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RESEARCH ARTICLE

Phytochelatins formation kinetics and Cd-induced growth inhibition in *Lolium perenne* L. at elevated CO_2 level under Cd stress

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Abstract

Elevated CO_2 levels may alleviate toxicities induced by environmental stresses in plants, such as heavy metals. To assess this possibility, seedlings of *Lolium perenne* L. were exposed to different Cd stress and CO_2 levels during hydroponic culture. The kinetics of growth, Cd accumulation, and thiol formation were investigated. Elevated CO_2 levels increased the growth rate from 30 to 75%, and decreased the Cd accumulation rate from 36 to 42%, leading to a decrease of Cd content in root and shoot. However, an increase in Cd transport from root to shoot was observed at elevated CO_2 under Cd stress. The production of phytochelatins (PCs) occurred earlier at elevated CO_2 level than at ambient CO_2 level after exposure to Cd stress. The mean SH/Cd ratio was relatively higher at elevated CO_2 level, but elevated CO_2 level significantly decreased thiol contents. The reduction of Cd contents, earlier production of PCs, and relatively higher SH/Cd ratio at the elevated CO_2 level alleviated Cd toxicity in root and shoot to some extent, causing significant yield increase of L. *perenne* after exposure to Cd stress. This study could provide an important data support and theoretical basis in understanding the effects of elevated CO_2 on plant growth, heavy metal accumulation, and thiol formation.

Keywords Elevated CO_2 level · Cd · Phytochelatins · Kinetics · *L. perenne*

Introduction

Heavy metal contamination, caused by mining, manufacturing, usage of synthetic products, and land application of industrial or domestic sludge, has becomes a serious environmental problem especially in some developing countries like China (Li et al. 2010; Luo et al. 2010). Cadmium (Cd) causes higher toxicity in plants than other heavy metals due to its solubility, mobility, absorption, and incorporation into tissues (Shi et al. 2009; Lou et al. 2013). However, plants have developed various ways to combat Cd-induced adverse effects, such as chelating by low molecular weight thiol such as cysteine (Cys), glutathione (GSH), and phytochelatins (PCs) which play crucial roles in detoxifying mechanisms (Yadav 2010; Sytar et al. 2013; Wu et al. 2013; Negrin et al. 2017). In addition, Cys and GSH were involved in PCs syntheses (Pal and Rai 2020; Wu et al. 2013). The syntheses of PCs are catalyzed by phytochelatin synthase (PCS) using GSH as substrate and metal ion as activator (Wang and Wang 2009; Branco et al. 2010; Etelvina and Victoria 2014), and dependent upon plant species, the toxic degree of metal ions and interactions among metals (Etelvina and Victoria 2014). It was reported that PCs production occurred earlier than any other physiological parameter and could be used as biochemical indicators/markers to assess metal toxicity to biota (Wang and Wang 2009; Branco et al. 2010; Ju et al. 2011). Morelli et al. (2009) found that the determination of PCs was a better approach than chemical analyses of metals.

The CO_2 level in the atmosphere has been continuously rising due to the ongoing combustion of fossil fuels, resulting

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in the well-known consequences of the greenhouse effect and global climate change (Li et al. 2010; Jia et al. 2010). Plants adapt to elevated CO_2 levels through alterations of physiological and physicochemical processes, and it was summarized that elevated CO_2 level increased net photosynthetic rate and carbon assimilation, but decreased photorespiration and oxidative stress (Jia et al. 2010). The effects of elevated CO_2 level on plant growth, development, and element uptake under Cu, Cd, or Cs stress have been investigated, and results generally demonstrated that elevated CO_2 level alleviated the toxicities of heavy metals in plants to some extent (Li et al. 2010; Jia et al. 2010). However, very little information is available regarding the effects of elevated CO_2 level on phytochelatins formation kinetics and Cd-induced growth inhibition in *L. perenne*.

On the basis of previous studies, it is hypothesized that elevated CO_2 could alleviate Cd toxicity through increasing growth rate, reducing Cd accumulation rate and favoring thiol formation in plants. Therefore, the object of the present study was to investigate the kinetics of growth, Cd accumulation, and thiol formation in *L. perenne* exposed to Cd stress under ambient and elevated CO_2 levels. The knowledge gained might constitute an important advancement in the understanding of the effects of elevated CO_2 on plant growth, heavy metals accumulation, and formation of thiol especially PCs, and the mechanisms of elevated CO_2 in terms of alleviation toxicities of heavy metals in plants, possibly providing evidence for potential phytoremediation practices.

Materials and methods

Plant growth and Cd exposure

Seeds of *L. perenne* were obtained from the Plant Protection Institute, Chinese Academy of Agricultural Sciences (CAAS), P.R. China. Seeds were soaked in 1% (w/v) sodium hypochlorite (NaClO) solution for 15 min, washed with deionized water several times, sown into moist mixture of vermiculite and perlite (v:v, 1:1), and kept in growth chamber at 25 °C, with 16/8 h photoperiod at 105 μmol·m^{-2} s^{-1} and a relative humidity of 60%.

One week after planting, the containers were filled with full Hoagland's solution, and the culture medium was renewed once a week. The Hoagland's solution consisted of 4 mmol·L^{-1} Ca $(NO_3)_2 \cdot 4H_2O$, 6 mmol·L^{-1} KNO_3, 2 mmol·L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mmol·L^{-1} $NH_4H_2PO_4$, 15 μmol·L^{-1} H_3BO_3, 1 μmol·L^{-1} $MnSO_4 \cdot 4H_2O$, 0.5 μmol·L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.2 μmol·L^{-1} $CuSO_4 \cdot 5H_2O$, 0.01 μmol·L^{-1} $(NH_4)_6Mo_7O_{24}$, and 100 μmol·L^{-1} Fe-EDTA. Solution pH was adjusted to about 6.5 by 0.1 mol·L^{-1} NaOH solution.

After growth for 2 weeks, healthy and uniform-sized seedlings were randomly transplanted into plastic containers (length 45 cm × width 15 cm × height 15 cm) with 10 L aerated hydroponic culture of half-strength Hoagland's solution at a density of 10 seedlings per container, and kept in a controlled environmental room under the same condition as described above.

After growth for 3 weeks in full Hoagland's solution, the seedlings were randomly sorted into two groups, and each set was randomly divided into three subsets, in which seedlings were exposed to 0, 20, or 80 μmol·L^{-1} of Cd. A solution Cd^{2+} was supplied with $2CdCl_2 \cdot 5H_2O$. Four independent repeats were performed with each pot.

During the experimental period, the two sets of seedlings were grown in two controlled environment rooms under the same conditions as described above with the exception of CO_2 level. The carbon dioxide concentration in one chamber was maintained at 760 ± 28 μL·L^{-1} as elevated CO_2, and in the other, maintained at 380 ± 12 μL·L^{-1} as ambient CO_2, using an automatically controlled CO_2 release facility connected to CO_2 cylinders.

Sample collection

One of the ten seedlings in each container was randomly collected and separated into the root and shoot after 0, 1, 3, 6, 12, 24, 48, 72, 144, and 216 h of exposure to the Cd solution and CO_2 level. The intensive monitoring of many time points was set to observe clearer change of dry weight of root and shoot of *Lolium perenne L.* seedlings. Root and shoot, respectively, were each cut into small pieces (less than 0.5 cm in length) with stainless steel scissors and randomly divided into several subsamples. One subsample (approximately 0.20 g) was wrapped with tin foil, frozen in liquid N_2, and stored in dark at − 80 °C for thiol analyses. The remaining subsample was dried in an oven at 75 °C for 72 h, weighed, and ground into a fine, homogeneous powder using a stainless steel cutter-blender (IKA T2500, Germany) for Cd assay.

Cadmium quantification

Aliquots (approximately 0.30 g) of fine homogeneous powder were digested with 8:1 (v/v) HNO_3/H_2O_2 mixture using a microwave digestion system (CEM MARS 240/50, USA), and analyzed for Cd content by an Atomic Absorption Spectrometer (ZEEnit 700, Analytik Jena, Germany). Calibration curves were established using a commercial standard solution at a concentration of 1000 mg·L^{-1} purchased from Sigma Chemical. The reliability of the digestion and analytical procedure was assessed using blanks and standards as QA/QC samples.

Extraction procedure and derivatization of thiol

The samples (approximately 0.20 g), previously stored in the dark at − 80 °C, were homogenized in liquid N_2 using mortar and pestle, and 1.8 mL of extraction buffer containing 0.1% (v/v) TFA and 5 mmol·L^{-1} DTPA were added. The homogenates were transferred to a 2-mL centrifuge tube, vigorously mixed, and centrifuged at 12,000*g* for 10 min at 4 °C. The resulting supernatants were immediately put into derivatization of thiol.

The supernatant (250 μL) was transferred and mixed with 650 μL of 200 mmol·L^{-1} HEPES buffer (pH about 9.0) in 5 mmol·L^{-1} DTPA and 25 μL of 20 mmol·L^{-1} TCEP in HEPES buffer. This reaction mix was pre-incubated at room temperature (25 °C) for 5 min and the derivatization was performed by incubating the mix in dark for 30 min at room temperature (25 °C) after adding 20 μL of 50 mmol·L^{-1} mBBr in ACN. The reaction was terminated by adding 100 μL of 1 mol·L^{-1} MSA. The derivatized samples were filtered with 0.20-μm nylon syringe filters (Millipore Corp. Bedford, MA, USA) for HPLC analysis. The whole protocol was conducted in darkness and speed.

HPLC analysis of thiol

The separation of derivatized thiol was performed using an Agilent Technologies 1200 series HPLC system (Agilent Technologies Inc., Hambruecker Landstrasse, Waghaeusel-Wiesental, Germany) consisting of a quaternary pump with degasser, thermostat for ALS/FC/Spotter, thermostatted column compartment, diode array detector, fluorescence detector, and autosampler fitted with a 100-μL loop. The column was an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 30 mm, 1.8 μm; Agilent Technologies Inc., Princeton, MN, USA). The temperatures of the sample capsule and the column oven were maintained at 4 °C and 25 °C, respectively. The excitation and emission wavelengths were set at 380 and 470 nm, respectively. Data were integrated using ChemStation software (Agilent Technologies Inc., Version B.03.02).

Derivatized samples (20, 50, or 100 μL) were run with linear gradient elution. Solvent A was 0.1% (v/v) TFA in water and solvent B was ACN. The gradient profile was described as 0–20 min, 8–26% B; 20–22 min, 26–100% B; 22–24 min, isocratic 100% B; 24–28 min, 100–8% B; 28–30 min, isocratic 8% B, and total analysis time was 30 min. The flow rate was 0.8 mL·min^{-1}. All solvents were filtered with a 0.2-μm nylon filter (Nylaflo; Pall Corp., Ann Arbor, MI, USA) and degassed before use. The identification was carried out by comparing the traces obtained from blank (extract buffer), sample extract and standards of Cys, GSH (Fluka, Milwaukee, WI, USA), and $PC_{2\text{-}6}$ (AnaSpec, San Jose, CA, USA), and the quantification was performed by external standard method.

Mathematical and statistical analysis

The root/shoot ratio is one measure to assess the overall health of plants and is obtained by comparing root dry weight to shoot dry weight. The tolerance index (Ti) (Jia et al. 2010) represents the ability of plants to survive exposed to Cd stress, and it is calculated according to the equation: Ti = average of root or shoot biomass exposed to Cd stress /average of root or shoot biomass in control. The translocation accumulation factor (TAF) represents the distribution of plant growth and heavy metal absorption in above ground and underground organs, as an important index to evaluate the remediation capacity of plants (Nie 2005), and TAF = Cd content in shoot × shoot biomass)/Cd content in root × root biomass (Lin et al. 2014). The SH/Cd ratio represents the chelating ability of SH in root or shoot and is calculated as total SH content in the root or shoot × fresh weight of root or shoot/ (Cd content in the root or shoot × dry weight of root or shoot).

All experiments were conducted with four replicates, and results are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using PASW Statistics (SPSS Inc., USA, and release 18.0.0) and the figure was drawn using Origin Pro (Origin Lab Corp., USA, v8.0724). Difference was significant at the 5% level.

Results

Plant growth

The growth of *L. perenne* seedlings was promoted under elevated CO_2 and was inhibited after exposure to Cd stress (Fig. 1 and Table 1). Regardless of CO_2 level and Cd concentration, the dry weight of *L. perenne* seedlings increased with time. During the time course, the average dry weight at elevated CO_2 respectively increased by 5.22%, 8.73%, and 9.31% in roots at Cd concentrations of 0, 20, and 80 mol·L^{-1} compared to the ambient CO_2 control. Meanwhile, the average dry weight at elevated CO_2 respectively increased by 7.89%, 10.88%, and 15.05% in shoots at Cd concentrations of 0, 20, and 80 mol·L^{-1} compared to the ambient CO_2 control.

Previous studies have shown that root growth of both *L. perenne* and *T. repens* increased under elevated CO_2 conditions (Jongen et al. 1995; Blagodatskaya et al. 2010). Shoot biomass at elevated CO_2 significantly increased after 48 h for the same Cd concentration treatment ($p < 0.05$). Meanwhile, after treatment with the same Cd concentration and exposure time, the increase rate (%) in shoot stimulated by elevated CO_2 was relatively greater than its in root ($p < 0.05$).

However, the growth inhibition of root and shoot was aggravated by longer exposure duration in each process, and more severity of growth inhibition was observed in root than

its in shoot at different CO_2 concentrations and Cd concentrations, the growth inhibition of root and shoot increased with the prolongation of exposure time, and more severity of growth inhibition was observed in roots than its in shoots (Fig. 2). That may be related to higher concentrations of Cd in the roots than in the shoots (Jia et al. 2011). The growth inhibition both in root and shoot became significant after 48 h of Cd exposure at ambient CO_2, and 72 h at elevated CO_2, respectively (Fig. 1). At elevated CO_2, root biomass increased significantly after 72 h of exposure to 20 μmol·L^{-1} and 80 μmol·L^{-1} Cd stress compared to that at ambient CO_2 ($p < 0.05$), and Cd control treatment did not increase. The biomass of shoot had significantly increased after 48 h at elevated CO_2 of exposure to Cd stress and Cd control treatment. During the time course, Ti in roots generally began to decrease below 1 after 48 h at ambient CO_2, and decreased with increasing of Cd concentration and treatment time (Table S1). At elevated CO_2, the Ti in shoot generally started to be less than 1 after 72 h, and decreased with increasing of Cd concentration and treatment time (Table S1). Ti in roots and shoots at elevated CO_2 was relatively greater than at ambient CO_2 after exposure to the same Cd concentration and treatment time (Table S1). Therefore, elevated CO_2 delayed the growth inhibition of *L. perenne* and increased the biomass of *L. perenne*.

Accumulation of Cd

The accumulation of Cd in root and shoot was dependent upon CO_2 level, Cd stress concentration, and exposure duration (Fig. 2 and Table 1). The Cd contents in root and shoot were lower at elevated CO_2 than the ambient CO_2 control for the same Cd concentration treatment, and the Cd contents in root were much higher than those in shoot at the same CO_2 level. During the first 6 h, there was no significant difference in the Cd content in shoot treated both with 20 and 80 μmol·L^{-1} Cd ($p > 0.05$) (Fig. 2a). By contrast, the Cd contents in root exposed to 80 μmol·L^{-1} Cd were higher than those exposed to 20 μmol·L^{-1} Cd (Fig. 2b). It should be noted that after 24 h of Cd exposure, there was a significant reduction in the Cd contents in root and shoot at elevated CO_2, compared to the ambient CO_2 control ($p < 0.05$). Additionally, the reduction of Cd contents could alleviate the Cd-induced growth inhibition. After exposure to 20 μmol·L^{-1} and 80 μmol·L^{-1} Cd stress, the accumulation of Cd in root and shoot significantly increased with the exposure duration irrespective of the CO_2 level. Furthermore, the effects of CO_2 level, Cd concentration, and exposure time on TAFs were analyzed in the present study (Table 1), and it was found that the alteration of TAFs has a positive correlation with CO_2 level ($r = 0.179$, $p < 0.05$), which meant that TAFs increased at elevated CO_2 under Cd stress.

Syntheses of thiol

At the start of treatment, the content of GSH in shoot was (287.9 ± 33.6) nmol/g shoot fresh weight (mean ± SD, $n = 24$), and the GSH took account of total thiols in shoot about 90%. Meanwhile, the content of GSH in root was (34.0 ± 1.0) nmol/g root fresh weight (mean ± SD, $n = 24$), and the GSH took account of total thiols in root about 40%. After treatment with Cd, the proportion of GSH in root and shoot significantly reduced; however, the proportion in root was nearly 2.0% for 216 h, and always more than 50% in shoot. The proportion of PC_2 in root firstly increased and then decreased, in shoot continually increased. In general, the proportion of PC_3 in root and shoot presented increasing trend under Cd stress, especially after treatment of 12 h, the proportion of PC_3 markedly increased, and that maintained about 70% of total thiols in root

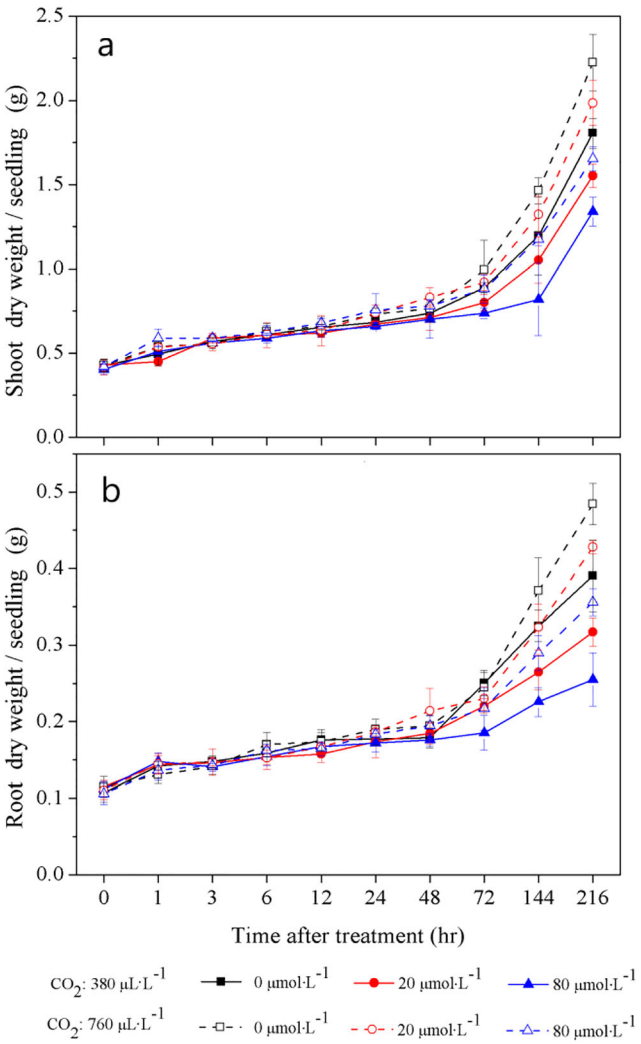

Fig. 1 Dry weight of root and shoot of *Lolium perenne* L. seedlings. Six-week-old seedlings were exposed to Cd at concentration of 0 (■), 20 (●), and 80 μmol·L^{-1} (▲) for 216 h at CO_2 level of 380 μL·L^{-1} (solid line) and 760 μL·L^{-1}(dash line), respectively. Points, means of four replicates. Vertical lines exceeding the symbols, SD

Table 1 Impacts of CO_2 level, Cd concentration, and exposure time on biomass, Cd contents, and thiols contents in *L. perenne* seedlings

	Biomass			Cd			Thiols in root								Thiols in shoot								SH:Cd	
	Root	Shoot	Ratio	Root	Shoot	TAFs	Cys	GSH	PC_2	PC_3	PC_4	PC_5	PC_6	Total	Cys	GSH	PC_2	PC_3	PC_4	PC_5	PC_6	Total	Root	Shoot
CO_2	**	**	*	**	**	**	*	*	**	**	**	**	*	**	**	**	**	**	**	**	ns	**	**	ns
Cd	**	**	ns	**	**	**	**	**	**	**	**	**	**	**	**	*	**	**	**	**	ns	**	**	**
Thiols	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
CO_2×Cd	ns	ns	ns	**	**	**	**	**	**	**	**	**	**	**	*	ns	**	**	**	**	**	*	ns	ns
CO_2×thiols	**	**	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Cd×thiols	**	**	ns	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
CO_2×Cd×thiols	ns	ns	ns	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	ns

**The difference is significant at the 0.01 level

*The difference is significant at the 0.05 level

ns, the difference is not significant at the 0.05 level

and 10% in shoot after treatment of 48 h. The proportion of PC_4 and PC_5 presented the same trend with PC_3 in roots and shoots. The proportion of PC_6 in roots firstly reduced and then slightly increased, and that altered slightly in shoots.

In most cases, the CO_2 concentration had a negative effect on thiol content (Figs. 3, 4, 5, 6, 7, 8, and Figs. S3 and S4, and Table 1). During the first 24 h, the total contents of SH (the SH is functional group which is composed of sulfur atom and a hydrogen atom) in root increased first and then reduced irrespective of CO_2 concentration with the exception of 80 μmol·L^{-1} Cd treatment at ambient CO_2. After 24 h of Cd exposure, the contents of total SH in root rapidly increased (Fig. 3a). Regardless of Cd concentration in solution, no significant changes were found in the total SH contents in shoot during the first 12 h and 24 h at ambient and elevated CO_2 level, and then continually increased (Fig. 3b).

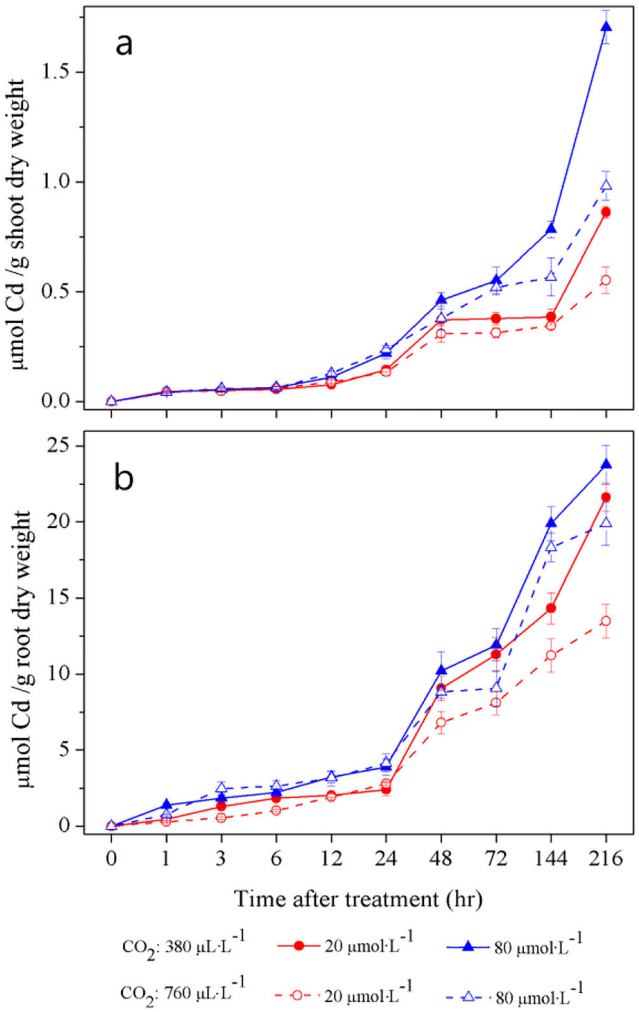

Fig. 2 Cd contents in root and shoot of *L. perenne* seedlings. Six-week-old seedlings were exposed to Cd at concentration of 20 (●) and 80 μmol·L^{-1} (▲) for 216 h at CO_2 level of 380 μL·L^{-1} (solid line) and 760 μL·L^{-1}(dash line), respectively. Points, means of four replicates. Vertical lines exceeding the symbols, SD

Regardless of Cd concentration, the contents of Cys in root exhibited a reduction trend during the first 12 h and 24 h at the ambient and elevated CO_2 level, respectively, then dramatically increased and reached a maximum at 72 h before decreasing rapidly (Fig. 4a). At the ambient CO_2 level, the maximum content of Cys in root was observed at 72 h exposure of Cd at three different Cd concentrations, whereas at elevated CO_2 level, it occurred after 72 h, 48 h, and 144 h exposure to 0 μmol·L^{-1}, 20 μmol·L^{-1}, and 80 μmol·L^{-1} Cd, respectively. The contents of Cys in shoot varied without significance during the first 12 h and 24 h at the ambient and elevated CO_2 level, respectively, and then increased to reach its peak, and finally decreased over time (Fig. 4b). Regardless of Cd concentrations, the peak contents were observed at 72 h exposure at the ambient CO_2 level, whereas it occurred at 48 h in Cd control and 144 h of Cd exposure at the elevated CO_2 level.

The GSH content exhibited a response pattern similar to the Cys content in most cases. At ambient CO_2 level, the root GSH content decreased during the first 12 h after Cd exposure, whereas at elevated CO_2 level, this trend appeared much less pronounced (Fig. 5a). At ambient CO_2 level, the GSH content dramatically increased after 24 h of exposure to 20 μmol·L^{-1} and 80 μmol·L^{-1} Cd stress, reached its maximum at 72 h, and then slightly decreased. However, the dramatic increase in the GSH content at elevated CO_2 level occurred after 12 h of exposure to Cd stress and the peak content was observed at 144 h. At ambient CO_2 level, the shoot GSH content was generally lower under Cd stress than the Cd control during the first 48 h; however, the variation trend became the opposite after 48 h exposure. By contrast, the shoot GSH content at elevated CO_2 level varied a little during the first 72 h, and afterward, it was generally higher under Cd stress than the Cd control (Fig. 5b).

There was little variation in root and shoot PC_2 content in the Cd control treatment at two CO_2 levels. At ambient CO_2 and under Cd stress, the root PC_2 content kept increasing until 72 h, subsequently, slightly decreased, whereas at elevated CO_2, it continuously increased during the time course (Fig. 6a, b). The shoot PC_2 content kept increasing with the time course irrespective of CO_2 levels and Cd concentrations.

An increase in root $PC_{3\text{-}6}$ contents was observed under 20 μmol·L^{-1} and 80 μmol·L^{-1} Cd stress regardless of CO_2 levels while in the Cd control treatment, very little variation was observed (Fig. S1a, S2a, S3a, and S4a). After exposure to 20 μmol·L^{-1} and 80 μmol·L^{-1} Cd stress, $PC_{3\text{-}5}$ contents in shoots tended to increase irrespective of Cd concentrations and CO_2 levels (Fig. S1b, S2b, and S3b), and PC_6 contents had no obvious regularity with time (Fig. S4b).

The proportion of thiol was significantly altered after exposure to Cd stress at the ambient and elevated CO_2 level, and it was noted that after treatment with Cd, the thiol in root were mainly PC_3 and PC_4, while the thiols in shoots were primarily GSH and PC_3. However, PC production occurred when the Cd

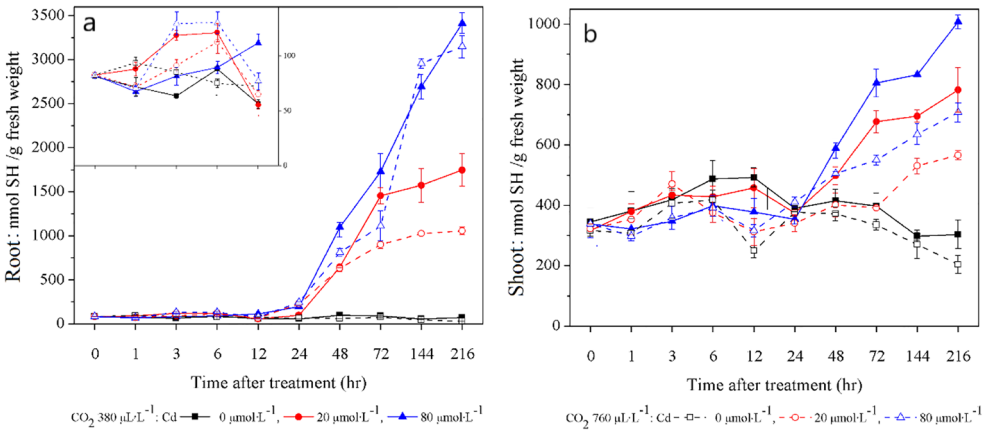

Fig. 3 SH contents in root and shoot of *Lolium perenne* L. seedlings. Six-week-old seedlings were exposed to Cd at concentration of 20 (●) and 80 μmol·L^{-1} (▲) for 216 h at CO_2 level of 380 μL·L^{-1} (solid line) and 760 μL·L^{-1}(dash line), respectively. Points, means of four replicates. Vertical lines exceeding the symbols, SD. In addition, it is hard to see the trend of the line from 0 to 12 h; thus, there is a magnified graph in the top left corner

concentration was 0 μmol·L $^{-1}$ due to the presence of other metal ions other than Cd (Figs. 7 and 8). During the time course, the proportion of Cys in roots dramatically decreased at 20 μmol·L^{-1} and 80 μmol·L^{-1} Cd stress and altered slightly in shoots after exposed to Cd stress.

Relationship of thiol and Cd

The binding ability of thiol including Cys, GSH, and $PC_{2\text{-}6}$ was evaluated by determining the molar ratio of thiol to Cd during the time course study. The SH/Cd ratio in roots decreased firstly and increase with ranging from 3.07 to 0.22, and in shoots, dramatically decreased during the time course from 69.64 to 4.57 (Table S1). During the time course, the overall mean of SH/Cd ratio in roots and shoots reduced with increasing Cd concentration at the same CO_2 level and was relatively higher at elevated CO_2 than at ambient CO_2 in roots, and no significant alteration was observed in shoots ($p > 0.05$).

The Cd contents and total SH contents in roots and shoots are plotted against each other in Fig. S5 exposure to Cd stress. The correlation between Cd and total SH contents in roots and shoots was calculated to estimate the importance of SH in the detoxification mechanism after exposure to Cd. Generally, the correlations of content of total SH and Cd were expressed as $C_{\text{total SH in fresh root}} = -126.279 + 130.272 C_{\text{Cd in dry root}}$ ($n = 160$, $r^2 = 0.894$, $p<0.01$) and $C_{\text{total SH in fresh shoot}} = 348.845 + 448.351 C_{\text{Cd in dry shoot}}$ ($n = 160$, $r^2 = 0.781$, $p<0.01$) in roots and shoots, respectively. There was a strong correlation between total SH content and Cd content in both root and shoot ($p<0.01$), indicating that the synthesis of thiols was closely related to Cd uptake.

Discussion

Increasing attention has been paid to single and/or interactive effects on plants of elevated CO_2 and salinity (Shimono et al. 2012; Zaghdoud et al. 2013), ozone (Clausen et al. 2011; Gillespie et al. 2011), cold (Tyagi et al. 2014), drought (Hebbar et al. 2013; Wang et al. 2017), organic pollutants (Hagedorn and Machwitz 2017), heavy metals (Li et al. 2010; Jia et al. 2010), or heat (Darbah et al. 2011; Pendall et al. 2016). Based on previous studies, it was found that elevated CO_2 could ameliorate the toxicity and damages induced by these environmental stresses to some extent, and the same result was obtained in present study.

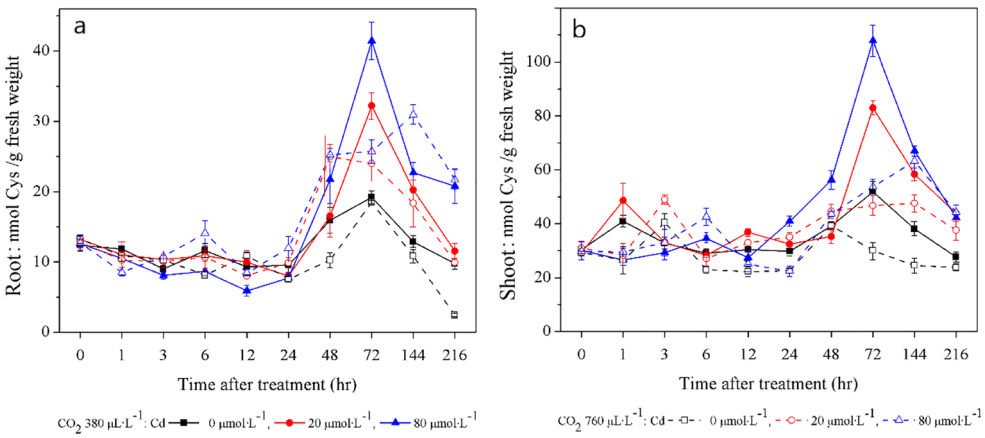

Fig. 4 Cys contents in root and shoot of *Lolium perenne* L. seedlings. Six-week-old seedlings were exposed to Cd at concentration of 20 (●) and 80 μmol·L^{-1} (▲) for 216 h at CO_2 level of 380 μL·L^{-1} (solid line) and 760 μL·L^{-1}(dash line), respectively. Points, means of four replicates. Vertical lines exceeding the symbols, SD

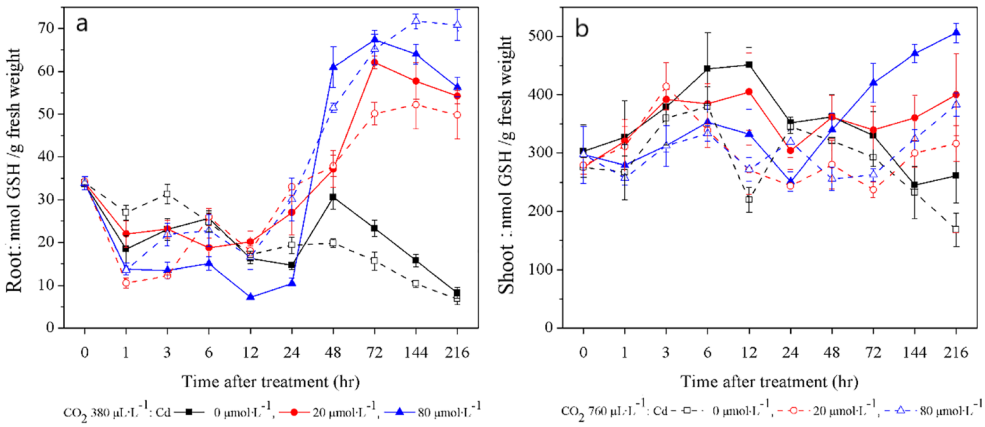

Fig. 5 GSH contents in root and shoot of *Lolium perenne* L. seedlings. Six-week-old seedlings were exposed to Cd at concentration of 20 (●) and 80 μmol·L^{-1} (▲) for 216 h at CO_2 level of 380 μL·L^{-1} (solid line) and 760 μL·L^{-1}(dash line), respectively. Points, means of four replicates. Vertical lines exceeding the symbols, SD

Elevated CO_2 stimulates plant growth

Cd toxicity is generally characterized by the inhibition of root and shoot biomass production, leaf chlorosis, and a loss of photosynthetic activity, as well as formation of free radicals (Jia et al. 2010; Dias et al. 2013; SALEHI 2014; Muradoglu et al. 2015). The effects of elevated CO_2 on plants were summarized in terms of increasing photosynthetic rate and carbon assimilation (Martínez-Lüscher et al. 2015), as well as reduction of photorespiration and oxidative stress (Rogers et al. 2004). The toxicity and damage induced by Cd in plants were ameliorated under the elevated CO_2 conditions, resulting in greater biomass production with Cd-induced damage (Li et al. 2010; Jia et al. 2010). This study showed that the growth of *L. perenne* was significantly inhibited after exposure to Cd at both ambient and elevated CO_2 levels. Inhibition of growth caused by Cd was greater in root than that in shoot, and the improvement in shoot was more than that in root at elevated CO_2, causing a reduction of root/shoot ratio with the increase of exposure duration (Table S1). On the contrary, the growth of *L. perenne* seedlings was improved at elevated CO_2 compared with those at ambient CO_2 when exposed to the same concentration of Cd (Table S1). It was found that the growth inhibition occurred earlier in root after exposure to Cd stress at both CO_2 levels, and the growth inhibition occurred later at elevated CO_2 levels after exposure to the same Cd concentration, which indicated that elevated CO_2 may increase tolerance of *L. perenne* to Cd stress.

Elevated CO_2 helps Cd transportation

Cd contents in roots and shoots were significantly reduced at elevated CO_2 level in comparison with those at ambient CO_2, which may be due to an increase in growth rate and a reduction of Cd concentration in *L. perenne*. (Table S1). The similar phenomenon has also been observed by Li et al. (2010) and Jia et al. (2010, 2011), which might be formed due to the dilution effect of elevated CO_2 (Loladze 2002). Elevated CO_2 could decrease essential microelements in plants (Yang et al. 2007; Zheng et al. 2008; Rajashekar 2018; Senghor et al. 2017). The reduction of Cd contents could ameliorate the Cd toxicity to plant, enhance the tolerance ability, and reduce the food safety risk to some extent (Jia et al. 2010). In present study, it was also found that translocation accumulation factor (TAF) increased at elevated CO_2 under Cd stress, and it was previously found that transport index (Ti) was higher under elevated CO_2 than under ambient CO_2, regardless of Cd concentrations and exposure times (Jia et al. 2011). Therefore, the very meaningful results were obtained that elevated CO_2 could increase Cd

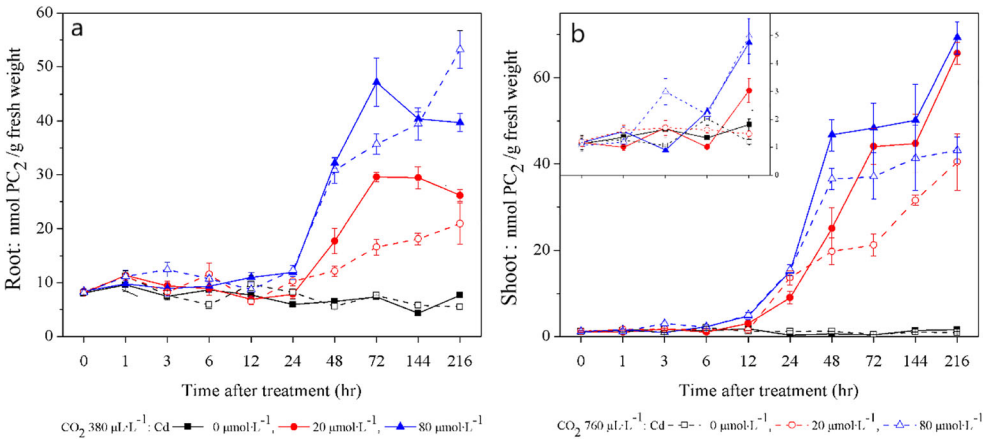

Fig. 6 PC_2 contents in root and shoot of *Lolium perenne* L. seedlings. Six-week-old seedlings were exposed to Cd at concentration of 20 (●) and 80 μmol·L^{-1} (▲) for 216 h at CO_2 level of 380 μL·L^{-1} (solid line) and 760 μL·L^{-1}(dash line), respectively. Points, means of four replicates. Vertical lines exceeding the symbols, SD

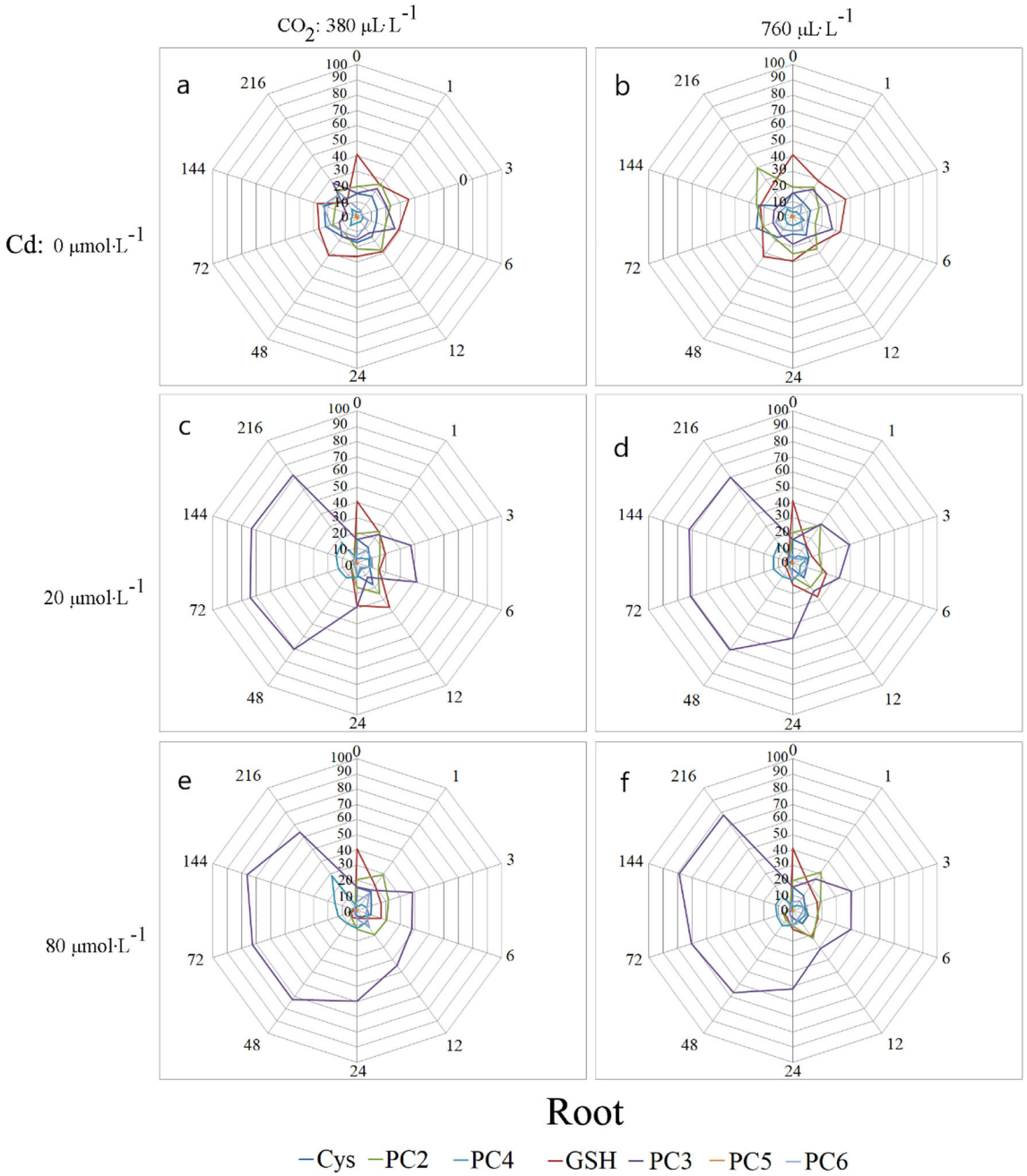

Fig. 7 Thiols proportion in of *Lolium perenne* L. seedlings. Six-week-old seedlings were exposed to Cd at concentration of 0, 20, and 80 μmol·L^{-1} for 216 h at CO_2 levels of 380 μL·L^{-1} and 760 μL·L^{-1}, respectively

translocation accumulation from root to shoot, indicating that L. *perenne* has potential for use in phytoremediation under elevated CO_2 levels, according to according to Nie (2005).

Elevated CO2 favors the synthesis of thiol especially PCs

There are many ways for plants to resist Cd stress (Pal and Rai 2020). Thiol including Cys, GSH, and PCs play important roles in detoxification under Cd stress (Sytar et al. 2013) by chelation of Cd and compartmentalization of thiol-Cd compounds into the vacuoles (Clemens and Peršoh 2009; Guo et al. 2012), whose syntheses are related to Cd concentration in plant tissues (Choppala et al. 2014). This study found that the contents and proportion of thiol, especially $PC_{2\text{-}6}$, increased after treatment with Cd in root and shoot at both CO_2 levels, which occurred earlier than growth inhibition occurred. In present study, the content of GSH in roots and shoots significantly reduced after treatment of Cd, but the proportion of GSH accounted for above 50% of total thiols

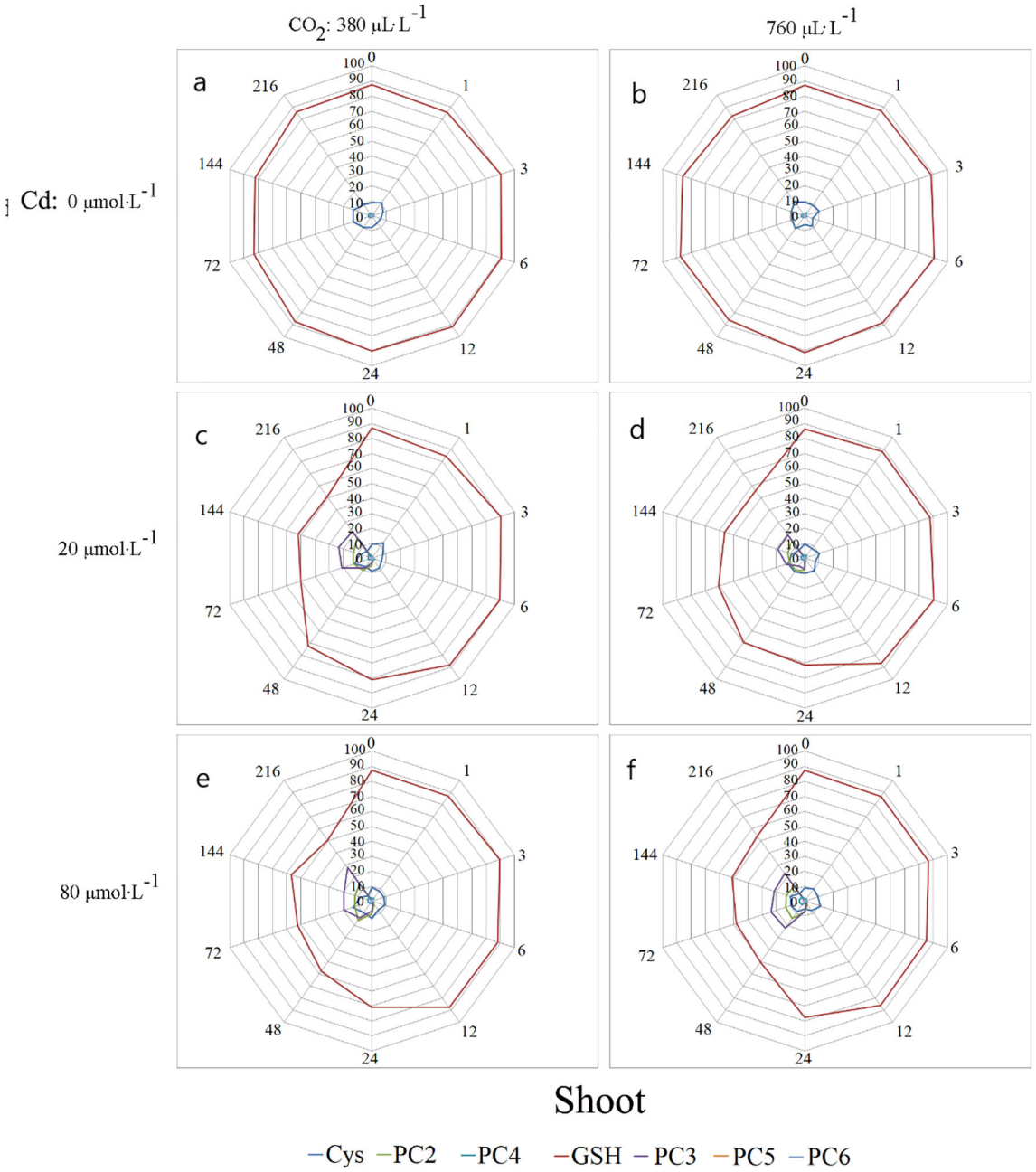

Fig. 8 Thiols proportion in shoot of *L. perenne* seedlings. Six-week-old seedlings were exposed to Cd at concentration of 0, 20, and 80 μmol·L^{-1} for 216 h at CO_2 levels of 380 μL L^{-1} and 760 μL·L^{-1}, respectively

in shoots. The content of PC_2 firstly increased and then decreased in roots under Cd stress, and continually increased in shoots. The content of PC_3, PC_4, and PC_5 in roots and shoots increased under Cd stress, especially after treatment of 12 h, which occurred earlier than growth inhibition occurred. The content of PC_6 in roots firstly reduced and then slightly increased after treatment of 72 h, and that altered slightly in shoots. The different alterations of GSH and $PC_{2\text{-}6}$ in roots and shoots indicated that different sulfhydryl compounds played different roles in detoxification in roots and shoots. In roots, the thiols of PC_2, PC_3, and PC_4 mainly performed the roles of detoxification after treatment of Cd, yet, GSH, PC_2, and PC_3 in shoots.

It has been proved that thiols especially PCs participate in Cd transportation from shoot to root and from root to shoot, and the transported PCs are possible to bind and aid in long-distance Cd transport (Gong et al. 2003; Chen et al. 2006). Under the elevated CO_2, higher molecular PCs (PC_4, PC_5, PC_6) were observed in both shoots and roots (Jia et al. 2011), which is possible to explain that the enhanced PCs products in roots under elevated CO_2 helped the transport of Cd from roots to above-ground parts. In present study, thiol

content was generally reduced at elevated CO_2, which may be caused by the dilution effect and lower Cd contents in plants. However, the production of PCs occurred earlier at elevated CO_2 than that at ambient CO_2, which was involved in the improvement of plant growth and earlier Cd transport from root to shoot.

It was found that Cd stress significantly altered the SH/ Cd ratio in both roots and shoots ($p < 0.01$) (Table 1), and elevated CO_2 significantly altered the SH/ Cd ratio in roots ($p < 0.01$), but no alteration was obtained in shoots ($p > 0.05$). In previous study, the PC-Cd ratios were not affected by elevated CO_2 in roots and shoots of *L. mutiforum* and *L. perenne* after growth for 58 days in Cd-contaminated soil (Jia et al. 2010). In shoot, the SH/Cd ratio was higher than 2.0, indicating that enough thiol were produced against Cd stress and elevated CO_2 scarcely altered the SH/Cd ratio to counter Cd toxicity in shoot. However, thiol in root could not effectively combat Cd stress, and additional ways such as cysteine and glutathione act, therefore, were involved in detoxification in *L. perenne* exposed to Cd at ambient and elevated CO_2 (Jia et al. 2010). The overall mean of the SH/Cd ratio in root was relatively higher at elevated CO_2 than at ambient CO_2, which may provide higher Cd binding ability of thiol in root and help Cd transport from root to shoot.

The PCs have been expected to be useful as biomarkers of environmental metal pollution, since they were identified (Grill et al. 1985), for the reason that the relationship between the metal uptake and the synthesis of PCs has a significant implication regarding metal toxicity. The similar results were obtained in present study that the synthesis of thiols was closely related to Cd uptake. However, in order to better understand the role of PCs in detoxification and utilize PCs as biomarkers, the PC kinetics-metal uptake-metal sensitivity relationship must be directly tested using quantitative experiments (Wu et al. 2016).

Conclusions

Elevated CO_2 resulted in a significant reduction of Cd content in root and shoot, and an increase of Cd translocation accumulation from root to shoot. The production of PCs occurred earlier than growth inhibition, which similarly occurred earlier at elevated CO_2 level than at ambient CO_2 level after exposure to Cd stress. Additionally, the average value of the SH/Cd ratio increased as the CO_2 concentration increased, and the reduction of Cd contents, earlier production of PCs, and relatively higher SH/Cd ratio due to elevated CO_2 level alleviated Cd toxicity in root and shoot to some extent. In summary, at elevated CO_2 level, not only Cd toxicity was alleviated in *L. perenne* to some extent, Cd translocation accumulation from root to shoot were obtained after exposure to Cd stress, but also the significant increases of yields of *L. perenne*. Those were shown that *L. perenne* has great potential for phytoremediation of Cd-contaminated environment combining with elevated CO_2 level.

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YQL—carrying out the experiment
HYL—chemical analysis and interpretation
HPP—statistical calculations
YX and XHJ—manuscript preparation and overall corrections

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Declarations

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Competing interests The authors declare no competing interests.

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