Effects of proline on copper transport in rice seedlings under excess copper stress

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Abstract

Copper exposure affects water flux and ion path in rice seedlings. To characterize the factors related to Cu transport in response to Cu exposure, rice (*Oryza sativa* cv. Taichung Native 1) seedlings grown by hyperculture were subjected to excess CuSO₄ treatments, and Cu transport, water flux (transpiration rate and xylem sap extrusion), and proline level were assessed. Excess Cu decreased the water flux of rice seedlings. In spite of the decrease in water flux, Cu upward transport increased steadily with time. The absorption of Cu in roots displayed a phase lag between 3 and 12 h of Cu exposure, while proline content in the rice roots decreased dramatically. The supplement of proline in culture medium increased water flux and Cu upward transport in mild Cu-treated rice seedlings. In extreme Cu treatment, however, proline supplement decreased Cu upward transport, as the extent of water flux raised by proline supplement was diminished. Proline supplement to Cu-treated rice seedlings not only reduced the Cu absorption in the roots but also the Cu exclusion, suggesting supplement of proline accompanied by Cu exposure induce a barrier of Cu influx and efflux in rice roots. Therefore, it was concluded that excess Cu leads to inadequate proline and results in the malfunction of copper transport barrier in rice roots.

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1. Introduction

Phytoremediation, defined as the use of plants to extract pollutants from the contaminated environment [1,2], is being considered as a promising technology for the recovery of soils contaminated with heavy metals. The accomplishment of the soil recovery through phytoremediation relies on several factors, including the extent of soil contamination, metal availability for uptake into roots, and plant ability to seize, absorb, and accumulate metals in their shoots [3]. Salt et al. [4] reported that wind boost metal flux to the shoots, while application of abscisic acid that block transpiration also reduce metal accumulation in the shoots. Thus, it implies that one strategy for increasing the efficiency of phytoextraction is to increase the metal translocation to the shoot by increasing plant transpiration. However, plant water transport was

often blocked by heavy metal stress and resulted in limiting transport of metals [5].

Proline accumulation is a widespread phenomenon among plants under water deficiency and has often been associated with osmoregulation [6]. Thus, it is reasonable to link the metabolism of proline and heavy metals stress in plants. In fact, many species, such as wheat [7], *Lemna minor* [8], *Silene vulgaris* [9], rice [10], have been found to accumulate proline in response to Cu exposure. However, most of the studies were focused on the shoots and not much attention has been paid on the roots. Besides, the studies on the root proline metabolism in response to heavy metals were ambiguous. Saradhi and Saradhi [11] detected the proline accumulation in the roots of *Cajanus cajan*, *Vigna mungo*, and *Triticum aestivum* as exposed to cadmium, cobalt, zinc, and lead. Chen and Kao [12] found Cd exposure increased the proline content in rice roots. However, Bassi and Sharma [7] observed a fluctuating pattern of proline accumulation in wheat roots in response to Zn and Cu exposure. Thomas et al. [13] reported that Cu exposure decreased the proline content in the roots of *Mesembryanthemum crystallinum*.

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Thus, the causes and the effects of proline metabolism in roots were remained to be elucidated.

Our previous studies showed Cu exposure induced proline accumulation in the detached leaves of rice seedlings [10]. Proline accumulation is also induced by Cd exposure in the roots of etiolated rice seedlings [12]. Thus, the present studies were aimed to test the assumption that Cu exposure might induce proline accumulation in the roots of rice seedlings. However, in the present report, we found that Cu exposure decreased proline contents in rice roots. Therefore, in order to understand the possible roles of proline reduction in rice roots, exogenous supply of proline in the rice culture medium was processed and the transport of water and Cu in rice seedlings were monitored.

2. Materials and methods

2.1. Plant material

The rice (*Oryza sativa* cv. Taichung Native 1) seedlings were grown as modified from Ref. [10] and were cultured on a stainless steel net floating on half-strength Johnson's modified nutrient solution (pH 4.8) in a 1 l plastic vessel. The nutrient solution was renewed every 3 days. The seedlings were grown for 10 days in a controlled room, where it had $300 \mu\text{M photons m}^{-2} \text{ s}^{-1}$ on the top of the canopy supplied by fluorescent/incandescent lamps with 14 h light period. Relative humidity was controlled to about 50% with a vaporizer. Temperature was maintained between 25 and 30 °C. The preparation of etiolated rice seedlings exactly followed the procedure in Ref. [12].

2.2. Copper exposure and proline supplement

Twenty rice seedlings with third leaf fully expanded were selected and transferred to a 100 ml beaker containing 40 ml of nutrient for 1 day recovery, then the new nutrient (40 ml) with various concentrations of CuSO_4 and L-proline were added to begin the treatment. After the treatment, seedlings were washed with 100 ml distilled water for three times, then were separated to shoots and roots by scissors and discarded the seeds. Finally, the plant tissues were quickly fixed in liquid nitrogen and stored in a -20°C freezer before analysis.

2.3. Cell wall preparation

The cell walls were prepared by homogenizing plant materials in cold phosphate buffer (50 mM, pH 5.8) using a mortar and pestle. The homogenate was centrifuged at $1000 \times g$ and washed at least four times with 50 mM phosphate buffer [14]. After washing, the supernatants were collected for determining the free Cu content, and the pellets were used for determining the cell wall bounded Cu content.

2.4. Determination of Cu, K, proline, and malondialdehyde (MDA)

For the determination of Cu and K, plant materials were placed in a digested bottle, and 10 ml 70% HNO_3 solution predigested under a hood for 20 min were added. With the lids covered, the samples were placed in a microwave oven (Chem Co, Mars-5) for 10 min [15]. The clear sample solutions then were filtered with Whatman #42 filter paper, and diluted to 25 ml in a volumetric flask. Copper and potassium concentrations in plant tissues were determined by Inductively Coupled Plasma-Atomic Emission Spectrometry (model: JY124), Jobin-Yvon, France. Proline was extracted and its concentration was determined by the method of Bates et al. [16]. Plant tissues were homogenized with 3% sulfosalicylic acid and the homogenates were centrifuged at $3000 \times g$ for 20 min. The supernatant was treated with acetic acid and ninhydrin, boiled for 1 h, and then the absorbance at 520 nm was determined. MDA, routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Ref. [17], where the level of lipid peroxidation products in the samples was expressed as 2-thiobarbituric acid reactive materials (aldehydes, mainly MDA and endoeroxides) [18].

2.5. Determination of relative water content, transpiration rate, and xylem sap extrusion rate

Relative water content, defined as the water content of leaf tissue as a percentage that of the fully turgid tissue, was determined according to the Ref. [19]. Twenty rice shoots were soaked in distilled water for 8 h, weighted to get the turgid weight, and then the plant shoots were dried in a 70°C oven to get the dry weight. The relative water content is defined as: $(\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100\%$.

Transpiration rates were calculated as the amount of nutrient in the beaker lost within 2 h. Beakers containing 40 ml of nutrient without plant were used as the blank. Xylem sap extrusion rates were calculated as the amount of xylem sap collected from 10 cutting stalks of rice seedlings. Within 1 h and in every 10 min after cutting, xylem saps were collected using a filter paper of $1 \text{ cm} \times 4 \text{ cm}$. The weight in weight of filter paper at final collection was counted as the amount of xylem sap extrusion.

2.6. Determination of Cu exclusion

Rice seedlings that treated with 0.5 mM of CuSO_4 for 8 h were washed with distilled water for three times, and then transferred to the nutrients with or without proline for 12 h in the light. After that, the amount of Cu in nutrients was counted as the rate of Cu exclusion in rice roots.

2.7. Data analysis

All the treatments in each experiment had three replicates or more. Every experiment was repeated at least two times,

and one was selected for the graphical representation. Comparison of two means was performed using the Student's *t*-test with $P = 0.05$. Comparison of multiple means was performed using the Duncan's multiple range test with $P = 0.05$.

3. Results

3.1. Copper absorption and transport

Rice seedlings exposed to excess Cu in the nutrient solution accumulated substantial amounts of Cu in their roots and shoots (Fig. 1). Approximately 95% of the Cu absorbed in rice seedlings was found in the roots (Table 1), where 40% of Cu absorbed was retained in the cell wall and 60% were in free form. Only 5% of Cu was found in the shoots, where 12% of those retained in the cell wall. Potassium contents were reduced in the roots as Cu concentrations in nutrient was higher than 0.1 mM, but potassium contents in the shoots were unaffected in all ranges of Cu treatment (Fig. 1).

The accumulation of Cu in rice shoots and roots was monitored during 24 h of extreme (0.5 mM) Cu treatment (Fig. 2). In the shoots, Cu accumulation appeared at 6 h of Cu exposure and accumulated constantly thereafter. In the roots, Cu content accumulated rapidly within 3 h of exposure, then a phase lag occurred from 3 to 12 h, and, subsequently, a rapid accumulation occurred after 12 h.

Table 1
Distribution of Cu content in rice shoots and roots

	Shoot ($\mu\text{mol g}^{-1}$ DW)	Root ($\mu\text{mol g}^{-1}$ DW)
Free form	0.96 ± 0.18	11.96 ± 0.86
Cell wall bounded	0.13 ± 0.05	7.98 ± 0.59
Total	1.09	19.94

3.2. Water flux and root growth

While Cu accumulated constantly in rice shoots upon extreme Cu treatment (Fig. 2), the rates of water transpiration and xylem sap extrusion in rice seedlings declined within 3 h (Fig. 3). Thereafter, the relative water content of shoot decreased after 12 h of treatment. The cease of root growth was significant at 24 h.

3.3. Proline content and lipid peroxidation

The Cu exposure increased proline content and lipid peroxidation in rice shoots, but both were decreased in the rice roots (Fig. 4). During 24 h of 0.5 mM Cu treatment (Fig. 5), the proline levels increased in the shoots within 6 h of Cu exposure, whereas it decreased gradually in the roots. Most of proline aggregated was observed in the rice root tips (Fig. 6), but copper exposure decreased proline contents in the root tips.

Fig. 1. Effect of CuSO_4 on copper and potassium contents in rice shoots and roots. Rice seedlings were treated with various concentrations of CuSO_4 for 24 h in the light. Vertical bars represent standard errors ($n = 3$).

Fig. 2. Time course of the CuSO_4 effect on copper contents in rice shoots and roots. Rice seedlings were treated with 0.5 mM of CuSO_4 in the light. Vertical bars represent standard errors ($n = 3$).

3.4. Proline supplement

The Cu exposure decreased water transpiration rate in rice seedlings, but the supplement of proline in the nutrient retarded the decrease of transpiration rate (Fig. 7). Meanwhile, the Cu contents in rice shoots were increased by proline supplement with mild Cu treatment (0.05 and 0.1 mM). However, at extreme Cu treatment (0.5 mM), the Cu content in the shoots was decreased by proline supplement. In the roots, proline supplement decreased the Cu content as nutrient Cu was higher than 0.05 mM. Potassium content was decreased only in the case of 0.5 mM Cu exposure, while proline supplement restrained the decrease. In mild Cu (0.1 mM) exposure, the extrusion rate of xylem sap decreased, the proline supplement restored the extrusion rate as the same as the control (Fig. 8). The supplement of proline alone increased the extrusion rate of xylem sap in rice seedlings. In Fig. 9, the supplement of proline on Cu-treated rice seedlings decreased the exclusion of Cu from rice roots into culture medium.

4. Discussion

The lifting of heavy metals from roots to shoots is of great concern to those who are interested in phytoremediation. Many reports showed that most Cu taken up was retained in

Fig. 3. Time course of the CuSO_4 effect on relative water content, transpiration rate, xylem sap, and root fresh weight in rice seedlings. Rice seedlings were treated with 0.5 mM of CuSO_4 in the light. Vertical bars represent standard errors ($n = 4$).

Fig. 4. Effect of CuSO_4 on proline and malondialdehyde (MDA) in rice shoots and roots. Rice seedlings were treated with various concentrations of CuSO_4 for 24 h in the light. Vertical bars represent standard errors ($n = 3$).

Fig. 5. Time course of the CuSO_4 effect on proline contents in rice shoots and roots. Rice seedlings were treated with 0.5 mM of CuSO_4 in the light. Vertical bars represent standard errors ($n = 3$).

the roots [20–22]. Liao et al. [23] suggested that the withholding of Cu by roots restricted the Cu translocation to xylem and shoots. Our results agree with the above findings and observed around 95% of the Cu taken up retained in the rice roots (Fig. 1). It is worth noting that about 40% of Cu in the root tissue was immobilized in the cell wall (Table 1). Even though, the remaining 60% of Cu taken up were not fully available for transport, only 5% of the Cu taken up was transported to the shoots. The explanations could be chelated by peptides [24], compartmented into vacuole [25] or simply blocked by endodermis [26]. The unusual amino acid nicotianamine (NA) was suggested to be essential for heavy metals transport in the phloem [27]. Furthermore, it has been also observed that in tomato plants, NA stimulate copper transport in the xylem [28]. Therefore, the restricted translocation of Cu in rice root might be due to the inadequate amount of NA.

Substantial evidences suggested that the translocation of heavy metals was driven mainly by water flux due to transpiration and root pressure [4,29–31]. In spite of the decrease in transpiration rate and root pressure (xylem sap extrusion rate) under Cu exposure (Fig. 3), the amount of Cu in the rice shoots increased constantly (Fig. 2), suggesting that the path of xylem could be substituted by another path. Hence, xylem was not the only path for Cu upward transport in rice seedlings. Similar results were also obtained by Thornton and Macklon [32]; they found that water flux only had a little effect on the uptake of Cu in ryegrass seedlings. In

Fig. 6. Effect of CuSO₄ on the distribution of proline in rice roots. Three days old etiolated rice seedlings were treated with various concentrations of CuSO₄ for 5 days in the dark. The sections of root tissue were cut from the root tip by every 2 mm. Each section contains the segments of 10 roots. Vertical bars represent standard errors ($n = 6$). Means labeled with different letters differ significantly ($P = 0.05$).

Fig. 7. Effect of proline supplement on transpiration rate, copper and potassium contents in rice seedlings exposed to copper. Rice seedlings were treated with 1 mM L-proline plus various concentrations of CuSO₄ for 24 h in the light. Vertical bars represent standard errors ($n = 3$). Means labeled with asterisk (*) differ significantly ($P = 0.05$).

Fig. 8. Effect of proline supplement on xylem sap extrusion in rice seedlings exposed to copper. Rice seedlings were treated with (or without) 1 mM L-proline and with (or without) 0.1 mM of CuSO_4 for 24 h in the light. Vertical bars represent standard errors ($n = 4$). Means labeled with different letters differ significantly ($P = 0.05$).

addition, numerous reports suggested that the transport of Cu and other metal ions via the path of phloem [33–35].

Proline is known to accumulate under heavy metal exposure and considered to involve in stress resistance [36,37]. Our previous studies showed that Cu was effective in inducing proline accumulation in the detached rice leaves [10]. To our surprise, the proline metabolism of rice seedlings in response to Cu exposure was different between shoots and roots (Figs. 4 and 5). Cu exposure increased the proline content and the degree of lipid peroxidation in rice shoots, but decreased in the rice roots. It was also observed that the responses of rice roots to Cu exposure were different from that of Cd exposure [12], where Cd increased the rice root proline content. Since the material used in the Cd experiment were etiolated rice seedlings, the experiment of Cu exposure on etiolated rice seedlings with the same growth condition as in Cd experiment was processed. The outcome showed that the root proline content was decreased in etio-

Fig. 9. Effect of proline supplement on copper exclusion in rice seedlings exposed to copper. Rice seedlings were pretreated with 0.5 mM of CuSO_4 for 8 h in the light, then transferred to the nutrients with or without 1 mM of L-proline for 12 h. Amounts of Cu in nutrients were counted as the copper exclusion ability in rice roots. Vertical bars represent standard errors ($n = 3$). Means labeled with asterisk (*) differ significantly ($P = 0.05$).

lated rice seedlings as exposed in excess Cu (Fig. 6), suggesting the existence of specificity among heavy metals to rice seedlings. Proline was found to relocate and accumulate in maize root tip during water stress treatment [38,39]. It is suspected that the proline content in the root tips could be raised by way of proline relocation even the total content of proline in rice root is decline. The result in Fig. 6 did not find the relocation of proline and, thus, excluded that suspicion.

The possible roles of proline in rice roots under Cu exposure were illustrated in the experiment of proline supplement (Fig. 7). In the mild Cu (0.05–0.1 mM) exposure, proline supplement increased the transport of Cu to shoots accompanied by the increases in water flux (transpiration and root pressure) (Figs. 7 and 8), suggesting that the increase in water flux increases Cu transport by way of xylem upon mild Cu exposure. Thus, the task of xylem on Cu upward transport cannot be excluded through it could be substituted by another path.

Proline has long been recognized as the protector against the stress environment [36,37,40]. The result in Fig. 8 showed the root pressure was maintained by proline supplement. It thus suggested that proline supplement under mild Cu exposure can prevent the root tissue from damage by Cu toxicity. Since proline supplement reduced the Cu content in the roots under mild Cu exposure (Fig. 7), it is plausible that proline supplement alleviates the burden of rice roots from mild Cu exposure through the enhancement of Cu upward transport. Furthermore, since the amounts of Cu reduced in the roots far beyond the amounts upward transport to shoots, proline supplement alleviates the burden may also through the reduction of Cu inward entry from nutrient.

When the amount of Cu in nutrient solution reached the extreme range (>0.5 mM), the supplement of proline, however, does not increase the Cu upward transport. In contrast, the amount of Cu in the rice shoots was reduced by proline supplement. In the meantime, the extent of water flow raised by proline supplement was diminished; suggesting proline supplement does not enhance the upward transport of Cu, but still can reduce the entry of Cu. Since the transpiration rates of rice seedlings under extreme Cu exposure were reduced enormously in contrast to the increasing amounts of Cu in the shoots, it was likely that, under extreme Cu exposure, the Cu transport mainly through the path of phloem and driven by the concentration gradient of Cu in the roots [41]. Hence, as the proline supplement reduced the Cu contents in the roots, the upward transport of Cu was reduced.

The supplement of proline reduced Cu contents in both mild and extreme Cu-treated rice roots. This was unlikely due to the increase of Cu transport to shoots, because the amount of Cu increased in the shoots could not compensate the amount of Cu decreased in roots. The studies in *Chlorella* cells by Wu et al. [42] showed that exogenous supply of proline to *Chlorella* cell culture medium after Cu treatment resulted in massive exclusion of absorbed Cu, hence the uptake of Cu in *Chlorella* cells was reduced. However, the present study in rice roots yielded contradictory results

(Fig. 9). The present study showed that the exclusion of Cu in rice roots was reduced by proline supplement. Thus, the less accumulation of Cu in rice roots resulted from proline supplement was unlikely due to the increase of Cu exclusion. Therefore, proline supplement in rice roots decreases not only Cu exclusion but also its uptake, suggesting that proline supplement on Cu-treated rice roots induced a barrier, by which the efflux and influx of Cu in rice roots were blocked.

Lignin was reported to form a hydrophobic matrix as it penetrates into cell wall, by which a barrier was created to obstruct the access of hydrophilic compounds [43]. Proline-rich (PRPs) and hydroxyproline-rich (HRGPs) glycoproteins are coupled with lignin deposition and likely function as foci for lignin polymerization [43]. The studies in antisense transgenic *Arabidopsis thaliana* indicated that proline deficiency in transgenic specifically affected the synthesis of PRPs and HRGPs [40]. It is likely that the deficiency of proline in rice roots resulted from Cu exposure might affect the synthesis of PRPs and HRGPs and led to the defect of cell wall lignification. In addition, the supplement of proline was able to overcome the shortage of proline in rice roots and probably gave enough resource for the synthesis of PRPs and HRGPs in rice roots under Cu exposure, by which the Cu influx decreased in the roots. In fact, a phase lag of Cu absorption occurred between 3 and 12 h of Cu exposure in accordance with the decline of proline content (supposed to be used for the synthesis of PRPs and HRGPs) (Fig. 2). Thereafter, as to the extent of proline deficiency and limiting lignin deposition, the absorption of Cu in rice roots increased dramatically after 12 h of Cu exposure.

Copper was reported to increase cell permeability, probably due to membrane damage, where rapid leakage of K has been widely interpreted as a symptom of Cu-induced oxidative damage to the plasma membrane [44–46]. In fact, a decrease of K content in rice roots in response to extreme Cu exposure is observed in Figs. 2 and 7. Copper is known to damage cell membranes by inducing lipid peroxidation [36,44]. Estimation of MDA is the most widely used method for the indication of oxidative stress, but Cherif et al. [47] reported that MDA cannot be related to lipid peroxidation in habituated sugarbeet plant cells due to the interferon-like pigments, sugars, and polyamines. Our results in Fig. 4 showed that MDA decreased in response to Cu exposure, perhaps due to the interferon like their reports. However, the existence of oxidative stress in rice roots induced by Cu exposure cannot be ruled out because several antioxidative enzyme activities and H₂O₂ levels have been found to increase in rice roots by Cu exposure [14].

In summary, Cu exposure decreased the water flux (transpiration rate and xylem sap extrusion) of rice seedlings. However, water flux was not the solo driving force for Cu translocation in rice seedlings. Proline content in the rice roots was decreased remarkably by Cu exposure. Proline supplement increased the Cu upward transport in mild

Cu-treated rice seedlings, but decreased the Cu transport in extreme Cu treatment. Proline supplement reduced the Cu absorption and exclusion in the roots of Cu-treated rice seedlings, indicating excess Cu leads to the inadequate of proline and results in the malfunction of copper transport barrier in rice roots.

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