Journal of Experimental Botany, Vol. 53, No. 379, pp. 2381–2392, December 2002
DOI: 10.1093/jxb/erf107

Journal of Experimental Botany

The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes

Henk Schat[1,3], Mercè Llugany[1,4], Riet Vooijs[1], Jeanette Hartley-Whitaker[2] and Petra M. Bleeker[1]

[1] *Department of Ecology and Physiology of Plants, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands*

[2] *Centre for Ecology and Hydrology, Merlewood, Grange-over-Sands, Cumbria LA11 6JU, UK*

Received 17 May 2002; Accepted 2 August 2002

Abstract

Using the γ-glutamylcysteine synthetase inhibitor, L-buthionine-[S,R]-sulphoximine (BSO), the role for phytochelatins (PCs) was evaluated in Cu, Cd, Zn, As, Ni, and Co tolerance in non-metallicolous and metallicolous, hypertolerant populations of *Silene vulgaris* (Moench) Garcke, *Thlaspi caerulescens* J.&C. Presl., *Holcus lanatus* L., and *Agrostis castellana* Boiss. et Reuter. Based on plant-internal PC-thiol to metal molar ratios, the metals' tendency to induce PC accumulation decreased in the order As/Cd/Cu > Zn > Ni/Co, and was consistently higher in non-metallicolous plants than in hypertolerant ones, except for the case of As. The sensitivities to Cu, Zn, Ni, and Co were consistently unaffected by BSO treatment, both in non-metallicolous and hypertolerant plants, suggesting that PC-based sequestration is not essential for constitutive tolerance or hypertolerance to these metals. Cd sensitivity was considerably increased by BSO, though exclusively in plants lacking Cd hypertolerance, suggesting that adaptive cadmium hypertolerance is not dependent on PC-mediated sequestration. BSO dramatically increased As sensitivity, both in non-adapted and As-hypertolerant plants, showing that PC-based sequestration is essential for both normal constitutive tolerance and adaptive hypertolerance to this metalloid. The primary function of PC synthase in plants and algae remains elusive.

Key words: Buthionine sulphoximine, heavy metal tolerance, *Holcus lanatus*, hyperaccumulator, metallophyte, phytochelatins, *Silene vulgaris*, *Thlaspi caerulescens.*

Introduction

Phytochelatins (PCs) are small metal-binding peptides with the structure $(\gamma\text{-glu-cys})_n$-gly, $(\gamma\text{-glu-cys})_n$-β-ala, $(\gamma\text{-glu-cys})_n$-ser, $(\gamma\text{-glu-cys})_n$-glu, $(\gamma\text{-glu-cys})_n$-gln or $(\gamma\text{-glu-cys})_n$, in which *n* varies from 2 to 11 (Grill *et al*., 1985, 1986*a*; Mehra and Winge, 1988; Meuwly *et al*., 1993; Klapheck *et al*., 1994). Their synthesis from glutathione (Grill *et al*., 1989), homo-glutathione, hydroxymethyl-glutathione (Klapheck *et al*., 1995) or γ-glutamylcysteine (Hayashi *et al*., 1991) is catalysed by a transpeptidase, named phytochelatin synthase, which is a constitutive enzyme requiring post-translational activition by heavy metals (Grill *et al*., 1989; De Knecht *et al*., 1995; Klapheck *et al*., 1995; Chen *et al*., 1997). Phytochelatin synthase (PCS) has been shown to be activated by a broad range of metals and metalloids, in particular Cd, Ag, Pb, Cu, Hg, Zn, Sn, Au, and As, both *in vivo* and *in vitro* (Grill *et al*., 1987; Maitani *et al*., 1996; Chen *et al*., 1997). The capacity to synthesize PCs is supposed to be present in all higher plants (Gekeler *et al*., 1989) and the majority of algae (Ahner *et al*., 1995). They also have been detected in several fungi, including *Schizosaccharomyces pombe*, *Candida glabrata*, and *Mucor racemosus* (Grill *et al*., 1986*b*; Mehra *et al*., 1988; Miersch *et al*., 2001). In addition, the nematode wurm, *Caenorhabdites elegans*,

[3] To whom correspondence should be addressed. Fax: +31 20 4447123. E-mail: hschat@bio.vu.nl
[4] Present address: Laboratorio de Fisiología Vegetal, Faculdad de Ciencias, Universidad Autónoma de Barcelona, E-08193 Bellaterra, Spain.
Abbreviations: BSO: L-buthionine-[S,R]-sulphoximine; PC: phytochelatin; GSH: glutathione; g-ECS: g-glutamylcysteine synthetase; GS: glutathione synthase; PCS: phytochelatin synthase.

appeared to possess a *PCS* gene, which restored PC synthesis and Cd tolerance in an *S. pombe* PCS knock-out strain, suggesting that functional *PCS* genes may be present in certain animals too (Clemens *et al*., 2001; Vatamaniuk *et al*., 2001).

There has been considerable debate concerning the function of PCs. They have been assumed to function in the cellular homeostasis or trafficking of essential heavy metal nutrients, particularly Cu and Zn (Thumann *et al*., 1991). However, the Cu and Zn exposure levels that are minimally required to induce PCs at considerable concentrations in plant cells are often far above the normal nutritional requirements, or even close to the toxicity thresholds (Schat *et al*., 2000). Moreover, PC-deficient mutants, such as *Arabidopsis cad1* (Howden *et al*., 1995), have never been reported to exhibit increased requirements for essential metal nutrients, and did not show considerably increased sensitivities to Cu or Zn (Howden and Cobbett, 1992). Conforming with these observations, ecotypic differences in Cu tolerance in *Arabidopsis thaliana* were shown to be correlated with type-2 metallothionein expression, rather than PC accumulation rates (Murphy and Taiz, 1995). However, *S. pombe* cells disrupted in their *PCS* gene were hypersensitive to Cu (Clemens *et al*., 1999), indicating that PC synthesis may be required for Cu detoxification in some organisms, at least.

On the other hand, there is convincing evidence that PCs are essential for normal constitutive tolerance to several non-essential metals, particularly Cd. First, disruption of the *PCS* gene in *S. pombe* resulted in hypersensitivity to Cd (Clemens *et al*., 1999; Ha *et al*., 1999). Second, expression of *PCS* cDNAs from wheat, *Arabidopsis*, and *S. pombe* dramatically increased Cd tolerance in *Saccharomyces cerevisiae*, even in mutants deficient in vacuole formation or vacuolar acidification (Clemens *et al*., 1999). Third, a number of Cd-hypersensitive *Arabidopsis* mutants appeared to be impaired in PC synthesis (Howden *et al*., 1995; Cobbett *et al*., 1998). In addition, tomato cell lines selected for hypertolerance to Cd exhibited enhanced PC synthesis under Cd exposure, due to increased γ-glutamyl cysteine synthetase (γ-ECS) activity (Chen and Goldsbrough, 1994). Furthermore, overexpression of bacterial γ-ECS or glutathione synthetase (GS) in *Brassica juncea* enhanced PC synthesis and Cd tolerance (Zhu *et al*., 1999*a*, *b*). PCs might also be required for tolerance to non-essential Hg and As. PC-deficient *Arabidopsis cad1* mutants were also hypersensitive to Hg (Howden and Cobbett, 1992). Inhibition of PC synthesis by treatment with the γ-ECS inhibitor, buthionine sulphoximine (BSO), enhanced Hg sensitivity in *Hydrilla verticillata* and *Vallisneria spiralis* (Gupta *et al*., 1998). Likewise, BSO-treated cell cultures of tobacco and *Rauvolfia serpentina* were found to be hypersensitive to As (Nakazawa *et al*., 2000; Schmöger *et al*., 2000).

Normal constitutive tolerance to Cd and, possibly, Hg and As, is apparently not entirely explained by the mere chelation of Cd by PCs in the cytosol. Several Cd-hypersensitive *S. pombe* mutants showed normal PC synthesis under Cd exposure, but appeared to be deficient in functional HMT1, an ABC-type transporter mediating the transport of Cd–PC complexes into vacuoles (Ortiz *et al*., 1992, 1995), or impaired in a further stabilization of vacuolar Cd-PC complexes through the incorporation of acid-labile sulphide (Speiser *et al*., 1992*b*; Juang *et al*., 1993). MgATP-dependent tonoplast transport and vacuolar accumulation of Cd–PC complexes have also been demonstrated in oat and tobacco, respectively (Vögeli-Lange and Wagner, 1989; Salt and Rauser, 1995). Also, acid-labile sulphide incorporation in Cd–PC complexes has been demonstrated in *B. juncea* and *Silene vulgaris* (Speiser *et al*., 1992*a*; De Knecht *et al*., 1994), suggesting that vacuolar compartmentalization and further stabilization of Cd–PC may be essential for normal Cd tolerance in plants too.

Naturally selected heavy metal hypertolerance, which is commonly found in plant populations from strongly metal-enriched soils, does not seem to be associated with enhanced PC synthesis. De Knecht *et al*. (1995) obtained equal capacities and activation constants for Cd-induced PC synthesis in crude protein extracts prepared from roots of Cd/Zn-hypertolerant and non-metallicolous *S. vulgaris*. The root PC concentrations measured *in vivo*, however, were much lower in the hypertolerant plants, even when compared at equal rates of Cd uptake (De Knecht *et al*., 1994). The *in vivo* acid-labile sulphide contents of the Cd–PC complexes and PC chain length distributions were identical, suggesting that possible differences in the stabilities of the complexes formed in both plant types were absent (De Knecht *et al*., 1994). Also, the reduced glutathione (GSH) concentrations in the roots responded similarly to Cd exposure, and the rates of PC degradation and GSH recovery after arresting the exposure were identical, suggesting that the lower PC accumulation in the hypertolerant plants resulted neither from a lower GSH availability, nor from a higher PC turnover rate (De Knecht *et al*., 1995). Finally, the fraction of exclusively acid-extractable root Cd was consistently higher in the hypertolerant plants (De Knecht *et al*., 1994). In the same species, Cu-induced and Zn-induced accumulation of PCs in roots also appeared to be much higher in non-metallicolous plants than in Cu-hypertolerant and Zn-hypertolerant plants respectively, both when compared at equal metal exposure levels and at equal rates of metal accumulation in the roots (De Vos *et al*., 1992; Harmens *et al*., 1993). Moreover, decreased PC accumulation was shown to co-segregate with Cu hypertolerance in crosses between non-metallicolous and Cu-hypertolerant plants (Schat and Kalff, 1992). Thus, although artificial over-expression of enzymes and transporters involved in the

PC-based metal sequestration machinery, such as γ-ECS, GS, PCS or HMT1, has been shown to increase metal tolerance or Cd tolerance, at least (Ortiz *et al*., 1995; Clemens *et al*., 1999; Zhu *et al*., 1999*a*, *b*), and although several examples of enhanced PC synthesis in cell lines artificially selected for Cd hypertolerance have been reported (Chen and Goldsbrough, 1994), there is no evidence of naturally selected enhanced PC synthesis in hypertolerant plant populations from Cd-, Zn-, or Cu-toxic environments. Moreover, BSO did not detectably enhance the response to a 40 μM Cd treatment in a hypertolerant ecotype of *S. vulgaris*, although it dramatically sensitized a non-metallicolous ecotype under identical conditions, suggesting that PC synthesis might not be required for naturally selected Cd hypertolerance in this species (De Knecht *et al*., 1992). In general, the strongly decreased rates of PC accumulation in Cu-, Cd-, and Zn-hypertolerant *S. vulgaris* mine populations (see above) might, in fact, result from increased activities of alternative PC-independent sequestration mechanisms leading to decreased cytoplasmic metal availabilities for PCS activation (De Knecht *et al*., 1995). On the other hand, naturally selected As hypertolerance in *Holcus lanatus* was found to be associated with enhanced rates of PC accumulation and increased PC-thiol to As molar ratios in roots, suggesting that PC synthesis might be essential for hypertolerance to As, at least (Hartley-Whitaker *et al*., 2001).

The present evidence with regard to the precise role for PCs in constitutive and naturally selected high-level metal tolerances is often fragmentory and ambiguous. Much of the evidence is based on single-concentration exposures. In this study, the complete dose–response curves were compared for root growth inhibition and PC accumulation imposed by Cu, Cd, Zn, Ni, Co, and As in metallicolous and non-metallicolous ecotypes of the pseudometallophytes *Silene vulgaris*, *Holcus lanatus*, *Agrostis castellana*, and the Zn hyperaccumulator, *Thlaspi caerulescens*. To assess the possible role for PCs in metal tolerance, the exposures were done with and without BSO in the nutrient solution.

Materials and methods

Plant materials

S. vulgaris seeds were collected from a copper mine near Marsberg (Germany), a zinc smelter waste deposit at Plombières (Belgium), and a non-metalliferous site at the Free University Campus (Amsterdam, The Netherlands). The population from Marsberg is Cu-hypertolerant, and shows low degrees of hypertolerance to Zn and Cd. The population from Plombières is hypertolerant to Zn and Cd, and shows pleiotropic hypertolerance to Ni and Co (Schat and Vooijs, 1997). More detailed site and population characteristics have been given in Schat *et al*. (1996). Seeds of *T. caerulescens* were collected from a Zn ore waste deposit near La Calamine (Belgium), a non-metalliferous site at Willerwiltz (Luxemburg), and from a serpentine hill (Monte Prinzera, Italy). From previous studies it appeared that the Zn tolerance of these populations, as estimated from threshold exposure levels for leaf chlorosis, varied strongly, decreasing in the order La Calamine (LC) > Monte Prinzera (MP) > Willerwiltz (W). Likewise, Ni tolerance decreased in the order MP >> LC > W, and Cd tolerance in the order LC >> W > MP. Zn, Ni, and Cd accumulation were much higher in MP and W than in LC (Assunção *et al*., 2001; AGL Assunção and H Schat, unpublished results). Seeds of *A. castellana* and *H. lanatus* were collected at an As-enriched gold mine waste dump near Jales (Portugal). Non-metallicolous *H. lanatus* was collected from the botanical garden of the Free University Campus (Amsterdam, The Netherlands).

Tolerance testing

Seeds were germinated on moist peat and 8-d-old seedlings were transferred to 1.0 l polyethylene pots (three plants per pot) with aerated MES-buffered nutrient solution composed as in Schat *et al*. (1996), or, in case of the grasses, with half-strength macronutrient concentrations. After 5 d of hydroponic culture, the solution was replaced by a fresh one of the same composition. Half of the pots were supplied with L-BSO at a 250 μM concentration (higher concentrations did not produce a further decrease of root GSH levels, as demonstrated in pilot experiments). After another 5 d period, the solutions were replaced again, and the metals were added at appropriate concentrations. All the metals were added as sulphate salts, except As, which was supplied as sodium arsenate. The test solutions were the same as during preculture, except for the Cu-spiked solutions, from which Fe-EDTA was omitted to prevent Cu-EDTA formation. Also, in the As-spiked solutions, the $NH_4H_2PO_4$ concentration was reduced to a 10 μM level, to prevent excessive competitive inhibition of arsenate uptake. The BSO treatment was maintained during metal exposure, except in the case of Cu, because Cu, by contrast with the other metals, appeared to be complexed by BSO in the nutrient solution, as shown by measurements with ion-specific electrodes (H Schat, unpublished results). The root elongation was measured after 4 d of exposure, using the charcoal staining method (Schat and Ten Bookum, 1992) in the case of *S. vulgaris* and *T. caerulescens*. Part of the plants were left unstained and used for PC and metal analysis. In the case of the grasses, the roots were cut off at the start of the metal treatment, and the length of the longest new root was measured after 4 d.

Growth chamber conditions were exactly as in Schat *et al*. (1996).

PC and metal analysis

Prior to harvest, the root systems were desorbed in an ice-cold 5 mM $Pb(NO_3)_2$ solution for 30 min. Roots and shoots were separated, immediately frozen in liquid nitrogen, lyophilized, and stored under vacuum until analysis. Twenty to 100 mg aliquots of ground dry material were digested in 2 ml of a 1:4 (v/v) mixture of 37% (v/v) HCl and 65% (v/v) HNO_3, in closed Teflon cylinders for 6 h at 140 °C. Metals in the digests were measured using a flame atomic absorption spectrophotometer (Perkin Elmer 2100), in the case of As with a coupled MHS-10 hydride system. Phytochelatins were extracted and measured by HPLC, using post-column derivatization with Ellman's reagent, exactly as described in De Knecht *et al*. (1994), except for samples of As-treated plants. The latter were analysed after pre-column derivatization with monobromobimane, exactly as in Sneller *et al*. (1999). Corrections for differential derivatization efficiences were made according to Sneller *et al*. (2000).

Statistics

The data were statistically analysed using two-way ANOVA after log-transformation of the data. Significance of the BSO×metal concentration interaction was used as a criterion for BSO-imposed hypersensitivity.

Results

Copper

PC-mediated Cu tolerance was tested in non-metallicolous and cupricolous *S. vulgaris* ecotypes (populations Amsterdam and Marsberg, respectively). Copper induced the accumulation of PCs in roots of both ecotypes. However, the threshold Cu exposure level required to induce significant PC accumulation was much higher in the cupricolous ecotype than in the non-metallicolous ecotype. Moreover, the cupricolous ecotype exhibited lower root-internal PC-thiol (PC-SH) to Cu molar ratios (Table 1). Shoot PC concentrations were negligible in both ecotypes (data not shown). In the roots, however, PCs were strongly decreased by BSO in both ecotypes (Table 1). Usually, the root copper concentrations were also decreased, though to a much lower degree, resulting in lower PC-SH to Cu molar ratios in the BSO-treated plants (Table 1). The root growth response to Cu, however, was completely unaffected by BSO, both in the non-metallicolous and the cupricolous ecotype (Fig. 1).

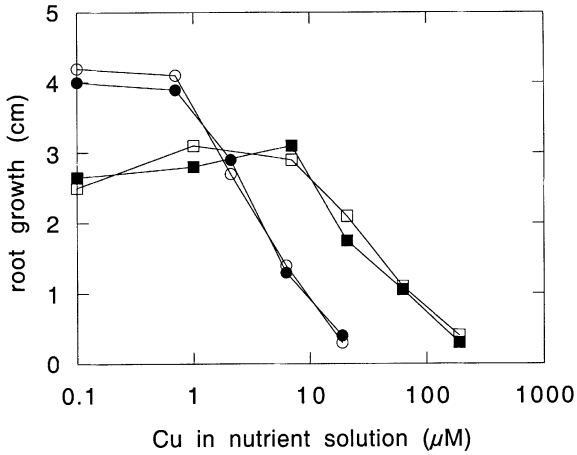

Fig. 1. Mean root elongation (n=15) throughout 4 d of exposure to Cu in BSO-treated (open symbols) and untreated (closed symbols) non-metallicous (circles) and cupricolous (squares) *S. vulgaris*. Standard errors varied between 2% and 8% of the means.

Cadmium

PC-mediated Cd tolerance was established in non-metallicolous and Cd/Zn-hypertolerant *Silene vulgaris* (populations Amsterdam and Plombières, respectively). Cd, like Cu, strongly induced the accumulation of PCs in roots (Table 2), but barely or not in shoots (data not shown). Again, the PC concentrations and the root-internal PC-SH to Cd ratios were higher in the non-metallicolous ecotype than they were in the hypertolerant calamine ecotype (Table 2). BSO strongly decreased root GSH and PC concentrations and, though to a much lower degree, root Cd concentrations, resulting in considerably decreased PC-SH to Cd molar ratios (Table 2). BSO significantly enhanced Cd-imposed root growth inhibition in the non-metallicolous ecotype (P <0.001), but did not detectably effect the root growth response of the hypertolerant ecotype (Fig. 2).

Using a similar experimental design, three *T. caerulescens* ecotypes with varying degrees of Cd tolerance and accumulation, originating from serpentine, calamine, and non-metalliferous soil (populations Monte Prinzera, La Calamine, and Willerwiltz, respectively) were compared. Cd induced considerable PC accumulation in the roots and shoots of all these ecotypes, though to different degrees. When compared at similar root-internal Cd concentrations, the root PC concentrations decreased in the order Monte Prinzera > Willerwiltz > La Calamine (Fig. 3), which is also the order of decreasing sensitivity to Cd (see Materials and methods). The same pattern was also found in shoots (data not shown). Cd-imposed root growth inhibition was not enhanced by BSO in either of the ecotypes (Fig. 4). At the end of the experiment, i.e. 4 d after Cd supply, there

Table 1. *Total phytochelatin thiol (PC-SH) concentrations and PC-SH to Cu molar ratios (means of three samples of three plants each; SE in parentheses) in desorbed roots of unpretreated and BSO-pretreated non-metallicolous (Amsterdam) and cupricolous (Marsberg)* S. vulgaris*, after a 4 d exposure to increasing Cu concentrations in the nutrient solution (nd=not determined)*

Population	Exposure (μM Cu)	PC-SH (μmol g^{-1} DW)		PC-SH:Cu (mol mol^{-1})	
		+BSO	–BSO	+BSO	–BSO
Amsterdam	0.1	<0.1	<0.1	nd	nd
	0.7	0.2 (0.07)	0.4 (0.11)	0.8 (0.23)	1.2 (0.40)
	2.1	3.2 (0.63)	9.7 (2.35)	1.4 (0.21)	3.7 (0.65)
	6.3	4.6 (0.41)	12.8 (1.98)	1.5 (0.47)	3.6 (0.15)
	18.9	1.8 (0.16)	2.8 (0.61)	nd	nd
Marsberg	0.1	<0.1	<0.1	nd	nd
	1.0	0.2 (0.08)	0.3 (0.08)	0.4 (0.04)	0.3 (0.07)
	7.0	0.5 (0.04)	1.1 (0.23)	0.8 (0.23)	1.4 (0.12)
	21.0	1.3 (0.24)	2.4 (0.37)	0.3 (0.06)	1.4 (0.16)
	63.0	2.7 (0.19)	7.9 (1.46)	0.3 (0.10)	1.3 (0.08)
	189.0	1.9 (0.45)	2.6 (0.09)	nd	nd

Table 2. *Total phytochelatin thiol (PC-SH) concentrations and PC-SH to Cd molar ratios (means of three samples of three plants each; SE in parentheses) in desorbed roots of non-metallicolous (Amsterdam) and calamine (Plombières)* S. vulgaris *after 4 d of exposure, with and without BSO, to increasing Cd concentrations in the nutrient solution (nd=not determined)*

Population	Exposure (μM Cd)	PC-SH (μmol g^{-1} DW)		PC-SH:Cd (mol mol^{-1})	
		+BSO	–BSO	+BSO	–BSO
Amsterdam	7.5	5.0 (1.07)	21.2 (2.07)	0.9 (0.08)	3.4 (0.40)
	15.0	4.1 (0.43)	24.7 (4.72)	0.6 (0.12)	2.7 (0.12)
	30.0	6.7 (1.24)	35.3 (2.91)	0.6 (0.03)	2.5 (0.31)
	60.0	9.2 (0.89)	40.6 (5.33)	0.7 (0.15)	2.6 (0.45)
	120.0	4.6 (0.39)	42.7 (2.71)	0.4 (0.10)	1.8 (0.06)
	240.0	nd	nd	nd	nd
Plombières	7.5	2.8 (0.20)	7.3 (0.83)	0.7 (0.02)	1.8 (0.25)
	15.0	3.0 (0.08)	9.3 (2.07)	0.5 (0.09)	1.7 (0.29)
	30.0	3.5 (0.53)	11.2 (0.99)	0.6 (0.05)	1.6 (0.08)
	60.0	6.1 (0.36)	16.(1.74)	0.6 (0.11)	1.7 (0.31)
	120.0	5.4 (1.14)	17.(3.01)	0.3 (0.07)	1.1 (0.16)
	240.0	9.3 (2.79)	26.(2.61)	0.3 (0.10)	0.7 (0.09)

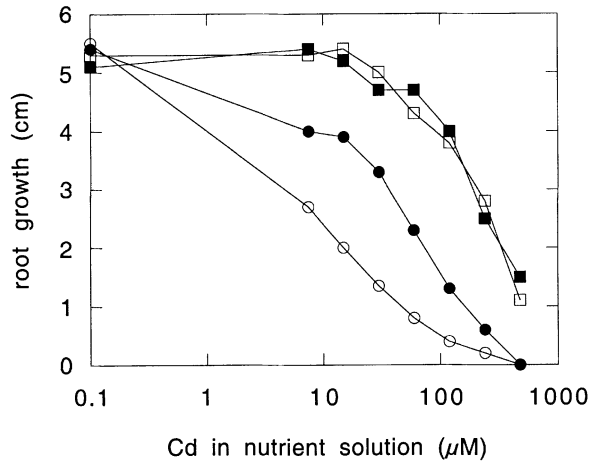

Fig. 2. Mean root elongation (n=15) throughout 4 d of exposure to Cd in BSO-treated (open symbols) and untreated (closed symbols) non-metallicolous (circles) and Cd-hypertolerant (squares) *S. vulgaris*. Standard errors varied between 3% and 14% of the means.

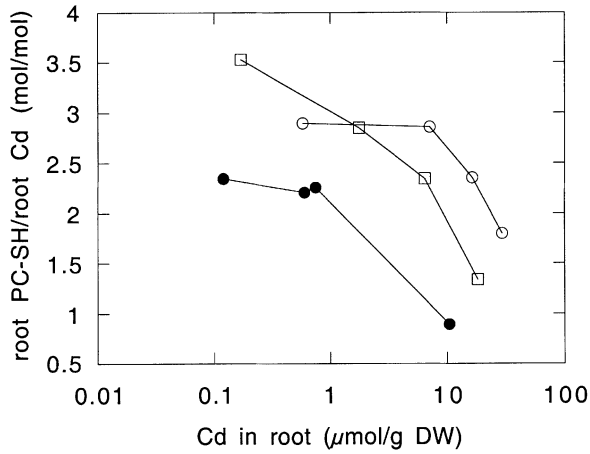

Fig. 3. Mean PC-thiol to Cd molar ratio (n=12) in roots, as a function of the root-internal Cd concentration, in *T. caerulescens* from calamine (closed circles), serpentine (open circles), and non-metalliferous soil (open squares), after 4 d of exposure to a series of Cd concentrations in the nutrient solution. Standard errors varied between 5% and 19% of the means.

were no visible effects of the treatments on shoot performance. However, the major sink for Cd in *T. caerulescens* is the shoot, and previous experiments (Assunção *et al*., 2001) clearly showed that after longer periods of exposure (>1 week) shoot performance responded much more sensitively to metal exposure than did root growth. Therefore, an additional longer-term experiment was performed with the serpentine and the calamine ecotype. After 2 weeks of exposure, the sensitizing effect of BSO was clearly apparent from the shoot performance of the serpentine ecotype. At 1 μM external Cd, the shoots of the BSO-treated plants were almost entirely bright yellow or white with necrotic parts, whereas those of the untreated plants were still more or less green. In addition, as indicated by the significance of the BSO×Cd interaction (P <0.01), BSO increased the shoot fresh weight response to the Cd treatment (Fig. 5). In the Cd-hypertolerant calamine ecotype, shoot fresh weight was only significantly decreased at the highest treatment level, i.e. 125 μM Cd, and the BSO×Cd interaction was not significant. Chlorosis was only apparent at the 25 μM and the 125 μM treatments, both with and without BSO. In both ecotypes, the root elongation response was unaffected by BSO (data not shown).

Arsenic

BSO-imposed effects on As tolerance were investigated in non-metallicolous *S. vulgaris* (Amsterdam) and *H. lanatus* (Amsterdam), as well as in *H. lanatus* and *A. castellana* from a strongly As-enriched gold mine waste deposit (Jales) (see Materials and methods). Arsenate caused a strong accumulation of PCs in *S. vulgaris*, albeit exclusively in roots, the root PC-SH to As molar ratios being

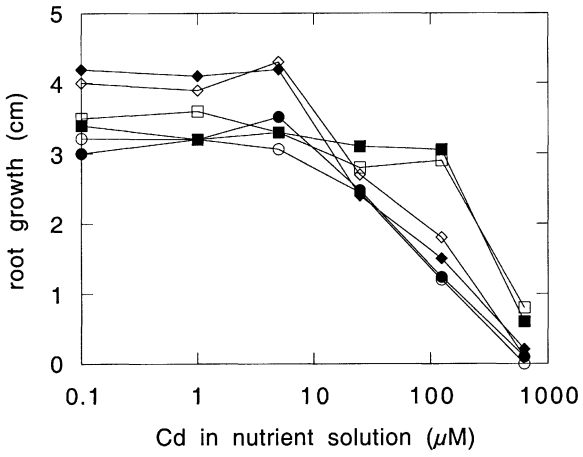

Fig. 4. Mean root elongation (n=12) throughout 4 d of exposure to Cd in BSO-treated (open symbols) and untreated (closed symbols) non-metallicolous (diamonds), serpentine (circles), and calamine (squares) *T. caerulescens*. Standard errors varied between 3% and 16% of the means.

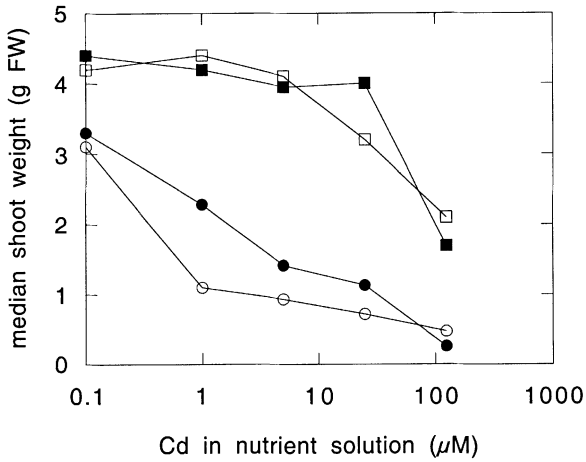

Fig. 5. Median shoot biomass (n=12) after 2 weeks of exposure to Cd in BSO-treated (open symbols) and untreated (closed symbols) serpentine (circles) and calamine (squares) *T. caerulescens*. Standard errors varied between 7% and 24% of the means.

above 3, except for the most toxic exposure levels (Table 3). The grasses also showed high PC accumulation rates, but the results were considered to be unreliable, owing to a variable recovery of the internal standard added to the samples, *N*-acetyl-L-cysteine (data not given). However, the PC-SH to As molar ratios obtained, though probably underestimated, were between 1.9 and 2.7, and did not vary with the degrees of As tolerance. BSO treatment dramatically decreased the root PC concentrations (Table 3), particularly in the grasses, where the root PC concentrations consistently remained below 0.1 μmol g^{-1} DW in the BSO treatment. In all the species tested, BSO strongly increased the root growth response to As, both in tolerant and non-tolerant ecotypes. In the case of the grasses, BSO completely arrested root growth, even at As exposure levels that did not cause root growth inhibition in the absence of BSO (Figs 6, 7). In fact, the root sytems of most of the plants seemed to have died off completely, and their leaves were wilted or largely necrotic, except for the younger ones, which showed some chlorosis, but remained turgid.

Zinc, Nickel, Cobalt

PC accumulation induced by Zn, Ni, or Co and possible PC-dependent tolerance to these metals were investigated in non-metallicolous and Zn-hypertolerant ecotypes of *S. vulgaris* (populations Amsterdam and Plombières, respectively) and *T. caerulescens* (populations Willerwiltz and La Calamine, respectively), as well as in a serpentine *T. caerulescens* ecotype (population Monte Prinzera) (see Materials and methods). Zn exposure caused a pronounced concentration-dependent accumulation of PCs in roots of non-metallicolous *S. vulgaris*, although the PS-SH to Zn molar ratios were far below unity (Table 4). The calamine ecotype exhibited lower root PC concentrations, in spite of a much higher root Zn accumulation rate, leading to dramatically decreased PC-SH to Zn molar ratios, as compared with the non-metallicolous ecotype (Table 4). In both ecotypes, the root growth response was unaffected by BSO (Fig. 8), although the BSO treatment effectively decreased the PC-SH to Zn molar ratios in both ecotypes (Table 4). PC accumulation was not apparent in shoots.

Zn-induced PC accumulation was also found in each of the *T. caerulescens* ecotypes, particularly in roots, but also in shoots. The PC concentrations were inconsiderable, however (less than 2 μmol g^{-1} dry weight), albeit much higher than in control plants (about 0.04 μmol g^{-1} dry weight), and did not increase with exposure levels within the range tested (25–1250 μM Zn). There were no obvious interecotypic differences in PC-SH to Zn molar ratio's. BSO did not detectably affect the growth response to Zn or the PC-SH to Zn molar ratios in either of the ecotypes (data not shown).

Ni and Co induced PC accumulation in both ecotypes of *S. vulgaris*, albeit exclusively in roots. The root PC concentrations, though being higher than in control plants (about 0.06 μmol g^{-1} dry weight), never exceeded 1 μmol g^{-1} dry weight, and tended to decrease with exposure level (5–80 μM Ni; 3–243 μM Co) in both ecotypes. The Zn-hypertolerant ecotype, which exhibits some pleiotropic hypertolerance to Ni and Co (Schat and Vooijs, 1997), consistently displayed much lower PC concentrations and lower PC-SH to metal molar ratios than did the non-metallicolous ecotype. BSO neither affected the PC-SH to metal molar ratios, nor the root growth response in either ecotype (data not shown).

PC accumulation under Ni exposure was also found in all of the three *T. caerulescens* ecotypes tested. The root PC concentrations were usually lower than 1 μmol g^{-1} dry

Table 3. *Total phytochelatin thiol (PC-SH) concentrations and PC-SH to As molar ratios (means of three samples of three plants each; SE in parentheses) in desorbed roots of non-metallicous* S. vulgaris *(population Amsterdam), after 4 d of exposure, with and without BSO, to increasing As concentrations in the nutrient solution (nd=not determined)*

Species (population)	Exposure (μM As)	PC-SH (μmol g^{-1} DW)		PC-SH:As (mol mol^{-1})	
		+BSO	–BSO	+BSO	–BSO
S. vulgaris (Amsterdam)	1	1.7 (0.32)	3.4 (0.19)	2.7 (0.40)	4.1 (0.27)
	8	3.7 (0.21)	8.6 (1.26)	1.9 (0.08)	3.1 (0.27)
	16	7.5 (0.51)	17.3 (2.50)	2.7 (0.12)	3.8 (0.19)
	32	6.8 (0.79)	27 4 (4.68)	1.1 (0.32)	2.7 (0.35)
	64	3.4 (0.64)	31.7 (6.07)	0.6 (0.01)	3.0 (0.58)
	128	<0.1	<0.1	nd	nd

weight in the non-metallicolous and the calamine ecotypes, and between 1 and 2 μmol g^{-1} in the serpentine ecotype, more or less irrespective of the level of exposure (15–450 μM Ni). The PS-SH to Ni molar ratios, however, were lower in the serpentine ecotype than in the other ones. Ni induced some PC accumulation in the shoot, up to 0.15 μmol g^{-1} dry weight, albeit exclusively in the serpentine ecotype. BSO did not affect the growth response to Ni in either of the ecotypes (data not shown). Cobalt-induced PC accumulation was only investigated in the calamine ecotype. The root PC concentrations were below 1 μmol g^{-1} and decreased with increasing exposure level (3–243 μM Co). The shoot PC concentrations were below the detection limit. BSO did not affect the growth response to this metal (data not shown).

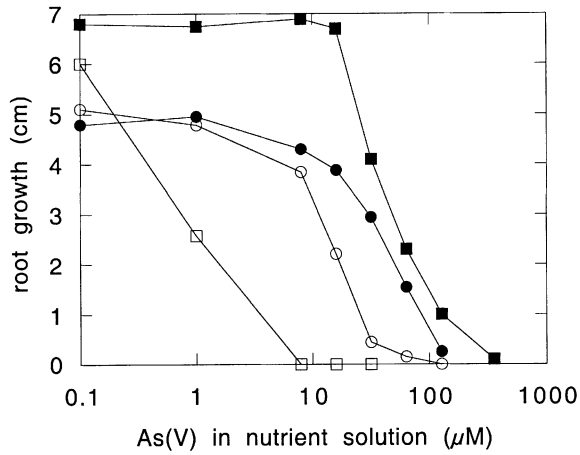

Fig. 6. Mean root elongation (n=15) throughout 4 d of exposure to arsenate in BSO-treated (open symbols) and untreated (closed symbols) non-metallicolous *S. vulgaris* (circles) and *H. lanatus* (squares). Standard errors varied between 2% and 15% of the means.

Discussion

The accumulation of PCs under copper stress has been demonstrated in a large number of algae and plants. However, more or less precise quantitative information with regard to the dose–effect relationships of this phenomenon is scarce. The threshold exposure levels for PC accumulation appeared, in general, to be lower for Cd than for Cu (Ahner and Morel, 1995; Rijstenbil and Wijnholds, 1996). In several studies Cu-induced PC accumulation was not apparent until the threshold exposure level for acute toxicity had been exceeded, suggesting that PCs are normally not involved in Cu sequestration under conditions of subtoxic exposure (De Vos *et al*., 1992; Rijstenbil *et al*., 1998; Rijstenbil and Gerringa, 2002). In the present study, the threshold Cu exposure levels for root growth inhibition and PC accumulation seemed to coincide, both in non-tolerant and Cu-tolerant *S. vulgaris* (Fig. 1, Table 1), suggesting that Cu did not induce PC accumulation until the capacity of the normal cellular homeostasis had been exhausted. The increased homeostatic capacity in the tolerant ecotype relies most probably on a combination of an enhanced capacity to efflux Cu from the root cells and a strong constitutive overexpression of a 2b-type metallothionein, *SvMT2b* (Van Hoof *et al*., 2001*a*, *b*). Also, the latter properties would be expected to reduce the activity of cytosolic Cu available for PCS activation, which would then explain the lower PC-SH to Cu molar ratios in the tolerant ecotype (Table 1). The absence of considerable PC accumulation under subtoxic exposure obviously explains the lack of effect of BSO on the threshold Cu exposure levels for root growth inhibition (Fig. 1). The absence of a clear effect of BSO on the slope of the dose–response curves is more difficult to explain. Cu–PC complexes seem to be highly stable (Mehra and Mulchandani, 1995; Leopold and Gunther, 1997), and intact Cu–PC complexes have been isolated from *S. vulgaris* roots (Verkleij *et al*., 1989), suggesting that PCs should contribute, at least to some extent, to Cu detoxification in Cu-stressed plants. One might argue that the BSO-imposed inhibition of PC synthesis was not complete, possibly through shoot-to-root transport of GSH (De Knecht *et al*., 1995). However, the PC-SH to Cu molar ratios in the treated plants were much decreased (Table 1), showing that the root performance under Cu-toxic conditions was not limited by the PC

Table 4. *Total phytochelatin thiol (PC-SH) concentrations and PC-SH to Zn molar ratios (means of three samples of three plants each) in desorbed roots of non-metallicolous (Amsterdam) and calamine (Plombières)* S. vulgaris*, after a 4 d exposure, with and without BSO, to increasing Zn concentrations in the nutrient solution (nd=not determined)*

Population	Exposure (μM Zn)	PC-SH (μmol g^{-1} DW)		PC-SH:Zn (mol mol^{-1})	
		+BSO	–BSO	+BSO	–BSO
Amsterdam	2	<0.1	<0.1	nd	nd
	25	2.1 (0.12)	6.1 (0.29)	0.21 (0.05)	0.34 (0.01)
	50	3.4 (0.23)	8.4 (0.13)	0.12 (0.03)	0.23 (0.03)
	100	3.7 (0.42)	8.6 (0.99)	0.08 (0.01)	0.22 (0.04)
	200	6.0 (1.41)	11.9 (1.15)	0.09 (0.02)	0.17 (0.02)
	400	5.8 (1.01)	14.9 (0.98)	0.11 (0.03)	0.20 (0.02)
Plombières	2	<0.1	<0.1	nd	nd
	200	0.3 (0.02)	0.5 (0.01)	0.008 (0.002)	0.014 (0.003)
	400	0.5 (0.12)	1.3 (0.11)	0.009 (0.003)	0.019 (0.002)
	800	2.1 (0.03)	3.4 (0.40)	0.004 (0.001)	0.017 (0.004)
	1600	3.4 (0.38)	5.0 (1.02)	0.008 (0.001)	0.014 (0.001)
	3200	2.1 (0.19)	2.7 (0.34)	0.005 (0.002)	0.008 (0.002)

synthetic capacity. Moreover, the PCS-deficient *cad1* mutant of *Arabidopsis thaliana* did not exhibit considerably increased sensitivity to Cu (Howden and Cobbett, 1992), although Cu has been shown to induce the accumulation of PCs in this species (Murphy and Taiz, 1995). Thus, most of the evidence available thus far suggests that PCs may not effectively contribute to Cu detoxification in most algae and higher plants, although they appear to do so in fission yeast (Clemens *et al*., 1999). The reason for this might lie in the presence or absence of more effective efflux- or MT-based alternative detoxification systems.

Cd has been shown to be a strong inducer of PC accumulation in a broad variety of algae and higher plants, as well as in several fungi, and PC-based Cd sequestration is generally considered to be essential for normal Cd tolerance in organisms with functional *PCS* genes (Howden and Cobbett, 1992; Ortiz *et al*., 1992; Speiser *et al*., 1992*b*; Cobbett *et al*., 1998; Clemens *et al*., 1999; Vatamaniuk *et al*., 2001). In agreement with this viewpoint, a strong Cd-induced PC accumulation and BSO-imposed hypersensitivity to Cd in non-metallicolous *S. vulgaris* was observed (Fig. 2). In the Cd-hypertolerant ecotype, however, the PC-SH to Cd molar ratios were much lower (Table 2), and BSO-imposed hypersensitivity to Cd was not apparent, irrespective of the level of exposure (Fig. 2), suggesting that Cd hypertolerance is achieved through enhanced activity of an as yet unknown PC-independent Cd sequestration mechanism decreasing the activity of cytoplasmic Cd available for PCS activation (De Knecht *et al*., 1992, 1995). The results obtained with *T. caerulescens* are basically in line with this. The degrees of Cd-imposed PC accumulation (Fig. 3) in the different ecotypes were inversely related to the levels of Cd tolerance. Apparently, the Cd-hypertolerant ecotype did not possess PC-dependent Cd tolerance, as shown by the absence of any BSO-imposed hypersensitivity (Fig. 4). In accordance with this, Ebbs *et al*. (2002) concluded that PC synthesis was not responsible for Cd tolerance in *T. caerulescens* from Prayon (Belgium), which exhibits a similar degree of Cd hypertolerance. However, BSO did significantly increase Cd sensitivity in the Cd-sensitive serpentine ecotype (Fig. 4), suggesting that PC-dependent constitutive Cd tolerance does occur in non-metallicolous ecotypes of this species. The major differences with *S. vulgaris* were that *T. caerulescens* showed considerable accumulation of PCs in the shoots, though less than in the roots, and that the responses to toxic Cd exposure and BSO were primarily apparent from shoot performance, rather than from root elongation, which is most probably due to the much higher rate of Cd translocation to the shoot.

Arsenic has been shown to induce high levels of PC accumulation in a variety of plant species (Grill *et al*., 1987; Maitani *et al*., 1996; Nakazawa *et al*., 2000; Schmöger *et al*., 2000). Intact As–PC complexes have been isolated from *S. vulgaris* (Sneller *et al*., 1999), and PC synthesis is supposed to be essential for As detoxification in plants (Schmöger *et al*., 2000; Hartley-Whitaker *et al*., 2001). In agreement with this, a strong As-induced PC accumulation was found in all the species tested. Moreover, BSO consistently produced hypersensitivity to As (Figs 6, 7). The relatively small BSO effect in *S. vulgaris*, as compared with the grasses (Fig. 6), might be due to the fact that BSO decreased root GSH to a much lower degree in *S. vulgaris* (to about 30% in unexposed controls) than it did in the grasses (to less than 5%). Arsenate, being a phosphate analogue, is taken up by phosphate transporters, and arsenate hypertolerance has been shown to be achieved through constitutive suppression of the high-affinity phosphate uptake system in a number of grass species, including *H. lanatus* (Meharg and Macnair, 1990, 1991*a*, *b*, 1992). This suppression would reduce the influx of As to a level that can be coped with by the constitutive PC-based detoxification machinery

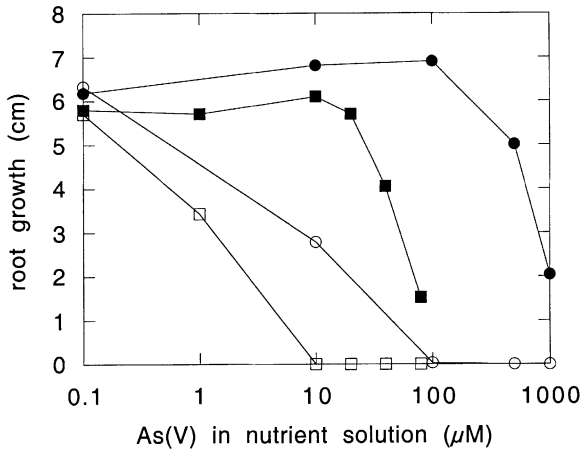

Fig. 7. Mean root elongation (n=15) throughout 4 d of exposure to arsenate in BSO-treated (open symbols) and untreated (closed symbols) metallicolous *A. castellana* (circles) and *H. lanatus* (squares). Standard errors varied between 4% and 18% of the means.

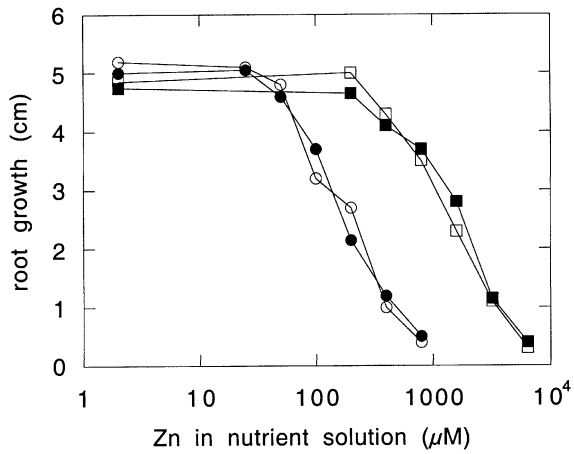

Fig. 8. Mean root elongation (n=15) throughout 4 d of exposure to Zn in BSO-treated (open symbols) and untreated (closed symbols) non-metallicolous (circles) and calamine (squares) *S. vulgaris*. Standard errors varied between 6% and 17% of the means.

(Hartley-Whitaker *et al*., 2001). These results support this hypothesis. Even extreme As hypertolerance, such as observed in the Jales population of *A. castellana*, was largely lost under BSO exposure (Fig. 7), suggesting that PC synthesis is equally essential for both normal constitutive tolerance and hypertolerance to As. Also in agreement with Hartley-Whitaker *et al*. (2001), tolerance-correlated decreases of PC-SH to As molar ratios were not observed in the mine ecotypes, suggesting that alternative, PC-independent sequestration mechanisms do not play any significant role in As hypertolerance.

Zn and, particularly, Ni and Co, are considered to be relatively weak activators of PCS, both *in vivo* and *in vitro* (Grill *et al*., 1988; Ahner and Morel, 1995; Klapheck *et al*., 1995). The stability of the Zn–PC complex is comparatively low (Maitani *et al*., 1996; Leopold and Gunther, 1997). The stabilities of Ni–PC and Co–PC complexes are unknown at present, but might be expected to be even lower, as suggested by the relatively low affinities of Ni and Co to other cysteine-based ligands (Perrin, 1979). Davies *et al*. (1991) reported that BSO did not increase Zn-imposed root growth inhibition in *Festuca rubra*. Also, the PC-deficient *Arabidopsis cad1* mutant did not exhibit considerably enhanced Zn-sensitivity (Howden and Cobbett, 1992). In agreement with this, low, but detectable PC accumulation rates and low PC-SH to metal molar ratios were observed in all the species and ecotypes under Zn, Ni, or Co exposure. BSO-mediated hypersensensitivity was consistently absent, both in *S. vulgaris* and *T. caerulescens*, suggesting that PC-based sequestration is not essential for the detoxification of either of these metals. Hypertolerance to Zn, Ni, and Co in *S. vulgaris*, and to Ni in *T. caerulescens* was again associated with decreased PC-SH to metal molar ratios, which may be taken to indicate that these hypertolerances are achieved through sequestration mechanisms that decrease the metals' availability for PCS activation in the cytoplasm, such as increased vacuolar transport in the case of Zn hypertolerance in *S. vulgaris* (Chardonnens *et al*., 1999). Comparable tolerance-related differences in PC accumulation were not found among the three distinctly Zn-tolerant *T. caerulescens* ecotypes, however. In particular, Zn, in contrast to Cd, induced very little PC accumulation in *T. caerulescens*, as compared to *S. vulgaris*. The reason for this is elusive, but might be related to the Zn hyperaccumulation trait.

To summarize, these results do not provide evidence in favour of a role for PCs in the detoxification of the essential metal micronutrients Zn, Ni, and Cu in plants, although Cu, when present at toxic concentrations, induced considerable PC accumulation and apparently formed stable complexes with PCs. Also, it is highly unlikely that PCs are essential in the detoxification of Fe, Mo, and Mn (Grill *et al*., 1989; Brune *et al*., 1995), suggesting that, in general, PCs might not be involved in the detoxification of excessively accumulated micronutrients in plants. On the other hand, this study confirms that PCs are required for the detoxification of certain non-essential metals. Taking further evidence from the literature into account, it might be hypothesized that, in plants and algae at least, the primary function of PCS would lie in the detoxification of non-essential metals and metalloids with relatively high affinities to sulphur, such as Cd, Hg (Howden and Cobbett, 1992; Gupta *et al*., 1998) and, particularly, As. Such metals, however, although being highly toxic and ubiquitous, are mostly present at negligible concentrations in the environment, which makes it difficult to believe that the conservation of functional PCS throughout the plant kingdom would be ultimately explained by toxic exposure to either of these metals. Moreover, in the case of Cd, these results clearly show a decreased dependency on PC-based

sequestration in hypertolerant *S. vulgaris* and *T. caerulescens*, suggesting that PC-mediated detoxification might not be the most effective strategy to cope with toxic exposure to this metal, at least.

In view of the spread of significantly homologous *PCS* genes over the animal, plant, and fungal kingdoms (Clemens *et al*., 1999, 2001; Vatamaniuk *et al*., 2001), it seems likely that they must have evolved from an ancient ancestral gene. *PCS* genes seem to have been lost in a number of animal and fungal lineages, possibly as a consequence of the evolution of more effective and more specific MT-based metal sequestration systems. One might argue that PCS originally functioned in Cu detoxification, as it still seems to do in *S. pombe* (Clemens *et al*., 1999). Its present function in plants and its ubiquitous occurrence throughout the plant kingdom are still enigmatic, however. Although the primary function of PCS does not seem to lie in the detoxification of excessively accumulated metal micronutrients, it cannot be excluded that it somehow functions in metal micronutrient homeostasis under non-toxic physiological conditions, particularly because plants exposed to normal nutritional micronutrient exposure levels appear to contain PCs at low, but detectable concentrations.

Acknowledgements

This work was supported by a grant from the Catalonian Generality (CIRIT, project BEAI 300151). The authors are indepted to Dr Alessandra Lombini, University of Bologna, who kindly provided the seeds of the Monte Prinzera population of *T. caerulescens*.

References

Ahner BA, Kong S, Morel FMM. 1995. Phytochelatin production in marine algae. 1. An interspecific comparison. *Limnology and Oceanography* **40,** 649–657.

Ahner BA, Morel FMM. 1995. Phytochelatin production in marine algae. 2. Induction by various metals. *Limnology and Oceanography* **40,** 658–665.

Assunção AGL, Da Costa Martins P, De Folter S, Vooijs R, Schat H, Aarts MGM. 2001. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell and Environment* **24,** 217–226.

Brune A, Urbach W, Dietz KJ. 1995. Differential toxicity of heavy metals is partly related to a loss of preferential extraplasmic compartmentation: a comparison of Cd-stress, Mo-stress, Ni-stress, and Zn-stress. *New Phytologist* **129,** 403–409.

Chardonnens AN, Koevoets PLM, Van Zanten A, Schat H, Verkleij JAC. 1999. Properties of enhanced tonoplast zinc transport in naturally selected zinc-tolerant *Silene vulgaris*. *Plant Physiology* **120,** 779–785.

Chen JJ, Goldsbrough PB. 1994. Increased activity of γ-glutamylcysteine synthetase in tomato cells selected for cadmium tolerance. *Plant Physiology* **106,** 233–239.

Chen JJ, Zhou JM, Goldsbrough PB. 1997. Characterization of phytochelatin synthase from tomato. *Physiologia Plantarum* **101,** 165–172.

Clemens S, Kim EJ, Neumann D, Schroeder JI. 1999. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO Journal* **18,** 3325–3333.

Clemens S, Schroeder JI, Degenkolb T. 2001. *Caenorhabdites elegans* expresses a functional phytochelatin synthase. *European Journal of Biochemistry* **268,** 3640–3643.

Cobbett CS, May MJ, Howden R, Rolls B. 1998. The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. *The Plant Journal* **16,** 73–78.

Davies KL, Davies MS, Francis D. 1991. The influence of an inhibitor of phytochelatin synthesis on root growth and root meristematic activity in *Festuca rubra* L. in response to zinc. *New Phytologist* **118,** 565–570.

De Knecht JA, Koevoets PLM, Verkleij JAC, Ernst WHO. 1992. Evidence against a role for phytochelatins in naturally selected increased cadmium tolerance in *Silene vulgaris* (Moench) Garcke. *New Phytologist* **122,** 681–688.

De Knecht JA, Van Baren N, ten Bookum WM, Wong Fong Sang HW, Koevoets PLM, Schat H, Verkleij JAC. 1995. Synthesis and degradation of phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*. *Plant Science* **106,** 9–18.

De Knecht JA, Van Dillen M, Koevoets PLM, Schat H, Verkleij JAC, Ernst WHO. 1994. Phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*. Chain length distribution and sulphide incorporation. *Plant Physiology* **104,** 255–261.

De Vos CHR, Vonk MJ, Vooijs R, Schat H. 1992. Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. *Plant Physiology* **98,** 853–858.

Ebbs S, Lau I, Ahner B, Kochian L. 2002. Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J&C Presl.). *Planta* **214,** 635–640.

Gekeler W, Grill E, Winnacker E-L, Zenk MH. 1989. Survey of the plant kingdom for the ability to bind heavy metals through phytochelatins. *Zeitschrift für Naturforschung* **C44,** 361–369.

Grill E, Gekeler W, Winnacker E-L, Zenk MH. 1986*a*. Homo-phytochelatins are heavy metal-binding peptides of homo-glutathione containing Fabales. *FEBS Letters* **205,** 47–50.

Grill E, Löffler S, Winnacker E-L, Zenk MH. 1989. Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proceedings of the National Academy of Sciences, USA* **86,** 6838–6842.

Grill E, Thumann J, Winnacker E-L, Zenk MH. 1988. Induction of heavy-metal binding phytochelatins by inoculation of cell cultures in standard media. *Plant Cell Reports* **7,** 375–378.

Grill E, Winnacker E-L, Zenk MH. 1985. Phytochelatins: the principal heavy-metal complexing peptides of higher plants. *Science* **230,** 674–676.

Grill E, Winnacker E-L, Zenk MH. 1986*b*. Synthesis of seven different homologous phytochelatins in metal-exposed *Schizosaccharomyces pombe* cells. *FEBS Letters* **197,** 115–120.

Grill E, Winnacker E-L, Zenk MH 1987. Phytochelatins, a class of heavy-metal-binding peptides from plants are functionally analogous to metallothioneins. *Proceedings of the National Academy of Sciences, USA* **84,** 439–443.

Gupta M, Tripathi RD, Rai UN, Chandra P. 1998. Role of glutathione and phytochelatin in *Hydrilla verticillata* Royle and *Vallisneria spiralis* L. under mercury stress. *Chemosphere* **37,** 785–800.

Ha SB, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS. 1999. Phytochelatin

synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *The Plant Cell* **11,** 1153–1163.

Harmens H, Cornelisse E, Den Hartog PR, Ten Bookum WM, Verkleij JAC. 1993. Phytochelatins do not play a key role in naturally selected zinc tolerance in *Silene vulgaris* (Moench) Garcke. *Plant Physiology* **103,** 1305–1309.

Hartley-Whitaker J, Ainsworth G, Vooijs R, Ten Bookum WM, Schat H, Meharg AA. 2001. Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiology* **126,** 299–306.

Hayashi Y, Nakagawa CW, Mutoh N, 1991. Two pathways in the biosynthesis of cadystins $(\gamma\text{-EC})_n$G in the cell-free system of the fission yeast. *Biochemistry and Cell Biology* **69,** 115–121.

Howden R, Cobbett CS. 1992. Cadmium-sensitive mutants of *Arabidopsis thaliana*. *Plant Physiology* **99,** 100–107.

Howden R, Goldsbrough PB, Andersen CR, Cobbett CS. 1995. Cadmium-sensitive, *ca*d1, mutants of *Arabidopsis thaliana* are phytochelatin-deficient. *Plant Physiology* **107,** 1059–1067.

Juang RH, McCue, Ow DW. 1993. Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulphinate *in vitro*. *Archives of Biochemistry and Biophysics* **304,** 392–401.

Klapheck S, Fliegner W, Zimmer I. 1994. Hydroxymethyl-phytochelatins [$(\gamma$-glutamylcysteine$)_n$-serine] are metal-induced peptides of the Poaceae. *Plant Physiology* **104,** 1325–1332.

Klapheck S, Schlunz S, Bergmann L. 1995. Synthesis of phytochelatins and homo-phytochelatins in *Pisum sativum* L. *Plant Physiology* **107,** 515–521.

Leopold I, Gunther D. 1997. Investigation of the binding properties of heavy-metal-peptide complexes in plant cell cultures using HPLC-ICP-MS. *Fresenius Journal of Analytical Chemistry* **359,** 364–370.

Maitani T, Kubota T, Sato K, Yamada T. 1996. The composition of metals bound to class III metallothioneins (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. *Plant Physiology* **110,** 1145–1150.

Meharg AA, Macnair MR. 1990. An altered phosphate uptake system in arsenate-tolerant *Holcus lanatus* L. *New Phytologist* **116,** 29–35.

Meharg AA, Macnair MR. 1991*a*. Uptake, accumulation and translocation of arsenate in arsenate-tolerant and nontolerant *Holcus lanatus* L. *New Phytologist* **117,** 225–231.

Meharg AA, Macnair MR. 1991*b*. The mechanisms of arsenate tolerance in *Deschampsia cespitosa* (L.) Beauv and *Agrostis capillaris* L. *New Phytologist* **119,** 291–297.

Meharg AA, Macnair MR. 1992. Suppression of the high-affinity phosphate uptake system: a mechanism of arsenate tolerance in *Holcus lanatus* L. *Journal of Experimental Botany* **43,** 519–524.

Mehra RK, Mulchandani P. 1995. Glutathione-mediated transfer of Cu(I) into phytochelatins. *Biochemical Journal* **307,** 697–705.

Mehra RK, Tarbet EB, Gray WR, Winge DR. 1988. Metal-specific synthesis of 2 metallthioneins and γ-glutamyl-transferase peptides in *Candida glabrata*. *Proceedings of the National Academy of Sciences, USA* **85,** 8815–8819.

Mehra RK, Winge DR. 1988. Cu(I) binding to the *Schizosaccharomyces pombe* γ-glutamyl-transferase peptides varying in chain lengths. *Archives of Biochemistry and Biophysics* **265,** 381–389.

Meuwly P, Thibault P, Rauser WE. 1993. γ-Glutamyl-cysteinylglutamic acid—a new homolog of glutathione in maize seedlings exposed to cadmium. *FEBS Letters* **336,** 472–476.

Miersch J, Tschimedbalshir M, Barlocher F, Grams Y, Pierau B, Schierhorn A, Kraus GJ. 2001. Heavy metals and thiol compounds in *Mucor racemosus* and *Articulospora tetracladia*. *Mycological Research* **105,** 883–889.

Murphy A, Taiz L. 1995. Comparison of metallothionein gene expression and non-protein thiols in 10 *Arabidopsis* ecotypes. *Plant Physiology* **109,** 945–954.

Nakazawa R, Ikawa M, Yasuda K, Takenaga H. 2000. Synergistic inhibition of the growth of suspension cultured tobacco cells by simultaneous treatment with cadmium and arsenic in relation to phytochelatin synthesis. *Soil Science and Plant Nutrition* **46,** 271–275.

Ortiz DFD, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow DW. 1992. Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *EMBO Journal* **11,** 3491–3499**.**

Ortiz DF, Ruscitti T, McCue KF, Ow DW. 1995. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane-protein. *Journal of Biological Chemistry* **270,** 4721–4728.

Perrin D. 1979. *Stability constants of metal-ion complexes. Part B. Organic ligands*. Oxford, UK: Pergamon Press.

Rijstenbil JW, Gerringa LJA. 2002. Interactions of algal ligands, metal complexation and availability, and cell responses of the diatom *Ditylum brightwelli* with a gradual increase in copper. *Aquatic Toxicology* **56,** 115–131.

Rijstenbil JW, Haritonidis S, Malea P, Seferlis M, Wijnholds JA. 1998. Thiol pools and glutathione redox ratios as possible indicators of copper toxicity in the green macroalgae *Enteromorpha* spp. from the Scheldt Estuary (SW Netherlands, Belgium) and Thermakos Gulf (Greece, N Aegean Sea). *Hydrobiologia* **385,** 171–181.

Rijstenbil JW, Wijnholds JA. 1996. HPLC ananlysis of non-protein thiols in planktonic diatoms: pool size, redox state and response to copper and cadmium exposure. *Marine Biology* **127,** 45–54.

Salt DE, Rauser WE. 1995. MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. *Plant Physiology* **107,** 1293–1301.

Schat H, Kalff MMA. 1992. Are phytochelatins involved in differential heavy metal tolerance or do they merely reflect metal-imposed strain? *Plant Physiology* **99,** 1475–1480.

Schat H, Ten Bookum WM. 1992. Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68,** 219–229.

Schat H, Vooijs R, Kuiper E. 1996. Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. *Evolution* **50,** 1888–1895.

Schat H, Van Hoof NALM, Tervahauta A, Hakvoort HWJ, Chardonnens AN, Koevoets PLM, Verkleij JAC, Ernst WHO. 2000. Evolutionary responses to zinc and copper stress in Bladder Campion, *Silene vulgaris* (Moench) Garcke. In: Cherry JH, Locy RD, Richter A, eds. *Plant tolerance to abiotic stresses: role of genetic engineering*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 343–360.

Schat H, Vooijs R. 1997. Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: a co-segregation analysis. *New Phytologist* **136,** 489–496.

Schmöger MEV, Oven M, Grill E. 2000. Detoxification of arsenic by phytochelatins in plants. *Plant Physiology* **122,** 793–801.

Sneller FEC, Van Heerwaarden LM, Kraaijeveld-Smit FJL, Ten Bookum WM, Koevoets PLM, Schat H, Verkleij JAC. 1999. Toxicity of arsenate in *Silene vulgaris*, accumulation and degradation of arsenate-induced phytochelatins. *New Phytologist* **144,** 223–232.

Sneller FEC, Van Heerwaarden LM, Koevoets PLM, Vooijs R, Schat H, Verkleij JAC. 2000. Derivatization of phytochelatins from *Silene vulgaris*, induced upon exposure to arsenate and cadmium: comparison of derivatization with Ellman's reagent and monobromobimane. *Journal of Agricultural and Food Chemistry* **48,** 4014–4019.

Speiser DM, Abrahamson SL, Banuelos G, Ow DW. 1992*a*. *Brassica juncea* produces a phytochelatin-cadmium-sulphide complex. *Plant Physiology* **99,** 817–821.

Speiser DM, Ortiz DF, Kreppel L, Scheel G, McDonald G, Ow DW. 1992*b*. Purine biosynthetic genes are required for cadmium tolerance in *Schizosaccharomyces pombe*. *Molecular and Cell Biology* **12,** 5301–5310.

Thumann J, Grill E, Winnacker E-L, Zenk MH. 1991. Reactivation of metal-requiring apoenzymes by phytochelatin-metal complexes. *FEBS Letters* **284,** 66–69.

Van Hoof NALM, Hassinen VH, Hakvoort HWJ, Ballintijn KF, Schat H, Verkleij JAC, Ernst WHO, Karenlampi SO, Tervahauta AI. 2001*a*. Enhanced copper tolerance in *Silene vulgaris* (Moench) Garcke populations from copper mines is associated with increase transcript levels of a 2b-type metallothionein gene. *Plant Physiology* **126,** 1519–1526.

Van Hoof NALM, Koevoets PLM, Hakvoort HWJ, Ten Bookum WM, Schat H, Verkleij JAC, Ernst WHO. 2001*b*. Enhanced ATP-dependent copper efflux across the root cell plasma membrane in copper-tolerant *Silene vulgaris*. *Physiologia Plantarum* **113,** 225–232.

Vatamaniuk OK, Bucher EA, Ward JT, Rea PA. 2001. A new pathway for heavy metal detoxification in animals—phytochelatin synthase is required for cadmium tolerance in *Caenorhabdites elegans*. *Journal of Biological Chemistry* **276,** 20817–20820.

Verkleij JAC, Koevoets PLM, Van't Riet J, Van Rossenberg MC, Bank R, Ernst WHO. 1989. The role of metal-binding compounds in the copper tolerance mechanism of *Silene vulgaris*. In: Hamer DH, Winge DR, eds. *Metal ion homeostasis: molecular biology and chemistry*. New York: Alan R Liss Inc, 347–358.

Vögeli-Lange F, Wagner GJ. 1989. Subcellular localization of cadmium and cadmium-binding peptides in tobacco. Implication of a transport function for cadmium-binding peptides. *Plant Physiology* **92,** 1086–1093.

Zhu YL, Pilon-Smits EAH, Jouanin L, Terry N. 1999*a*. Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. *Plant Physiology* **119,** 73–79.

Zhu YL, Pilon-Smits EAH, Jouanin L, Terry N. 1999*b*. Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. *Plant Physiology* **121,** 1169–1177.