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The possible role of metallothioneins in copper tolerance of *Silene cucubalus*

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Abstract. Growth and copper-binding of a copper-tolerant and a copper-sensitive population of *Silene cucubalus* (L.) Wib. have been studied. The copper-tolerant plants showed a much lower uptake and a proportionally higher transport of copper from root to shoot. A copper-binding protein with an apparent M_r of 8500 resembling metallothionein has been isolated from the roots of copper-treated plants of the tolerant population. After 20 d, the protein was observed to be inducible upon copper supply in the copper-tolerant plants, but not yet in the sensitive ones. This could be an indication of a difference in metal-regulated synthesis of the protein. Ion-exchange chromatography of the 8500 protein yielded a major copper-containing fraction eluting at high ionic strength. Other characteristics such as UV absorption and amino-acid composition resembled strongly those of metallothioneins. The involvement of metallothioneins in the detoxification of copper within Cu-tolerant plants is discussed in relation to other mechanisms.

Key words: Copper (uptake, tolerance) – Metallothionein – Protein, Cu-binding – *Silene* – Translocation (Cu).

Introduction

A number of higher-plant species have evolved ecotypes with the ability to grow in soils containing high concentrations of heavy metals (Antonovics et al. 1971; Ernst 1974). There are many types of heavy metal and various hypotheses have been put forward to explain the mechanisms of resistance: i) exclusion from the plant (Baker 1978), ii) compartmentation of the metals in vacuoles (Ernst 1969, 1982; Mathys 1977; Brookes et al. 1981) and cell walls (Turner 1970), iii) the evolution of metal-tolerant enzymes (Wainwright and Woolhouse 1975) and iv) of specific metal-binding proteins similar to metallothioneins (Rauser and Curvetto 1980; Lolkema et al. 1983, Rauser 1984).

Despite the fact that tolerance to one metal does not necessarily confer tolerance to other heavy metals (Gregory and Bradshaw 1965), it is still uncertain whether different mechanisms are at work with respect to different metals of whether different mechanisms complement each other with respect to one metal. Because of the co-occurence of different heavy metals at the same locality, multiple and co-tolerances exist (Allen and Sheppard 1971; Walley et al. 1974; Cox and Hutchinson 1980) and these have hampered the debate on the specificity of tolerance mechanisms. Consequently, we have studied the effect of copper on a copper-sensitive and a copper-tolerant population of *Silene cucubalus,* because copper-tolerance does not seem to be managed by copper compartmentation in the vacuole system (Ernst 1974). In this context it is interesting that metallothionein-like-proteins may be implicated in copper detoxification in tolerant plants.

Abbreviation: DEAE = diethyloaminoethyl

Materials and methods

Plant material. Seeds were collected from two populations of *Silene cucubalus* (L.) Wib., a copper-sensitive one from the botanical garden of the Free University, Amsterdam, The Netherlands, and a copper-tolerant one from a copper mine waste near Imsbach, FRG.

Plant culture. Seeds were sown on garden soil and 10d after germination transplanted to tanks (five plants per tank) filled with 5 l aerated nutrient solution, containing per liter: KNO_3 (4 mmol); $Ca(NO_3)_2.4H_2O$ (1.5 mmol); $NH_4H_2PO_4$ (1 mmol); $MgSO_4.7H_2O$ (0.5 mmol); KCl (50 μmol); H_3BO_3 (25 μmol); $MnSO_4.H_2O$ (2 μmol); $ZnSO_4.7H_2O$ (2 μmol); $(NH_4)_6Mo_7O_{24}$ (0.5 μmol); Fe-ethylene diamine tetraacetic acid (EDTA) (20 μmol); and $CuSO_4.5H_2O$ (0.1 μmol). The pH was adjusted to 5.5. Growth took place in a greenhouse under the following conditions: 70 ± 5% relative humidity, 12/12 h light/dark cycle, and a day/night tem-

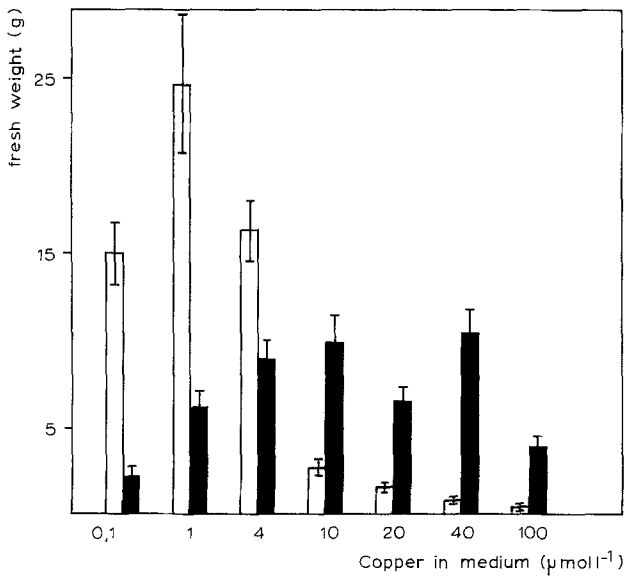

Fig. 1. Biomass production of a sensitive (*open columns*) and a copper-tolerant (*filled columns*) population of *Silene cucubalus*, expressed as fresh weight per plant after a growth period of 20 d on a range of Cu-concentrations in the nutrient solution. Columns represent the average of 13 plants ± SE

perature of 22/15°C. After an initial growth period of 18 d without excess copper, the nutrient solution was replaced by another one containing 0.1, 1, 4, 10, 20, 40 or 100 μmol l^{-1} $CuSO_4$. After a further 20-d period, during which the medium was replaced three times and the pH was kept constant at 5.5, the experiment was finished. Roots were blotted between paper towels prior to weighing. As a reference to the study on metal-binding proteins, ten plants of the tolerant population were also grown in nutrient solution with $ZnSO_4$ (200 μmol l^{-1}) and in nutrient solution with both $ZnSO_4$ (200 μmol l^{-1}) and $CuSO_4$ (40 μmol l^{-1}).

Copper analysis. Six plants of both populations were harvested from each copper treatment. Roots and shoots were separated and dried at 80°C. Roots were washed five times in large volumes of deionized H_2O before drying. After wet-ashing of the dried material in HNO_3- $HClO_4$ (7:1, v/v), the copper concentration was determined by atomic absorption spectrophotometry (Perkin-Elmer 4000; Überlingen, FRG).

Chromatography. Roots from both tolerant and sensitive plants from the experiments with a copper concentration of 1 μM (control treatment) and 40 μM (copper treatment), being the optimum concentrations of the sensitive and the tolerant population, respectively, were harvested, washed in deionized H_2O and homogenized in NaH_2PO_4-Na_2HPO_4 (50 mM; pH 7.0) with 2-mercaptoethanol (5 mM) using a blender. The slurry was strained through four layers of cheesecloth. The filtrate was centrifuged at 20000 *g* for 30 min, the supernatant concentraded by ultrafiltration (Amicon Diaflo YC 05; Oosterhout, The Netherlands) and applied to a column (16/70) containing Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden). Elution (24 ml h^{-1},5-ml fractions) was carried out with NaH_2PO_4-Na_2HPO_4 (5 mM; pH 7.0) at room temperature. Fractions were collected on a Pharmacia Frac 100 fraction collector. The effluents were monitored at 254 nm. Copper in the fractions was directly determined by atomic absorption spectrophotometry. One aliquot of the tolerant copper-treated material was chromatographed on a Sephadex G-50 fine column equilibrated in the same 5 mM phospate buffer and calibrated with Cytochrome c, RNase A, myoglobin and chymothrypsiongen having MWs of

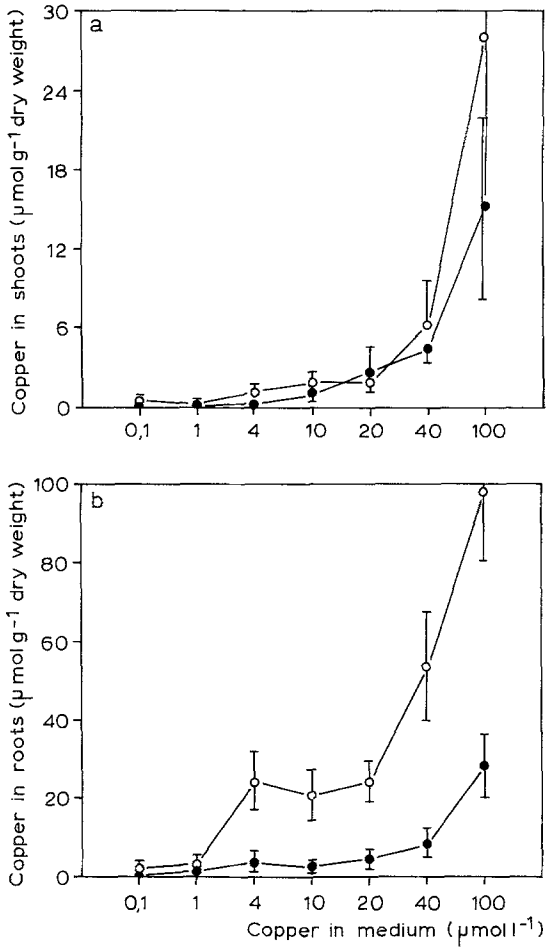

Fig. 2a,b. Copper concentrations in the shoots (**a**) and in the roots (**b**) of a copper-sensitive (○–○) and a copper-tolerant (●–●) population of *Silene cucubalus* on a range of Cu concentrations in the nutrient solution after a growth period of 20 d. Vertical bars, SD; N = 6

$12.4 \cdot 10^3$, $13.7 \cdot 10^3$, $17.8 \cdot 10^3$ and $25 \cdot 10^3$, respectively. The copper-containing peak C of the coppertreated tolerant group (Fig. 5) was pooled, concentrated by ultrafiltration, dialysed against 5 mM 2-amino-2-(hydroxymethyl)-1.3-propanediol-(Tris)HCl and chromatographed on a Sephadex-diethylaminoethyl (DEAE)-A 25 ion-exchange column (16/12) equilibrated in 5 mM Tris-HCl, pH 8.6 according to Rauser and Curvetto (1980). The absorption spectrum of the copper-containing material of the DEAE fractionation was determined after desalting and dialysis against 50 mM Tris-HCl, pH 7.8 (Bartolf et al. 1980).

Amino-acid analysis. Fractions of the DEAE column containing material eluted at high ionic strength were pooled, dialysed against 5 mM Tris-HCl, pH 8.6 and hydrolysed in 6 N HCl for 24 h at 110°C in evacuated sealed tubes. The amino-acid composition was determined with an automated amino-acid analyzer (Beckmann 119 CL; München, FRG).

Results

The biomass production of both populations was different on both low and high copper concentrations (Fig. 1). The sensitive population had a

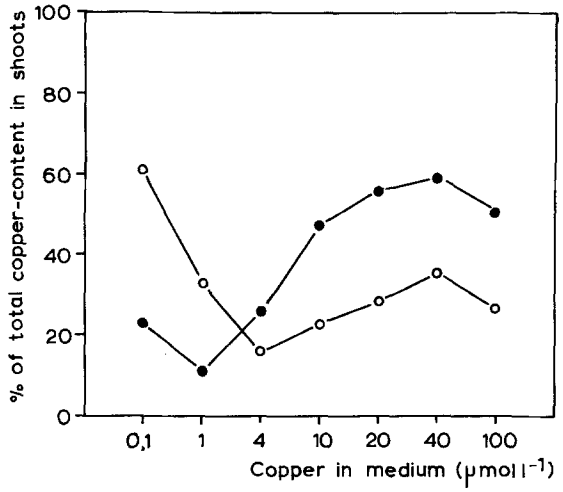

Fig. 3. Percentage of copper content in the shoots of a sensitive (○–○) and a copper-tolerant (●–●) population of *Silene cucubalus* on a whole-plant basis after a growth period of 20 d on a range of Cu concentrations in the nutrient solution

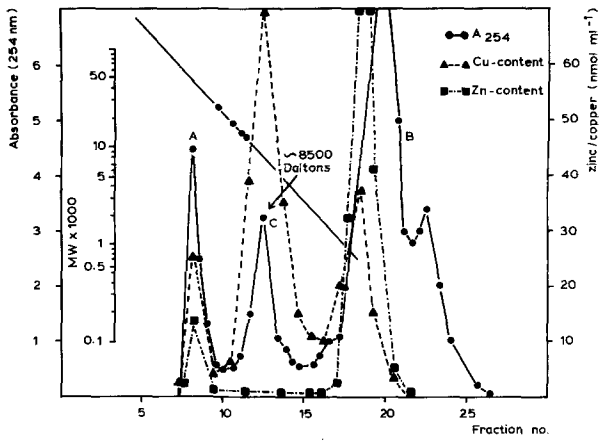

Fig. 5. Sephadex G-50 elution profile of root extract of a Cu-tolerant *Silene cucubalus* grown on 40 μM Cu in the nutrient solution.

growth maximum at 1 μmol Cu l^{-1}, whereas growth was inhibited by 4 μmol Cu l^{-1} and higher concentrations. In contrast to this, the tolerant population gave a positive growth reaction with an increase of the copper supply up to a maximum at 40 μmol Cu l^{-1}.

The copper concentration of the roots of the tolerant population was very low compared with that of the sensitive one (Fig. 2b). The copper concentrations of the shoots did not differ between sensitive and tolerant plants (Fig. 2a), resulting in a higher proportion of the total content on a whole-plant basis in the tolerant plants (Fig. 3).

At the copper concentration giving rise to maximum biomass (40 μmol Cu l^{-1}), the endogenous copper concentration of the tolerant plants was in roots 4 times and in shoots 20 times that of the sensitive ones at their biomass maximum (1 μmol Cu l^{-1}).

Apart from the above differences in uptake and transport behaviour, a marked difference between the two populations was detected with regard to the G-75 gel-filtration profiles (Fig. 4). Copper was eluted from the columns in three small peaks in both populations in the control treatment (1 μM). Two of the copper peaks corresponded with two

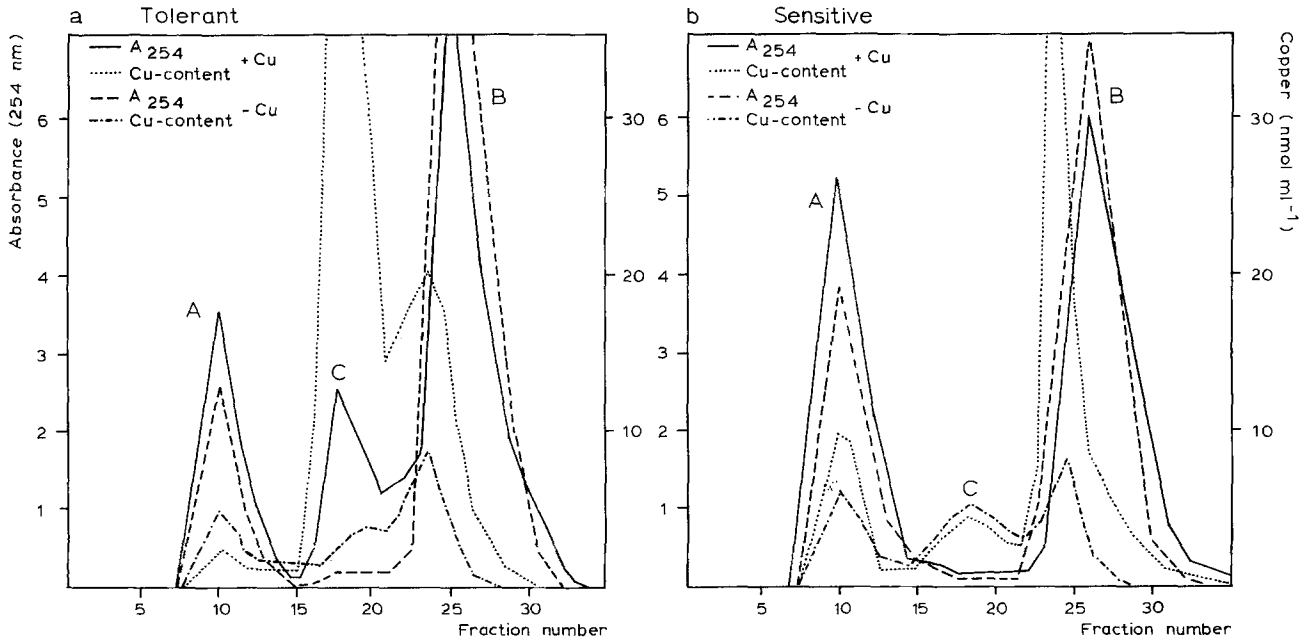

Fig. 4a,b. Sephadex G-75 gel-filtration profiles of root extracts of a copper-tolerant (**a**) and a sensitive (**b**) population of *silene cucubalus* after growth on 1 μM (–Cu). and on 40 μM (+ Cu) Elution was at 24 ml h^{-1} with 5 mM phosphate buffer (pH 7.0), 5-ml fractions

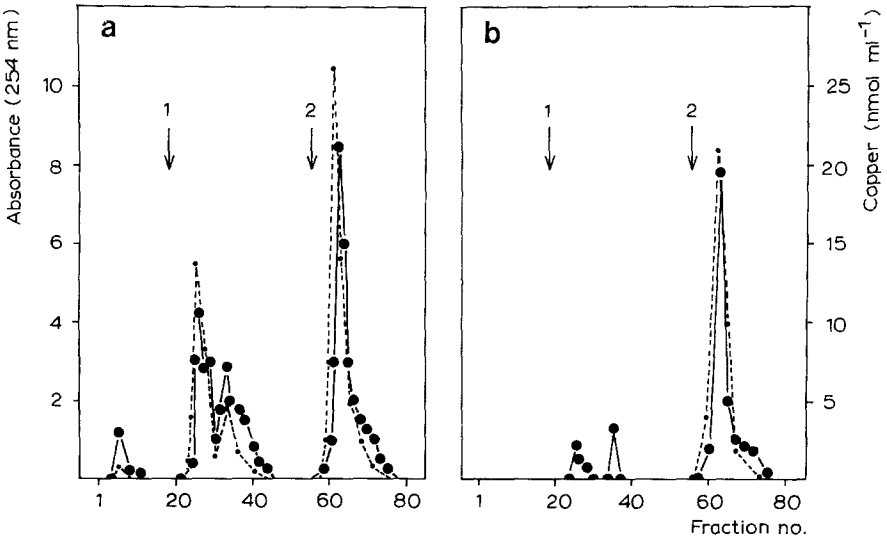

Fig. 6a, b. Ion-exchange chromatography of (**a**) root extract of a Cu-tolerant *Silene cucubalus* and (**b**) Mr 8500 material derived from gel filtration on Sephadex G-75. Elution at 24 ml h^{-1}, 3-ml fractions. *Arrows 1* and *2* indicate the addition of, respectively, 200 and 500 mM NaCl to the elution buffer

major UV-absorbance peaks (A and B). After growth on 40 μM Cu, however, most of the eluted copper of the sensitive plants seemed to be bound in the low-molecular-weight fraction B, while most of the eluted copper of the tolerant population was associated with a new, inducible peak of UV-absorbing material (peak C). Indeed, 72% of all the eluted copper was associated with peak C in the tolerant copper-treated plants, while in the sensitive plants 70% of it eluted in peak B. Up to 35% of the total content of the tolerant roots eluted from the column, against only 10% of the sensitive ones. The amount of C-peak material increased with increasing copper-supply and its proportion in the G-75 elution profile at least doubled in the roots of tolerant plants grown on 100 μmol Cu l^{-1}. The inducibility of the C-peak was specific to copper. Root material of tolerant plants grown on 200 μM zinc did not produce a peak C. Most of the zinc eluted in peak B. Growth on both copper and zinc (40 and 200 μM respectively) did result in a copper-containing peak C but again all the zinc was associated with peak B (Fig. 5). Molecular-weight estimation on calibrated columns revealed an M_r of the C-peak material of about 8500 (Fig. 5).

After chromatography of a total root extract on DEAE-A 25, two major copper-containing peaks were observed eluting from the column at relative low and high ionic strengths. (Fig. 6). When the M_r 8500 material from the G-75 fractionation was chromatographed on DEAE-A 25, only one copper-containing peak was observed eluting after addition of 500 mM NaCl to the elution buffer (Fig. 6). The absorption spectrum of the material eluting at high ionic strength showed a relatively high absorption at 254 nm and a relatively low absorption at 280 nm. Acidification to pH 1 resulted in a decrease in absorption at 254 nm which in the case of metallothioneins is believed to be due to the breaking of Cu-mercaptide bonds (Kägi and Nordberg 1978). The amino-acid composition of C-peak material, listed in Table 1, was characterized by a rather high content of half-cystine especially when compared with the metal-containing part of the B peak. Acidic amino acids comprised a large proportion of the total amino acids and together with cysteine, glycine and serine they accounted for 61.4% of the residues, whereas the aromatic and basic amino acids accounted for a very low proportion.

Table 1. Amino-acid composition of DEAE high-ionic-strength fraction (C) and of G-75 metal-containing part of fraction B (B) of *Silene cucubalus* grown on excess Cu (mol %)

Amino-acid composition of copper-containing fractions		
	C	B
Cysteine[a]	9.3	1.9
Aspartate	8.5	8.9
Glutamate	19.9	7.0
Glycine	14.0	10.1
Serine	9.8	7.3
Threonine	3.5	3.8
Proline	1.9	3.9
Alanine	7.9	6.2
Valine	3.3	3.6
Methionine	3.0	5.2
Isoleucine	2.9	3.4
Leucine	4.2	4.2
Tyrosine	1.6	3.0
Phenylalanine	1.3	6.9
Lysine	5.1	8.6
Histidine	3.1	8.0
Arginine	1.2	8.4

[a] Determined as half-cystine

Discussion

The increasing biomass production with increasing copper supply of the tolerant population (Fig. 1)

indicates a considerable demand for copper. This is comparable to a similar growth-optimum curve described by Ernst (1982) in relation to zinc-tolerant populations of *Silene cucubalus*. In these populations the zinc demand is believed to be a consequence of the compartmentation of the metal in the vacuole system (Ernst 1974). This mechanism does not seem to play a role in copper tolerance of the population described here. The low uptake of the tolerant plants is in agreement with former results of Ernst (1972) on this population, but from the results of other copper-tolerant ecotypes of *S. cucubalus* it was concluded that partial exclusion is not the physiological basis of copper tolerance (Ernst 1972, 1974).

The high proportion of the total amount of copper in the shoots of the tolerant plants is rather striking. It is in contrast to the opinion that copper should be excluded from the leaves because of its inhibitory function of photosynthesis (Graham 1981). Restricted translocation from roots to shoots is also reported in *Agrostis stolonifera* (Wu et al. 1975) and *S. maritima* (Baker 1978). Accumulation into the shoots, however, can provide a means of getting rid of a large proportion of the copper during the annual loss of nearly all shoot material. Apart from a slower uptake by the roots and the proportionally higher transport to the shoots, it seems that the tolerant population can cope with a high internal copper concentration since the root and shoot concentrations of the tolerant plants at maximum growth are, respectively, 4 and 20 times that of those of the sensitive plants at their maximum growth. This means that, at least in the roots of tolerant plants, an effective cellular detoxification mechanism must exist.

Since further attention in this study was focused on roots only, possible accumulation of copper in plastocyanin, which can account for a copper storage of 50–80% of the total content of leaves (Hewitt 1983), has not been considered. The G-75 elution profile of the roots of the copper-treated tolerant plants (Fig. 4a) resembles that of cadmium-treated tomato roots (Bartolf et al. 1980) and cabbage and tobacco (Wagner and Trotter 1982).

The molecular-weight estimate of the C-peak material (Fig. 5) is consistent with that of the estimates of Cd-thioneins of cabbage and tobacco (Wagner and Trotter 1982), tomato (Bartolf et al. 1980) and of low-molecular-weight Cd-binding components of the roots of bean plants (Weigel and Jäger 1980). The M_rs of metallothioneins are in the range of 6000–7000 (Kägi et al. 1974). The increased M_r of the metallothionein of Cu-tolerant *S. cucubalus* may be explained by the general findings that gel-filtration estimates tend to give higher values (Kägi and Nordberg 1978).

The elution profile of the DEAE ion-exchange chromatography of the total root extract resembles that of the fractionation of Cu-thionein from the roots of *A. gigantea* (Rauser and Curvetto 1980). The G-75-derived C-peak material adsorbed strongly to DEAE, which is also a feature of metallothioneins (Kägi et al. 1974; Rauser and Curvetto 1980). The typical UV absorption of the DEAE high-ionic-strength material is another characteristic feature of metallothioneins. The acidic amino acids together with cysteine, glycine and serine make up 67,5% of the residues in the Cu-thionein of *A. gigantea* (Rauser and Curvetto 1980) and 59.8% in the 2Cu- thionein of yeast (Weser et al. 1977), whereas this percentage is 61,4% in the C-peak material of *S. cucubalus* of the present study. The evidence, therefore, that the copper-binding protein C is a metallothionein is quite strong. Conclusive evidence can only follow after further purification. The cysteine content of 9.3% is not as high as the half-cystine content of the Cu-thionein of *A. gigantea* (Rauser and Curvetto 1980) but this might be caused by the fact that the latter material was, after ion-exchange separation, further purified by electrophoresis. Further research will be focused on the biological function of the metallothionein in the copper-resistance of the investigated population of *S. cucubalus*. The increase of C-peak material after increasing copper supply to the tolerant plants is comparable to that of *Neurospora crassa* (Lerch 1980). This is consistent with the suggestion that heavy metals might be able to regulate metallothionein synthesis at a transcriptional level (Premakumar et al. 1975; Kägi et al. 1981). Recently, Fogel and Welch (1982) proposed that Cu-resistance in a yeast strain is mediated by a gene-amplification mechanism whereby sensitive and resistant strains do not differ from each other with regard to the kind of DNA sequence at the locus, but rather in the number of repeats. Resistance levels are proportional to the number of repeats. The observation that the G-75 profiles of the sensitive plants (Fig. 4b) always showed copper peaks in the M_r 8500 region (C peak) might be an indication of some copper-binding protein also in the sensitive population. From a number of reports (Dabin et al. 1978; Bartolf et al. 1980; Weigel and Jäger 1980; Rauser 1981; Casterline and Barnett 1982; Wagner and Trotter 1982), it can be concluded that metal-treated plants possessing Cd- and Cu-binding components such as described above, do not necessarily have to be resistant to the metal.

We therefore suggest that the difference in metallothionein between tolerant and sensitive plants is based on regulation and-or amplification rather than on qualitative differences. The low copper uptake and the relatively high translocation to the shoot of the tolerant population support the idea that apart from a possible detoxification by metallothioneins, other physiological mechanisms can contribute to the extremely high degree of copper-resistance as well. The loss of energy of the resistant population expressed as a lower biomass production at the growth maximum (Fig. 1) could be a further indication of a drastic change in cell metabolism.

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