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Glutathione in adaptation of *Arabidopsis thaliana* to cadmium stress

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Abstract

The role of glutathione (GSH) in the adaptation of wild type *Arabidopsis thaliana* plants to Cd stress was investigated. The nutrient solution (control or containing 50 or 100 µM Cd) was supplemented with buthionine sulfoximine (BSO; 50, 100, 500 µM, to decrease the GSH content in plants) or GSH (50, 100, 500 µM, to increase its content in plants) in order to find how GSH content could regulate Cd stress responses. BSO application did not influence plant biomass, while exogenous GSH (especially 500 µM) reduced root biomass. BSO (500µM) in combination with Cd (100 µM) increased Cd toxicity on root growth (by over 50 %), most probably due to reduced GSH content and phytochelatin (PC) accumulation (by over 96 %). On the other hand, combination of exogenous GSH (500 µM) with Cd (100 µM) was also more toxic to plants than Cd alone despite a significant increase in GSH and PC accumulation (up to 2.7 fold in the roots). This fact could indicate that the natural content of endogenous GSH in wild type *A. thaliana* plants is sufficient for Cd-tolerance. A decrease in this GSH content led to decreased Cd-tolerance of the plants but an increase in GSH content did not enhance Cd-tolerance, and it showed even toxic effect on the plants.

Additional key words: BSO, GSH labelling with monochlorobimane, phytochelatin.

Introduction

Tripeptide glutathione (γ-Glu-Cys-Gly, GSH) is the most abundant low molecular mass thiol compound in the plants and other organisms. Due to its high content in cells and the chemical reactivity of the thiol group, glutathione is particularly suitable to serve a broad range of biochemical functions in organisms (Maughan and Foyer 2006, Rouhier *et al.* 2008, Szalai *et al.* 2009). This compound is involved in storage and transport of reduced sulphur, synthesis of proteins and nucleic acids, regulation of growth, development and cell cycle, vacuolar sequestration of pigments, scavenging peroxides produced during cellular metabolism or oxidative stress. Glutathione also plays an important role in plant adaptation to many environmental stresses such as heavy metals, xenobiotics, pathogen attack, drought, atmospheric pollution, temperature extremes, and salt stress. The GSH level alters in response to different types of biotic and abiotic stresses and it has been suggested that GSH may act both as a stress sensor and as a part of a very compact signal transduction system (Xiang *et al.* 2001, Maughan and Foyer 2006, Szalai *et al.* 2009).

There are several possible ways for GSH to be involved in heavy metal tolerance and sequestration. It protects cells from the oxidative stress induced by heavy metals (Semane *et al.* 2007). GSH is also suggested to be a direct ligand for metals in the cytosol and their transport form into the vacuole. Furthermore, GSH is a substrate for synthesis of phytochelatins (PC), adaptive thiol peptides of the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2 - 11), which are directly involved in heavy metal detoxification and homeostasis. Their synthesis is catalyzed by phytochelatin synthase (PCS, γ-Glu-Cys dipeptidyl transpeptidase, EC 2.3.2.15. The enzyme is active only in the presence of metal(loid) ions, including Cd, Ag, Bi, Pb, Zn, Cu, Hg, Au and As, but it appears that not metal ions themselves but rather metal glutathione thiolates (*e.g.* Cd-(GS)_2) are responsible for that enzyme catalytic activation (Vatamaniuk *et al.* 2000). Both Cd-(GS)_2 and free GSH act as γ-Glu-Cys acceptors and donors in PC synthesis. From *in vitro* experiments it

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Abbreviations: BSO - buthionine sulfoximine; DTNB - 5,5'-dithiobis-2-nitrobenzoic acid; GSB - glutathione S-bimane; GSH - reduced glutathione; GSSG - oxidized glutathione; MCB - monochlorobimane; PC - phytochelatin; ROS - reactive oxygen species.

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results that Cd, Cu, Pb and Hg are transferred from metal-glutathione complex to PCs (n = 2, 3, and 4), however, metal-GSH complexes have not yet been demonstrated in *in vivo* plants.

Phytochelatins are produced in the cytosol, where they have a high affinity for binding with heavy metals, particularly Cd. These metal-PC complexes (LMM - low molecular mass) are then transported into the vacuole, sequestering thus the metals from sensitive enzymes. Once in the vacuole, LMM complexes are converted to high molecular mass (HMM) complexes under incorporation of sulphide, PC and more Cd ions. This system provides plants with a moderate level of resistance against Cd and Cu (Xiang *et al.* 2001).

The role of GSH and PCs in heavy metal detoxification and tolerance was well characterized in *A. thaliana* mutants or transgenic plants with an altered level of GSH or PC synthesis. Cd-sensitive mutants *cad1* and *cad2* were either GSH- and PC deficient due to a mutation in γ-glutamyl-cysteine synthetase (*cad2*) or only PC deficient due to a mutation in PC synthase gene (*cad1*) (Howden and Cobbett 1992, Howden *et al.* 1995, Cobbett *et al.* 1998). Genetically transformed *Arabidopsis* plants with modified expression of γ-glutamyl-cysteine synthetase varied in the GSH level ranging between 3 and 200 % of the level in wild type plants (Xiang *et al.* 2001). Transgenic plants with low GSH levels, limiting thereby their capacity to produce PC, were hypersensitive to Cd, but plants with an elevated GSH level did not increase metal resistance. Transgenic *A. thaliana* plants overexpressing *Arabidopsis* PC synthase (*AtPCS1*) exhibited an enhanced PC accumulation, but, surprisingly, they were hypersensitive to Cd (Lee *et al.* 2003). Apart from studying mutants or transgenic plants with altered GSH and/or PC levels, the exogenous application of GSH, as well as application of the GSH biosynthesis inhibitor, buthionine sulfoximine (BSO, the selective inhibitor of γ-glutamyl-cysteine synthetase) is also an effective tool for understanding how the level of GSH regulates heavy metal stress responses.

In the present study, we analyzed the physiological consequences of changes in GSH content in wild-type of *Arabidopsis thaliana* plants under Cd stress after application of exogenous GSH to the nutrient solution or by blocking GSH synthesis using BSO. Additionally, GSH was visualized in intact roots of *Arabidopsis* after labelling with monochlorobimane (MCB) to give fluorescent glutathione S-bimane (GSB) and imaging by means of confocal laser scanning microscopy. The aim of the study was also to examine the effect of BSO and GSH themselves on plant growth.

Material and methods

Plants, cultivation and treatments: Wild-type plants of *Arabidopsis thaliana* (L.) Heynh cv. Columbia were cultivated on Hoagland nutrient solution as described earlier (Wójcik and Tukiendorf 2003). The plants were treated for 14 d separately with Cd (50 or 100 μM) (in the form of $Cd(NO_3)_2 . 4 H_2O$), BSO (50, 100 or 500 μM) or GSH (50, 100 or 500 μM), and also Cd/BSO or Cd/GSH combinations using concentrations 50/50, 100/100, 100/500 (μM/μM).

Glutathione determination by confocal laser scanning microscopy: GSH contents were visualized in intact *Arabidopsis* roots after *in situ* conjugation with monochlorobimane (MCB) to give a fluorescent glutathione S-bimane (GSB) adduct. Apical fragments of roots (approximately 5 mm in length) were incubated in 100 μM MCB for 1 h. After incubation, roots were placed in a drop of deionised water on a microscope slide and covered with a coverslip.

Root samples were imaged using a confocal laser scanning microscope (*LSM 5 Pascal*, *Zeiss*, Jena, Germany). The GSB conjugate was excited at 458 nm by an argon laser (*Lasos Laser Technic*, Jena, Germany) with beam transmission 19 % (giving a power of 49 μW at the sample). The fluorescence emission for GSB was collected at 475 nm using *HFT 458* filter. Samples were observed with a *Zeiss* 5×/0.15 or 10×/0.3 *Plan-Neoflual* lens without digital zoom. The pinhole was 317 μm. Images of GSB fluorescence in roots were analyzed in term of fluorescence intensity and distribution. The length of the zone with the highest fluorescence intensity was measured by manually drawn lines along the root using *LSM 5 Pascal* software (*Zeiss LSM* image examiner). Fluorescence intensity of different root samples was analyzed by comparing their histograms prepared using the same software. The level of auto-fluorescence was examined under the same imaging conditions after root incubation in deionised water for 1 h. GSH-bimane adducts could not be visualized in leaves due to a very high level of leaf autofluorescence.

Thiol peptides analysis by HPLC and enzymatic assay: GSH and PC contents in fresh root and shoot extracts were determined using HPLC method as described earlier (Wójcik and Tukiendorf 2005). Total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) were measured in root and leaf samples frozen with liquid nitrogen by the method of Anderson (1985). GSH content was additionally determined by subtraction of GSSG value from the total glutathione content.

Cd determination in plant tissues: To remove cell wall-bound metals, the roots were desorbed prior to their harvest in ice-cold 10 mM $CaCl_2$ solution for 0.5 h. Roots and shoots were separately dried at 105 °C and wet digested in $HNO_3/HClO_4$ (4:1, v/v) at 220 °C (*Velp Scientica* digester, model *DK20*, Usmate, Italy).

Cadmium content was determined by inductively coupled plasma atomic emission spectrophotometry (ICP-AES) (*Leeman Labs*, model *PS950*, Hudson, USA) using Cd atomic spectroscopy standard solution.

Statistical analysis: Statistical analysis was carried out using one-way *ANOVA* (for determination of the significant differences in GSH content in control plants and also in GSH or PC accumulation in Cd-treated plants under different BSO or GSH treatments), or two-way *ANOVA* (for determination of the significant differences in Cd × BSO or Cd × GSH interaction significance in plant fresh mass). Data of growth parameters were obtained from 4 - 5 independent replications (at least 10 plants for each replication) and data of Cd and thiol peptides accumulation from 3 - 4 replications (5 plants for each replication). Images of GSB fluorescence were taken from at least 20 root samples in each treatment, and the length of the zone exhibiting the most intensive fluorescence was measured and averaged.

Results

In *A. thaliana* roots, grown for 2 weeks in control nutrient solution, the highest amounts of GSH, visualized as its fluorescent glutathione S-bimane derivative (GSB), were detected in the apical fragments at a distance of approximately 734 ± 70 µm (*n* = 20) from the root apex. Quantitative determination of GSH is possible using this method, but it requires a very complex calibration procedure, which was not the aim of this work. We wanted to examine whether the distribution of GSH in roots changes after plant exposition to BSO, exogenous application of GSH or Cd. At maintaining constant analysis parameters we could compare relative amounts of GSH in root tissues. The application of 500 µM BSO for 14 d decreased GSB fluorescence (Fig. 1). At the same time the length of the zone, giving the highest GSB fluorescence, did not change significantly (826 ± 66 µm). In roots of plants exposed to 500 µM exogenous GSH or 100 µM Cd the level of GSB fluorescence was similar to control, but the highest GSB fluorescence was detected at shorter distance from the root tip (370 ± 27 or 363 ± 41 µm, respectively).

Application of BSO decreased, and exogenous GSH increased content of GSH in *A. thaliana* roots and shoots (Table 1). Concentration 500 µM BSO reduced GSH content by 74 and 67 % in roots and in shoots, respectively ($P < 0.01$). However, application of 500 µM GSH to the solution resulted in over 3-fold and 2.5-fold increase in GSH content in roots and in shoots, respectively. Application of 50 µM BSO and 50 or 100 µM GSH almost never (except for 100 µM GSH treatment in roots) significantly affected the GSH level in plants.

Cadmium did not significantly change GSH content in plants, except for 100 µM Cd at which an increase in shoot GSH content was found (Table 2). Phytochelatin accumulation was found in Cd-treated but not in control plants.

Application of 50 or 100 µM BSO simultaneously with 50 or 100 µM Cd, respectively, had no significant influence on GSH content and PC accumulation in roots and shoots (data not shown). Addition of 500 µM BSO together with 100 µM Cd significantly decreased GSH

Table 1. GSH content [nmol(SH) g^{-1}(f.m.)] in control plants and those grown in the presence of BSO or GSH in the solution. Values correspond to mean values ± SEM of 3 - 4 measurements using HPLC method and 3 measurements using enzymatic method (total *n* = 6 - 7). *Asterisks* indicate significant differences in relation to control at ** - $P < 0.01$, *** - $P < 0.001$.

	Control	BSO concentration [µM]			GSH concentration [µM]		
		50	100	500	50	100	500
Roots	194.46±26.12	98.74± 5.70**	103.88±11.82**	51.55±2.17***	329.75±50.74	476.88±40.02**	693.84±41.27***
Shoots	248.08±30.76	184.62±10.66	195.86±11.32	81.90±6.11**	382.63±52.16	414.75±55.30	597.30±51.14**

Table 2. GSH and PC content [nmol(SH) g^{-1}(f.m.)] in plants treated with Cd and Cd/BSO or Cd/GSH concentrations. Values represent means ± SEM, *n* = 3 - 4. The values followed by the same letter are not statistically different at $P < 0.05$.

Variant	Roots		Shoots	
	GSH	PC	GSH	PC
100 µM Cd	307.78 ± 56.26ab	310.64 ± 86.45a	283.79 ± 39.79ab	637.13 ± 55.18a
100 µM Cd + 500 µM BSO	76.38 ± 11.20a	-	169.02 ± 26.15a	93.58 ± 5.40a
100 µM Cd + 500 µM GSH	923.46 ± 113.18b	841.49 ± 48.58a	744.18 ± 98.22b	724.01 ± 33.44

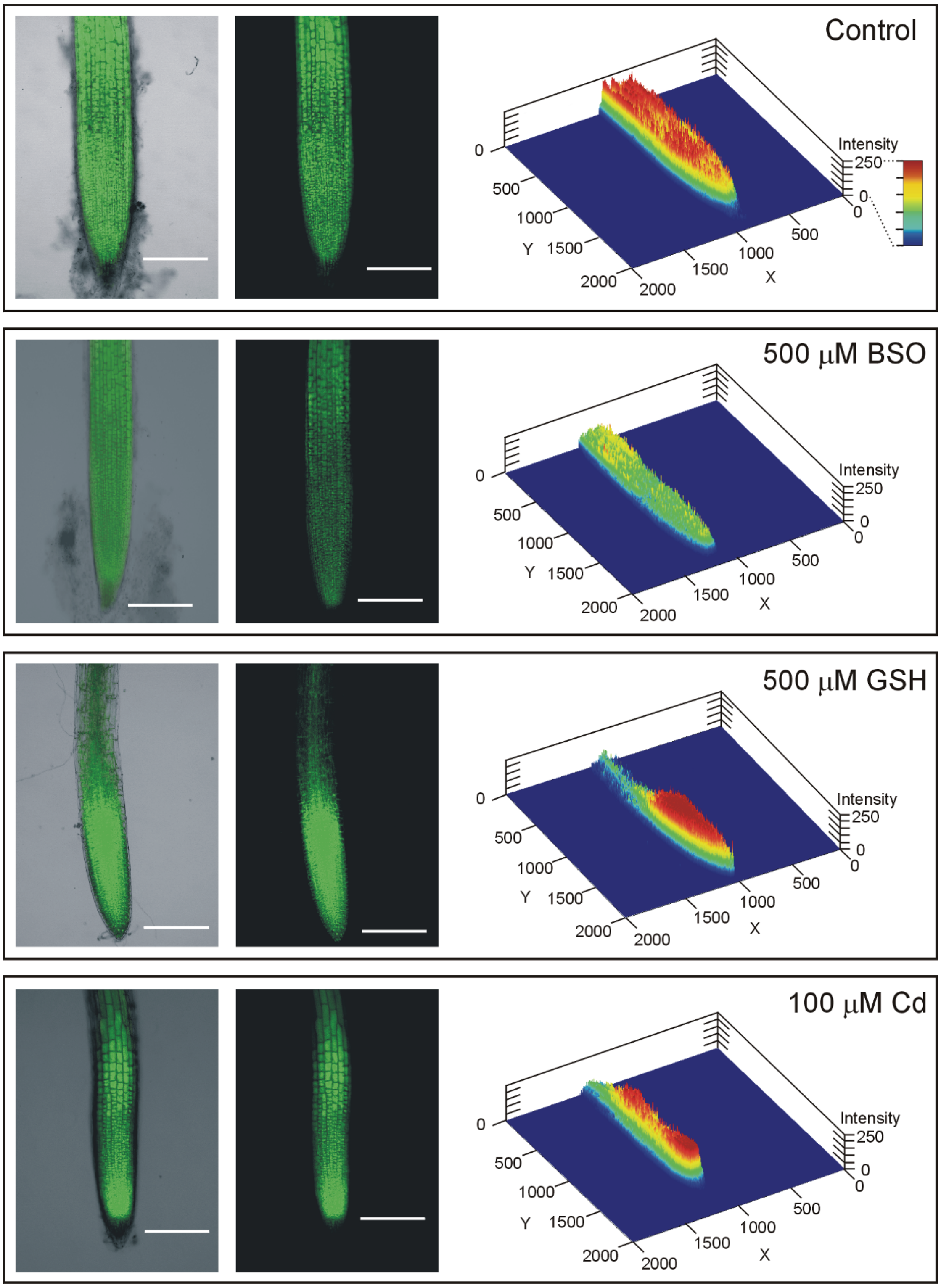

Fig. 1. Visualization of glutathione S-bimane conjugates in intact roots of *Arabidopsis thaliana*. Images were collected from control plants and these treated with 500 µM BSO, 500 µM GSH or 100 µM Cd for 14 d, using confocal laser scanning microscopy after root incubation with MCB. In each panel the first image presents the pattern of GSB fluorescence overlapping the non-confocal view of root contrasted by using the Nomarsky technique for better visualization of the root outline. The second image presents a pure pattern of GSB fluorescence. Fluorescence intensity and distribution in each root image is presented in concurrent histograms. All images presented were collected using a 10× lens. *Bars* = 200 µm.

content and drastically reduced PC accumulation in comparison to plants treated with 100 µM Cd (Table 2).

Addition of 50 or 100 µM of exogenous GSH to the nutrient solution of Cd-treated plants did not significantly influence GSH content in root and shoot tissues (data not shown), in contrast to 500 µM GSH which induced GSH content increase in plants (Table 2). At the same time this exogenous GSH concentration increased PC accumulation in roots up to 2.7 fold, under 100 µM Cd/500 µM GSH treatment, compared to 100 µM Cd treatment. However, significant increase in PC accumulation in shoots was not found under any GSH treatments.

Table 3. The portion of GSH and GSSG in the total glutathione pool [%] of plants grown for 14 d in the control solution or in the presence of BSO, GSH, Cd or Cd/BSO or Cd/GSH combinations. GSH content was averaged from 3 - 4 measurements using HPLC method and 3 measurements using enzymatic method ($n = 6 - 7$), while GSSG and total glutathione contents were determined using enzymatic method ($n = 3$).

Variant [µM]	Roots GSH	GSSG	Shoots GSH	GSSG
Control	100	0	100	0
500 BSO	100	0	100	0
500 GSH	83.4	16.6	88.2	11.8
100 Cd	76.3	23.7	74.8	25.2
100 Cd + 500 BSO	100	0	85.0	15.0
100 Cd + 500 GSH	70.7	29.3	71.9	28.1

In both roots and shoots of control plants, similarly as in BSO treated plants, the oxidized glutathione form (GSSG) was undetectable (Table 3). However, plant treatment for 14 d with Cd (100 µM) or exogenous GSH (500 µM) as well as with Cd in combination with GSH or BSO (both 500 µM) resulted in significant accumulation of GSSG, with the exception of Cd/BSO treated roots. The endogenous glutathione redox status (defined as GSH/(GSH+GSSG) ratio) was the lowest in Cd/GSH treated plants (0.71) as compared to plants exposed to Cd (0.75 - 0.76) and GSH alone (0.83 in roots and 0.88 in shoots).

Cd negatively influenced plant growth (Fig. 2*A*,*B*). Fresh mass reduction by 42 - 50 % at 100 µM Cd and by 22 - 33 % at 50 µM Cd in comparison to control was found (Fig. 2*A*). BSO application did not change significantly plant fresh mass in contrast to exogenous GSH; increased GSH concentrations progressively decreased fresh mass of roots (by 74 % at 500 µM GSH, $P < 0.001$) and shoots (by 40 % at 500 µM GSH, $P < 0.01$) (Fig. 2*A*). Both BSO and GSH applied at their highest concentration (500 µM) together with 100 µM Cd increased Cd toxicity only in root biomass. Lower BSO and GSH concentrations did not modify significantly Cd toxicity. Root fresh mass of plants exposed to 100 µM Cd/500 µM BSO combination was lower in comparison to plants exposed to 100 µM Cd alone by 64 % ($P < 0.001$). Exogenous GSH at 100 µM Cd/500 µM GSH treatment decreased root fresh mass by 45 %, in comparison to 100 µM Cd treated plants ($P < 0.05$). At the same time shoot fresh mass of Cd-treated plants was not influenced either by BSO or by exogenous GSH (Cd × BSO or Cd × GSH interaction, $P > 0.05$).

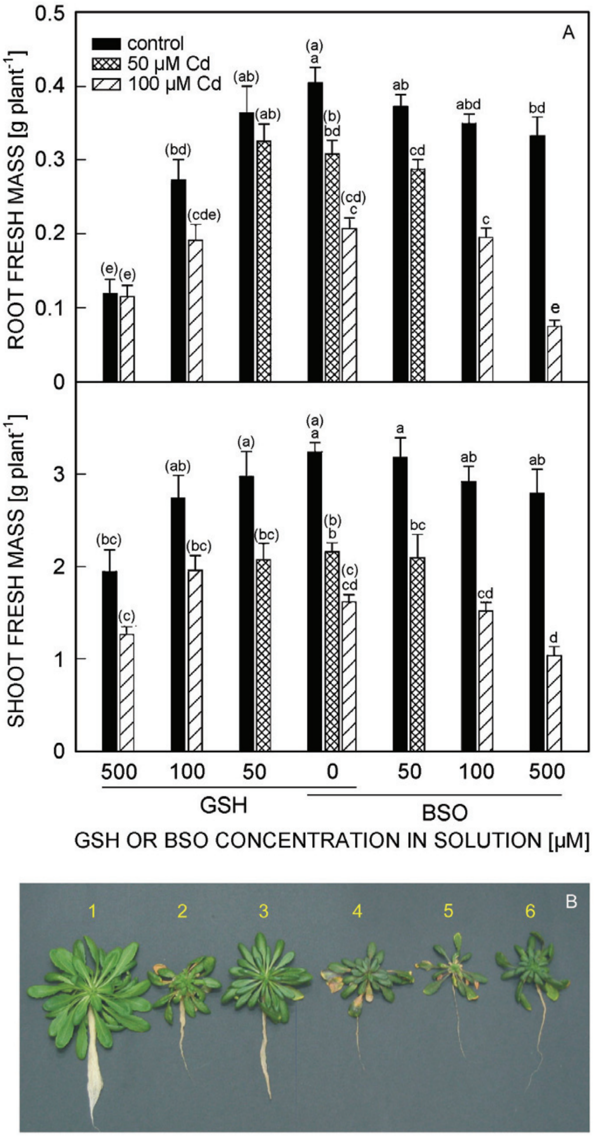

Fig. 2. Fresh mass (*A*) of control plants and those treated with BSO, GSH, Cd or Cd/BSO or Cd/GSH combinations for 14 d. Means ± SE, $n = 10 - 40$. The values followed by the same letters are not statistically different at $P < 0.05$. Letters in parenthesis refer to the significance of Cd/GSH treatments, and without parenthesis to the significance of Cd/BSO treatments. General appearance (*B*) of control (*1*), and treated plants: 100 µM Cd (*2*), 500 µM BSO (*3*), 100 µM Cd/500 µM BSO (*4*), 100 µM Cd/500 µM GSH (*5*) and 500 µM GSH (*6*).

Plants of *A. thaliana* cultivated in the presence of

50 and 100 μM Cd for 14 d accumulated 863.33 ± 198.49 and 951.32 ± 141.46 mg(Cd) kg^{-1}(d.m.) in roots, and 657.36 ± 24.95 and 907.11 ± 49.36 mg(Cd) kg^{-1}(d.m.) in shoots, respectively. Neither BSO or exogenous GSH influenced significantly Cd accumulation in plant roots and shoots during the experiment (data not shown).

Discussion

Studies on *Arabidopsis* have contributed markedly to understanding of mechanisms of detoxification and sequestration of heavy metals and metalloids (*e.g.* Cobbett *et al.* 1998, Xiang *et al.* 2001, Cobbett and Meagher 2002). As it appears from those studies carried out mainly on different mutants or transgenic plants, PC synthesis is an important mechanism of Cd detoxification and tolerance in *A. thaliana*. However, PC synthesis and accumulation strongly depend on available GSH and it was suggested that the GSH content was a limiting factor for PC synthesis.

GSH distribution and quantification in *Arabidopsis* root was described by Fricker *et al.* (2000). The highest fluorescence signal (produced by GSB conjugate) was found in the epidermal cell layer and decreased radially into the centre of the root (being the lowest within the vascular tissue). The average fluorescence within each cell type (apart from the cap cells) decreased also with the distance from the meristem on passing through the elongation zone around 400 μm from the tip to the region of root-hair initiation around 800 μm from the tip. The latter is closely in line with our results. The decrease in the fluorescence signal along the root is due not only to lower amount of GSH itself but also to changes in root anatomy and cell ultrastructure, including mainly an increase in the cell volume with cell expansion, which dilutes the signal. Cell wall thickening additionally lowers the signal. Changes in the root anatomy caused by Cd or GSH toxicity, relying among other things on a significant decrease in the elongation zone, are the reason for the observed shortening of the zone of the most intensive GSB fluorescence. In the roots of plants treated with BSO, no such anatomical perturbations were observed, and the pattern (but not intensity) of GSB fluorescence was similar to control roots. In *Arabidopsis* roots exposed to 1 mM BSO for 12 h, GSH labelling with MCB was completely abolished (Fricker *et al.* 2000). The fact that we could detect GSH in plants grown for 14 d at 500 μM BSO (both using HPLC or enzymatic methods and GSB visualization with confocal laser scanning microscopy) indicated that GSH was formed *de novo* during the assay period.

The important role of GSH in Cd detoxification is shown in our present study on *A. thaliana* plants with an altered GSH content due to application of BSO or exogenous GSH. BSO added together with Cd (500/100, μM/μM) significantly reduced GSH content and PC accumulation in plants (Table 2) and resulted in enhancement of Cd toxicity on plant growth (Fig. 2). Since BSO did not significantly influence Cd accumulation in plants, the toxicity symptoms could be caused by lack of a sufficient amount of GSH and PC to detoxify the intracellular Cd. The results are consistent with those obtained for other plant species (Gussarsson *et al.* 1996, Schat *et al.* 2002, Ben Ammar *et al.* 2008) and wild type of *A. thaliana* plants treated with BSO in the presence of Cd (Howden and Cobbett 1992, Howden *et al.* 1995). Investigations of *Arabidopsis* mutants or transgenic plants with low GSH levels or inhibited PC synthase confirmed that their hypersensitivity to Cd was due to limited capacity of these plants to produce PC (Vernoux *et al.* 2000, Xiang *et al.* 2001). Similarly, increased Cd tolerance was observed in transgenic *Brassica juncea* plants overexpressing enzymes of GSH biosynthesis, thus exhibiting elevated PC accumulation (Zhu *et al.* 1999a,b). On the contrary, *Arabidopsis* mutants or transgenic plants with elevated GSH content or PC synthesis did not show enhanced Cd-resistance in comparison with wild type plants (Xiang *et al.* 2001, Lee *et al.* 2003, Peterson and Oliver 2006). Moreover, overexpression of PC synthase in *A. thaliana* resulted in Cd-hypersensitivity (Lee *et al.* 2003). In accordance with this, a strong increase in GSH content and PC accumulation found in our plants treated with Cd and exogenous GSH was accompanied by declined plant growth. Our results are also in agreement with those of Lee *et al.* (2003) who found that application of exogenous GSH (1 mM) to Cd-treated (50 μM) wild type *A. thaliana* plants reduced their root growth (by 50 % in comparison with plants treated with Cd alone). The exogenous GSH-imposed increase in Cd toxicity which we observed cannot be explained by enhanced Cd accumulation in plants because GSH application did not significantly change Cd content in roots and shoots. It was rather caused by GSH treatment itself, since the fresh mass of plants exposed to 500 μM GSH was almost the same as that of plants simultaneously exposed to 100 μM Cd and 500 μM GSH. These results suggest that the natural level of endogenous GSH in wild type of *A. thaliana* plants is sufficient to provide an appropriate level of PC synthesis and Cd-tolerance. Similarly, Lee *et al.* (2003) assumed that the normal level of *Arabidopsis* PC synthase expression was sufficient to synthesize the required PCs in response to the supplemented Cd.

In the present study we also examined the effect of BSO and GSH on non Cd-treated *A. thaliana* plants. BSO was found to decrease GSH content in control plants without any significant toxic effect on the plant biomass. On the other hand, exogenous GSH application (500 μM) increased the GSH content in plant tissues, but simultaneously an inhibition of plant growth was found.

The BSO-imposed decrease in GSH content with no influence on *A. thaliana* plant growth was also described earlier (May and Leaver 1993), and inhibition of *A. thaliana* root growth under exogenous GSH treatment was found, as well (Lee *et al.* 2003, Espunya *et al.* 2006). In the experiments of Sánchez-Fernández *et al.* (1997), depletion of GSH by BSO application decreased the number of meristematic cells undergoing mitosis as well as hair length and density, while exogenous application of micromolar concentrations of GSH had the opposite effect. In BSO treated plants the root length was maintained at the control value through increased cell elongation, while the roots of plants exposed to exogenous GSH shortened due to inhibition of cell elongation. Similarly, Vernoux *et al.* (2000) reported that BSO (2500 µM) added to the growth medium of *Arabidopsis* seedlings inhibited root apical meristem activity. Those authors suggested that there must exist a threshold of the GSH content below which developmental effects are observed in roots. They found complete root growth inhibition (inhibition in cell division in root meristem) in *rml1* mutants, in which the intracellular concentration of GSH was only 2.7 % of that in the wild type. Cobbett *et al.* (1998), however, did not observe any perturbation of root cell division in *cad2-1* mutants (*cad 2* is allelic to *rml1* gene), in which the intracellular concentration of GSH was 15 to 30 % of that in the wild type. In our plants treated with 500 µM BSO for 14 d the intracellular content of GSH was reduced to 26.5 % of that in the roots of control plants. This suggested that in these plants such a threshold of GSH content was not exceeded and thus root growth could steadily proceed.

The toxicity of exogenous GSH to plant growth is still not well understood. Since GSH rapidly and spontaneously oxidizes in the medium (Anderson 1985, Jamaï *et al.* 1996), it may be assumed that much of the GSH added to the nutrient solution of our plants became oxidized to GSSG. However, both GSH and GSSG can be easily taken up by plants, with the latter being even a preferential form for the uptake (Sánchez-Fernández *et al.* 1997, Senda and Ogawa 2004, Belmonte *et al.* 2006). This is probably because the transport of GSH across the membrane requires a transporter that has high affinity for GSSG but lower for GSH (Jamaï *et al.* 1996). Although GSSG transported into cells is rapidly reduced to GSH by glutathione reductase, the local imbalances in the redox state of the GSH pool may lead to disruption in the cell redox balance and cause oxidative stress. This may explain, at least partially, the observed toxicity. Indeed, in GSH treated plants (500 µM) the contribution of GSSG to the total glutathione pool reached 17 % in roots and 12 % in shoots after 14-d exposure, whereas in control plants no GSSG was detected. Interestingly, a decreased GSH redox status was also found in Cd, Cd/GSH and Cd/BSO (shoots only) treated plants. Similar observations were also reported earlier for plants treated with Cd, BSO or exogenous GSH (Senda and Ogawa 2004, Belmonte *et al.* 2006, Semane *et al.* 2007, Markovska *et al.* 2009). The lowest GSH redox status in Cd/GSH treated plants, as compared to plants treated with GSH or Cd solely, could express cumulative effect of Cd and GSH on GSH oxidation. The observed increase in GSSG content may be due to involvement of GSH in the scavenging of ROS *via* ascorbate-GSH cycle. This is supported by the fact that ROS have been shown to accumulate upon Cd or BSO exposure (Armstrong and Jones 2002, Skórzyńska-Polit *et al.* 2003/4, Maksymiec and Krupa 2006). However, data for exogenous GSH exposure are, to our best knowledge, not yet available.

Similar growth inhibition, as in the presence of exogenous GSH, in *Arabidopsis* was also observed after plant exposure to Cys and other components of PC (including Cys, Glu, γ-Glu-Cys, γ-Glu-Gly, Cys-Gly, supplied at 1 mM) as well as in plants with genetically modified high PC level (Lee *et al.* 2003). Thus, in our plants the excess of intracellular GSH and PC could be the reason of growth inhibition. Any imbalance, both deficiency and excess, of cellular thiol compounds, with GSH being one of the most important, influences cell metabolism. Perturbation of normal GSH content could increase the susceptibility to oxidative stress, although it could also disturb redox signalling in important processes or redox controlled gene expression. Additionally, changes in the spatial tissue distribution of GSH, especially in root meristem, could alter the ability of plants to detect redox changes and the subsequent redox dependent modulation of growth (Espunya *et al.* 2006).

Glutathione plays an important role in Cd-tolerance of wild type *A. thaliana* plants, mainly as a substrate for PC synthesis. The natural content of endogenous GSH seems to be sufficient for Cd detoxification, as supported by the fact that reduction of this content (under BSO treatment) led to enhanced Cd-sensitivity, but increased GSH content did not enhance Cd-tolerance and it was even toxic for the plants.

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