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Response of wild type of *Arabidopsis thaliana* to copper stress

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Abstract

Wild type of *Arabidopsis thaliana* plants were cultivated hydroponically in Hoagland and Arnon nutrient solution and treated with copper (5 - 100 μM) for 2, 4, 7 and 14 d. A progressive decrease of the root length and biomass was observed at increasing Cu concentration in the nutrient solution. Roots accumulated higher amounts of Cu than shoots at all Cu treatments. Changes of cell and chloroplast ultrastructure of Cu-treated plants were also observed. Cu application did not induce formation of Cu-phytochelatin complexes. Changes in glutathione and glutathione disulfide content observed in roots and shoots of Cu-treated plants suggest their participation in amelioration of metal-induced oxidative stress.

Additional key words: copper toxicity and tolerance, glutathione, glutathione disulfide, phytochelatin.

Introduction

Copper is known to be an essential micronutrient for plants, but it can also be a toxic element, at a tissue concentration only slightly higher than optimal. Cu excess induces a wide range of biochemical effects and metabolic disturbances that are responsible for a strong inhibition of plant growth and plant yield decrease. Cu concentrations in the environment are low: 20 - 30 mg kg^{-1} in non-contaminated soils and less than 2 μg kg^{-1} in natural waters. In contaminated soils and waters Cu concentration can reach 2000 mg kg^{-1} and 500 - 2000 μg kg^{-1}, respectively (Fernandes and Henriques 1991).

Tolerance of plants to Cu-rich environments is achieved by one or more of the following mechanisms: excretion, exclusion, retention in the roots, immobilization in the cell wall, accumulation in organelles, production of intracellular metal-binding chelators (Ernst *et al.* 1992). Following Cu uptake through high-affinity transporters in plasmalemma such as COPT1, Cu ions can be chelated in cytosol by specific Cu chaperones (CCH1) and delivered to Cu pumps (RAN1 protein) for its metal transport into Golgi apparatus (Clemens 2001).

However, the best known intracellular metal-binding chelators are phytochelatins (PCs) and metallothioneins (MTs). Metallothioneins are cysteine-rich proteins required for heavy metal binding and tolerance in animals and microorganisms. Many recent studies indicate that plants also posses functional metallothionein genes. At present, 64 MT-II genes from different plant species and tissues have been described and 4 of the MT genes have been transcribed in *Arabidopsis* (Zhou and Goldsbrough 1995, Rauser 1999). Although plants harbour MT-like genes, the exposure of plant cells to heavy metals generally results in the synthesis of GSH-derived metal-chelating peptides called phytochelatins. PCs are class III of MTs and form metal-binding complexes, which contribute to Cd, Pb, Hg, Cu, Zn, As detoxification and to Cu and Zn homeostasis. PCs are derived from glutathione by the action of constitutively present PC synthase. Recently, PC synthase encoding gene has been cloned by several laboratories (Cobbett 1999). PC synthase is activated by heavy metal ions that are complexed by induced PCs via thiolate coordination.

Several lines of evidence suggest that GSH plays a crucial role in defence against potentially toxic metal ions by sequestering them prior to the synthesis of MTs or PCs. It is suggested that Me-GSH complex may be the

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Abbreviations: GSH - glutathione; GSSG - glutathione disulfide; PC - phytochelatin.
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actual substrate for PC synthase reaction, or metal ions may be transferred directly to the newly synthesized PCs. It was shown that GSH can be an effective donor of Cu(I) and perhaps of other metals to MTs and PCs (Mehra and Mulchandani 1995). Both peptides are known to play a role in heavy metal tolerance in *Arabidopsis*. The Cu-hypersensitive mutant (*cup1-1*) of *A. thaliana* is characterized by increased accumulation of Cu (2 to 3-fold concentration of the wild type). The increase in Cu accumulation by this mutant is consistent with the observation that the induction of *MT2a* gene is expressed and correlated with Cu tolerance (Van Vliet *et al.* 1995). The Cd-sensitive mutants *cad1* and *cad2* of *A. thaliana* are deficient in PCs because of a loss of PC synthase activity or a decrease in GSH level, respectively. These mutants confirm the importance of PCs for Cd detoxification. Additionally, each of these mutants is only slightly more sensitive to Cu than the wild type. This suggests that PCs play a relatively minor role in Cu detoxification (Howden *et al.* 1995a,b). The mechanism of metal detoxification is more complex than simple chelation of the metal ion by PCs. The metal ion must activate PC synthase, be chelated by the PCs and transported to the vacuole to form more stable complexes with sulfide or organic acids. This sequence of events is required for Cd but not always for Cu detoxification. Although PCs are induced by Cu *in vivo*, PC synthase is activated by Cu *in vitro* and PCs can chelate Cu *in vitro*, it is still unknown whether PCs effectively chelate Cu *in vivo* or whether Cu-PC complexes, if formed, can be sequestered in the vacuole (Cobbett 2000).

This work was carried out to investigate the effect of copper on some growth parameters and metal detoxification in the wild type of *A. thaliana* plants grown in hydroponic culture supplied with different Cu concentrations.

Materials and methods

Plants and growth conditions: *Arabidopsis thaliana* (L.) Heynh (wild type, cv. Columbia) seeds were sown in moist, doubly autoclaved garden soil and after 10 d the seedlings were transferred into pots filled with the same soil. After 7 weeks the plants were transferred by ones into pots filled with 0.5 dm^3 of Hoagland and Arnon nutrient solution. $CuSO_4 . 5\ H_2O$ (*Sigma,* Germany) was added to the nutrient solution after a 2 d acclimation phase at concentrations 5, 10, 25, 50, 100 µM. The plants were analysed after 2, 4, 7 and 14 d since the metal addition. The plants were grown in a vegetation room at day/night temperature of 23/18 °C with 11-h photoperiod, photosynthetic photon flux density of 140 µmol m^{-2} s^{-1} and relative humidity of 60 %.

The inhibition rate of root elongation was expressed as index of tolerance (IT) - a comparison of root elongation of metal treated plants in relation to control plants. The lowest copper concentration totally inhibiting root growth (EC_{100}) was determined by the Norit test. Before the metal was added, roots were stained black with activated charcoal (*Sigma*). At Cu concentrations not inhibiting totally root growth, the adhering fragments were of bright colour and differed distinctly from the dark zone above them. However, at metal concentrations totally inhibiting roots elongation these organs remained black.

Thiol peptides analysis by HPLC method: Roots and shoots were weighed and ground in a cooled mortar with a double volume of 0.1 M HCl. Homogenates were centrifuged at 13 000 *g* and the obtained supernatants were used for chromatographic analyses.

A *Beckman* (Fullerton, USA) chromatograph (*model 126/166*) with a *Supelco* precolumn (4.6 × 10 mm) and column (4.6 × 250 mm) both filled with *Ultrasphere C-18* were used. Samples were separated in acetonitrile (ACN) linear gradient (0 - 20 %) in 0.05 % trifluoroacetic acid (TFA) for 40 min at a flow rate of 1 cm^3 min^{-1}. Next, the column was washed with 50 % ACN and equilibrated with 0.05 % TFA. Separated peptide fractions flowing out of the column were directed into a mixer (0.01 cm^3) and mixed with 200 µM Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB, *Sigma,* Germany) in 0.05 M potassium-phosphate buffer, pH 7.6 (0.5 cm^3 min^{-1}). Absorbance of the products of DTNB reaction with -SH groups was measured at 405 nm using a *Beckman* detector (*model 166*). The retention times and peak areas were determined with a computer programme *Gold Nouveau* (*Beckman*).

Electron microscopy: Leaf segments of Cu-treated (50 µM for 14 d) and control plants were fixed in a mixture of 2 % glutaraldehyde in 50 mM Na-PIPES buffer, pH 7.5, for 4 h. The samples were further dehydrated in a graded ethanol series and embedded in *LR-White* epoxy resin. Ultrathin sections were cut with glass knives on a *Reichert Ultracut S* ultramicrotome (Vienna, Austria), examined and photographed using a *Tesla BS 500* (Brno, Czech Republic) electron microscope.

Glutathione disulfide was estimated by the GSSG recycling method according to Anderson (1985) with GSSG as a standard. The shoot and root extracts in 5 % sulfosalicylic acid were treated prior to analysis with 2-vinylpyridine for minimized GSH oxidation.

Copper determination: Plant samples for copper determination were prepared in Tris-glycine buffer, pH 8.5, and copper content was estimated spectophotometrically using diethyldithio-carbamine at 448 nm.

Statistics: The results of the growth parameters were the means of over ten measurements in 3 - 10 independent repetitions ± SE. Analyses of metalopeptides in the extracts of the plant organs were performed 2 times (using 3 - 5 plants for each analysis).

Results

Copper tolerance in a wild type of *A. thaliana* was measured by determining the concentration which completely inhibited root growth (EC_{100}). By using Norit test it was found that the lowest Cu concentration causing total inhibition of *A. thaliana* roots was 150 µM. This concentration was not used in further experiments. Other Cu concentrations (5 - 100 µM) caused a decrease in root elongation. The index of tolerance (IT) reached a value of 0.5 at 10 µM Cu after 4 d (Fig. 1). Exposure of *A. thaliana* plants to increasing Cu concentration significantly affected shoot and root fresh mass.

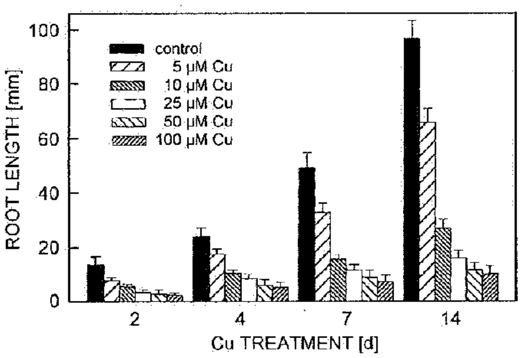

Fig. 1. Root length of control and Cu-treated *A. thaliana* plants. Means ± SE, $n = 25$.

Shoot and root biomass was found to decrease with increasing time of exposure to the metal and its concentration in the medium (Fig. 2). Below 5 µM Cu no significant effects of copper on shoot and root biomass were measured. The application of copper to the root medium did not visibly affect plants until day 4, but after this period the symptoms of copper toxicity started. The oldest leaves became chlorotic and partially necrotic. These changes were particularly distinct at higher Cu concentrations (50 and 100 µM). Cu-stress appeared also in changes of root morphology: reduction of lateral roots, their thickening, unnatural curving as well as darkening of their tissues.

In roots of Cu exposed *A. thaliana* plants, Cu concentration increased almost linearly with time up to the 1 300 µg g^{-1}(f.m.) (Fig. 3). Most of the metal was retained in roots, and after 14 d at 100 µM Cu its amount was over 5 times higher than in shoots. However, Cu accumulation in shoots effected the ultrastructure of leaf mesophyll cells as well as their chloroplasts. Mesophyll cells displayed a more or less circular shape with relatively few, partially disturbed chloroplasts. The cells of control were elongated, chloroplasts were crowded at the cell periphery, and their internal membrane system was organized in big grana consisting of many highly ordered thylakoids. The ultrastructural differences of chloroplasts of Cu-polluted plants concern the quantity and distribution of the internal membrane system. They contain also large starch grains and are rather circular than oval-shaped (Fig. 4).

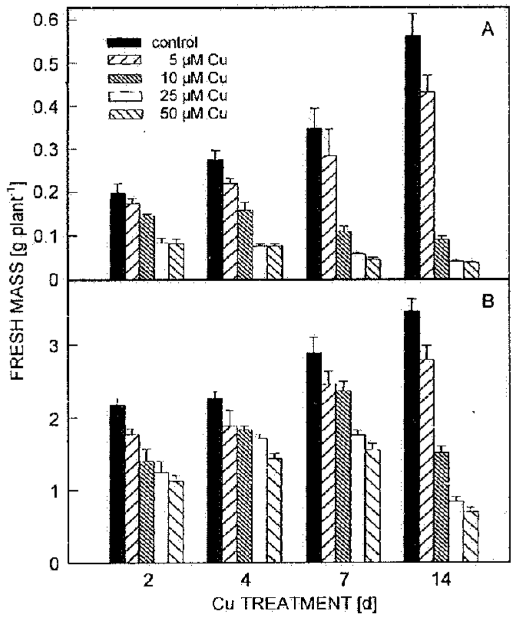

Fig. 2. Fresh mass of roots (*A*) and shoots (*B*) of control and Cu-treated *A. thaliana* plants. Means ± SE, $n = 25$.

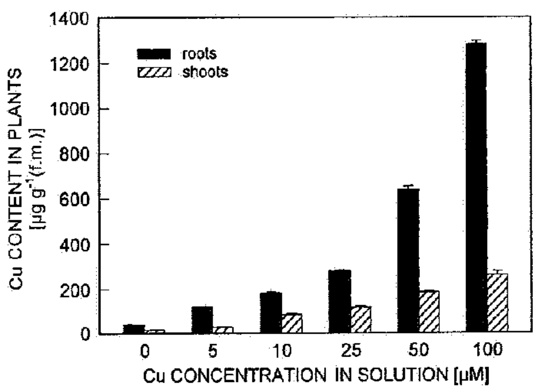

Fig. 3. Cu content in *A. thaliana* plants after 14 d of growth in the presence of copper. Means ± SE, $n = 5$.

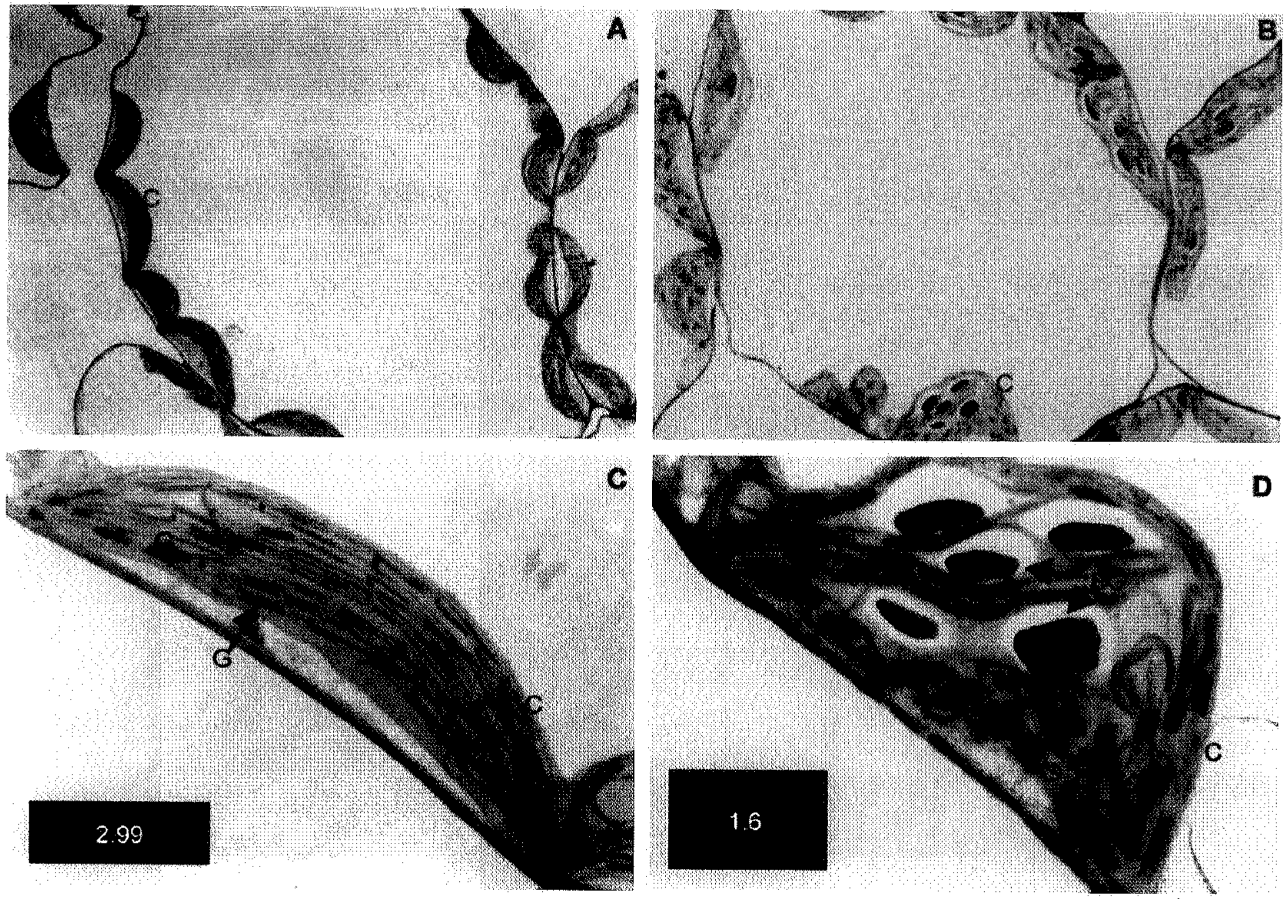

Fig. 4. Cross section of mesophyll cells of control (*A*) and Cu-treated (14 d, 50 μM) (*B*) *A. thaliana* leaves. Comparison of mesophyll chloroplasts of control (*C*) and Cu-treated (*D*) plant leaves. C - chloroplast, SG - starch grains, G - grana stacks. *Bars* and numbers in Fig. 4 *C* and *D* represent the ratio of chloroplast length to its width, which are means of 3 replications (50 - 70 measurements in each). *A* - ×2 200, *B* - ×2 500, *C* - ×10 000, *D* - ×10 000.

Increase in intracellular heavy metal concentration induces in plants accumulation of thiol peptides involved in metal detoxification. In our experiments, application of 5 - 50 μM Cu did not induce formation of PC complexes between 2 and 14 d after metal exposure. Moreover, GSH content decreased in roots and increased in shoots. The content of glutathione, precursor of phytochelatin synthesis, in roots and shoots changed independently of Cu concentration. At extended Cu exposure and its higher concentration GSH disappeared (Fig. 5*A*) and GSSG production in roots was found. In roots, 5 - 50 μM Cu caused GSSG increase from 148 to 328 % of control. Although GSSG content in shoots was lower (from 20 to 130 % of control) and GSH content of Cu-treated plants was much higher than in the control (Fig. 5*B*) phytochelatin synthesis in these organs also did not occur.

Discussion

In higher plants Cu causes various effects depending strongly both on the plant growth stage at which the metal was applied and the time of its action. Phytotoxic effect of Cu on metabolism and growth of higher plants is very often bigger than other (Zn, Fe, Cd) heavy metals (Fargašová 2001). A common feature of the action of excess Cu in most plants is biomass decrease of roots and their length which result from direct metal action on cell division (Jiang *et al.* 2001). Root growth is the parameter most widely used in determining heavy metal toxicity/tolerance in plants. In our experiments with *A. thaliana*, root growth stop index was estimated at 150 μM Cu. The results obtained by Van Vliet *et al.* (1995) showed that the growth of the *A. thaliana* seedlings was enhanced at the presence of 2 μM Cu. In our experiments, the biomass started to diminish at 5 μM Cu.

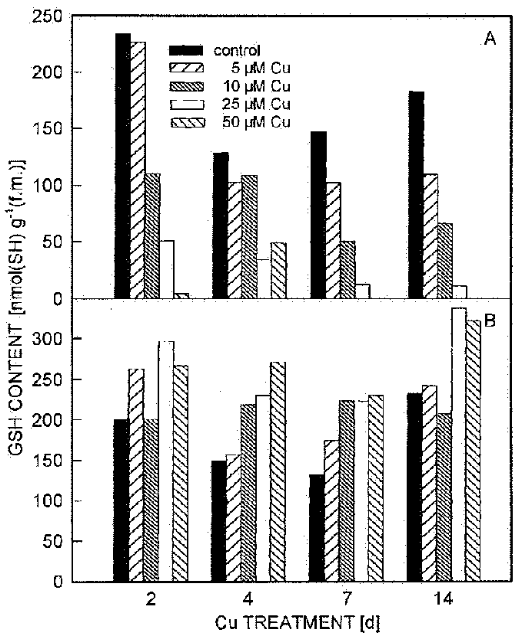

Fig. 5. Glutathione (GSH) content in roots (*A*) and shoots (*B*) of control and Cu-treated *A. thaliana* plants. Means of 2 replications (5 - 10 plants in each).

Copper usually accumulates in the root apoplast at cell walls and cell membranes, which prevents its absorption into the cytosol and its transport to the plant shoot (Ernst *et al.* 1992). This mechanism is clearly shown by the final shoot and root Cu concentration in *A. thaliana* plants after Cu treatment: 200 and 1 300 μg(Cu) g^{-1}(f.m.), respectively.

Our studies confirm the destructive effect of Cu on the ultrastructure of chloroplasts in mesophyll cells. The changes observed (thylakoid dilation, grana destruction and starch grains accumulation) are characteristic of chloroplast senescence. Similar observations were described by Eleftheriou and Karataglis (1989) for wheat growing under field conditions, by Reboredo and Henriques (1991) for *Halimione portulacoides* and by Angelov *et al.* (1993) for pea plants.

Induction of phytochelatins is a general answer of higher plants to heavy metal exposure. Binding of heavy metal ions to PCs molecules starts very quickly, especially in plant roots. Phytochelatins seem to be a useful early warning system for heavy metal stress in plants. Since PCs induction is observed only in plants grown under conditions of excess heavy metals, it can be a biomarker indicating internal excess of heavy metal content, including Cu (Keltjens and van Beusichem 1998).

In our studies with Cu-treated *A. thaliana* plants, PCs induction was not found. Copper (20 μM) was a poor elicitor of PCs also in maize seedlings (Tukendorf and Rauser 1990). Moreover, at extended of Cu exposure the amount of PCs decreased in roots. Similar results were obtained in the experiments of Cu-treated spinach plants (Tukendorf 1993). The reason of this decrease is that copper may catalyze oxidation of cellular thiols. Binding Cu ions to PCs seems to play only a transient role in heavy metal detoxification. In *Silene vulgaris* plants grown on a medieval copper mining dump, Cu-complex disappeared in roots between 7 and 14 d after Cd and Cu exposure (Leopold *et al.* 1999).

Phytochelatin synthesis induced by many heavy metals is associated with a rapid depletion of total GSH in plant tissues. However, PCs production induced by Cu in maize seedlings was accompanied by a 45 % increase in total GSH (Tukendorf and Rauser 1990), and by 40 % in spinach plants in comparison to control (Tukendorf 1993). One interpretation is that increased GSH, besides PCs, can serve to complex Cu like in hepatoma cells in which about 60 % of cytoplasmic Cu occurs as Cu-GSH complexes (Freedman *et al.* 1989).

GSH is a well-known antioxidant playing a major role in the defence against many stresses (heavy metals, excess sulfur, heat, cold, drought, xenobiotics, ozone, pathogen attack, oxidative stress) (Rennenberg and Brunold 1994). The metal-induced increase in GSH in plants may be connected with the susceptibility of cells to oxidative stress, especially in the case of the redox-cycling metal copper.

The increase in GSSG content in *A. thaliana* plants was similar to the content of this peptide in the roots of copper-treated *Silene cucubalus* plants (de Vos *et al.* 1992). These results indicate an increased oxidation of GSH *in vivo*. Copper is known to catalyze not only GSH oxidation but also other cellular thiols. Therefore, copper may be capable of catalyzing also oxidation of the phytochelatin-SH groups, which could explain the sharp decline in the content of PCs in plants exposed to Cu.

We conclude that Cu tolerance in wild type of *A. thaliana* plants does not depend on the production of phytochelatins.

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