and PC synthase in Indian mustard was observed to increase the synthesis of PCs and confer resistance to Cd[11]. On the other hand, high concentration of Cd generates oxidative stress possibly by inducing reactive oxygen species (ROS) like the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen and hydroxyl radicals (·OH)[12]. ROS may lead to the unspecific oxidation of proteins and membrane lipids or cause DNA alterations, thus affecting cell viability[13]. Changes in ROS level have been observed in many Cd-treated plants[13−15]. Plant cells have developed a wide range of antioxidative defensive mechanisms including both non-enzymatic antioxidants like GSH and ascorbate (ASA), and enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POD)[16,17]. Arguments remain concerning these mechanisms mentioned, however, because some researchers believe that oxidative stress is not the primary cause of Cd toxicity[18].

In this study, *Brassica chinensis* L., one of the staple and most popular vegetables in China, was chosen to investigate its Cd-accumulating capability and its biochemical responses under Cd stress. PCs, GSH and several typical antioxidative enzymes (including SOD, CAT, APX, and POD) were investigated at the molecular level to determine the relationship between them and Cd accumulation, and/or any synergistic detoxifying Cd effects among them. These results are crucial to an understanding of the inherent protection mechanisms of plants against adventitious toxicants.

1 Materials and methods

1.1 Plant material and growth conditions

B. chinensis seeds (F1 Beauty Crown from Japan) were geminated on filter papers in Petri dishes. In three days after germination, seedlings were carefully transferred to 100 mL pots filled with modified one-quarter-strength Hoagland culture solution[19]. *B. chinensis* seedlings were allowed to grow in hydroponics for two weeks before treatment with Cd started. Appropriate amounts of $3CdSO_4 \cdot 7H_2O$ were added into the culture solution to achieve 0, 5, 25, 50, 100 and 200 μmol/L Cd concentrations in the culture solutions. The seedlings were grown at a controlled temperature (25±1 ℃) with a 16 hours/day white light (700 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and humidity of about 60%. The culture solution was renewed every two days. After six days of exposure, the roots of seedlings were immersed in ice-cold 20 mmol/L EDTA solution for 15 min to displace extracellular Cd. The seedlings were then rinsed with ultrapure water (Millipore, Bedford, MA, USA), and blotted to remove excess water before further examination.

Soils taken from cropland was air-dried, mixed, sieved and then appropriate amounts of $3CdSO_4 \cdot 7H_2O$ were added into the soil to achieve 2, 20 and 200 mg/kg(soil) Cd concentrations. *B. chinensis* seeds were germinated in those soils. 30 days later, the seedlings were collected and treated as in hydroponics.

1.2 Cd accumulation and distribution in *B. chinensis*

After harvesting, appropriate amounts of cultured roots and shoots of *B. chinensis* were first dried at 80℃ in a conventional electric oven for 24 h, and then weighed and digested in a mixture of 5∶1 HNO_3∶$HClO_4$ (*v*/*v*). Finally, the digests were diluted with ultrapure water to 25 mL for Cd determination using inductively coupled plasma mass spectrometry (ICP-MS, Elan-DRC II, PerkinElmer-SCIEX, Canada).

Cd subcellular distribution was analyzed according to Yang et al.[20]. Briefly, fresh shoots of *B. chinensis* cultured at 200 μmol/L Cd were homogenized using a mortar and pestle with an ice-cold 10 mmol/L Tris-HCl buffer at pH 7.4. Homogenates were centrifuged at 600 g for 5 min and the resulting pellet contained mainly cell walls. The resulting supernatant was then centrifuged at 2000 g for 10 min and the resulting pellet contained mainly chloroplast and nucleolus. The resulting supernatant was further centrifuged at 12000 g for 15 min and the resulting pellet contained mainly mitochondria, while main cytoplasm was in the supernatant. The Cd concentrations in the four fractions were determined using ICP-MS as described above.

1.3 Thiol peptides analysis using HPLC

The fresh roots and shoots were ground in liquid nitrogen and homogenized in an ice-cold and nitrogen-saturated 0.1 N HCl. Homogenates were centrifuged at 20000 g for 15 min at 4℃, and then the supernatants obtained were used for thiol peptide analysis by HPLC using a system similar to that described by Grill et al.[21]. Thiol peptides were separated on a C_{18} reverse phase column (Shimadzu, Japan) using a 2 to 20% acetonitrile gradient in 0.02% (*v/v*) trifluoroacetic acid over 20 min. The content of thiol peptides was measured using on-line postcolumn derivatization with 5,5-dithio-bis-2-nitrobenzoic acid, and detected at 410 nm. All PC data (ΣPC) were reported as the molar concentration of a sum of the Cys units of PC variants with *n* from 2 to 6. The assignments of the respective peaks were performed with electrospray ionization mass spectrometry (ESI-MS) and ESI-MS/MS (ESQUIRE-LC, Bruker Daltonics, Germany) after separation without postcolumn derivatization.

1.4 Analysis of H_2O_2 and lipid peroxidation

Five hundred milligrams of fresh shoots were ground in liquid nitrogen and suspended in 2.5 mL of 50 mmol/L sodium phosphate buffer at pH 6.8. Then the H_2O_2 content in the shoots was measured according to the method of Gay and Gebicki[22]. The level of lipid peroxidation in the shoots was determined by estimation of malondialdehyde (MDA) content following the method of Dhindsa et al.[23].

1.5 Enzyme assay

Fresh shoots were ground in liquid nitrogen and homogenized with an extraction buffer of 100 mmol/L sodium phosphate (pH = 7.5) containing 1 mmol/L EDTA and 1% polyvinylpyrrolidone at 4℃. The homogenate was centrifuged at 25000×*g* for 20 min at 4 ℃. For analysis of APX, an extraction buffer containing 5 mmol/L ASA was used. Soluble protein was determined in enzyme extracts according to the method of Bradford[24] with BSA as a standard. The enzyme activities were determined according to the following methods: SOD of Beyer and Gridovich[25]; CAT of Chance and Maehly[26]; POD of Shah et al.[27]; and APX of Chen and Asada[28]. All solutions used for analytical and enzymatic investigations were prepared with ultrapure water.

2 Results

2.1 Uptake and distribution of Cd in *B. chinensis*

Cd uptake by *B. chinensis* was measured over a Cd concentration range from 5 to 200 μmol/L in the culture solutions. These relatively high concentrations were selected because the Cd concentrations in Cd-contaminated soil were from 6.2 to 35.7 μmol/L[29]. The results showed that the Cd concentrations of the roots and shoots of *B. chinensis* showed a linear increase in response to the increase of Cd concentrations in the culture solutions (Figure 1). *B. chinensis* accumulated 150.0±17.8 mg/kg(dry weight, DW) in shoots and 536.7 ± 34.8 mg/kg(DW) in roots when exposed to 5 μmol/L Cd, and the bioaccumulation coefficients of *B. chinensis* (concentration of Cd in dry tissue/concentration of Cd in culture solution) were up to 268 for the shoot and 957 for the root. It should be noted that *B. chinensis* still survived even in the culture solution containing 200 μmol/L Cd, and the Cd distribution in the subcelluar fractions of these *B. chinensis* shoots was 22.0±8.7 mg/kg in the cell wall fraction (37.2%), 3.3±0.7 mg/kg in the chloroplast and nucleolus fraction (6.0%), 2.4±0.4 mg/kg in the mitochondria fraction (4.4%) and 29.5±7.8 mg/kg in the cytoplasm fraction (52.4%). Figure 2 shows that the Cd concentration accumulated in the shoots of soil-cultured *B. chinensis* was 261.7 mg/kg(DW) Cd when exposed to 200 mg/kg(DW) Cd.

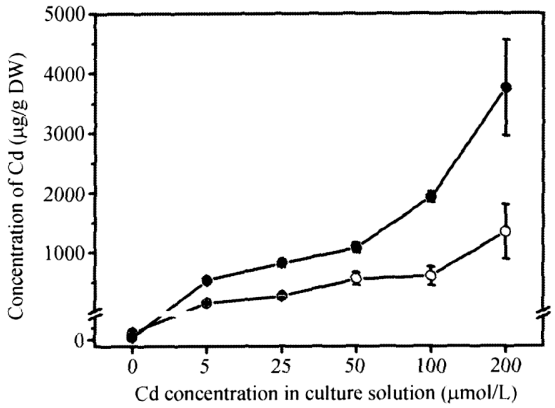

Figure 1 Cd uptake in the roots (●) and the shoots (○) of *B. chinensis* under exposure to different concentrations of Cd for 6 d. Error bars represent standard deviation of 9 repetitions from triplicate cultivations. DW, dry weight.

2.2 Generation of PCs under Cd exposure

The synthesis of Cd-binding peptides was induced when *B. chinesis* seedlings were exposed to Cd in the culture

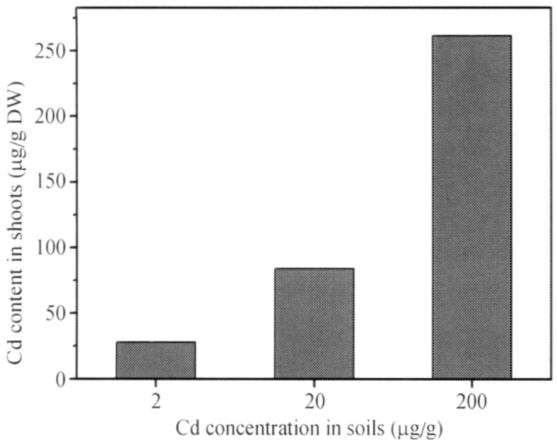

Figure 2 Concentration of Cd in the shoots of soil-cultured *B. chinensis* under different Cd exposure.

solutions (Figure 3(a)—(c) and Table 1). Figure 3(a), (b) and (c) show the HPLC chromatograms with postcolunm derivatization for the control roots, the Cd treated shoots and the Cd treated roots of *B. chinensis*, respectively. Besides GSH peaks, PCs [(γ-Glu-Cys)$_n$-Gly] peaks with *n* from 2 to 5 in shoots and from 2 to 6 in roots were found under Cd stress. In addition, des-Glu-PC_3 was also identified in both shoots and roots, which was also identified previously in maize[30]. It has been suggested that the unique N-terminal amine bond of PC_3 is hydrolyzed by γ-Glutamyl transpeptidase[31]. PCs were not detected in the control *B. chinensis* (Figure 2(a) and Table 1). The predominant components of PCs were PC_3 and PC_4 in the shoots, and PC_3, PC_4 and PC_5 in the roots (Table 1). In roots, the level of PC_2 was almost unchanged over the entire Cd concentration range tested. The fresh-weight ratios of PC units to Cd were found to be from 1.0 to 3.2 in the shoots, and 1.4 to 3.2 in the roots. The results also showed that the GSH pool varied considerably in response to Cd stress (Table 1): GSH increased significantly in both shoots and roots followed by depletion along with the increase of Cd concentrations in the culture solutions.

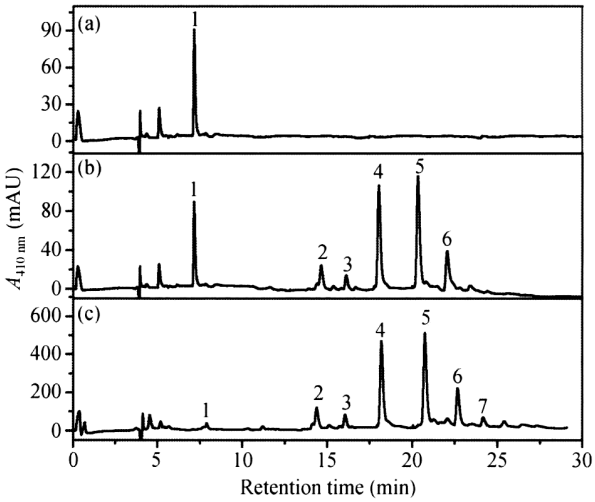

Figure 3 HPLC chromatograms of phytochelatins and related thiol peptides in the control root (a), the Cd-treated shoots (b) and the Cd-treated roots (c) of *B. chinensis*. The identified peaks are: 1, GSH; 2, PC_2; 3, des-Glu-PC_3; 4, PC_3; 5, PC_4; 6, PC_5; 7, PC_6, and some unidentified SH-peptides.

2.3 Oxidative stress and antioxidative response under Cd stress

H_2O_2 and MDA are two major end products of heavy metal stress. H_2O_2 showed an increase trend under Cd exposure from 5 to 200 μmol/L Cd (Table 2). It was

Table 1 Levels of GSH and PC variants induced in the roots and the shoots of *B. chinensis* under exposure to different concentrations of Cd

Plant tissue	Cd (μmol/L)	GSH (nmol/g FW)	PCs (nmol/g FW)						ΣPC (nmol/g FW)
			PC_2	PC_3	des-Glu-PC_3	PC_4	PC_5	PC_6	
Shoot	0	13.6 ± 1.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	57.2 ± 1.1	6.0 ± 0.4	8.3 ± 0.3	0.4 ± 0.2	4.7 ± 0.1	0.7 ± 0.1	n.d.	60.3 ± 3.1
	25	46.9 ± 1.8	10.3 ± 2.7	46.3 ± 2.0	1.1 ± 0.3	35.1 ± 3.4	1.6 ± 0.4	n.d.	311.4 ± 27.3
	50	64.0 ± 5.2	44.6 ± 3.2	69.9 ± 4.6	1.3 ± 0.1	52.2 ± 4.1	3.3 ± 0.3	n.d.	528.4 ± 38.5
	100	57.3 ± 9.8	47.4 ± 9.9	98.5 ± 7.8	2.3 ± 0.5	68.4 ± 7.4	4.3 ± 0.7	n.d.	692.4 ± 77.7
	200	26.9 ± 2.0	79.9 ± 0.8	123.8 ± 4.9	3.8 ± 0.9	73.7 ± 1.7	4.7 ± 0.2	n.d.	861.2 ± 26.8
Root	0	10.4 ± 1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	27.4 ± 1.3	7.3 ± 0.9	58.8 ± 2.9	0.7 ± 0.2	58.6 ± 2.2	13.5 ± 0.9	2.2± 0.2	508.0 ± 24.6
	25	58.0 ± 8.2	12.6 ± 0.8	107.1 ± 4.6	5.8 ± 0.7	124.8 ± 2.6	35.7 ± 2.1	4.0 ± 0.4	1065.6 ± 38.7
	50	152.2 ± 23.2	11.7 ± 2.2	130.6 ± 6.0	3.4 ± 1.0	149.1 ± 2.6	47.8 ± 6.9	4.6 ± 0.6	1288.6 ± 70.6
	100	53.8 ± 21.0	9.5 ± 0.7	157.2 ± 3.4	5.4 ± 0.3	158.7 ± 4.3	41.5 ± 5.8	6.8 ± 0.2	1390.2 ± 35.7
	200	26.6 ± 3.5	9.5 ± 0.6	223.6 ± 7.8	4.3 ± 0.5	199.2 ± 3.8	37.3 ± 0.8	5.6 ± 0.2	1719.8 ± 45.2

Values represent the mean of 9 repetitions from triplicate cultivations ± SD. FW, fresh weight. ΣPC, molar concentrations of a sum of the Cys units of the detected PC variants. n.d., Not detected.

Table 2 Levels of H_2O_2 and MDA in the shoots of *B. chinensis* under exposure to different concentrations of Cd

Oxidative stress	Cd (μmol/L)					
	0	5	25	50	100	200
H_2O_2 μmol/g FW	120.33 ± 14.06	101.02 ± 14.60	128.20 ± 17.21	145.91 ± 49.77	180.30 ± 28.41	238.89 ± 13.37
MDA nmol/g FW	4.66 ± 0.18	5.13 ± 0.18	5.23 ± 0.38	5.14 ± 0.57	4.82 ± 0.52	6.28 ± 0.48

Values represent the mean of 9 repetitions from triplicate cultivations ± SD. FW, fresh weight.

Table 3 The activities of the antioxidative enzymes SOD, CAT, POD and APX in the shoots of *B. chinensis* under exposure to different concentrations of Cd

Enzyme activity (Units mg^{-1} protein min^{-1})	Cd (μmol/L)					
	0	5	25	50	100	200
SOD	31.74 ± 3.48	33.36 ± 3.02	34.20 ± 6.99	30.37 ± 1.74	25.83 ± 2.29	26.20 ± 3.40
CAT	238.44 ± 25.45	329.77 ± 59.64	264.28 ± 29.04	306.82 ± 25.77	358.10 ± 68.21	284.46 ± 34.59
POD	0.20 ± 0.04	0.21 ± 0.01	0.25 ± 0.06	0.32 ± 0.02	0.35 ± 0.04	0.30 ± 0.04
APX	0.78 ± 0.22	1.44 ± 0.26	2.25 ± 0.14	1.33 ± 0.14	1.20 ± 0.09	1.68 ± 0.18

Values represent the mean of 9 repetitions from triplicate cultivations ± SD.

nearly 200% at 200 μmol/L Cd compared to the control. The content of MDA showed some fluctuation in the range from 5 to 100 μmol/L Cd but a significant 34.7% increase at 200 μmol/L Cd (Table 2).

The ability of plants to metabolize ROS depends largely on the induction of the antioxidative activity of SOD, CAT, APX and POD. In an attempt to understand the role of antioxidative enzymes in plants under Cd stress, the activities of SOD, CAT, APX and POD were estimated spectrophotometrically (Table 3). SOD activity, responsible for elimination of O_2^- in cells, showed no significant difference with and without Cd stress. However, small decreases in SOD activities (by 17.4% and 18.6%) were observed when the plants were treated with 100 and 200 μmol/L Cd, respectively. CAT, APX and POD, three potent scavengers of H_2O_2, function at different locations inside the cell to maintain H_2O_2 at a steady level. Table 3 indicates that the activities of these three enzymes were stimulated by Cd. The activity of CAT showed a significant increase during Cd treatment and there was a nearly 50% increase at 100 μmol/L Cd. However, it decreased at 200 μmol/L Cd, but was still higher than the control. POD activity gradually increased with the increasing concentration of Cd in the culture solutions; the maximum occurred at 100 μmol/L Cd, which was about a 75% increase compared with the control. APX activity was highly stimulated from 5 to 25 μmol/L Cd (up to 289% of the control at 25 μmol/L Cd), and then significantly suppressed by increasing concentration of Cd in the culture solutions. However, it was still higher compared with the control.

3 Discussion

It is well known that crop and crop-related species of the family Brassicaceae are able to accumulate Cd in their roots and shoots[32,33]. *Thlaspi caerulescens*, a Cd hyperaccumulator of the family Brassicaceae, was observed to accumulate Cd in its shoots up to 1140 mg/kg on a shoot dry weight basis, when growing in a solution containing 200 μmol/L Cd[34]. *Brassica juncea* L. was found to take up Cd ranging from 200 to1200 mg/kg when hydroponically exposed to 25 μmol/L Cd[35]. Our results showed that *B. chinensis* accumulated 261.7 mg/kg DW in the shoots when the Cd concentration was 200 mg/kg in the soils. Obviously, *B. chinensis* is a potential hyperaccumulator of Cd according to the definition that the threshold is 100 mg Cd /kg DW in shoots of natural soil-grown plants[36].

Figure 4(a) and (b) illustrates a consistent relationship between Cd concentrations in the plants with ΣPC and GSH concentrations. The results showed that Cd hyperaccumulation in *B. chinensis* was associated with enhanced rates of PC generation, which suggested that PC synthesis might be essential for hyperaccumulation and hypertolerance to Cd. Furthermore, increased PC accumulation is due to increasing uptake of Cd into the cytoplasm, which in turn induced PC synthase and the enhanced level of GSH that is the precursor of PCs synthesis. Under 200 μmol/L Cd exposure, PCs seemed to be saturated by Cd while GSH concentration was decreased

(Figure 4(a) and (b)), which demonstrated that Cd accumulation was beyond the capacity for Cd detoxification by *B. chinensis*. In the roots, the almost unchanged level of PC_2 under Cd exposure at different concentrations might be responsive to the PC biosynthetic pathway, in which it was also a precursor of higher species of PCs besides GSH[37]. Grill et al. reported incubation with PC synthase, and only PC_2 led to the synthesis of PC_4[21]. Theoretically, Cd is chelated through the thiol group in PCs with the molar ratio of thiol to Cd being 4. When PCs were intentionally saturated with Cd, a ratio of 1.01 was obtained[38]. The molar PC:Cd ratios from 1.00 to 3.32 in the shoots and 1.37 to 3.22 in the roots obtained in this study demonstrated that the PC content in *B. chinensis* was sufficient for the chelation of significant amounts of Cd. This has been evidenced by the reconstituted $Cd_{1\text{-}2}PC_2$ and $Cd_{1–3}PC_{3–5}$ complexes *in vitro*[39]. Some studies have shown a similar correlation between PC and Cd: the ratio in *Rauvolfia. serpentina* was 3.78[38], and in maize was 1.01[40]. The results obtained in this study suggested a PC-mediated Cd tolerance mechanism in *B. chinensis*.

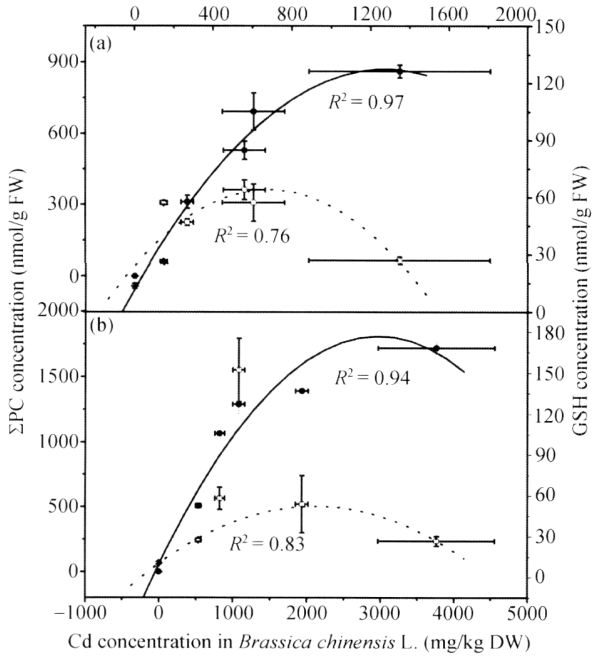

Figure 4 Correlations between Cd concentrations and ΣPC concentrations (●), Cd concentrations and GSH concentrations (○) in the shoots (a) and in the roots (b). The solid line shows the relationship between ΣPC concentrations and Cd concentrations; the dotted line shows the relationship between GSH concentrations and Cd concentrations. ΣPC data are reported as molar concentrations of a sum of the Cys units of the detected PC variants. Error bars represent standard deviation of 9 repetitions from triplicate cultivations. DW, dry weight. FW, fresh weight.

GSH is also a major non-protein thiol peptide in plants, which has many functions in plant metabolism. For this reason, its concentration is controlled by a complex homeostatic mechanism where the availability of sulphur seems to be required. In this study, Cd was added in the form of $CdSO_4$ which enhanced sulphur absorption and favored Cd accumulation through binding with PCs. The results for GSH showed a significant change under the exposure of different Cd concentrations in this study (Table 1). The increased level of GSH resulted from the induction by Cd of the transcription genes of GSH biosynthesis such as γ-glutamylcysteine synthetase and GSH synthetase while the decrease of GSH level was a common response to Cd caused by an increased consumption of GSH for the production of PCs[40,41]. Besides, being the precursor of PCs, GSH is an important component of the active oxygen scavenging system of the cell. GSH as an antioxidant, together with ASA and antioxidative enzymes such as SOD, CAT, APX and POD, controls the cellular concentration of ROS.

In the present study, the enhanced level of H_2O_2 and lipid peroxidation dependent on concentrations of Cd indicated Cd induced oxidative damage to *B. chinensis* under Cd stress. Similar results have also been found in *A. thaliana*, *Nicotiana tabacum* and *Pisum sativum* L. cv. Azad[42,43,13]. In contrast with Cu, Cd does not seem to act directly on the production of ROS[44]. The enhanced oxidative stress might be due to the decline of the antioxidant GSH and antioxidative enzymes, activating Ca-dependent systems and affecting Fe-mediated processes[45]. The increased lipid peroxidation observed could probably be ascribed to the harmful effect of an over production of H_2O_2 or its poisonous ROS derivatives in various compartments[46]. SOD is considered as the first plant antioxidative defense and is charged with the dismutation of O_2^- to H_2O_2 and O_2, and thus maintains O_2^- at a steady level[47]. Nevertheless, the results obtained in this study did not show a serious change of SOD activity under Cd stress (Table 3). Observations of SOD activity in different plant species under several stress conditions suggest that different mechanisms may be involved in oxidative stress injury[48,49].

Recent studies also envisage GSH and H_2O_2 as the central components of signal transduction of both environmental and abiotic stress in plants[50], and H_2O_2 has been shown to act as a signaling molecule in the activa-

tion of cellular defenses including CAT, POD and APX[51,52]. Previous reports showed a varying response involving either an increase or a decrease in these enzyme activities when exposed to Cd[53−55]. The responses observed in the present study showed, in general, an increase of enzyme activities at low Cd concentration and a reduction at high Cd concentration. The great increase in CAT activities in *B*. *chinensis* shoots compared with the control suggested that the stimulation of their scavenging mechanisms for removing H_2O_2 resulted from Cd stress. The enhanced level of POD activities suggested that Cd has triggered secondary metabolites such as the lignin in *B*. *chinensis*. The decline of CAT and POD activities observed at 200 μmol/L Cd is ascribed to inactivation of enzymes by ROS, decrease in the synthesis of enzymes and/or change in their protein contents[56].

APX is one of the most widely distributed antioxidative enzymes in plant cells. In this study, APX activity increased remarkably from 5 to 25 μmol/L Cd (Table 3). However, as Cd concentration in the culture solution increased, the pronounced Cd accumulation found in *B*. *chinensis* shoots seemed to adversely affect APX activity. The reason is that APX-mediated detoxification of H_2O_2 is coupled with ASA oxidation and then oxidized ASA is regenerated via the oxidation of GSH[57]. So, the depletion of GSH led to the decline of APX activity (Figure 4(a), Table 3). Although the decreased activity of APX was compensated for the increased activity of CAT and POD, the oxidative stress seemed to overwhelm the antioxidative defense and overproduced H_2O_2 at 200 μmol/L Cd because of its very low affinity for H_2O_2 (K_m = 0.047−1.1 mol/L), and peroxidase has a higher affinity with ASA than guaiacol[58]. APX, with its high affinity for H_2O_2, probably accounts for most of the H_2O_2 removal. Figure 5 shows the correlations between ΣPC and H_2O_2, as well as between APX and H_2O_2. The results suggested that APX and PCs supplemented each other in Cd detoxification. The loss of APX activities or the decrease of PC synthesis might lead to H_2O_2 accumulation.

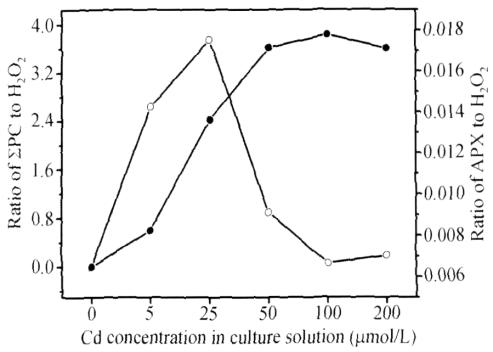

Figure 5 Relationship between ΣPC-H_2O_2 (●) and APX-H_2O_2 (○) under exposure to different concentrations of Cd. ΣPC data are reported as molar concentrations of a sum of the Cys units of the detected PC variants. Data represent the mean values of 9 repetitions from triplicate cultivations.

In summary, results obtained in this study suggest two important pathways involved in Cd hyperaccumulation and tolerance in *B*. *chinensis*. When *B*. *chinensis* is stressed by high Cd concentrations, some of the Cd induces PC synthesis while the rest induces oxidative stress. PC synthesis requires the synthesis of GSH by γ-GluCys synthetase and GSH synthetase, followed by binding of Cd and the synthesis of PCs by PC synthase. Then PCs combine with free Cd to form Cd-PC chelates and are transported into the vacuole[37]. At the same time, the excessive Cd induced ROS. O_2^- is dismuted by SOD, and the resulting H_2O_2 is metabolized by APX, CAT and POD. ASA is converted to dehydroascorbate (DHA) by H_2O_2 through a reaction catalyzed by APX, while DHA is reduced to ASA nonenzymically or enzymically by GSH[16]. These reactions result in reduced free Cd damage (low MDA levels) and enhanced Cd accumulation and tolerance, showing that GSH is the pivotal component in these two detoxification pathways. The decrease of GSH under high concentration Cd exposure leads to the loss of detoxification capacity and intrinsic H_2O_2 accumulation, and this would cause premature cell death.

The authors are grateful to Prof. John Hodgkiss for improving the English of the manuscript.

1 Tyler G, Balsberg-Pahlsson A M, Bengtsson G, et al. Heavy metal ecology and terrestrial plants, micro-organisms and invertebrates. Water Air Soil Pollut, 1989, 47(3-4): 189−215

2 Wagner G J. Accumulation of cadmium in crop plants and its consequences to human health. Adv Agron, 1993, 51: 173−212

3 Vögeli-Lange R, Wagner G J. Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves. Plant Physiol, 1990, 92(44): 1086−1093

4 Rauser W E. Phytochelatins and related peptides. Structure, biosynthesis, and function. Plant Physiol, 1995, 109(4): 1141−1149

5 Tomsset A B, Thurman D A. Molecular biology of metal tolerances in plants. Plant Cell Environ, 1988, 11(5): 383－394
6 Rauser W E. Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin and metallothioneins. Cell Biochem Biophys, 1999, 31(1): 19－48
7 Cobbett C S. Phytochelatin biosynthesis and function in heavy-metal detoxification. Curr Opin Plant Biol, 2000, 3(3): 211－216
8 Howden R, Andersen C R, Goldsbrough P B, et al. A cadmium-sensitive, glutathione-deficient mutant of *Abrabidopsis thaliana*. Plant Physiol, 1995, 107(4): 1067－1073
9 Cobbett C S, May M J, Howden R, et al. The glutathione-deficient cadmium-sensitive mutant, *cad2-1*, is deficient in γ-glutamylcysteine synthetase. Plant J, 1998, 16(1): 73－78
10 Ha S B, Smith A P, Howden R, et al. Phytochelatin synthase genes in Arabidopsis and the yeast *Schizosaccaromyces pombe*. Plant Cell, 1999, 11(6): 1153－1163
11 Zhu Y L, Pilon-Smits E A H, Tarun A S, et al. Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. Plant Physiol, 1999, 121(4): 1169－1177
12 Hendry G A F, Baker A J M, Ewart C F. Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus*. Acta Bot Neerl, 1992, 41(3): 271－281
13 Dixit V, Pandey V, Shyam R. Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J Exp Bot, 2001, 52(358): 1101－1109
14 Skórzyńska-Polit E, Drążkiwicz M, Krupa Z. The activity of the antioxidative system in cadmium-treated *Arabidopsis thaliana*. Biol Plant, 2003, 47(1): 71－78
15 Wójcik M, Kórzyńska-Polit E, Tukiendorf A. Organic acids accumulation and antioxidant enzyme activities in *Thlaspi caerulescens* under Zn and Cd stress. Plant Growth Regul, 2006, 18(2): 145－155
16 Noctor G, Foyer C H. Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol, 1998, 49: 249－279
17 Asada K. The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol, 1999, 50: 601－639
18 De Vos R C H, Vonk M J, Vooijs R, et al. Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiol, 1992, 98(3): 853－858
19 Feldmann K A, Marks M D. *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: A non-tissue culture approach. Mol Gen Genet, 1987, 208(1-2): 1－9
20 Yang J R, Bao Z P, Zhang S Q. Distribution and chemical binding forms of Cd, Pb in plant cell. Chi Environ Sci (in Chinese), 1993, 13(4): 263－268
21 Grill E, Winnacker E L, Zenk M H. Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc Natl Acad Sci USA, 1987, 84(2): 439－443
22 Gay C, Gebicki J M. A critical evaluation of the effect of sorbitol on the ferric-xylenol organe hydroperoxide assay. Anal Biochem, 2000, 284(2): 217－220
23 Dhindsa R S, Dhindsa P, Thorpe T A. Leaf senescence correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J Exp Bot, 1987, 32(1): 93－101
24 Bradford M M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem, 1976, 72: 248－254
25 Beyer W F, Fridovich Y. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem, 1987, 161: 559－566
26 Chance B, Maehly A C. Assay of catalases and peroxidases. Methods Enzymol, 1955, 2: 764－775
27 Shah K, Kuma R G, Verma S, et al. Effect of cadmium on lipid peroxidation, superoxide anion generation and activities antioxidant enzymes in growing rice seedlings. Plant Sci, 2001, 161(6): 1135－1144
28 Chen G X, Asada K. Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol, 1989, 30(7): 987－998
29 Salt D E, Wagner G J. Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a Cd/H antiport activity. J Biol Chem, 1993, 268(17): 12297－12302
30 Chassaigne H, Vacchina V, Kutchan T M, et al. Identification of phytochelatin-related peptides in maize seedlings exposed to cadmium and obtained enzymatically in vitro. Phytochemistry, 2001, 56(7): 657－668
31 Martin M N, Slovin J P. Purified γ-Glutamyl transpeptidases from tomato exhibit high affinity for glutathione and glutathione S-conjugates. Plant Physiol, 2000, 122(4): 1414－1426
32 Nanda-Kumar P B A, Dushenkov V, Motto H, et al. Phytoextaction: The use of plants to remove heavy metals from soils. Environ Sci Technol, 1995, 29(5): 1232－1238
33 Salt D E, Prince R C, Pickering I J, et al. Mechanisms of cadmium mobility and accumulation in Indian mustard. Plant Physiol, 1995, 109(4): 1427－1433
34 Brown S L, Chaney R L, Angle J S, et al. Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. Soil Sci Soc Am J, 1995, 59(1): 125－133
35 Ishikawa S, Noriharu A E, Mutakami M, et al. Is *Brassica juncea* a suitable plant for phytoremediation of cadmium in soils with moderately low cadmium contamination?－Possibility of using other plant species for Cd-phytoextration. Soil Sci Plant Nutr, 2006, 52(1): 32－42
36 Baker A J M, McGrath S P, Reeves D R, et al. Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. Boca Raton: CRC Press, 2000. 171－188

37 Zenk M H. Heavy metal detoxification in higher plants— A review. Gene, 1996, 179(1): 21－30
38 Strasdeit H, Duhme A K, Kneer R, et al. Evidence for discrete $Cd(SCys)_4$ units in cadmium phytochelatin complexes from EXAFS spectroscopy. J Chem Soc Chem Commun, 1991, 16: 1129－1130
39 Chen L Q, Guo Y F, Yang L M, et al. SEC-ICP-MS and ESI-MS/MS for analyzing *in vitro* and *in vivo* Cd-phytochelatin complexes in a Cd-hyperaccumulator *Brassica chinensis*. J Anal At Spectrom, 2007, 22(11): 1403－1408
40 Meuwly P, Rauser W E. Alteration of thiol pools in roots and shoots of maize seedlings exposed to cadmium: Adaptation and developmental cost. Plant Physiol, 1991, 99(1): 8－15
41 Xiang C B, Oliver D J. Glutathione metabolic genes coordinately respond to heavy metals and jasmoni acid in *Arabidopsis*. Plant Cell, 1998, 10(7): 1539－1550
42 Cho U H, Seo N H. Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. Plant Sci, 2005, 168(1): 113－120
43 Boominathan R, Doran P M. Cadmium tolerance and antioxidative defenses in hairy roots of the cadmium hyperaccumulator, *Thlaspi caerulescens*. Biotechnol Bioeng, 2003, 83(2): 158－167
44 di Toppi L S, Gabbrielli R. Response to cadmium in higher plants. Environ Exp Bot, 1999, 41(2): 105－130
45 Okamoto O K, Pinto E, Latorre L R et al. Antioxidant modulation in response to metal-induced oxidative stress in algal chloroplasts. Arch Environ Contam Toxicol, 2001, 40(1): 18－24
46 Bowler C, Montagu M V, Inze D. Superoxide dismutase and stress tolerance. Ann Rev Plant Physiol Plant Mol Biol, 1992, 43: 83－116
47 Alscher R G, Erturk N, Heath L S. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Bot, 2002, 53(372): 1331－1341
48 Yu Q, Rengel Z. Drought and salinity differentially influence activities of superoxide dismutase in narrow-leafed lupins. Plant Sci, 1999, 142(1-2): 1－11
49 Yu Q, Rengel Z. Waterlogging influences plant growth and activities of superoxide dismutases in narrow-leafed lupin and transgenic tobacco plants. J Plant Physiol, 1999, 155(3): 431－438
50 Foyer C H, Lopez-Delgado H, Dat J F, et al. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. Physiol Plant, 1997, 100(2): 241－254
51 Prasad T K, Anderson M D, Martin B A, et al. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell, 1994, 6(1): 65－74
52 Karpinski S, Reynolds H, Karpinska B, et al. Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science, 1999, 284(5414): 654－657
53 Gallego S M, Benavides M P, Tomaro M L. Effect of cadmium ions on antioxidative defense system in sunflower cotyledons. Biol Plant, 1999, 42(1): 49－55
54 Srivastava M S, Tripathi R D, Govindarajan R, et al. Phytochelatin synthesis and response of antioxidants during cadmium stress in *Bacopa monnieri* L. Plant Physiol Biochem, 2006, 44(1): 25－37
55 Schützendübel A, Schwanz P, Teichmann T, et al. Cadmium-induced changes in antioxidative systems, hydrogen peroxide content and differentiation in Scots pine roots. Plant Physiol, 2001, 127(3): 887－892
56 Verma S, Dubey R S. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci, 2003, 164(4): 645－655
57 Metwally A, Safronova V I. Belimov A A, et al. Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. J Exp Bot, 2005, 56(409): 167－178
58 Dalton D A, Hanus F J, Russell S A, et al. Purification, properties, and distribution of ascorbate peroxidase in legume root nodules. Plant Physiol, 1987, 83(4): 789－794