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Glutathione Depletion Due to Copper-Induced Phytochelatin Synthesis Causes Oxidative Stress in *Silene cucubalus*

C. H. Ric De Vos*[1], Marjolein J. Vonk, Riet Vooijs, and Henk Schat

Department of Ecology and Ecotoxicology, Free University of Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

ABSTRACT

The relation between loss of glutathione due to metal-induced phytochelatin synthesis and oxidative stress was studied in the roots of copper-sensitive and tolerant *Silene cucubalus* (L.) Wib., resistant to 1 and 40 micromolar Cu, respectively. The amount of nonprotein sulfhydryl compounds other than glutathione was taken as a measure of phytochelatins. At a supply of 20 micromolar Cu, which is toxic for sensitive plants only, phytochelatin synthesis and loss of total glutathione were observed only in sensitive plants within 6 h of exposure. When the plants were exposed to a range of copper concentrations for 3 d, a marked production of phytochelatins in sensitive plants was already observed at 0.5 micromolar Cu, whereas the production in tolerant plants was negligible at 40 micromolar or lower. The highest production in tolerant plants was only 40% of that in sensitive plants. In both varieties, the synthesis of phytochelatins was coupled to a loss of glutathione. Copper at toxic concentrations caused oxidative stress, as was evidenced by both the accumulation of lipid peroxidation products and a shift in the glutathione redox couple to a more oxidized state. Depletion of glutathione by pretreatment with buthionine sulfoximine significantly increased the oxidative damage by copper. At a comparably low glutathione level, cadmium had no effect on either lipid peroxidation or the glutathione redox couple in buthionine sulfoximine-treated plants. These results indicate that copper may specifically cause oxidative stress by depletion of the antioxidant glutathione due to phytochelatin synthesis. We conclude that copper tolerance in *S. cucubalus* does not depend on the production of phytochelatins but is related to the plant's ability to prevent glutathione depletion resulting from copper-induced phytochelatin production, *e.g.* by restricting its copper uptake.

In plants, both essential and nonessential heavy metals induce the formation of thiol-rich peptides, (γ-glutamylcysteinyl)$_n$-glycines with $n = 2$ to 11, also known as metal-binding compounds or phytochelatins (8, 26). Experiments with BSO,[2] an inhibitor of γ-glutamylcysteine synthetase, showed that glutathione serves as a precursor in the phytochelatin biosynthesis and that phytochelatins are involved in the detoxification of heavy metals *in vivo* (17, 23, 25). It has recently been demonstrated that the formation of phytochelatins is catalyzed by a specific γ-glutamylcysteine dipeptidyl transpeptidase, called phytochelatin synthase, which is activated in the presence of metal ions and uses GSH as a substrate (9). Phytochelatins are the major if not the only thiol-rich compounds induced in metal-exposed plants (8, 26), although it has been reported that copper induces metallothionein-like compounds as well (27). Phytochelatins probably play a central role in the homeostatic control of metal ions in plants (26). They may also be involved in the physiological mechanism of metal tolerance of selected cell lines and intact plants (4, 12, 22, 25, 27).

Phytochelatin synthesis induced by cadmium is associated with a rapid depletion of total glutathione in plant cell suspensions (4, 23) and intact plants (15, 20, 28). By contrast, phytochelatin production induced by copper was accompanied by an increase of total glutathione in maize (28). However, because the discrimination between reduced and oxidized glutathione was not always made in these studies, it is still unknown whether the metal-induced changes in glutathione involve changes in GSH, GSSG, or both.

GSH is a well-known antioxidant playing a prominent role in the defense against free radicals in plants (1). In animal cells, a depletion of GSH may cause oxidative stress, especially in the presence of redox-cycling compounds (7, 16). The metal-induced depletion of glutathione in plants due to phytochelatin synthesis may therefore increase the susceptibility of cells to oxidative stress, especially in the case of the redox-cycling metal copper. In addition, copper may catalyze the oxidation of cellular thiols, resulting in the production of free radicals and subsequent lipid peroxidation, as was demonstrated in animal cells (11, 21).

In this paper, we report the effect of copper on the level of nonprotein thiols in the roots of both copper-sensitive and tolerant *Silene cucubalus*. This species is known to produce phytochelatins upon exposure to heavy metals, including copper (8, 29). In both tomato cell suspension and tobacco leaves, the accumulation of nonprotein thiols upon cadmium supply corresponded to the production of phytochelatins (23, 30). Based on these results, the amount of acid-soluble SH compounds other than GSH was taken as a measure of phytochelatin-SH in the present study. The experiments were carried out to determine the role of phytochelatins in the mechanism of copper tolerance in *S. cucubalus* and to investigate the role of GSH, on the one hand, in the production of phytochelatins and, on the other hand, in the prevention of oxidative stress.

[1] Present address: Centre for Plant Breeding and Reproduction Technology, P.O. Box 16, 6700 AA Wageningen, The Netherlands.

[2] Abbreviations: BSO, buthionine sulfoximine; TBA-rm: 2-thiobarbituric acid-reactive material; SH, sulfhydryl.

MATERIALS AND METHODS

Growth and Harvest of Plants

Silene cucubalus (L.) Wib. (= *Silene vulgaris* [Moench] Garcke) plants were obtained from seeds that were collected from the copper-sensitive population Amsterdam and the copper-tolerant population Imsbach and were precultured on a nutrient solution as described previously (5). Sensitive and tolerant plants are resistant to copper concentrations up to 1 and 40 μM in their nutrient solution, respectively, which was determined from the effect of copper on the growth of roots (6). After preculture (day 0), the plants were transferred to a fresh nutrient solution from which copper and FeEDTA were omitted and which was buffered with 2 mM Mes at pH 5.0 (adjusted with KOH). Copper was then added as $CuSO_4$ at the appropriate concentrations. Control plants were grown without supply of copper. The nutrient solutions were refreshed at day 2. Following this culture procedure, the concentrations of free copper ions in the nutrient solution were nearly constant during the experiments, as was determined with a Cu^{2+} electrode (model 94–29 SC, reference electrode model 90–01 00; Orion Res. Inc., Boston).

At harvest, the roots of intact plants were washed with 5 mM $Pb(NO_3)_2$ at 0°C for 30 min to remove the adhering copper (10). After washing, the roots of three plants were pooled, quickly frozen in liquid nitrogen, and lyophilized. The dried material was stored under vacuum at room temperature. This storage did not affect the thiol content.

Extraction and Assays of Nonprotein Thiols

Nonprotein thiols were extracted by grinding 20 mg dry weight in 2 mL 5% (w/v) sulfosalicylic acid + 6.3 mM diethylenetriaminepentaacetic acid (pH < 1) at 0°C with quartz sand in a mortar. The homogenate was centrifuged at 10,000*g* for 10 min (4°C). The clear supernatants were collected and immediately used for the determination of thiols.

The level of total acid-soluble SH compounds was determined with Ellman's reagent. Three-hundred microliters of supernatant was mixed with 630 μL of 0.5 M K_2HPO_4 and 25 μL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (final pH 7). The absorbance at 412 nm was read after 2 min (ϵ_{412} = 13,600). Values were corrected for the absorbance of supernatant and 5,5′-dithiobis(2-nitrobenzoic acid). GSH and GSSG were assayed by the GSSG-recycling method (2). GSSG was used as a standard. The final concentration of glutathione reductase (Sigma, type III from bakers' yeast) was 0.5 unit/mL in the assay of total glutathione (GSH + GSSG), and 1 unit/mL in the assay of GSSG. Each extract was assayed twice, and the variation between replicate assays was always less than 3%. The level of phytochelatin-SH was calculated by subtracting the amount of GSH from the amount of total acid-soluble SH compounds.

The recovery and oxidation of acid-soluble thiols were determined with GSH and GSSG as external standards (treated as described for thiol extraction from root material) and internal standards (added to root material prior to homogenization).

Copper Analysis and Lipid Peroxidation

The copper content of roots was calculated from the copper concentration in the acid supernatant, which was directly determined by atomic absorption spectrophotometry (Perkin-Elmer 4000, Überlingen, Germany). The amount of lipid peroxidation products in lyophilized root material was measured as the amount of TBA-rm absorbing at 532 nm (5).

RESULTS

Preliminary experiments were carried out to determine the recovery and oxidation of SH compounds during the extraction of thiols from the roots (data not shown). A pronounced loss and a rapid oxidation of the internal GSH standard was observed when the root material was not lyophilized prior to homogenization, or when the lyophilized material was homogenized in a solution with pH 3 or higher. The recoveries of the external standards were more than 95% under these conditions, indicating that the loss and oxidation of thiols were caused by compounds in the root extracts. The addition of the antioxidant ascorbic acid or polyvinylpolypyrrolidone to bind phenolic acids could not prevent this loss and oxidation of thiols. By contrast, with the procedure described in "Materials and Methods," which includes lyophilization, the recovery of the internal standards added to root material of both copper-treated and control plants from either the sensitive or the tolerant population was more than 97%, suggesting that thiol oxidation did not take place. Furthermore, copper added during thiol extraction neither affected the recovery and assay of thiols nor caused the oxidation of the internal GSH standard, indicating that differences in thiol levels between plants are not caused by possible differences in copper contents.

The effect of a supply of 20 μM Cu, toxic for sensitive plants only, on the levels of acid-soluble SH compounds and total glutathione (GSH + GSSG) was studied in a 3-d experiment (Fig. 1). In sensitive plants, this copper treatment resulted in

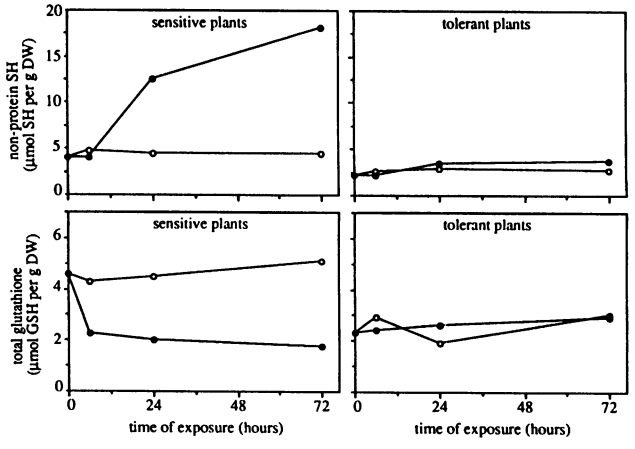

Figure 1. Effect of copper supply on the levels of acid-soluble SH compounds and total glutathione (GSH + GSSG) in the roots of copper-sensitive (left figures) and -tolerant (right figures) *S. cucubalus*. ○, Controls (no copper added); ●, high copper-treatment (20 μM Cu). Roots of three plants were pooled.

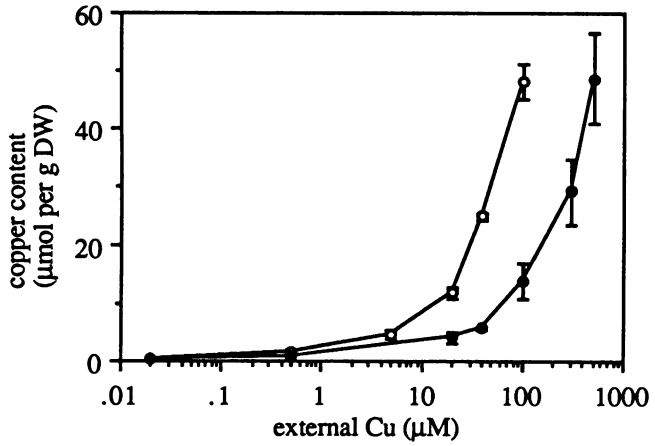

Figure 2. Copper contents of roots of copper-sensitive (O) and copper-tolerant (●) *S. cucubalus*. Values are means with SD (n = 3).

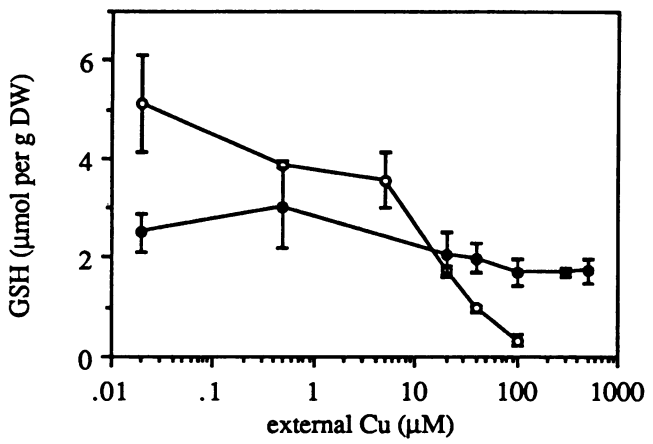

Figure 4. Copper-induced GSH depletion in the roots of copper-sensitive (O) and -tolerant (●) *S. cucubalus*. Values are means with SD (n = 3).

a marked increase of the level of nonprotein SH compounds after more than 6 h of exposure. However, as the level of total glutathione was decreased by 50% after 6 h of metal supply, copper apparently induced the formation of nonprotein SH compounds other than glutathione, *i.e.* phytochelatins, within 6 h of exposure. In contrast to these changes in sensitive plants, the levels of nonprotein SH compounds and total glutathione in tolerant plants were not affected by this copper treatment.

In subsequent experiments, the plants were exposed to copper for 3 d. At each metal concentration supplied, the copper content in the roots of sensitive plants was higher than that of tolerant plants (Fig. 2), suggesting a restricted uptake of copper in tolerant plants.

The content of phytochelatin-SH (Fig. 3) in sensitive plants increased up to 25 μmol SH g^{-1} dry weight at 20 μM Cu and declined strongly at higher metal concentrations. The level of phytochelatin-SH in these plants was already increased at 0.5 μM Cu, which is a nontoxic concentration for these plants. By contrast, the level of phytochelatin-SH in tolerant plants was significantly increased only at concentrations that were toxic for that variety, *i.e.* at 40 μM Cu or higher. The highest level in these plants was 10 μmol SH g^{-1} dry weight at 300 μM Cu.

In both sensitive and tolerant plants, the production of phytochelatins was coupled to a decrease of GSH (Fig. 4). At toxic concentrations, copper also induced oxidative damage, as was demonstrated by an increase of TBA-rm (Fig. 5) and by a shift of the GSH/GSSG couple to a more oxidized state (Fig. 6). Both GSH depletion and oxidative stress occurred at lower external copper concentrations in sensitive plants than in tolerant ones. In both varieties, oxidative stress was apparent at about 2.5 μmol GSH g^{-1} dry weight or lower, corresponding to a depletion of 60% or more in sensitive plants and a depletion of 20% or more in tolerant ones, compared with the levels in the controls.

The relation between glutathione depletion and oxidative stress was further examined in metal-sensitive plants pretreated with BSO for 7 d to inhibit the glutathione synthesis

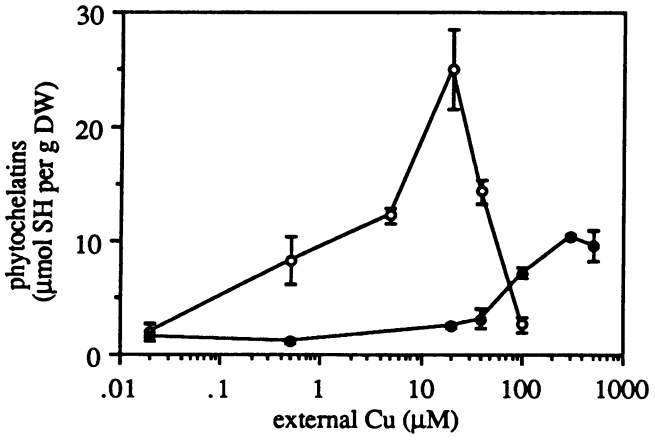

Figure 3. Copper-dependent phytochelatin production in the roots of copper-sensitive (O) and -tolerant (●) *S. cucubalus*. Values are means with SD (n = 3).

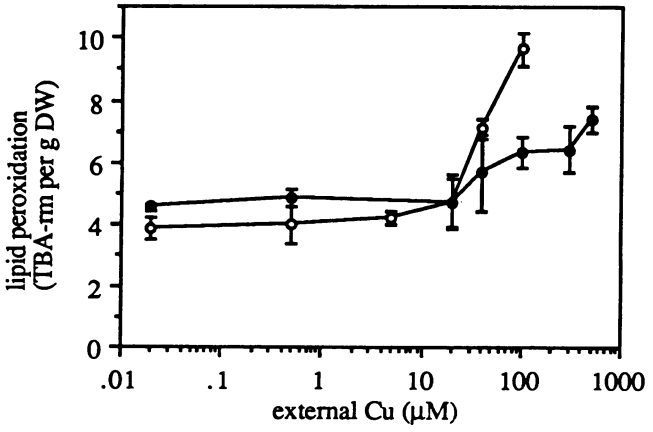

Figure 5. Copper-induced lipid peroxidation in the roots of copper-sensitive (O) and tolerant (●) *S. cucubalus*. Values are means with SD (n = 3).

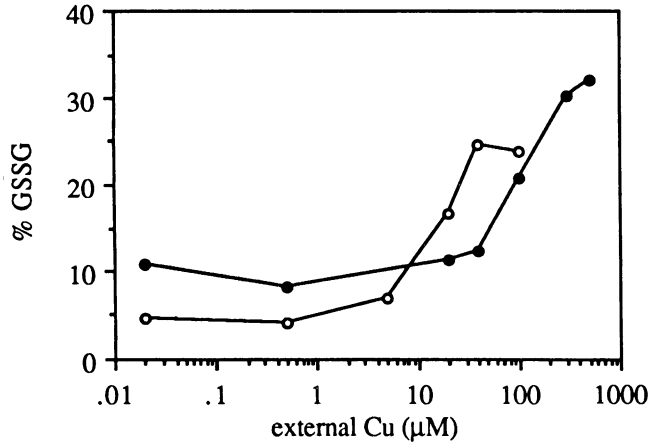

Figure 6. Effect of copper on the glutathione redox state, expressed as GSSG in percentage of total glutathione, in the roots of copper-sensitive (○) and -tolerant (●) *S. cucubalus*.

(Table I). As was determined with cation-specific electrodes, BSO did not affect the availability of cadmium, but it strongly bound copper ions forming nontoxic complexes (log K = 4.56, in 5 mM $NaNO_3$ and 2 mM Mes/KOH, pH 5). Because BSO did not further decrease the glutathione level in the roots after the 7 d pretreatment (J.A. De Knecht, unpublished results), it was omitted from the nutrient solution during the subsequent exposure to copper. In plants not treated with BSO, the supply of either cadmium or copper resulted in an increase of phytochelatin-SH, in both cases together with a decrease in GSH. However, only copper caused an increase in the GSSG content, the oxidation state of the glutathione couple (GSSG as percentage of total), and lipid peroxidation products as well. BSO decreased the levels of both GSH and GSSG, due to its inhibition of GSH synthesis, without affecting the glutathione redox state or lipid peroxidation. The metal-induced production of phytochelatins was strongly inhibited by BSO, especially in the case of copper. The level of GSH in BSO-treated plants decreased to only 17% of that in the controls upon exposure to either cadmium or copper. However, the oxidation state of the glutathione couple and the level of lipid peroxidation products were affected only in the presence of copper. The copper-induced oxidation of glutathione and peroxidation of lipids were significantly stimulated by the pretreatment with BSO (Student's t tests, $P < 0.01$). The sulfhydryl reagent *N*-ethylmaleimide neither affected the levels of thiols nor induced lipid peroxidation.

DISCUSSION

Both the rate of phytochelatin production upon an excessive supply of copper (20 μM, Fig. 1) and the maximum level of phytochelatin-SH (Fig. 3) were higher in the roots of copper-sensitive *S. cucubalus* than in the roots of tolerant plants. This result indicates that copper tolerance in this species is not based on an elevated production of phytochelatins. Copper in phytochelatins is bound as Cu^+ (18), and the stability of this Cu^+-thiolate complex depends on the chain length of the phytochelatins (13). Therefore, one may argue that copper tolerance depends on the production of specific phytochelatins that form a more stable complex with copper. However, phytochelatin synthesis in tolerant plants was negligible at 40 μM Cu or less (Figs. 1 and 3), which concentrations have no effect on the root growth of this variety but severely inhibit that of sensitive plants (6), demonstrating that phytochelatins are not responsible for the elevated no-effect level for copper on root growth in tolerant plants. Also, in both sensitive and tolerant plants the production of phytochelatins was most pronounced at copper concentrations that are toxic, *i.e.* more than 1 μM Cu in the case of sensitive plants and more than 40 μM Cu in the case of tolerant ones, suggesting that phytochelatin synthesis cannot prevent copper toxicity. These results indicate that phytochelatins, regardless of their chain length, are not primarily involved in copper tolerance (*cf.* 24). In contrast with this conclusion, Salt *et al.* (22) suggested that phytochelatins play a crucial role in copper tolerance in *Mimulus guttatus,* because they observed that BSO increased the sensitivity of tolerant plants to copper. However, this result does not imply that the mechanism of copper tolerance depends on phytochelatin synthesis (see below). Moreover, as copper and BSO were supplied simultaneously, the results of Salt *et al.* (22) might have been influenced by the formation of Cu-BSO complexes in the nutrient solution.

Both copper and cadmium caused a marked decrease in glutathione (Table I). GSH is a substrate for phytochelatin synthase (9), so the depletion of glutathione can be ascribed to the metal-induced phytochelatin synthesis in the root cells (*cf.* 15, 20). In contrast with our results, Tukendorf and Rauser

Table I. *Acid-Soluble Thiols and Lipid Peroxidation (TBA-rm) in the Roots of Copper-Sensitive Silene cucubalus*

The preculture with BSO lasted 7 d, after which the plants were exposed to the various reagents for another 3 d. Final concentrations of reagents in the nutrient solution were: 20 μM Cu, 30 μM Cd, 100 μM NEM, and 500 μM BSO. Data are means ± SD (n = 3). ND = not determined.

Preculture	Treatment	Phytochelatins	GSH	GSSG		TBA-rm
		μmol SH g^{-1} dry wt	μmol g^{-1} dry wt	nmol g^{-1} dry wt	% of total	$A_{532-600}$ g^{-1} dry wt
Normal	Control	3.5 ± 1.6	4.7 ± 0.6	173 ± 18	7 ± 0	4.1 ± 0.8
Normal	NEM	3.4 ± 1.9	4.8 ± 0.6	190 ± 17	7 ± 1	4.2 ± 0.2
Normal	Cu	20.4 ± 4.4	1.9 ± 0.2	257 ± 16	21 ± 1	5.8 ± 0.5
Normal	Cd	40.6 ± 9.3	2.1 ± 0.4	50 ± 15	7 ± 2	ND
BSO	BSO	1.5 ± 0.5	1.4 ± 0.5	91 ± 12	12 ± 2	4.3 ± 0.8
BSO	Cu	2.3 ± 1.3	0.8 ± 0.2	164 ± 27	30 ± 2	9.1 ± 0.4
BSO	BSO + Cd	12.7 ± 1.4	0.7 ± 0.1	29 ± 4	8 ± 1	4.1 ± 0.5

(28) reported that copper caused an increase of total glutathione in the roots of maize. Because these authors did not discriminate between GSH and GSSG, their result may be explained by the copper-induced accumulation of GSSG in the roots (*cf.* Table I). The apparent differences among plant species with regard to the effect of copper on the level of total glutathione may then depend on differences in the *in vivo* activity of glutathione reductase.

In both sensitive and tolerant plants, the induction of oxidative stress by copper was coupled to a lowered GSH content (Figs. 4–6) and was significantly increased after pretreatment of plants with BSO (Table I). By contrast, even at a comparably low level of GSH, oxidative stress was not observed with the redox-inactive metal cadmium (Table I). Obviously, GSH plays an important role in the prevention of damage by copper specifically. The observed relation between GSH content and oxidative damage suggests that the production of phytochelatins by copper, in contrast with that by cadmium, may cause oxidative stress as soon as the cytoplasmic concentration of GSH, being both a precursor of phytochelatins and an important antioxidant, falls below a critical level. As a consequence, phytochelatin synthesis induced by copper is potentially harmful for both copper-sensitive and -tolerant plants. The BSO-induced increase in sensitivity of tolerant *M. guttatus* to copper (22) may therefore be ascribed to the depletion of GSH itself rather than to the inhibition of phytochelatin synthesis.

The increase of the GSSG content in the roots of copper-treated sensitive plants (Table I) indicates an increased oxidation of GSH *in vivo*. Copper is known to catalyze not only the oxidation of GSH, but also that of other cellular thiols (11, 14, 21). Therefore, copper may be capable of catalyzing the oxidation of the phytochelatin-SH groups as well, which might explain the sharp decline in the level of phytochelatins in sensitive plants exposed to more than 20 μM Cu (Fig. 3). This decline of phytochelatin-SH was especially apparent at a low GSH content and a high oxidation state of the glutathione couple (Figs. 3, 4, and 6), so phytochelatin oxidation is possibly coupled to a low reduction state of the cell. This suggestion is supported by the fact that the Cu^+-thiolate binding of phytochelatins is air-labile (18). Oxidation of the Cu^+-thiolate binding will release the copper ion, which may result in an increased toxicity. In addition, the copper-mediated oxidation of thiols may severely affect the activity of enzymes, *e.g.* by the formation of mixed disulfides (3). At a comparably high level of oxidative stress in the plants, the level of phytochelatin-SH declined in sensitive plants only (Figs. 3 and 6), suggesting that copper possibly affected the phytochelatin-synthesis machinery in sensitive plants as well.

The loss of glutathione upon metal exposure may be compensated for by GSH synthesis in the root cells themselves (20) and/or by transport from other root compartments and plant parts such as the leaves (19). In *S. cucubalus,* both the constitutive level of glutathione and the copper-induced production of phytochelatins were lower in tolerant plants than in sensitive ones (Figs. 1, 3, and 4), suggesting a lower capacity of copper-tolerant plants to synthesize GSH and to compensate for the metal-induced loss of GSH in the roots.

A fast copper-induced synthesis of GSH and phytochelatins, therefore, is not a plausible system of copper tolerance. Our data are more in support of a model in which phytochelatins are produced as a reaction to the influx of copper ions in the cells. The restricted synthesis of phytochelatins in copper-tolerant plants then suggests a mechanism that keeps copper out of the root cells. In accord with this hypothesis, at nontoxic levels for tolerant plants, *i.e.* less than 40 μM Cu, the uptake of copper by tolerant plants was lower than that by sensitive plants (Fig. 2), and the production of phytochelatins was negligible (Figs. 1 and 3). Also, tolerant plants possibly have a higher trigger point for the onset of phytochelatin synthesis, because at a comparable copper content of the roots the level of phytochelatin-SH in sensitive plants was higher than that in tolerant ones (Figs. 2 and 3). The constitutive difference between tolerant and sensitive plants in the level of GSH itself is probably not involved in copper tolerance, because it was found that plants from another copper-tolerant population of *S. cucubalus* have the same constitutive glutathione level as sensitive plants (data not shown).

In conclusion, our data indicate that copper tolerance in *S. cucubalus* is not related to an elevated production of phytochelatins or glutathione, but to another mechanism, *e.g.* a restriction of copper uptake (*cf.* 6). If the rate of copper uptake by the root cells is too high, the production of phytochelatins results in a depletion of cytoplasmic GSH, causing copper-mediated oxidative damage.

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