

## Modulation of Exogenous Glutathione in Phytochelatins and Photosynthetic Performance Against Cd Stress in the Two Rice Genotypes Differing in Cd Tolerance

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Received: 7 August 2010 / Accepted: 7 December 2010 /  
Published online: 30 December 2010  
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**Abstract** Greenhouse hydroponic experiments were conducted using Cd-sensitive (Xiushui63) and tolerant (Bing97252) rice genotypes to evaluate genotypic differences in response of photosynthesis and phytochelatins to Cd toxicity in the presence of exogenous glutathione (GSH). Plant height, chlorophyll content, net photosynthetic rate (Pn), and biomass decreased in 5 and 50  $\mu\text{M}$  Cd treatments, and Cd-sensitive genotype showed more severe reduction than the tolerant one. Cadmium stress caused decrease in maximal photochemical efficiency of PSII (Fv/Fm) and effective PSII quantum yield [Y(II)] and increase in quantum yield of regulated energy dissipation [Y(NPQ)], with changes in Cd-sensitive genotype being more evident. Cadmium-induced phytochelatins (PCs), GSH, and cysteine accumulation was observed in roots of both genotypes, with markedly higher level in PCs and GSH on day 5 in Bing97252 compared with that measured in Xiushui63. Exogenous GSH significantly alleviated growth inhibition in Xiushui63 under 5  $\mu\text{M}$  Cd and in both genotypes in 50  $\mu\text{M}$  Cd. External GSH significantly increased chlorophyll content, Pn, Fv/Fm, and Y(II) of plants exposed to Cd, but decreased Y(NPQ) and the coefficient of non-photochemical quenching (qN). GSH addition significantly increased root GSH content in plants under Cd exposure (except day 5 of 50  $\mu\text{M}$  Cd) and induced up-regulation in PCs of 5  $\mu\text{M}$ -Cd-treated Bing97252 throughout the 15-day and Xiushui63 of 5-day exposure. The results suggest that genotypic difference in the tolerance to Cd stress was positively linked to the capacity in elevation of GSH and PCs, and that alleviation of Cd toxicity by GSH is related to significant improvement in chlorophyll content, photosynthetic performance, and root GSH levels.

**Keywords** Cadmium · Chlorophyll fluorescence · Glutathione · Photosynthesis · Phytochelatin · Rice (*Oryza sativa* L.)

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## Abbreviations

Ci	Intercellular CO <sub>2</sub> concentration
Cys	Cysteine
Fo, Fm, and Fv	Initial, maximal, and variable fluorescence
Fv/Fm	Maximum efficiency of photosystem II photochemistry
Gs	Stomatal conductance
GSH	Reduced glutathione
PCs	Phytochelatin
PCS	Phytochelatin synthase
Pn	Net photosynthetic rate
PSII	Photosystem II
qN	Coefficient of non-photochemical quenching
Tr	Transpiration rate
Y(II)	Effective PSII quantum yield
Y(NPQ)	Quantum yield of regulated energy dissipation

## Introduction

High cadmium (Cd) content in soil results in inhibition of plant growth and yield reduction, and even causes human health problems via food chain. Cereals, especially rice, as a staple food in East Asia, is a major source of Cd intake. Moreover, Cd has a very long biological half-life ( $\approx 30$  years) in the human body and is mainly accumulated in human kidneys and causes pulmonary emphysema and renal tubular damage [1]. Therefore, understanding the mechanism of rice tolerance to Cd stress would be important to develop varieties with improved Cd tolerance and minimized Cd accumulation.

Plant photosynthetic apparatus seems to be particularly sensitive to Cd [2]. Larsson et al. [3] found that seedlings of *Brassica napus* exposed to Cd under high light intensity showed a significant decrease in chlorophyll content and photochemical quantum yield of photosynthesis. In several plant species, light and dark reactions of photosynthesis are reported to be inhibited by heavy metals at different target sites, and photosystem II (PSII) was particularly affected [4]. Chlorophyll *a* fluorescence is an indicator of efficiency of energy conversion at PSII reaction centers and sheds light on the mode of action of a given xenobiotic [5]. Recently, pulse amplitude modulated (PAM) fluorescence and saturation pulse technique have been used to measure toxicity-induced changes in effective PSII quantum efficiency [6].

On the other hand, in most non-hyperaccumulators, phytochelatin (PCs) are widely accepted as a major product for plant detoxification and tolerance to heavy metal stress. Phytochelatin is the most common name for such glutathione-related metal-complexing peptides with general structure  $[(\gamma\text{-Glu-Cys})_n\text{-Gly}]$ ,  $n=2\text{--}11$  and occur in plants. Ha et al. [7] demonstrated that inactivation of phytochelatin synthase (PCS) genes which led to PC deficiency resulted in Cd hypersensitivity in both *Arabidopsis* and yeast. However, little is known about whether such difference for PCs exists among rice genotypes differing in Cd tolerance. If difference in Cd tolerance among plant genotypes is intrinsically associated with activation of PCs synthesis and function of photosynthesis system under stress conditions, we would hypothesize existence of Cd-induced changes in PCs accumulation and photosynthesis/chlorophyll fluorescence.

Due to its unique redox and nucleophilic properties, reduced glutathione ( $\gamma\text{-Glu-Cys-Gly}$ , GSH) is involved in cellular defense against toxic action of xenobiotics, oxyradicals,

salinity, acidity as well as metal cations. Glutathione can act as a first line of defense against metal toxicity through complexing metals before induced synthesis of PCs reaches to an effective level [8]. Glutathione is also an immediate substrate for synthesis of PCs. Xiang et al. [9] reported that genetically modified *Arabidopsis* plants with low GSH levels were hypersensitive to Cd due to their limited capacity to make phytochelatins. Therefore, the question remains whether external GSH can act as a regulator in preventing Cd stress.

Our previous study found significant difference in response to Cd toxicity among rice genotypes with different Cd tolerance [10]. The present study reported genotypic difference in photosynthesis, chlorophyll fluorescence, and PC production. The role of external GSH against Cd stress was also discussed. We aimed to provide a basis for developing strategies to reduce risks associated with Cd toxicity and maintaining sustainable plant production.

## Materials and Methods

### Plant Material and Experimental Design

Hydroponic experiments were carried out in Huajiachi Campus, Zhejiang University, Hangzhou, China. Two *Japonica* unwaxy rice genotypes (*Oryza sativa* L.) were used: Bing97252 and Xiushui63 Cd tolerant and sensitive, respectively [10]. Healthy seeds were surface sterilized by soaking in 1.5% H<sub>2</sub>O<sub>2</sub> for 30 min and fully rinsed by deionized water. After soaked in deionized water at room temperature for 2 days at 25±1°C, seeds were germinated for 1 day at 35°C. Healthy and germinated seeds were sowed in sterilized sand bed and kept in an incubator at 30°C day/28°C night under 80% relative humidity. At the second leaf stage (10 days old), uniform healthy plants of different genotypes were selected and transplanted to different 5-L plastic containers filled with 4.5 L of International Rice Research Institute nutrient solution for rice. The container was covered by a polystyrol plate with seven evenly spaced holes (two plants per hole) and placed in a greenhouse. On the seventh day after transplanting, Cd (as CdCl<sub>2</sub>) and GSH were added to corresponding containers to form five treatments: basal nutrient solution (control), 5 µM Cd (Cd1), 5 µM Cd+50 µM GSH (Cd1+GSH), 50 µM Cd (Cd2), and 50 µM Cd+50 µM GSH (Cd2+GSH). The experiment was laid in split-plot design with treatment as main plot and with seven replicates. The nutrient solution pH was adjusted to 5.1 by 0.1 M NaOH or HCl, and the solution was renewed every 5 days.

Cysteine (Cys), GSH, and PC concentrations of shoot and root were determined after 5, 10, and 15 days of treatment. After 15 days of treatment, the second fully expanded leaves were selected to measure chlorophyll content and other photosynthetic parameters. Meanwhile, plants were uprooted and plant height was simultaneously measured, and then dried at 80°C for 2 days and weighed.

### Determination of Chlorophyll Content, Chlorophyll Fluorescence, and Photosynthetic Parameters

Chlorophyll content, chlorophyll fluorescence, and photosynthetic parameters were measured on the second uppermost fully expanded leaf with seven replicates, with three plants per replicate for chlorophyll content and two plants for chlorophyll fluorescence and photosynthetic parameters. Chlorophyll content of leaves was determined according to acetone/ethanol mixture method of Chen [11]. Measurement of net photosynthetic rate (P<sub>n</sub>), stomatal conductance (G<sub>s</sub>), transpiration rate (Tr), and intercellular CO<sub>2</sub> concentration (C<sub>i</sub>)

was conducted with a LI-6400 portable photosynthesis system (Li-COR, Lincoln, NE, USA). Chlorophyll fluorescence parameters were performed using pulse-modulated chlorophyll fluorometer and ImagingWin software application (IMAGING-PAM; Walz, Effeltrich, Germany). After 20-min dark adaption, leaves were illuminated under a high saturating light pulse with a frequency of 0.05 Hz that lasted for 260 s. The initial fluorescence ( $F_0$ ) was determined by a measuring beam ( $<0.05 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR). The maximal fluorescence ( $F_m$ ) was determined using saturating pulse ( $2,500 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR). Intensities of actinic light setting was  $280 \mu\text{mol mol}^{-2} \text{s}^{-1}$  PAR. Variable fluorescence ( $F_v$ ) was calculated from the formula:  $F_v = F_m - F_0$ . Maximal photochemical efficiency of PSII ( $F_v/F_m$ ) was calculated according to Genty et al. [12] by ImagingWin software automatically. False-color images of  $F_v/F_m$  were recorded, stored, and compared using ImagingWin. Each pulse of high saturating light induced a change of other fluorescence parameters, which were calculated and stored in kinetics curves by ImagingWin software. Other calculated fluorescence parameters included effective PSII quantum yield [ $Y(II) = (F_m' - F)/F_m'$ ] [13], quantum yield of regulated energy dissipation ( $Y(NPQ)$ , [13]), and coefficient of non-photochemical quenching ( $q_N$ ) [14]. To account for variations in photosynthetic parameters across the surface of individual plants, data presented are average values obtained across at least seven individual experiments.

#### Determination of Cys, GSH, and PCs

Extraction and analysis of PCs and other low molecular weight SH were performed according to Zhang et al. [15] with minor modification for four replicates with three plants per replicate. In brief, plants were rinsed with deionized water and roots were immersed in  $\text{Na}_2\text{EDTA}$  for 15 min, fully rinsed by deionized water, then separated into roots and shoots (stems and leaves). Tissues (about 0.2 g) were ground into powder with liquid  $\text{N}_2$ , and then powdered samples were homogenized in 2 mL 0.1% (w/v) trifluoroacetic acid (TFA, Sigma), 6.3 mM diethylenetriamine pentaacetic acid (DTPA, Sigma). The homogenate was centrifuged at  $10,000 \times g$  for 10 min at  $4^\circ\text{C}$ . Supernatant (250  $\mu\text{L}$ ) was mixed with 450  $\mu\text{L}$  200 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[3-propane sulfonic acid] (HEPPS, Sigma) buffer containing 6.3 mM DTPA (pH 8.2), and 10  $\mu\text{L}$  25 mM monobromobimane [mBBr, Sigma, dissolved in acetonitrile (ACN)]. Derivatization was then carried out for 30 min in the dark at room temperature. The reaction was terminated by adding 300  $\mu\text{L}$  of 1 M methanesulfonic acid (Sigma). Then, samples were stored in the dark at  $4^\circ\text{C}$  for HPLC analysis within 2 or 3 days. Reagent blanks without sample were used to identify reagent peaks. All reagents were of HPLC grade.

By using a binary gradient of mobile phase A (0.1% TFA) and B (100% ACN) at room temperature, samples were analyzed on an Agilent 1100 HPLC system with fluorescence detector at 380 nm/470 nm (excitation/emission). A C18 column (Agilent XDB-C18, 5  $\mu\text{m}$ ,  $4.6 \times 250$  mm) was adopted; flow rate was set at  $1 \text{ mL min}^{-1}$ . Derivatives (20  $\mu\text{L}$ ) were run with linear gradient (12–25% B for 15 min, then 25–35% B for 14 min, and subsequently 35–50% B for 21 min). Before injecting a new sample, the column was cleaned (5 min, 100% B) and equilibrated (10 min, 12% B). Post time was 5 min. All solvents were degassed before use. Retention time and concentrations of Cys, GSH, and PCs were checked with mixed standard of Cys, GSH,  $\text{PC}_2$  ( $[\gamma\text{-Glu-Cys}]_2\text{-Gly}$ ),  $\text{PC}_3$  ( $[\gamma\text{-Glu-Cys}]_3\text{-Gly}$ ), and  $\text{PC}_4$  ( $[\gamma\text{-Glu-Cys}]_4\text{-Gly}$ ).  $\text{PC}_2$ ,  $\text{PC}_3$ , and  $\text{PC}_4$  standard were obtained from Shanghai Science Peptide Biological Technology Co., Ltd.

Statistical analyses were performed with Data Processing System statistical software package [16] using two-way ANOVA followed by Duncan's multiple range test (SSR) to evaluate significant treatment effects at significance level of  $p \leq 0.05$ .

## Results and Analysis

### Plant Height and Biomass Accumulation

Rice plants exposed to Cd1 (5  $\mu\text{M}$  Cd) showed a decrease ( $p < 0.05$ ) in plant height, and shoot and root dry weights (Table 1). Growth reduction was more severe with the increase in Cd level in media. The severity of Cd toxicity symptoms significantly differed between two genotypes. Xiushui63 was more affected, Cd toxicity symptoms of leaf necrotic patches also appeared more rapidly and severely, and plant height was also more inhibited. With higher Cd treatment, growth of Xiushui63 was inhibited. With 5  $\mu\text{M}$  Cd treatment, shoot DW was less affected in Bing97252 (30% of decrease in Bing97252 vs. 40% in Xiushui63). Reduction of root DW in Cd2 treatment (50  $\mu\text{M}$  Cd) did not have genotypic variation. Presence of exogenous GSH in media alleviated the impact of Cd toxicity to plants but the effect varied with combinations of genotype and Cd level in media (Table 1). In Cd1+GSH, addition of 50  $\mu\text{M}$  GSH increased plant height and shoot dry weight by 16% and 15%, respectively, over Cd1 in Xiushui63, while no significant effect was observed in Bing97252. When plants were exposed to the media with 50  $\mu\text{M}$  Cd, presence of GSH effectively increased plant growth in both genotypes when compared with those grown in

**Table 1** Effect of Cd and GSH in nutrient media on plant height, biomass, and photosynthesis parameters of two rice genotypes exposed to Cd for 15 days

Treatment	Plant height (cm)	Shoot DW (mg)	Root DW (mg)	Chlorophyll content (mg g <sup>-1</sup> FW)	Pn ( $\mu\text{M}$ CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	Tr (mM H <sub>2</sub> O m <sup>-2</sup> s <sup>-1</sup> )	Gs (mM H <sub>2</sub> O m <sup>-2</sup> s <sup>-1</sup> )	Ci ( $\mu\text{M}$ CO <sub>2</sub> M <sup>-1</sup> )
Xiushui63								
Control	45.3 a	490 a	102 a	4.29 a	20.1 a	5.20 a	460 a	362 c
Cd1	30.7 c	295 c	87 b	3.61 b	13.2 c	3.53 bc	269 b	356 c
Cd1+GSH	35.5 b	338 b	95 ab	3.72 b	17.4 b	5.86 a	495 a	381 b
Cd2	17.8 e	99 e	27 d	2.17 d	0.7 e	2.87 c	207 b	430 a
Cd2+GSH	24.5 d	181 d	39 c	2.51 c	6.4 d	3.73 b	213 b	354 c
Bing97252								
Control	58.4 a	667 a	126 a	4.53 a	19.8 a	4.92 b	752 a	401 a
Cd1	49.1 b	465 b	98 b	4.09 c	17.3 b	5.14 b	571 c	386 a
Cd1+GSH	47.4 b	490 b	116 ab	4.30 b	19.1 a	6.66 a	656 b	381 a
Cd2	28.3 d	144 d	34 d	3.18 e	11.2 d	3.36 c	244 d	316 b
Cd2+GSH	34.1 c	311 c	70 c	3.52 d	13.5 c	3.29 c	212 d	326 b

Data were means of seven independent replicates ( $n=7$ , each replicate containing three plants) for plant height and biomass, three replicates ( $n=3$ , each replicate containing three plants) for chlorophyll content, and seven replicates ( $n=7$ , each replicate containing two plants) for photosynthesis parameter measurements. Different letters indicate significant differences ( $p < 0.05$ ) among the treatments within each genotype. Control, Cd1, Cd1+GSH, Cd2, and Cd2+GSH represent the basic nutrition solution (BNS), BNS+5  $\mu\text{M}$  Cd, BNS+5  $\mu\text{M}$  Cd+50  $\mu\text{M}$  GSH, BNS+50  $\mu\text{M}$  Cd, and BNS+50  $\mu\text{M}$  Cd+50  $\mu\text{M}$  GSH, respectively

Pn net photosynthetic rate, Gs stomatal conductance, Tr transpiration rate, Ci intercellular CO<sub>2</sub> concentration

Cd2 treatment. Improvement of plant growth was more significant in Bing97252 than in Xiushui63 for shoot (2.16-fold vs. 1.84-fold increase) and root dry weight (2.06-fold vs. 1.44-fold increase).

### Chlorophyll Content

Leaf chlorophyll content was decreased by 16% and 10% in Xiushui63 and Bing97252, respectively, in Cd1 when compared with control (Table 1). This reduction was 50% and 30% in Xiushui63 and Bing97252, respectively, under Cd2. In Cd1+GSH, leaf chlorophyll content in Bing97252 increased significantly over Cd1, but no evidence of such an effect was observed in Xiushui63 (Table 1). This GSH mitigating effect was apparent for both genotypes in Cd2 treatments.

### Photosynthetic Parameters

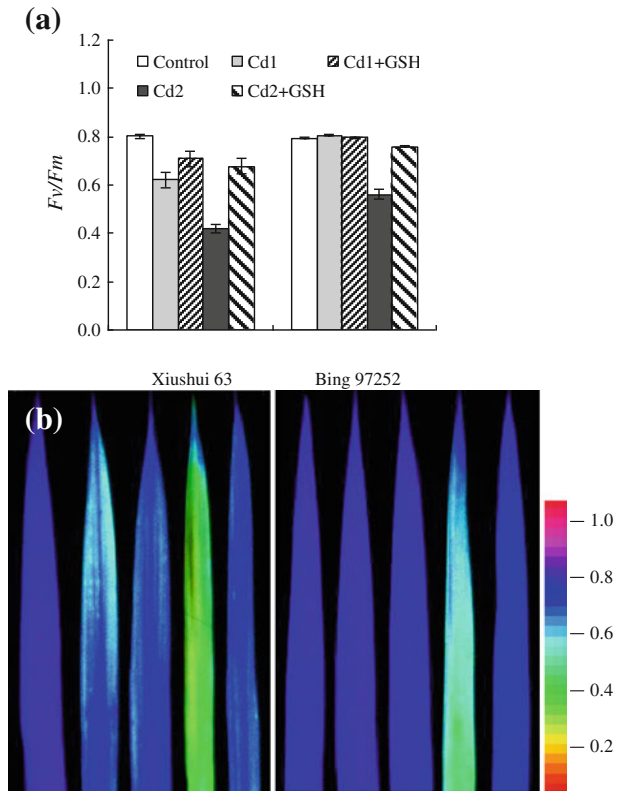
Cadmium stress induced a significant reduction in Pn in both genotypes, being 34% and 13% lower in Xiushui63 and Bing97252, respectively, in Cd1 than control (Table 1). In Cd2, Pn was inhibited in Xiushui63, whereas it decreased by 50% in Bing97252. Presence of GSH alleviated Cd-induced decline for Pn. Pn was 32% higher for Xiushui63 in Cd1+GSH than in Cd1. For Bing97252, Pn in Cd1+GSH was 30% higher than in Cd1 treatment, and there was no difference from control. Mitigating effect of GSH for Pn was more pronounced in Cd2, especially for Xiushui63. Transpiration rate in Xiushui63 decreased when grown in Cd1 and Cd2, but only decreased in Cd2 in Bing97252. Glutathione significantly restored Tr in both Cd levels in Xiushui63 (Table 1). In Bing97252, however, Tr was only increased in Cd1+GSH. Cadmium stress significantly decreased Gs in leaves (Table 1). Stomatal conductance reduction was 42% and 24% in Cd1 treatment in Xiushui63 and Bing97252, respectively, and 55% and 68% in Cd2 treatment. Addition of GSH recovered Gs in Cd1 but not in Cd2. No difference for Ci was observed between Cd1 and control for both genotypes (Table 1). In Cd2, Ci was higher than control for Xiushui63 but lower than control for Bing97252. GSH effect was opposite for Xiushui63 between Cd1 and Cd2 treatments. Addition of GSH had no effect on Ci in Bing97252.

### Chlorophyll Fluorescence Parameters of PSII

After 15-day Cd exposure, Fv/Fm ratio tended to decrease with increasing Cd level in media in Xiushui63 (cf. reduced by 23%, 48% in Cd1, Cd2 relative to control) (Fig. 1). In Bing97252, Fv/Fm ratio was only reduced at Cd2 (cf. 29% lower than control). Glutathione addition restored the respective values almost to control level. False-color image application was useful to understand changes of Fv/Fm induced by different treatments (Fig. 1b). Leaf color shifted from blue to green along with reducing Fv/Fm ratio, but that was recovered to the level almost comparable to control when plants were grown in GSH added media (blue). Images also revealed that Cd stress damages started in basal part of leaves, and then spread to leaf lamina, with the effect of Cd stress on Fv/Fm being lighted in leaf apex.

Figure 2 shows kinetics of three chlorophyll fluorescence parameters: Y(II), Y(NPQ), and qN. Effective PSII quantum yield at the first pulse is the highest (equal to the value of Fv/Fm) then dropped quickly. With a continually high saturating light pulse, Y(II) rose and then reached a steady condition (Fig. 2a, b). Cadmium treatments inhibited the rising of Y(II). For leaves from plants grown with GSH-supplied treatments, kinetic curves suggested

**Fig. 1** Maximal photochemical efficiency of PSII (Fv/Fm; **a**) and false-color images of Fv/Fm **(b)** of Xiushui63 (*left*) and Bing97252 (*right*) after grown for 15 days in different nutrient media with or without Cd and GSH. These measurements were taken after leaves had 20 min of dark adaption. Leaves from *left* to *right* represent basic nutrition solution (BNS), BNS+5  $\mu$ M Cd, BNS+5  $\mu$ M Cd+50  $\mu$ M GSH, BNS+50  $\mu$ M Cd, and BNS+50  $\mu$ M Cd+50  $\mu$ M GSH, respectively



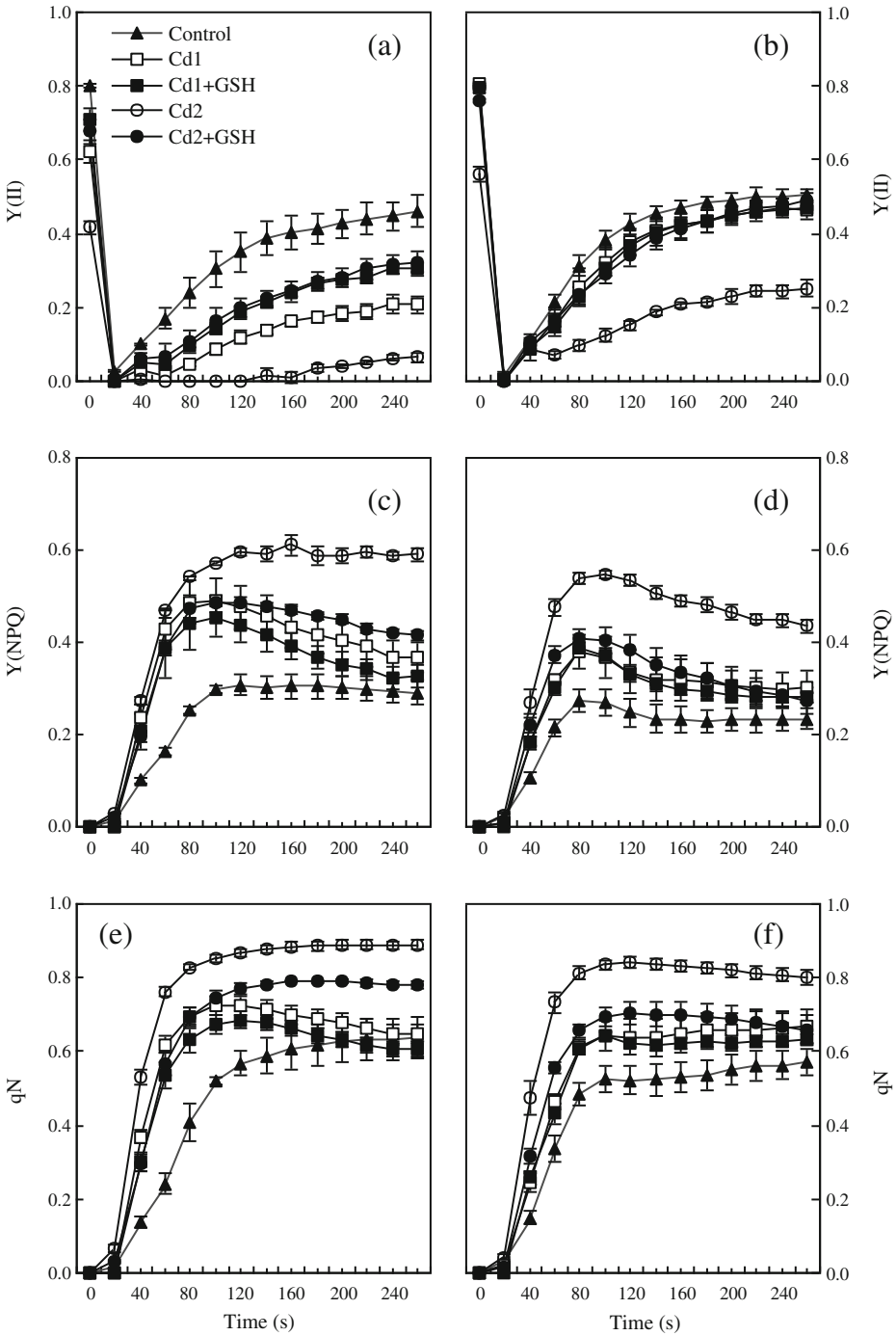
shortened rising time and improved height in Xiushui63 compared with respective Cd treatments (Fig. 2a). Comparing treatments at last pulse with control, Y(II) was decreased by 55% with Cd1 and 86% with Cd2 in Xiushui63. Nevertheless, only Cd2 caused significant reduction of Y(II) (by 50%) in Bing97252. Application of GSH remarkably alleviated inhibition caused by Cd, especially in Xiushui63 (Fig. 2a, b).

The dynamics of Y(NPQ) showed that non-photochemical quenching was increased mostly in Cd2 (Fig. 2c, d). Y(NPQ) at last pulse in Cd2 was 1.04- and 0.86-fold higher than control in Xiushui63 and Bing97252, respectively. Exogenous GSH decreased Y(NPQ) of plants grown in Cd2 (cf. 30% in Xiushui63 and 37% in Bing97252), but this was not the case for those in Cd1. However, no genotypic difference was found for dynamics of qN (Fig. 2e, f).

### GSH Accumulation

Change of GSH concentration in roots and shoots of two genotypes under Cd stress is shown in Fig. 3. Glutathione concentration of control of both genotypes at day 10 in both roots and shoots is significantly lower than that at 5 or 15 days. Cd stress increased root GSH concentration (Fig. 3a, b). This increase was relatively larger for Xiushui63 after 10 and 15 days of exposure and for Bing97252 after 5 and 10 days of exposure. Exogenous GSH in Cd treatments significantly increased root GSH concentrations at 10 days after exposure onwards in both genotypes and was more pronounced in Bing97252. However,



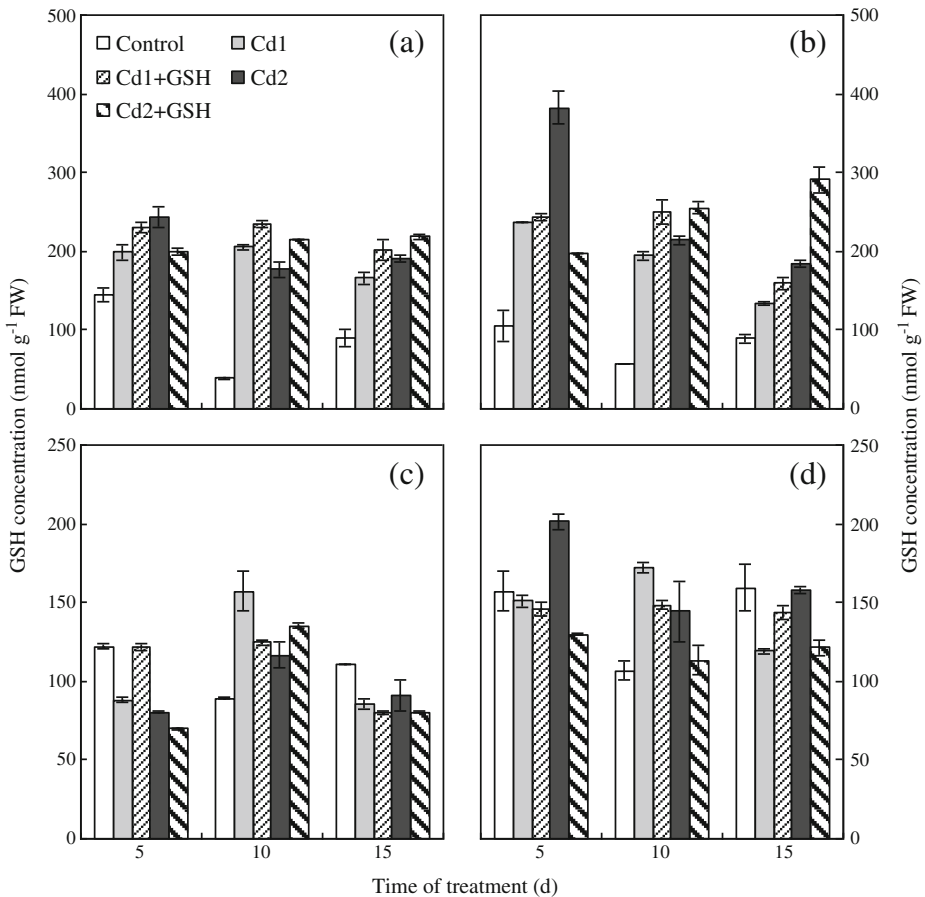




◀ **Fig. 2** Kinetic curves of fluorescence parameters of Xiushui63 (*left panel*) and Bing97252 (*right panel*) after 15-day Cd exposure. Before measurements were made, leaves were dark adapted for 20 min; for details, refer to experimental procedures.  $Y(II)$  effective PSII quantum yield,  $Y(NPQ)$  quantum yield of regulated energy dissipation,  $qN$  coefficient of non-photochemical quenching. *Error bars* refer to SD values ( $n=7$  with two plants per replicate)

significantly lowered GSH level in Cd2+GSH, but still higher than control, was observed at day 5 in both genotypes.

Shoots of Bing97252 contained more GSH than those of Xiushui63 (Fig. 3c, d). Genotypic response was different in Cd stress condition. After 5-day exposure, GSH content in Cd1 and Cd2 (5 and 50  $\mu$ M Cd) was decreased by 28% and 34% in Xiushui63 against control. However, in Bing97252, GSH content remained unchanged in Cd1 and was increased by 28% in Cd2. Similar trends between two genotypes were observed at 10 and 15 days of Cd1 and Cd2 treatments: increased at 10 days but decreased or unchanged at 15 days. In Cd1+GSH, GSH addition brought back shoot GSH content to control level at

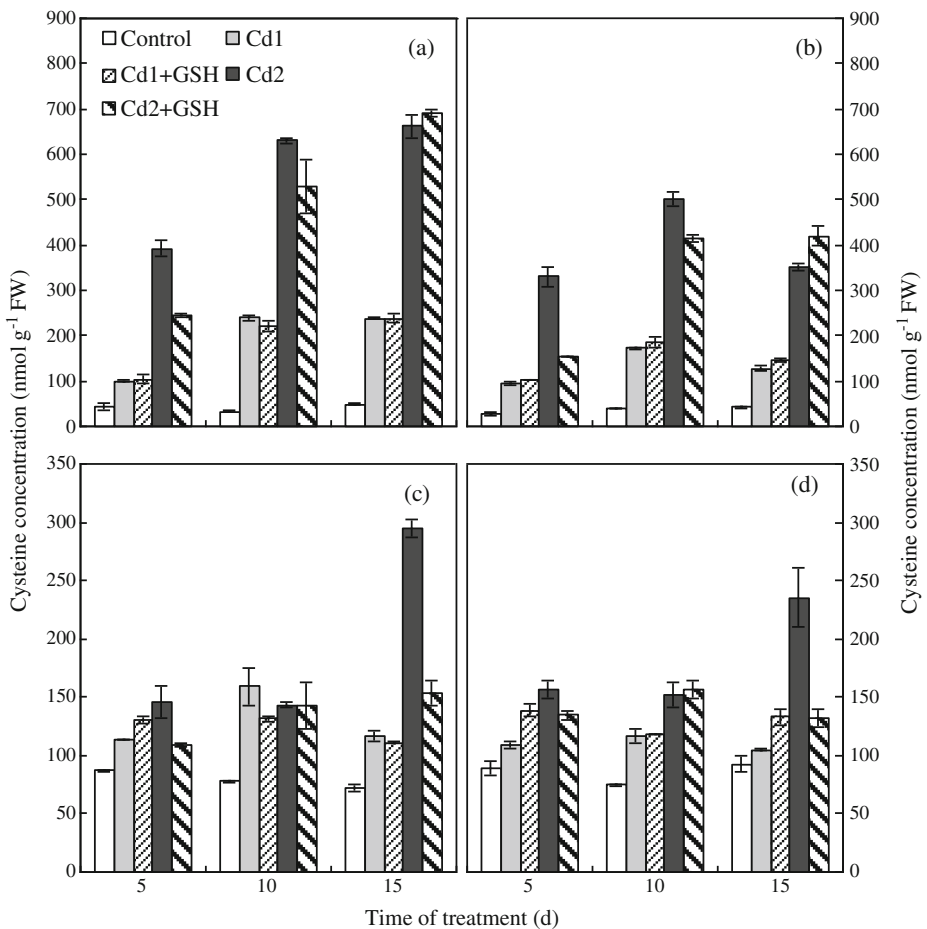


**Fig. 3** Changes in GSH concentrations in roots (a, b) and shoots (c, d) of Xiushui63 (*left panels*) and Bing97252 (*right panels*). *Error bars* refer to SD values ( $n=4$  with two plants per replicate)

5 days after treatment in Xiushui63 and at 15 days in Bing97252. However, there was a decline trend for shoot GSH content in Cd2+GSH except the data point for Xiushui63 at 10 days of exposure.

#### Production of Cysteine and Phytochelatin

There was a genuine root and shoot increase in Cys concentration for both genotypes in Cd treatments compared with controls (Fig. 4). At 5-day Cd exposure, Cys increase in root of Bing97252 was higher than that of Xiushui63 (cf. 3.59- and 12.50-fold in Bing97252 vs. 2.32- and 9.10-fold in Xiushui63, with Cd1 and Cd2 treatment, compared with control, respectively). Addition of GSH did not change root Cys concentration in both genotypes in Cd1; however, it decreased Cys concentration at 5 and 10 days of exposure in Cd2. In shoot, Cys concentration in Cd2+GSH was lower than in Cd2 at 5 days and 15 days after exposure, but it still remained higher than control (Fig. 4c, d).



**Fig. 4** Changes in cysteine concentrations in roots (a, b) and shoots (c, d) of Xiushui63 (left panels) and Bing97252 (right panels). Error bars refer to SD values ( $n=4$  with two plants per replicate)

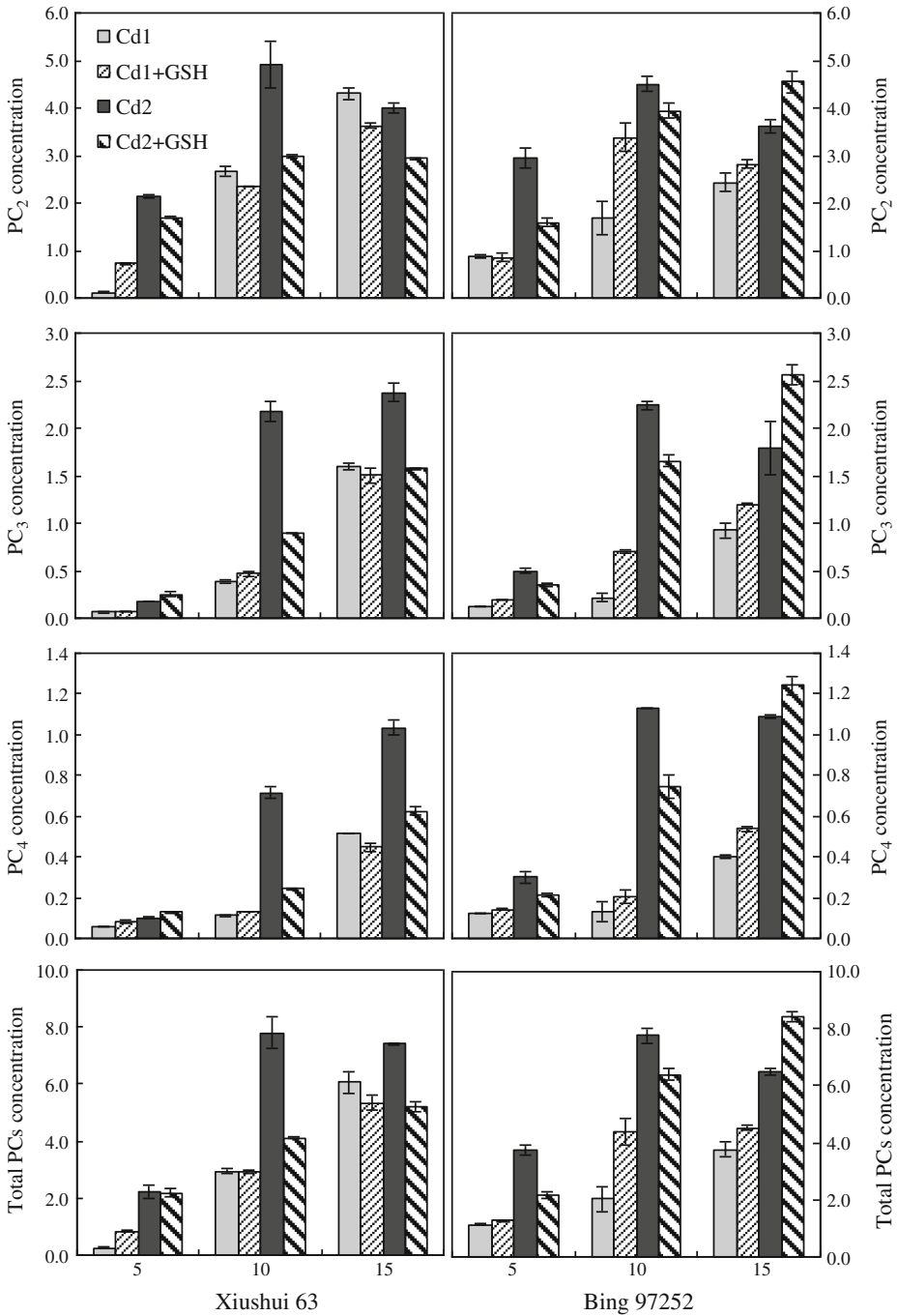
Phytochelatin contents were below detection limits in shoots and in roots of plants grown in control conditions (data not shown). Three main PC forms (PC<sub>2</sub>, PC<sub>3</sub>, and PC<sub>4</sub>) in roots were investigated (Fig. 5). In Cd1 treatment, PC contents of roots in Cd1 treatment increased drastically along with time of exposure. At 5-day exposure, it was 3.7-fold higher in Bing97252 than in Xiushui63 due to the most part of the increase in PC<sub>2</sub> in Cd1 treatment. In Cd2 treatment, PC contents increased sharply and reached peak at 10 days.

Addition of GSH in Cd1 boosted root PC concentrations of Xiushui63 at day 5 and then remained the same levels as Cd1. On the contrary, PC concentrations reached peak at 10-day exposure in Bing97252. Addition of GSH in Cd2 reduced PC concentration in Xiushui63 at 10–15 days after exposure. However, in Bing97252, it was lower at 5 and 10 days but markedly higher at 15 days when compared with those observed in Cd2.

## Discussion

This study confirmed our previous finding that Xiushui63 was more sensitive to Cd stress than Bing97252 [10]. The Cd-sensitive genotype showed greater reduction of plant growth, leaf chlorophyll content, and Pn (Table 1). Our preliminary measurements were done in control treatment adding just GSH and no Cd, and no differences were found with control plants (data not shown). In our previous study [17], GSH addition decreased Cd concentration in shoots and roots of both the two rice genotypes at 50  $\mu\text{M}$  Cd level, and roots of Xiushui63 and shoots of Bing97252 at 5  $\mu\text{M}$  Cd level. Higher Cd concentration in roots of tolerant genotype Bing97252 did not cause higher Cd in shoots. Moreover, Bing97252 had lower shoot/root ratio of Cd accumulation in Cd2 treatment. It may be suggested that restricted distribution of Cd was related to Cd tolerance in rice. However, GSH improved Cd translocation in some conditions. Considering dramatically reduced shoot and root Cd concentration in Cd2+GSH treatment, exogenous GSH seemed to act by reducing Cd uptake rather than translocation.

The negative impact of Cd in photosynthesis is well documented. The evidence in this study demonstrated that reduction of chlorophyll content should be an important reason for the sharp decline in Pn, but the extent of the negative effect of Cd stress on photosynthetic apparatus differed between genotypes. For instance, reduction of Pn in 5  $\mu\text{M}$ -Cd-treated plants ranged from 34% of Xiushui63 to 13% of Bing97252, respectively. In 50  $\mu\text{M}$  Cd treatments, Bing97252 could still maintain more than half of photosynthesis capacity as control, but in Xiushui63 no Pn was measured (completely inhibited). However, 50  $\mu\text{M}$  Cd almost completely inhibited Pn in Xiushui63, yet chlorophyll content decreased just by 50%. A similar effect was observed in sugar beet and tomato [18, 19]. Moreover, addition of GSH did not affect chlorophyll contents at high Cd levels but restored Pn to appreciable levels. It seems that a high level of Cd inhibits plant growth and Pn in a different way. As to stomatal conductance (Gs) and transpiration rate (Tr), 5  $\mu\text{M}$  Cd caused more reduction in Xiushui63 than in Bing97252, and deleterious effects become pronounced in 50  $\mu\text{M}$ -Cd-treated plants. It may be suggested that more than 5  $\mu\text{M}$  Cd would induce closing of stomata resulting in reduction in Gs. Concerning intracellular CO<sub>2</sub> concentration (Ci), 5  $\mu\text{M}$  Cd had no effect on two genotypes even though Pn was inhibited. Also, 50  $\mu\text{M}$  Cd significantly increased intracellular CO<sub>2</sub> in Xiushui63 but reduced in Bing97252 (Table 1). These results suggested that not stomatal limitation but decrease of chlorophyll content contributed to the decrease in Pn [20]. In addition, further studies are needed to test if the effects on photosynthesis at low Cd concentrations are due to a Cd-induced Fe deficiency



**Fig. 5** Changes in PC<sub>2</sub>, PC<sub>3</sub>, PC<sub>4</sub>, and total PC (sum of PC<sub>2</sub>, PC<sub>3</sub>, and PC<sub>4</sub>) concentrations ( $\mu\text{mol} - \text{SH g}^{-1}$  FW) in roots of Xiushui63 (left panels) and Bing97252 (right panels). Error bars refer to SD values ( $n=4$  with two plants per replicate)

while at high Cd reflects true Cd toxicity and that can also explain differences when adding GSH.

Cadmium is thought to act at PSII on both oxidizing (donor) and reducing (acceptor) sides. Moreover, PSII reaction centers and PSII electron transport are affected by interaction with Cd, metal-impairing enzyme activity, and/or protein structure. Chlorophyll *a* fluorescence carries information on efficiency of energy conversion at PSII reaction centers. The light-induced changes in fluorescence parameters are highly dynamic. When dark-adapted sample is illuminated, fluorescence yield first rises and then drops again (dark–light induction curves; Kautsky effect). Kinetics analysis reveals that characteristic changes in quantum yield and non-photochemical quenching accompany changes in fluorescence yield. In this experiment, changes in photosynthetic function of rice leaves treated with different levels of Cd were determined using various chlorophyll fluorescence parameters (Figs. 1 and 2). After 15-day Cd exposure, decreasing trend in Fv/Fm ratio and Y(II) with increasing Cd level in medium was observed, with Xiushui63 being even more suppressed. Similar results were observed in soybean [21], barley [22], and rice [20] of Fv/Fm value being decreased significantly under Cd stress. More decreased Fv/Fm and Y(II) in Xiushui63 represented PSII of Xiushui63 was more sensitive to Cd, indicating that relatively higher protective capacity of PSII should be an important tolerant mechanism for tolerant rice genotype. The photosynthetic decline of Cd-stressed plants may therefore be a consequence of damage to PSII reaction centers influenced photosynthetic electron transport or associated chlorophylls. Quantum yield of regulated energy dissipation reflects either all light-induced quenching processes or only rapidly reversible processes, usually considered to be regulatory [13]. Fast fluorescence induction kinetic measurements provided detailed information on response of leaves of rice plants when exposed to Cd stress. A dose-dependent increase in Y(NPQ) was observed through dynamics analysis, especially in Xiushui63. It indicated that photon flux density was more excessive at higher levels of Cd in the sensitive rice genotype. The study shows that the negative effect of Cd on Pn is attributed to complex physiological disturbances, mainly including inhibition of chlorophyll content, and reduction in Fv/Fm and PSII. Exogenous GSH could significantly alleviate inhibition in Xiushui63 in 5  $\mu\text{M}$  Cd and both genotypes in 50  $\mu\text{M}$  Cd. Addition of GSH significantly enhanced Pn, Fv/Fm, and Y(II) of plants under 5 or 50  $\mu\text{M}$  Cd stress, Tr and Gs under 5  $\mu\text{M}$  Cd level, but decreased Y(NPQ) and qN, indicating that exogenous GSH could effectively contribute to improving photosynthesis rate, especially in PSII. Meanwhile, GSH caused less Cd reaching shoots.

Cadmium is a strong inducer of PC accumulation in a broad variety of algae and plants as well as in several fungi, and PC-based Cd sequestration is generally considered to be essential for normal Cd tolerance in organisms [23]. In agreement with this viewpoint, strong Cd-induced PC accumulation was observed in Cd-stressed plant roots (Fig. 5), although no PCs were detected in control plants. Furthermore, an immediately faster increase in PC level on day 5 of 5  $\mu\text{M}$  Cd treatment in Bing97252 was observed compared with that in Xiushui63. Addition of GSH to 5  $\mu\text{M}$  Cd medium induced up-regulation in PC concentrations of Xiushui63 on day 5, and after that tend to be downregulated. This suggests that PC especially its effectively instantaneous production is a strategy to cope with toxic Cd exposure in respect to PC-dependent Cd tolerance in rice roots.

Cysteine is a precursor of GSH and other thiol compounds. Rauser and Meuwly [24] reported that Cys-rich complex can bond Cd in roots of maize seedlings from 19% after 1 day of exposure to 59% by day 7. In some algae (e.g., *Trebouxia* sp.), increased levels of Cys content were found in metal-tolerant ecotypes when compared to wild type also in metal untreated cells. In the present study, a sharp increase in Cys was observed at Cd, a

situation that exists in both genotypes. Increased rate was even much higher than that of GSH, indicating that thiol compounds play an important role as response to Cd toxicity in rice. In addition, as an antioxidant and a precursor of PCs, GSH was largely induced by Cd in roots. In this work, addition of GSH to Cd-stressed plants significantly increased root GSH concentrations, except at 5-day 50  $\mu\text{M}$  Cd exposure. Furthermore, both absolute content and increased rate of GSH in Cd-tolerant genotype Bing97252 after 5 days of Cd single exposure, compared with control, was higher than the sensitive genotype Xiushui63. Therefore, we conclude that tolerance to Cd stress was linked to the capacity to achieve instantaneous elevation of intracellular GSH as well as PCs.

**Acknowledgments** This work was supported by the Key Research Foundation of Zhejiang Bureau of Science and Technology (2009C12050). We thank Dr. Shiming Liu, from CSIRO Plant Industry of Australia, for his valuable remarks and comments to improve this manuscript. We also thank Ms. Jingqun Yuan from Analysis and Measurement Center of Zhejiang University for her excellent technical assistance with Agilent 1100 HPLC system; Ms. Jianghong Zhao and Mr. Yuanlong Li, from 985-Institute of Agrobiolgy and Environmental Science (985-IAES) of Zhejiang University, for their kind help with our experiment.

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