Cu and Cd induced Cytotoxicity Involving Lipid Peroxidation and Sulfhydryl Compounds in the Hyperaccumulator and Nonaccumulator Varieties of *Commelina Communis*

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***Abstract*—The ability to accumulate Cu and Cd was investigated in Cu hyperaccumulator and nonaccumulator varieties of *Commelina communis*. Furthermore, the role of malondialdehyde (MDA), glutathione (GSH), and phytochelatin (PC) in the detoxification mechanisms used by the hyperaccumulator *C. communis* to cope with heavy metals were investigated. Results showed that Cu and Cd contents of both leaves and roots in the hyperaccumulator were higher than those in the nonaccumulator. However, the hyperaccumulator variety could have a more powerful ability to transport Cu and Cd from roots to shoots, thus decreasing the toxicity risk. Meanwhile, the MDA, GSH, and PC contents in the hyperaccumulator were significantly lower than those in the nonaccumulator under Cu and Cd stress, indicating that the former can utilize these sulfhydryl compounds to reduce toxicity caused by metal ions. Thus, the hyperaccumulator can tolerate heavy metal-induced toxicity better than the nonaccumulator.**

***Index Terms*—Heavy metals, malondialdehyde, glutathione, phytochelatin.**

I. Introduction

Cu is an essential micronutrient for plants, a component of several electron transport enzymes, and is involved in catalyzing redox reactions in mitochondria and chloroplasts [1]. However, Cu also induces toxicity at tissue concentrations slightly above its optimal levels [2]. It can produce a mass of reactive oxygen species, damage cell membranes, and accumulate peroxide [3]. Glutathione (GSH) plays an important role in the heavy metal detoxification mechanism of plants [4]. GSH is a ubiquitous molecule with several roles in cell metabolism, including reactive oxygen species processing, redox state regulation, transport of amino acids, and sulfur storage [5], [6]. A function of GSH could be the chelation of toxic Cd (II) ions during their transport through the cytoplasm [7]. GSH is also used to synthesize phytochelatin (PC) [8]-[10]. Numerous physiological studies have indicated that the roles of PC include heavy metal detoxification [11] and maintenance of the homeostasis of intracellular levels of essential metal ions [12]. Malondialdehyde (MDA) is often used as an indicator of peroxidation of membrane lipids in plants. Under stressed conditions, measurements of MDA levels are routinely used as an index of lipid peroxidation.

Commelina communis is commonly known in China as an annual multibranched herb with erect stems in its upper part and creeping stems in its lower part. Many studies have been performed to illustrate the hyperaccumulating mechanism of the hyperaccumulator variety of *C. communis*. Reports that describe the reaction of MDA, GSH, and PC to Cu, however, number much less. Moreover, scholars widely hypothesize that the hyperaccumulator can accumulate more than one kind of heavy metal. As such, two of varieties of *C. communis* were studied in our experiment as to whether or not they could also accumulate Cd in addition to Cu. The movement of MDA, GSH, and PC in the two varieties was further investigated under Cu- and Cd-induced stress. The study is an attempt to understand the mechanism by which the hyperaccumulator variety can tolerate higher levels of Cu.

Manuscript received January 14, 2013; revised March 14, 2013.

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II. Materials and Methods

A. Plant Material and Culture

Copper hyperaccumulator and nonaccumulator varieties of *Commelina communis* [13] were collected from copper-contaminated areas and used in the following experiments. The height of the collected *C. communis* was about 15 cm. Both the copper hyperaccumulator and the nonaccumulator varieties of *C. communis* were placed in 1/10-strength Hoagland's medium (Holagland and Arnon 1938) [14] for 60 days at day/night temperatures of 25/18 °C. Then, the plants were transferred into two nutrient solutions containing 100 μM $CuSO_4$ or 100 μM $CdSO_4$. After 30 days of treatment, the plants were harvested, dried at 60 °C to constant weight and weighed.

B. Cu and Cd Accumulation

Leaves were washed with distilled water. Roots were quickly rinsed with distilled water, and then transferred to vessels containing ice-cold desorption solutions (2 mM Tris-MES, 5 mM $CaCl_2$) for 15-min to remove metal ions adhering to the root surface. All samples were oven-dried separately at 60 °C for 48 h. Once dried, 0.10 g samples were digested in $HClO_4/HNO_3$ (1:3 v/v). Final volumes were adjusted to 10 mL by 2% HNO_3. Metals were measured by using ICP-AES.

DOI: 10.7763/IJESD.2013.V4.365

C. Malondialdehyde

The level of lipid peroxidation was expressed as MDA content and was determined based on its thiobarbituric acid (TBA) reactive metabolites similar to the methodology of Sharma et al. [15] but with minor modifications.

D. Glutathione and Phytochelatins

Quantitation of GSH and PCs by HPLC. Approximately 0.5 g of plant tissue was ground with a mortar and pestle in 0.1 N HCl (0.5 mL). After the sample was vortexed and incubated on ice for 10 min, insoluble material was removed by centrifugation (Beckman, Microfuge). The extract (220 ml) was mixed with 60 mL of 2mM N-acetyl-Cys (included as a standard) and filtered through a 0.2 mm membrane. This mixture (250 mL) was analyzed by HPLC using post column derivatization with Ellman's reagent (DTNB) to detect GSH, PCs and their homologs.

III. Results and Discussion

A. Cu and Cd Concentrations

In order to examine how *C. communis* accumulates heavy metals, the heavy metal content of the leaves and roots was determined. For each species subjected to Cu treatment, as shown in Fig. 1, the Cu concentration in the hyperaccumulator was always higher than in the nonaccumulator.

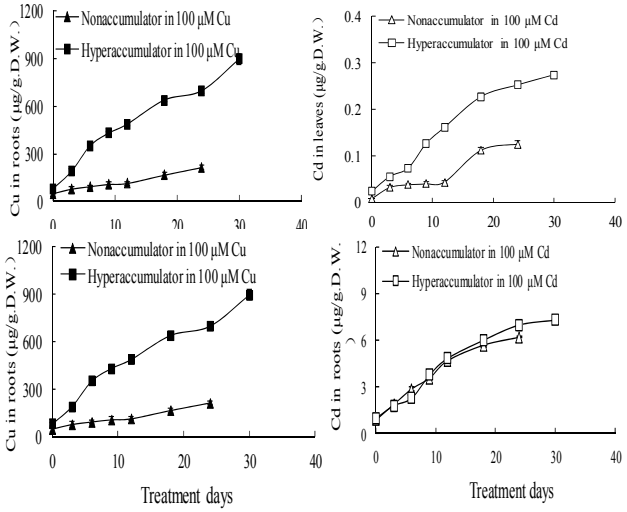

Fig. 1. Changes in heavy metal contents in the hyperaccumulator and nonaccumulator varieties of *Commelina communis*.

The Cu concentration in the hyperaccumulator leaves increased at all times; in the nonaccumulator, it did not change from days 12 to 24. For leaves subjected to Cd treatment, the Cd concentration was found to be minimal; the maximum content determined was only 0.27 μg/g dry leaves in the hyperaccumulator, while it was only 0.12 μg/g dry leaves in the nonaccumulator. Thus, the hyperaccumulator variety of *C. communis* hardly accumulated Cd. This finding is in accordance with the research of Wei et al. [16].

The concentrations of Cu and Cd in the roots of plants are also shown in Fig. 1. The results indicated that the roots could uptake and accumulate large amounts of both Cu and Cd. Heavy metal concentrations in the roots of both hyperaccumulator and nonaccumulator species showed a rapid increase in response to metal treatments. Compared with the metal concentrations in different tissues, Cu concentrations in the roots of the hyperaccumulator and nonaccumulator were typically about 1 and 2 times higher than those in the leaves of the plants, respectively. Hyperaccumulator varieties transported more metal from roots to leaves, which implied hyperaccumulators could store metal ions in the leaves, and then decrease the toxicity risk. Hyperaccumulators such as *C. communis* could utilize defense strategies against metal stress, e.g. compartmentalisation of metals in vacuoles [17].

However, Cd concentrations in the roots of the hyperaccumulator and nonaccumulator were typically 26-42 and 50-119 times higher than those in the leaves of the plants, respectively. Furthermore, no significant differences in Cd concentrations in the roots of the two species were observed, which implied that the hyperaccumulator variety of *C. communis* was not a Cd hyperaccumulator. A reasonable explanation may be that the Cu transporter in the plant was different to the Cd transporter. The heavy metal ATPases, the natural resistance associated macrophage proteins (Nramps) and the cation diffusion facilitators (CDFs), the ZIP family, and the cation antiporters have now been identified in plants as likely to be involved in heavy metal transport [18]. The major Cu transporters in plants are the heavy metal ATPases, while major Cd transporters could be other transporters. Grennan [19] reported that the Cd-hyperaccumulator used components of the Zn transport system for Cd transport.

B. Plant Growth

The biomass of the hyperaccumulator showed no significant differences in nutrient medium and 100 μM Cu treatments (Table I). Comparing the changes in plant biomass, biomass production by the hyperaccumulator variety was largely unaffected by the 100 μM Cu treatment (decrease of 5.1% from control; Table I), while growth was suppressed by the 100 μM Cd treatment (inhibition of 35.5% from control; Table I). However, the data presented here (Table I) indicates that exposure to Cu and Cd resulted in a deleterious effect on the growth of the nonaccmulator variety. The biomass of the nonaccmulator was markedly decreased by Cu and Cd treatments (reduction of 25.2 and 31.1% from control, respectively; Table I).

TABLE I: Dry Weight of Plants Grown in Control Nutrient Solution and Supplemented with Cu or Cd for 30 Days

Treatment	Hyperaccumulator			Nonaccumulator		
	Time		Growth rate (%)	Time		Growth rate (%)
	0 d	24 d		0 d	30 d	100
Control	32.5± 2.3	49.1 ± 1.2	51.1	25.1 ± 3.6	35.3 ± 2.3	40.6
100 μM Cu	32. 6±2.3	47.6 ± 3.0	46.0	25.3 ± 3.6	29.2 ± 2.2	15.4
100 μM Cd	32.6 ± 2.3	37.7 ± 3.4	15.6	25.2 ± 3.6	27.6 ± 2.8	9.5

The data of dry weight are means ± s.e. from three replicates.

C. A Malondialdehyde

Malondialdehyde (MDA) content showed fluctuations (Fig. 2) in the leaves, but the general trend was increases in MDA with increasing days in both Cu and Cd treatment.

MDA content in the hyperaccumulator was always lower than in the nonaccumulator. Specifically, when the nonaccumulator was exposed to 100 μM Cu or Cd for 3 days, the MDA content increased. However, in the hyperaccumulator, the increase in MDA content was delayed until the 6th day. MDA content then began to decline, but increased again after the 24th day in the Cu solution. In the Cd solution, the same pattern was observed, except that MDA content began to increase on the 18th day of Cd treatment. Total change in MDA content was different for the hyperaccumulator and the nonaccumulator. In general, the changes were 24% and 50% in the Cu solution and Cd solution, respectively. However, the hyperaccumulator showed less damage in the Cu solution than in the Cd solution.

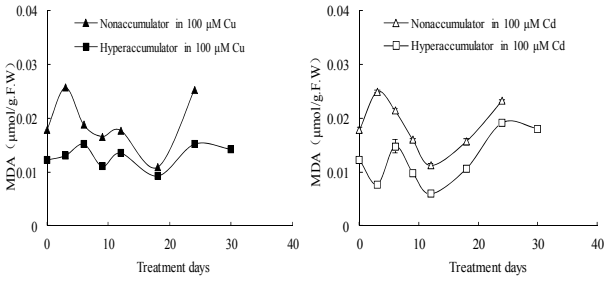

Fig. 2. Changes in MDA content in the hyperaccumulator and nonaccumulator varieties of *Commelina communis*.

MDA, a decomposition product of polyunsaturated fatty acids and hydroperoxides, is often utilized as a suitable biomarker for lipid peroxidation, [20] an effect of oxidative damage. Nonetheless, lipids are not the only targets for MDA action. In fact, MDA can damage DNAs by forming adducts for deoxyguanosine and deoxyadenosine [21]. In our experiment, MDA content in the hyperaccumulator was usually steady, and the change was only 24% under Cu stress. Under Cd stress, on the other hand, MDA content changed by 50%. Furthermore, the changes in MDA content were not sensitive to the increase of Cu and Cd concentrations in the hyperaccumulator until the increases in metal concentration were more than 3.6 and 3.1 fold at the sixth day of treatment, respectively. This indicates that the hyperaccumulator could better endure Cu stress. Meanwhile, MDA content in the nonaccumulator underwent larger changes than in the hyperaccumulator. This suggests that the degree of lipid peroxidation was stronger in the nonaccumulator, which can result in irreversible damage to tissue development and function. For the hyperaccumulator, the concentrations of Cu and Cd in leaves increased about 3.6-fold and 4.0-fold after 6 d treatment, respectively.

D. Glutathione and Phytochelatins

Nemours study reported that one of the important heavy metal accumulation mechanisms could be the GSH-phytochelatins-mediated metal resistance [22]. Glutathione (GSH) plays several roles in cell metabolism such as redox state regulation, oxidative stress control, and protection against xenobiotics and heavy metals. GSH is also used to synthesize phytochelatins. GSH is synthesized in two steps catalysed by g-glutamylcysteine synthetase (g-ECS) and glutathione synthetase. g-ECS is feedback inhibited by GSH, which has led to the proposal that this enzyme acts as the rate-limiting step in the pathway. Phytochelatins are synthesized by phytochelatin synthase (PCS), which is active when two GSH molecules plus a heavy metal form a thiolate. Phytochelatins can be transported into the vacuole and form stable high molecular weight complexes around a metal ion crystallite core to reduce metal toxicity [11].

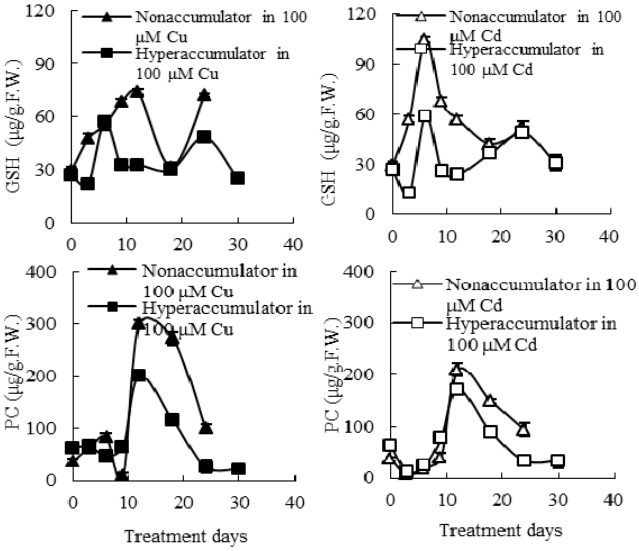

Fig. 3. Changes in GSH and PC content in the hyperaccumulator and nonaccumulator varieties of Commelina communis.

With the increase in treatment days, the GSH content in all the samples changed, as shown in Fig. 3. In the nonaccumulator variety of *C. communis*, the GSH content in both Cu and Cd treatments increased significantly before 12 d, increasing by about 3.5-fold, from 29 μg/g fresh leaves to 104 μg/g fresh leaves, and then, the GSH content continually decreased. However, the GSH content increased again and the plants were dead at 24 d. In the hyperaccumulator variety, on the other hand, GSH content was slightly increased and reached a peak at six days of only two-fold in both 100 μM Cu and 100 μM Cd solutions. Sun et al. [23] observed that no PC was detected in *Sedum alfredii* in a mine population; instead, Zn and Pb treatments resulted in an increase in GSH, indicating that GSH might be involved in Zn and Pb accumulation and tolerance. Abiotic stresses have been previously shown to enhance GSH accumulation in the leaves and roots of various species [24], [25]. Under Cu or Cd stress, the level of GSH in the nonaccumulator was always higher than that in the hyperaccumulator, which correlated with the changes in MDA content. Thus, we may infer that under Cu or Cd stress, the nonaccumulator produces higher levels of GSH to alleviate the effects of heavy metal toxicity. Data on the hyperaccumulator was not similar to that of the nonaccumulator, as it was found that the hyperaccumulator did not produce as much GSH. This suggests that the hyperaccumulator had strongly endured the effects of the heavy metals.

PCs, one of the major defense mechanisms (heavy metal-binding ligands in plant cell) that chelate and sequestrate heavy metals, play an important role in plant detoxification. In our experiment, PC changes were measured in both Cu and Cd treatments (Fig. 3). Under Cu or Cd stress, the PC content in the nonaccumulator changed

significantly. On day 12, it had increased seven-fold under Cu stress, and five-fold under Cd stress. In the hyperaccumulator, on the other hand, PC content only increased about two-fold for both Cu and Cd treatments. After 12 days, the PC content began to decline. Overall, the PC content in both treatments was higher in the nonaccumulator than in the hyperaccumulator. This may be due to some anti-heavy metal mechanism in the species.

Our data showed that GSH content was higher under Cu or Cd stress in the hyperaccumulator when the PC content was lower; an inverse proportional relationship also existed between the two. A reduction in GSH was initially observed in the hyperaccumulator after exposure to the Cd or Cu solution. A common response to heavy metal exposure is the increased consumption of GSH for PC production. However, in bryophytes [7], in response to an excessive heavy metal supply, the synthesis of GSH was enhanced but no PC synthesis could be detected. Thus, GSH may play a favorable role in the avoidance of heavy metal stress and detoxification, especially in the first few days after heavy metal introduction. Our data showed that the PC contents did not initially change significantly, and thus, GSH may be the more important detoxification substance in hyperaccumulator species. The consumption of GSH within plant tissues indicates an attempt to maintain cellular GSH metabolism and to minimize the consequences of oxidative stress [26]. GSH is the metabolic precursor of heavy metal-chelating PCs, and the increase of GSH might be attributed to the fact that its synthesis is a demand-driven process. PC-formation may have triggered its own synthesis [27], [28].

Plants have evolved a variety of mechanisms to control and respond to the uptake of and endurance against heavy metals. In this experiment, under Cu and Cd stress, GSH increased sharply in the first several days after exposure in the nonaccumulator, but the PC content changed slowly. Mendoza-Cózatl and Moreno-Sánchez [22] observed that the rate of GSH synthesis under unstressed conditions is mainly controlled by the demand for GSH. Under Cd stress, another GSH-consuming branch, the PC was activated, thus increasing GSH demand. Thus, the GSH concentration increased in Cd. During this time, PCs may not yet have exerted any detoxification efforts against the heavy metal, and thus, GSH played an important role in plant defense against oxidative stress by increasing tolerance in the nonaccumulator. The high concentrations of Cd and Cu (to a lesser extent) could induce the synthesis of cysteine-rich PCs. It is interesting to speculate whether or not higher GSH levels result in enhanced PC production, as observed in other heavy metal-treated plants [29]. In our experiments, the PC content increased rapidly after 12 days, while the GSH content sharply decreased. Higher Cd concentrations certainly induced a decrease in GSH content and an increase in PC concentration [4]. The intracellular level of GSH regulated PC synthesis. In water cultures, Leopold [30] found that the synthesis of PC molecules and the binding of heavy metal ions to these peptides seem to be only transient processes in *Silene vulgaris* under Cu and Cd stress. This shows that PCs are not responsible for the development of heavy metal-tolerant phenotypes. This finding needs to be further studied.

IV. Conclusion

Experimental results showed that the Cu and Cd contents of leaves and roots were higher in hyperaccumulator than in nonaccumulator varieties. However, the hyperaccumulator variety could have a more powerful ability to detoxify and transport Cu and Cd from roots to shoots, thus decreasing the toxicity risk. Thus, the hyperaccumulator can tolerate heavy metal-induced toxicity better than the nonaccumulator. Although the hyperaccumulator only accumulated Cd to a limited extent, it has a stronger ability to tolerate Cd toxicity than the nonaccumulator.

Acknowledgment

The study was supported by the National Natural Science Foundation of China (Grant No. 21007003) and Fundamental Research Funds for the Central Universities (FRF-BR-09-009A) and CNPC Innovation Fund (2009D-5006-04-02).

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