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In seedlings of the heavy metal accumulator *Brassica juncea* Cu^{2+} differentially affects transcript amounts for γ-glutamylcysteine synthetase (γ-ECS) and metallothionein (MT2)

Holger J. Schäfer, Steffen Greiner, Thomas Rausch, Angela Haag-Kerwer*

Botanisches Institut, Im Neuenheimer Feld 360, D-69120 Heidelberg, Germany

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Abstract Glutathione (GSH) is the precursor of the phytochelatins (PC), which in plants and fungi are involved in heavy metal sequestration. The regulatory enzyme γ-glutamylcysteine synthetase (γ-ECS) catalyzes the first step in GSH biosynthesis. For the heavy metal accumulator *Brassica juncea* L. a partial γ-ECS cDNA was cloned by RT-PCR. Treatment of suspension-cultured dark grown seedlings with micromolar concentrations of $CuSO_4$ resulted in a strong increase of γ-ECS mRNA in roots and shoots, concomitant with an increase of GSH and phytochelatins. A significant up-regulation of γ-ECS mRNA was observed at 25 μM $CuSO_4$ (shoot growth: −11%), whereas maximum up-regulation was obtained at 100 μM $CuSO_4$ (shoot growth: −60%). Unexpectedly, metallothionein 2 (MT2) mRNA was decreased in response to the $CuSO_4$ treatments. $CdSO_4$ at a concentration of 50 μM caused a 72% reduction in shoot growth without affecting the amounts of γ-ECS- and MT2 mRNAs. $ZnSO_4$ at a concentration of 500 μM did not reduce growth but induced transient increases of γ-ECS- and MT2 mRNAs. The implications of the results with respect to differential regulation of γ-ECS and MT2 during heavy metal exposure are discussed.

Key words: Heavy metal stress; *Brassica juncea*; γ-Glutamylcysteine synthetase; Metallothionein; Gene expression; Copper; Cadmium; Zinc

1. Introduction

In plants heavy metal tolerance and hyperaccumulation are thought to be the result of complexation and sequestration of heavy metal ions by specific cysteine-rich polypeptides, known as metallothioneins (MTs) and phytochelatins (PCs), respectively [1,2]. While MTs are gene products, PCs are synthesized enzymatically from glutathione (or related peptides). The tripeptide glutathione (GSH), a product of primary sulfur metabolism [3], is not only involved in PC synthesis after heavy metal exposure but plays also an important role in countering oxidative stress and may be conjugated to diverse xenobiotic compounds as a prerequisite for their vacuolar sequestration [4].

When induced to synthesize PCs in response to heavy metal stress plants often show a pronounced reduction of their cellular GSH pools [5,6], with a concomitant increase of GSH synthetase activity [7]. These observations support a precursor product relationship between GSH and PCs and indicate the substantial demand for GSH during heavy metal stress-induced PC synthesis. GSH synthesis involves two ATP-dependent steps: The first reaction from L-glutamic acid and L-cysteine to γ-glutamylcysteine is catalyzed by the enzyme γ-ECS, while in the second step glycine is added via GSH synthetase [3]. The rate-limiting step of GSH synthesis is thought to be catalyzed by γ-ECS, as the activity of this enzyme is (i) feedback-regulated by GSH, (ii) dependent on cysteine availability, and (iii) inhibited by heavy metals [7,8]. Furthermore, γ-ECS was shown to play a major role in the regulation of PC synthesis [9,10].

Recently, the heavy metal accumulating species *Brassica juncea* has received attention due to its possible use for phytoremediation of heavy metal-polluted soils [11]. In particular, PCs have been shown to accumulate in roots of *B. juncea* upon exposure to Cd, where they are thought to be involved in Cd binding and sequestration [12,13]. Conversely, little is yet known on how this heavy metal accumulator responds to stressful concentrations of copper, an otherwise essential element. We hypothesized that both heavy metal ions could induce differential responses, although in principle excess of Cu and Cd could both induce the same sequestering mechanisms. Thus, it was of primary interest to analyze the expression of key factors for PC and MT formation, namely γ-ECS and MT2, during Cu stress as compared to Cd stress, using concentrations which lead to a comparable growth reduction. Here we have determined growth response and transcript amounts for γ-ECS and MT2 in dark-grown *B. juncea* seedlings after exposure to Cu and Cd, and compared the effects to those of the much less toxic Zn.

2. Materials and methods

2.1. Plant material

Seeds of *B. juncea* L. (variety Vittasso) were surface-sterilized with 2% sodium hypochlorite and 0.1% Triton X-100 for 3 min. After extensive rinsing with sterilized distilled water seeds were germinated in Murashige and Skoog liquid medium and grown under continuous shaking (120 rpm) in the dark for 5 days. For heavy metal exposure $CuSO_4$, $CdSO_4$, or $ZnSO_4$ (for final concentrations: see figure legends) were added from concentrated stock solutions after the 5th day of culture. Seedlings were harvested at 5, 22 and 46 h after heavy metal treatment. After the different incubation times shoot length was determined, and tissue samples for isolation of total RNA were shock-frozen in liquid nitrogen. For the preparation of *B. juncea* L. cDNA sand-grown 4-week-old plants were stressed simultaneously with 1 ppm Cd, 3 ppm Ni, 14 ppm Pb, 0.6 ppm Cu, and 3 ppm Zn, supplied as a single dose on the 28th day. Thereafter, the plants were cultivated for an additional 10 days before harvest. Preparation of *A. thaliana* cDNA was from 7-day-old dark grown seedlings grown in suspension in Murashige Skoog medium.

2.2. Determination of GSH and phytochelatin contents

Plant material frozen in liquid nitrogen was extracted in 5% 5-sulfosalicylic acid, 6 mM diethylenetriaminepentaacetic acid as described in [14]. After centrifugation the supernatant was used for determina-

*Corresponding author. Fax: (49) (06221) 545859.
E-mail: ahaag@botanik1.bot.uni-heidelberg.de

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tion of total non-protein thiols with Ellman's reagent [14], and enzymatic GSH determination according to Anderson [15]. Total phytochelatin concentration expressed as GSH equivalents was estimated from the difference between total non-protein thiols and GSH.

2.3. Cloning of partial cDNAs for B. juncea γ-ECS and A. thaliana MT2

Total RNA from mature leaves of heavy metal-stressed *B. juncea* L. plants was used for first strand cDNA synthesis with Moloney murine leukemia reverse transcriptase (GIBCO-BRL). A partial γ-ECS cDNA was obtained by PCR with 5′-CCTATGAAGTATGATCAAA-TAGC-3′ (sense) and 5′-AGAATTGCCGAAATGTCATTCC-3′ (antisense) as primers. Both primers were designed according to the *A. thaliana* γ-ECS sequence (GenBank accession no. Z29490). The size of the amplified product is 697 bp. The partial cDNA clone has been submitted to the GenBank/EMBL Data Bank (accession no. X95563). The cDNA clone for *A. thaliana* γ-ECS has been described by May and Leaver [16]. A partial MT2 cDNA for *A. thaliana* was obtained from seedling cDNA with the primers described by MT2 [17]. Amplification products were cloned into the Eco RV site of the SK-II$^+$ pBluescript vector (Stratagene). The identity of both partial cDNA clones was confirmed by dideoxy sequencing.

2.4. Isolation of total RNA and northern blot analysis

Isolation of total RNA followed the protocol of Logemann et al. [18]. Alkaline northern blot analysis, probe labeling by PCR, hybridization and non-radioactive detection were performed according to Löw and Rausch [19]. Hybridization temperature was 40°C for both probes (probe sizes: γ-ECS, 697 bp; MT2, 298 bp). High stringency washes were based on ≥65% homology.

3. Results and discussion

3.1. Cloning a partial B. juncea γ-ECS cDNA

For the analysis of differential responses of *B. juncea* seedlings to Cu and Cd stress we have cloned a partial cDNA for γ-ECS by RT-PCR. Sequence comparison of the partial *B. juncea* γ-ECS cDNA clone (651 bp of coding region) with the enzyme of *A. thaliana* revealed 91 and 96% identity at the nucleotide and protein level, respectively. Expression of MT2, a gene previously shown to be induced by heavy metals in *A. thaliana*, was assessed with a heterologous probe [17]. Transcript size of *B. juncea* γ-ECS, as detected with the homologous cDNA probe, was about 1.85 kb in leaves and roots (Fig. 1), being slightly smaller than γ-ECS of *A. thaliana* [16].

Recently, the identity of the *A. thaliana* γ-ECS clone isolated by complementation of a γ-ECS-deficient *E. coli* mutant [16] has been challenged. In particular, doubts were raised as to the specificity of the enzyme assay used for activity determination in the crude extract of complemented *E. coli* cells [20]. However, it could be shown that the same *A. thaliana* γ-ECS clone was able to complement a γ-ECS-deficient yeast mutant (M. May, personal communication), partially restoring the GSH level; also, a specific HPLC-based enzyme assay has now been used leaving no doubt about the identity of the original *A. thaliana* γ-ECS clone and the partial γ-ECS clone from *B. juncea* used in the present study.

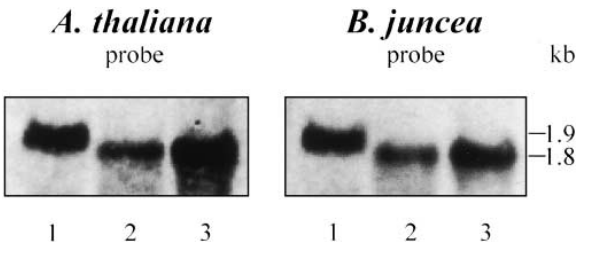

Fig. 1. Transcript size of γ-ECS mRNA in *A. thaliana* (1, total seedlings), and *B. juncea* leaf (2) and root tissue (3). cDNA probes were from *A. thaliana* (left) or *B. juncea* (right). The partial cDNA clone from *B. juncea* was obtained by RT-PCR. 5 μg of total RNA were loaded per lane.

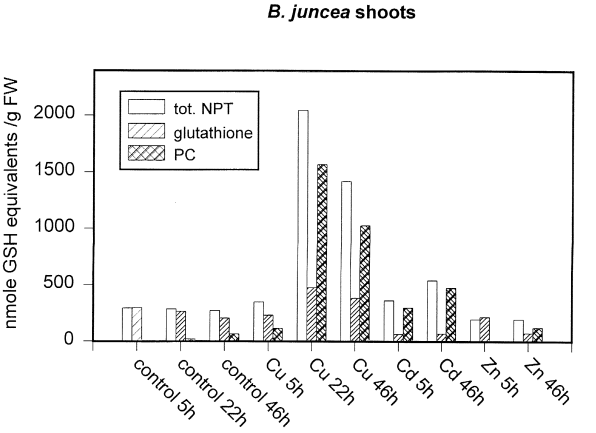

Fig. 2. Concentrations of total non-protein thiols (tot. NPT), GSH and phytochelatins (PC) in the shoots of dark grown *B. juncea* seedlings after treatment with 100 μM $CuSO_4$, 50 μM $CdSO_4$, and 500 μM $ZnSO_4$, respectively, for the indicated time intervals. Values are expressed as GSH equivalents.

With the MT2-probe of *A. thaliana* a single transcript of approx. 580 bp was detected in total RNA samples of *B. juncea* seedlings under stringent conditions, indicating expression of a homologous MT2 gene in *B. juncea* (Figs. 2 and 3).

3.2. Effects of Cu, Cd and Zn on growth of B. juncea seedlings

The range of heavy metal concentrations supplied to the nutrient solution of 5-day-old dark-grown *B. juncea* seedlings and the growth responses of the shoots are presented in Table 1. Seedlings treated with 25 μM $CuSO_4$ and 500 μM $ZnSO_4$ showed shoot growth comparable with controls, whereas shoot growth of seedlings treated with 100 μM and 200 μM $CuSO_4$, as well as with 50 μM $CdSO_4$, was significantly reduced ($p = 0.05$). The relative growth responses of the shoots of *B. juncea* seedlings are similar with those found for roots in various *A. thaliana* ecotypes [21].

3.3. Glutathione, phytochelatins and γ-ECS transcripts in heavy metal-stressed B. juncea seedlings

To compare the effect of Cu and Cd treatments on GSH and PC concentrations we chose metal concentrations which caused similar growth reductions (see Table 1), i.e. 100 μM Cu and 50 μM Cd. Upon exposure to 100 μM $CuSO_4$ *B. juncea* seedlings reacted with a strong increase of total non-protein thiol compounds (Fig. 2). Both GSH content and total phytochelatin concentration were strongly increased after 22 h of $CuSO_4$ treatment. Following a treatment with 50 μM $CdSO_4$ the *B. juncea* seedlings also showed a significant though less pronounced increase of the phytochelatin concentration, however, this increase was accompanied by a decrease of the GSH content. In the seedlings exposed to 500 μM $ZnSO_4$ no significant changes of GSH and phytochelatin concentrations were detected. These results indicate qualitative differences in the non-protein thiol composition in response to the different metal ion treatments. The Cd-induced decrease of GSH content could be the consequence of a direct inhibition of GSH synthetase, as this enzyme has previously

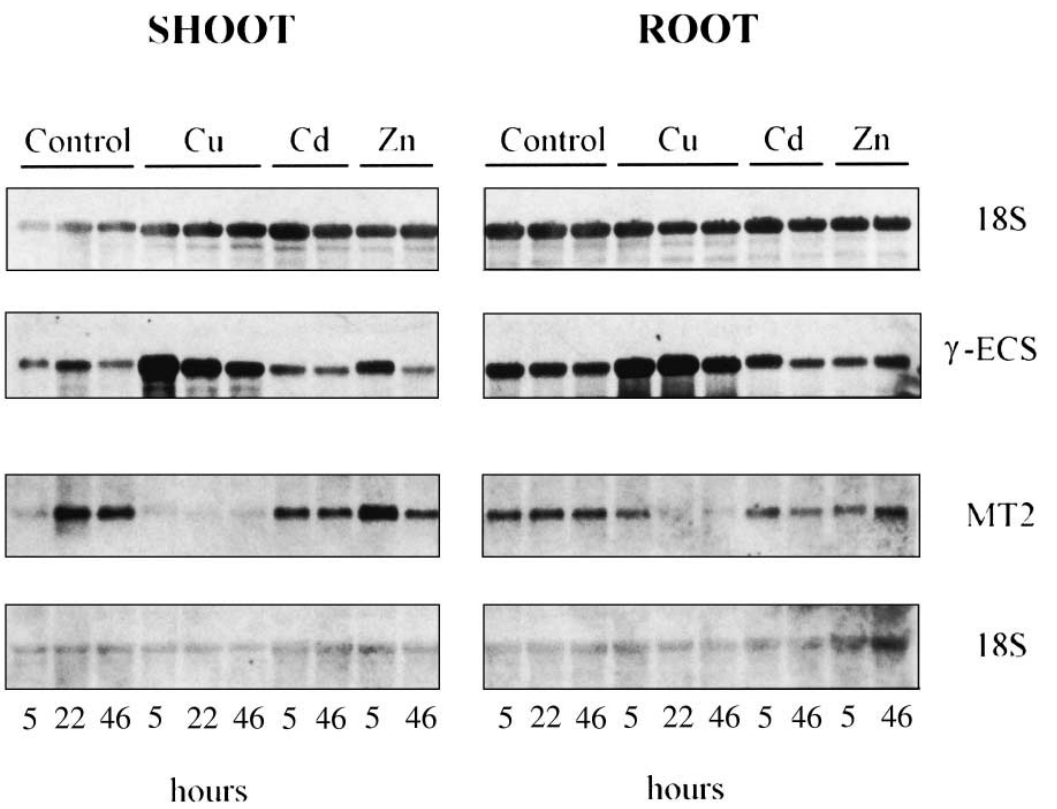

Fig. 3. Northern blot analysis of γ-ECS- and MT2 mRNA in shoot and root tissue of dark grown *B. juncea* seedlings after treatment with 200 μM $CuSO_4$, 50 μM $CdSO_4$, and 500 μM $ZnSO_4$, respectively. Total RNA was extracted from frozen material of controls and stressed seedlings 5, 22 and 46 h after heavy metal application. 5 μg of total RNA were loaded per lane. Equal loading was confirmed with a 18S rRNA probe.

been shown to be very sensitive to Cd, in contrast to γ-ECS which is much less sensitive [7].

In response to Cu exposure transcript amounts for γ-ECS were strongly increased in shoot and root tissue (Figs. 3 and 4). The Cu effect indicates that de novo synthesis of GSH plays a major role in the Cu stress response [22]. The observation that after Cu-treatment the contents of GSH and PC increase in parallel does, however, not prove a direct precursor-product relationship since recently Meuwly et al. [23] have shown that at least in some plants γ-glutamylcysteine may be the direct precursor for both GSH and PCs. The induced formation of PCs indicates complexation and sequestration of excess Cu, a process earlier claimed to counter excess levels of Cd, Ni and Zn [5]. Recently, Murphy and Taiz [24] found greater amounts of non-protein thiols in certain *A. thaliana* ecotypes with high Cu tolerance. In preliminary experiments with *A. thaliana* seedlings we observed a similar Cu-induced increase of γ-ECS transcript amounts (data not shown), suggesting that increased expression of γ-ECS is not confined to the heavy metal accumulating species *B. juncea*. It is noteworthy, that GSH may also counter oxidative stress resulting from the Cu treatment [3]. As Cu may inhibit γ-ECS activity [8] the up-regulation of transcript amount may be an essential step to maintain a sufficient rate of GSH synthesis.

In contrast to Cu, cadmium has been known as a potent inducer of PC formation [12,13]. We have stressed *B. juncea* seedlings with 50 μM $CdSO_4$, a concentration leading to a similar growth inhibition as compared to 100 μM $CuSO_4$. Surprisingly, under our experimental conditions transcript amounts for γ-ECS did not change significantly after Cd exposure (Fig. 3), however, Cd effects on enzyme activity and/or turnover cannot be excluded. In particular, GSH synthetase has been shown to be particularly sensitive towards Cd, whereas γ-ECS is much less sensitive [7]. Furthermore, assuming that γ-glutamylcysteine may be a direct precursor for PC synthesis (see above) the steady concentration of the immediate GSH precursor could be lowered after the onset of PC formation.

Table 1
Growth response of shoots of *B. juncea* seedlings to heavy metal exposure

Treatment	Shoot growth rate (mm h^{-1})	% of control
Control	0.53[a]	100
Cu 25 μM	0.43[a]	89
Cu 100 μM	0.18[b]	40
Cu 200 μM	0.08[b]	17
Cd 50 μM	0.11[b]	28
Zn 500 μM	0.48[a]	89

Shoot lengths were measured 5, 22 and 46 h after heavy metal application (n=12). Growth rate was deduced by linear regression.
[a,b]Data are significantly different at the level of $p = 0.05$.

Table 2
Summary of Cu, Cd and Zn effects on shoot growth and transcript amounts for γ-ECS and MT2 in seedlings of *B. juncea*

Response of seedling	Heavy metal exposure		
	100 μM $CuSO_4$	50 μM $CdSO_4$	500 μM $ZnSO_4$
Shoot growth, % of control	40	28	89
γ-ECS mRNA	strong increase	no effect	transient increase
MT2 mRNA	strong decrease	no effect	transient increase

When seedlings were challenged with 500 μM $ZnSO_4$, a concentration at which seedling growth is barely affected (Table 1), transcript levels for γ-ECS were moderately and transiently increased (Fig. 3).

3.4. Expression of MT2 in heavy metal-stressed *B. juncea* seedlings

To monitor stress-induced metallothionein formation we assessed transcript amounts for MT2 [17,24]. Metallothioneins are thought to play a role in Cu detoxification [10], and in *A. thaliana* MT2 mRNA has been shown to be Cu-inducible [15,18,22,25]. Surprisingly, in *B. juncea* seedlings exposure to $CuSO_4$ led to a significant decrease of MT2 mRNA (Figs. 3 and 4). This decrease was more pronounced in shoots than in roots. While 50 μM $CdSO_4$ led to a similar reduction in shoot growth as compared to 100 μM $CuSO_4$ (Table 1), transcript amounts for MT2 were not affected (Fig. 3). Thus, it seems unlikely that the Cu-induced reduction of MT2 mRNA is the result of a general growth repression. The absence of Cd effects on γ-ECS- and MT2 mRNA amounts in *B. juncea* seedlings may reflect a different threshold concentration required for causing effects at the gene expression level. Also additional chelating mechanisms may be involved (complexation by organic acids [26] or amino acids [27]). Again, Zn exposure led to a transient induction of MT2 mRNA in the shoot (Fig. 3), indicating that GSH and/or PC as well as MTs participate in cellular homoeostasis of this essential element.

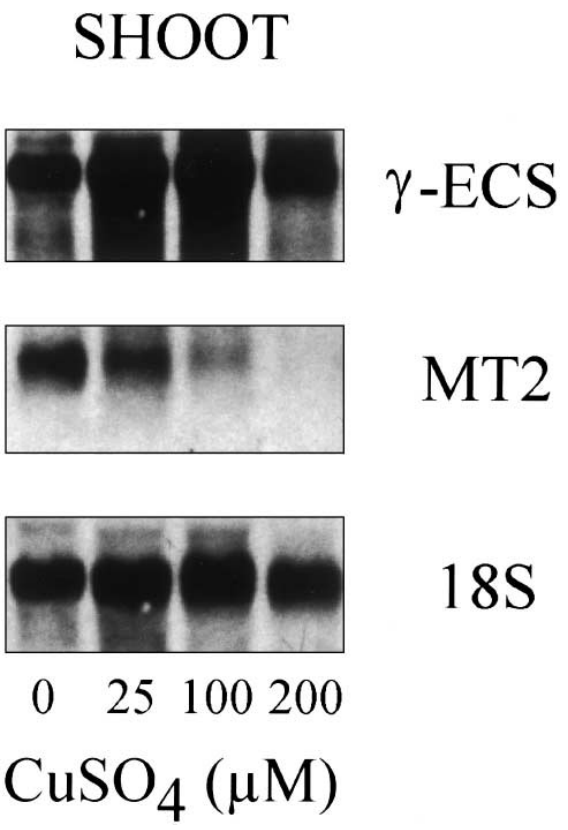

Fig. 4. Expression of γ-ECS- and MT2 mRNA in shoot of dark grown *B. juncea* seedlings 22 h after treatment with 25 μM, 100 μM and 200 μM $CuSO_4$, respectively. 5 μg of total RNA were loaded per lane.

In conclusion our data show that while in etiolated *B. juncea* seedlings Cu (100 μM) and Cd (50 μM) lead to similar growth reductions transcript amounts for γ-ECS and MT2 are (differentially) affected only by the Cu treatment (Table 2). We hypothesize that in response to Cu stress heavy metal quenching by PCs is favored over sequestration by MTs. In addition, the increase in GSH content may be required for countering a Cu-induced oxidative stress. Conversely, for the treatment with the toxic Cd ion the significant growth inhibition is not accompanied by changes in γ-ECS and MT2 expression although the changes in non-protein thiols indicate that metabolic changes have occurred. However, the response to Cd exposure strongly depends on plant development since in leaves of 6-week-old *B. juncea* plants 25 μM $CdSO_4$ clearly affects γ-ECS transcript levels (H.J. Schäfer, A. Haag-Kerwer and T. Rausch, manuscript in preparation).

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