Plant Biotechnology Journal (2004) **2**, pp. 469–476 doi: 10.1111/j.1467-7652.2004.00092.x

Increased cysteine availability is essential for cadmium tolerance and accumulation in *Arabidopsis thaliana*

José R. Domínguez-Solís[1], M. Carmen López-Martín[1], Francisco J. Ager[2], M. Dolores Ynsa[2], Luis C. Romero[1] and Cecilia Gotor[1,*]

[1]*Instituto de Bioquímica Vegetal y Fotosíntesis, Centro de Investigaciones Científicas Isla de la Cartuja, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, Avda. Américo Vespucio s/n, 41092 Sevilla, Spain*
[2]*Centro Nacional de Aceleradores, Avda. Thomas Edison s/n, 41092 Sevilla, Spain*

Received 2 March 2004;
revised 16 April 2004;
accepted 27April 2004.
**Correspondence* (fax 34-95-4460065;
e-mail gotor@ibvf.csic.es)

Summary

Employing genetic transformation using an *Atcys-3A* cDNA construct expressing the cytosolic *O*-acetylserine(thiol)lyase (OASTL), we obtained two *Arabidopsis* lines with different capabilities for supplying cysteine under metal stress conditions. Lines 1-2 and 10-10, grown under standard conditions, showed similar levels of cysteine and glutathione (GSH) to those of the wild-type. However, in the presence of cadmium, line 10-10 showed significantly higher levels. The increased thiol content allowed line 10-10 to survive under severe heavy metal stress conditions (up to 400 μM of cadmium in the growth medium), and resulted in an accumulation of cadmium in the leaves to a level similar to that of metal hyperaccumulator plants. Investigation of the epidermal leaf surface clearly showed that most of the cadmium had accumulated in the trichomes. Furthermore, line 10-10 was able to accumulate more cadmium in its trichomes than the wild-type, whereas line 1-2 showed a reduced capacity for cadmium accumulation. Our results suggest that an increased rate of cysteine biosynthesis is responsible for the enhanced cadmium tolerance and accumulation in trichome leaves. Thus, molecular engineering of the cysteine biosynthesis pathway, together with modification of the number of leaf trichomes, may have considerable potential in increasing heavy metal accumulation for phytoremediation purposes.

Keywords: *O*-acetylserine, cadmium stress, cysteine biosynthesis, sulphur assimilation, trichome cells.

Introduction

Cysteine biosynthesis is the final step in the sulphate assimilation pathway and is catalysed by *O*-acetylserine(thiol)lyase (OASTL, EC 4.2.99.8). This enzyme incorporates sulphide into *O*-acetylserine (OAS), the latter being produced through the acetylation of serine by the action of serine acetyltransferase (SAT, EC 2.3.1.30). Both OASTL and SAT activities have been demonstrated to be localized in three cellular compartments involved in protein synthesis: the cytosol, plastid and mitochondrion (Hell *et al*., 2002). In the *Arabidopsis thaliana* genome, five different SAT genes (Howarth *et al*., 2003) and at least seven different OASTL genes (Jost *et al*., 2000) have been identified. Some evidence has suggested that the isoforms may have specific contributions to particular metabolic pathways. Thus, certain OASTL isoforms exhibit β-cyanoalanine synthase enzyme activity and are involved in cyanide detoxification (Hatzfeld *et al*., 2000; Yamaguchi *et al*., 2000), whereas the cytosolic isoform ATCYS-3A seems to play a role in cadmium (Cd) tolerance (Domínguez-Solís *et al*., 2001).

Cysteine is the precursor molecule for the synthesis of glutathione (GSH), the predominant non-protein thiol, which plays an important role in plant stress responses. GSH has been implicated in plant responses to toxic levels of heavy metals, as it is the precursor for the synthesis of phytochelatins (PCs), the thiolate peptides involved in the detoxification of Cd and other heavy metals. PCs bind toxic heavy metal ions, and these complexes are transported into the vacuole by an ABC-type transporter (Cobbett, 2000). Today, the contamination of soils and water by toxic heavy metals presents a major environmental and human health problem. The

potential removal of these toxic metals by phytoremediation technology has stimulated interest in the investigation of the physiological and molecular mechanisms of plant adaptation to high levels of heavy metals.

We have shown previously that the *A. thaliana* OASTL gene *Atcys-3A* and the SAT gene *Sat-5* are induced in leaf lamina, root and stem cortex and trichomes in response to Cd treatment (Domínguez-Solís *et al*., 2001; Howarth *et al*., 2003). Additionally, the over-expression of *Atcys-3A* in *A. thaliana* resulted in increased Cd tolerance (Domínguez-Solís *et al*., 2001). These results suggest that specific OASTL and SAT isoforms may play a role in increasing cysteine production under conditions of heavy metal stress when increased biosynthesis of GSH is required. In this work, we further investigate the relationship between cysteine biosynthesis and heavy metal tolerance.

Another important aspect in the metal detoxification process is the sequestration of the PC–metal complexes in the vacuoles; however, little is known about the specific cell types in which storage is predominant. Optimization of the phytoremediation process requires a greater understanding of the points of accumulation of heavy metals in leaves so that their storage capacity may be increased. In this respect, we have evidence to show that *Arabidopsis* trichomes possess substantial storage capacity, and can act as sinks for toxic molecules that have been detoxified via mechanisms involving GSH (Gutiérrez-Alcalá *et al*., 2000). In the present study, we evaluated the role of leaf trichomes in heavy metal storage and the possibility of manipulating their storage capacity.

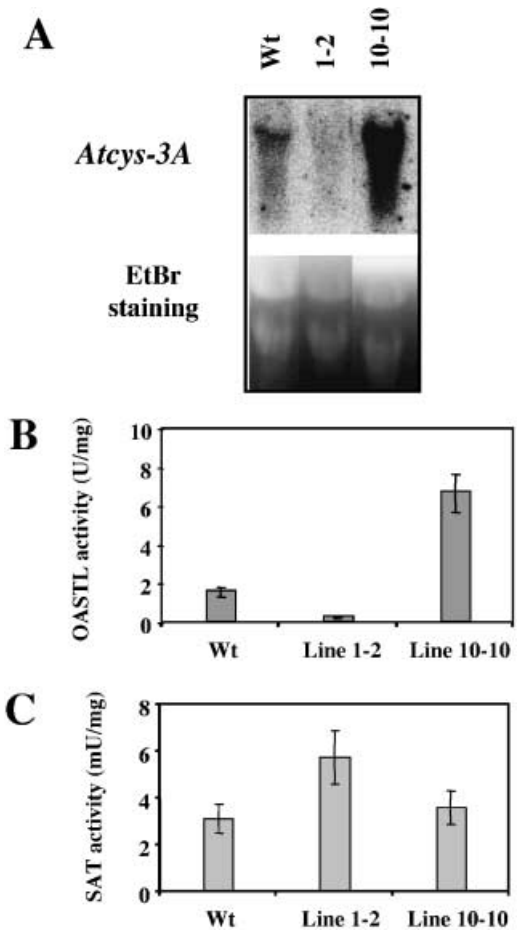

Figure 1 *Arabidopsis* transgenic lines 1-2 and 10-10. (A) Northern blot analysis. Total RNA isolated from leaves was hybridized with a gene-specific probe for *Atcys-3A*. As a control for RNA loading, ethidium bromide (EtBr) staining of the gel is also shown. (B) *O*-Acetylserine(thiol)lyase (OASTL) and (C) serine acetyltransferase (SAT) activities. Crude extracts were prepared from leaves and used as sources of enzymes for activity measurements, as indicated in 'Experimental procedures'. The values shown represent the averages of at least five independent experiments, and the error bars represent standard deviations. Wt, wild-type.

Results

Arabidopsis transgenic lines with different levels of *Atcys-3A* gene expression

We have shown previously that a primary response of *Arabidopsis* plants to Cd stress is the induction of genes responsible for cysteine biosynthesis (Domínguez-Solís *et al*., 2001; Howarth *et al*., 2003). In an effort to increase the rate of cysteine biosynthesis in *Arabidopsis*, we over-expressed an *Atcys-3A* cDNA encoding one of the cytosolic isoforms of OASTL. Ten independent transgenic lines were obtained and used for an initial characterization. Northern blot analysis showed divergent transcript abundance levels amongst the transgenic lines compared with the wild-type. Some lines accumulated more *Atcys-3A* mRNA than the wild-type, whereas the level of *Atcys-3A* expression in other lines was lower than that observed in the wild-type (data not shown). Two representative homozygous lines were selected for the studies presented in this work. One of these, line 1-2, showed the lowest amount of transcript, lower than that observed in the wild-type, suggesting the possible presence of a gene silencing mechanism in this line (Figure 1A). The other, line 10-10, showed up to a nine-fold increase in *Atcys-3A* mRNA accumulation compared with that of the wild-type, and may be considered as an over-expressing line (Figure 1A). When the total OASTL enzyme activity was determined in these two lines, the level of enzyme activity correlated with *Atcys-3A* mRNA accumulation. Line 1-2 showed about an 80% decrease in activity compared with that of the wild-type, while the OASTL activity in line 10-10 was increased four-fold relative to that of the wild-type (Figure 1B). It should be noted

that a general reduction in growth in line 1-2 was observed when compared with that of the wild-type under non-stress conditions (data not shown). We routinely obtained about a 30% reduction of line 1-2 seed production with respect to line 10-10 when plants were harvested after the same period of growth.

SAT activity was also measured in the transgenic lines and compared with the activity level in the wild-type. Although the SAT activity in line 10-10 remained unchanged compared with that of the wild-type, line 1-2 showed about a two-fold increased level of enzyme activity relative to that of the wild-type (Figure 1C). It has been shown that the cytosolic SAT isoform is feedback inhibited by low concentrations of cysteine (Noji *et al*., 1998). Our results show that a significant reduction in cytosolic OASTL protein induces an increase in SAT activity, probably due to an important decrease in the cytosolic content of cysteine below the threshold of the feedback regulation.

We were interested in determining the responses of the two transgenic lines at the level of the rate of thiol biosynthesis when subjected to heavy metal stress. To this end, mature plants were exposed to cadmium chloride treatment and the cysteine and GSH contents in leaf tissue were determined. At the high concentration of 250 μM $CdCl_2$, line 1-2 behaved similarly to the wild-type, and a depletion of cysteine and GSH levels was observed. In line 10-10, however, the cysteine level remained unchanged, whilst the GSH content increased significantly (Figure 2). In the absence of Cd, the concentrations of GSH in the wild-type and the two lines were similar. These results suggest that constitutive over-expression of *Atcys-3A* provides the plant with a sufficient amount of GSH to respond to Cd, and therefore GSH depletion is not observed under conditions of severe heavy metal stress.

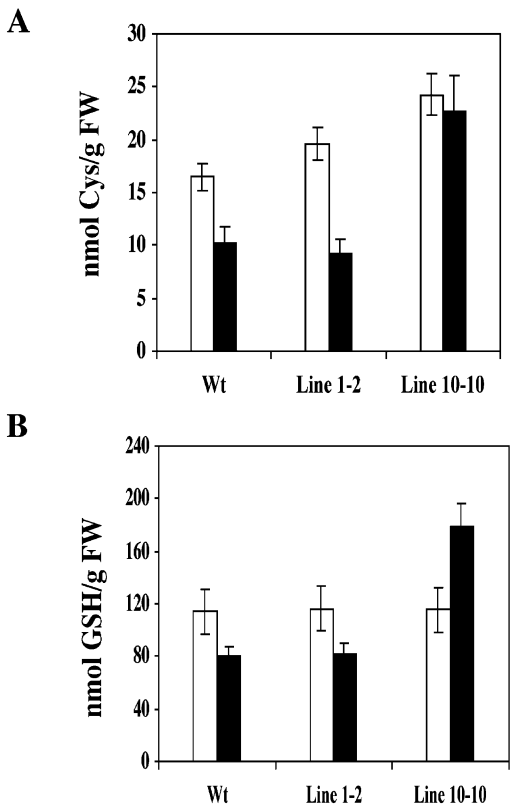

Figure 2 Cysteine (Cys) and glutathione (GSH) contents in wild-type (Wt) and transgenic lines 1-2 and 10-10 following cadmium treatment. Mature plants were incubated in the absence (open bars) and presence (dark bars) of 250 μM $CdCl_2$ for 18 h, and leaves were collected for Cys and GSH determination. The values represent the averages of at least four independent experiments, and the error bars represent standard deviations. FW, fresh weight.

Cadmium tolerance of plants with increased cysteine availability

Two parameters, seedling fresh weight and root length, were measured in an effort to determine whether high cysteine availability can induce an improved tolerance for Cd. We have observed previously that the exogenous addition of cystine to wild-type plants is sufficient to support their growth in the presence of 250 μM $CdCl_2$ (Domínguez-Solís *et al*., 2001). The exogenous addition of cystine instead of cysteine was used to avoid reaction in the medium between the heavy metal and the free thiol group of cysteine. We wanted to compare this condition with that of the over-expressing transgenic line in different concentrations of Cd. We observed that line 10-10 and the wild-type plus exogenous cystine were able to survive in the presence of over 400 μM $CdCl_2$, whilst line 1-2 and the wild-type without exogenous cystine died, with the weight of a dead seedling being around 2 mg (Figure 3A). The EC_{50}, defined as a 50% reduction in the mean seedling fresh weight, was observed at 300 μM $CdCl_2$ for line 10-10 and at 200 μM $CdCl_2$ for the wild-type and line 1-2. The analysis of root length revealed that line 10-10 and the wild-type plus exogenous cystine behaved similarly at concentrations of Cd up to 250 μM. However, higher Cd concentrations inhibited the root growth of the wild-type plus exogenous cystine to the same extent as line 1-2 and the wild-type without exogenous cystine (Figure 3B).

The improved tolerance of line 10-10 and the wild-type plus exogenous cystine at low concentrations of Cd may be due to enhanced metal accumulation. To test this hypothesis, we exposed mature plants to 250 μM $CdCl_2$ for 14 days, and determined the Cd content in leaves by atomic emission spectrometry. We observed an increase in Cd accumulation in both line 10-10 and the wild-type plus exogenous cystine

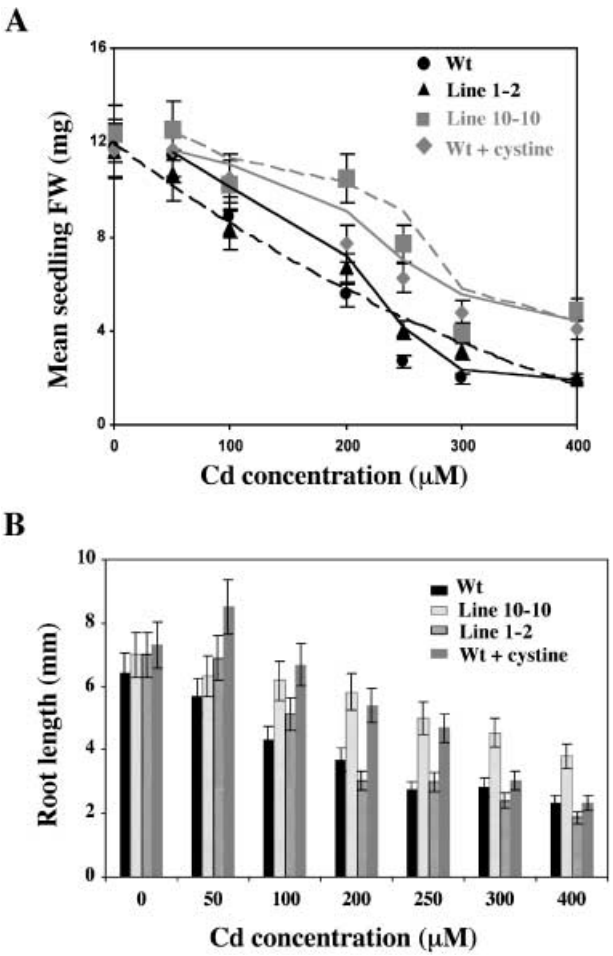

Figure 3 Effect of increased cysteine availability on plant tolerance to cadmium. Five-day-old seedlings germinated on Murashige–Skoog (MS) medium were transferred to medium containing $CdCl_2$ at different concentrations, including 200 μM of cystine where indicated. After an additional 6 days of growth, total seedling fresh weights (FW) (A) and root lengths (B) were determined. Each data point represents the mean of nine independent experiments, in which 10 seedlings were weighed or their roots measured. Error bars represent the standard deviation. Curve fitting in (A) showing the trend of the data was calculated by non-linear regression using the program Microsoft Excel 98. Wt, wild-type.

(Table 1). However, the transgenic line proved to be a more efficient accumulator plant, the highest level of Cd accumulation being 72% more than that of the wild-type. As described in the previous experiments, line 1-2 behaved similarly to the wild-type, being able to accumulate the same amount of Cd ions.

It has been shown previously that an increased tolerance to Cd results when tobacco seedlings are grown in the presence of calcium (Ca) (Choi *et al*., 2001). In an effort to further improve the threshold of tolerance of line 10-10, we examined the effect of Ca when added to the growth medium. When wild-type seeds were germinated in the presence of both Cd and Ca, no apparent phenotypic symptoms of heavy

Table 1 Cadmium concentration in leaves of *Arabidopsis* plants

Plant	mg Cd/g dry weight
Wt	0.39 ± 0.02 (100)
Line 10-10	0.67 ± 0.06 (172)
Line 1-2	0.38 ± 0.03 (99)
Wt + cystine	0.52 ± 0.00 (133)

Mature plants were incubated with 250 μM $CdCl_2$ for 14 days and 200 μM cystine where indicated. Leaves were then collected for Cd determination, as described. Values ± standard deviation are shown from five independent measurements. Values in parentheses show the percentage of Cd accumulated relative to the amount accumulated by the wild-type (Wt).

metal toxicity were observed. Wild-type seedlings looked as healthy as the wild-type seedlings growing in the absence of Cd (data not shown). Thus, we grew line 10-10 seedlings in Cd-contaminated medium in the absence or presence of Ca for 2 weeks and quantified the amount of Cd accumulated by the seedlings. Surprisingly, we observed a lower Cd accumulation in the presence of Ca, 75% less Cd than in the absence of Ca (data not shown). Furthermore, we found a 4.6-fold higher accumulation of Cd in seedlings than in mature plants (data not shown), suggesting that the developmental stage of the plant is an important parameter in determining the levels of metal accumulation.

Trichome as the main point of cadmium accumulation in *A. thaliana* leaves

Previous studies performed in different plant species have suggested trichomes as a possible location of Cd accumulation in leaves (Ager *et al*., 2002; Choi *et al*., 2001; Küpper *et al*., 2000; Salt *et al*., 1995). To investigate the involvement of *A. thaliana* trichomes in Cd detoxification, we performed energy-dispersive X-ray (EDX) analysis of leaf trichomes in combination with proton-induced X-ray emission (PIXE) microanalysis. Figure 4 shows a trichome on the upper surface of a mature leaf, obtained by scanning electron microscopy (SEM), and the EDX dot maps with the distribution of Cd, Ca and K. Cd preferentially accumulated in the trichome base underneath the branching point, Ca was highly abundant in the middle and upper parts of trichome cells, whilst K was most abundant in the epidermal leaf surface.

We quantified the concentration of different elements in trichomes and epidermal cells from wild-type plants following Cd treatment using PIXE microanalysis. The amount of Cd accumulated inside the trichome was 10-fold higher than that found in the epidermis. Interestingly, the concentrations of phosphorus and sulphur increased significantly in the

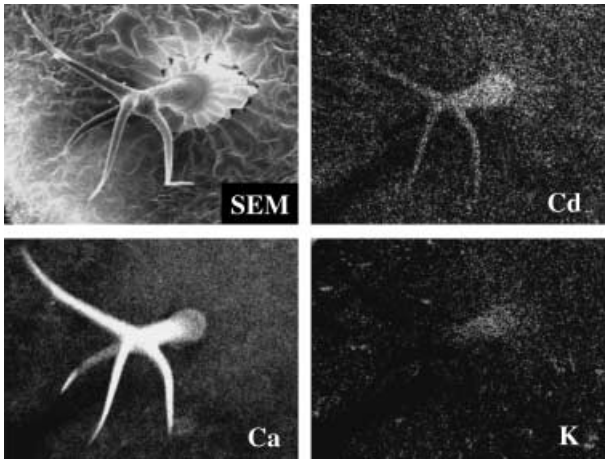

Figure 4 Leaf trichome of *Arabidopsis thaliana* treated with 250 μM Cd under scanning electron microscopy (SEM), and the energy-dispersive X-ray analysis two-dimensional distribution patterns for Cd, Ca and K.

Table 2 Concentration of cadmium in leaf trichomes quantified by proton-induced X-ray emission (PIXE) microanalysis

Plant	Cd content (μg/g)
Wt	9185 ± 780
Line 10-10	31030 ± 3408
Line 1-2	2015 ± 1700

Plants were treated with 250 μM $CdCl_2$ for 14 days and the leaves were collected for Cd quantification by PIXE microanalysis. Standard deviations are shown.

trichome when the plant was treated with the heavy metal (data not shown). A similar quantification analysis was performed in leaf trichomes of the transgenic lines following Cd treatment. Line 10-10 accumulated 3.4-fold more Cd in its trichomes than the wild-type, whereas trichomes of line 1-2 showed a reduced capacity for Cd accumulation (Table 2). These results highlight the important physiological significance of trichome cells in acting as sinks during metal detoxification. Furthermore, an increased rate of cysteine biosynthesis also leads to the enhanced accumulation of Cd in leaf trichomes.

Discussion

Plants are exposed during their vital cycle to adverse environmental conditions that negatively affect growth, development or productivity. The presence of toxic compounds, such as heavy metals, is one factor that can cause damage to plants. Plants have developed defence mechanisms to cope with these adverse conditions. Many of these responses involve the synthesis of thiol-containing molecules. It is well known that Cd detoxification involves the chelation of the heavy metal by a family of peptide ligands, the PCs (Cobbett, 2000), which is dependent on the synthesis of the precursor thiol molecules cysteine and GSH. We have demonstrated previously in *Arabidopsis* that the plant responds to Cd toxicity by inducing the genes responsible for cysteine biosynthesis (Domínguez-Solís *et al*., 2001; Howarth *et al*., 2003). An increased expression of genes involved in sulphur assimilation and GSH and PC synthesis in response to Cd treatment has also been shown in *Arabidopsis* (Harada *et al*., 2002; Xiang and Oliver, 1998) and other plant species, as well as in *Brassica juncea* (Heiss *et al*., 1999).

By employing genetic transformation techniques, we obtained plants with different capabilities for the supply of cysteine under stress conditions. The transgenic lines 1-2 and 10-10, grown under standard conditions, showed similar levels of cysteine and GSH compared with the wild-type. However, under conditions of Cd stress, line 10-10 was able to respond more efficiently, showing significantly higher levels of cysteine and GSH than line 1-2 and the wild-type. Thus, the machinery involved in cysteine synthesis in line 10-10 plants seems capable of supplying the required thiol precursor for PC synthesis more efficiently. This allows line 10-10 plants to survive under severe heavy metal stress, even in the presence of 400 μM $CdCl_2$ in the growth medium. A similar Cd tolerance can also be achieved by the exogenous application of thiol-related compounds to wild-type plants, indicating once again that increased cysteine availability is the main factor responsible for imparting Cd tolerance in *Arabidopsis*. Interestingly, increased synthesis of PCs in *Arabidopsis* leads to Cd hypersensitivity (Lee *et al*., 2003), suggesting that the manipulation of cysteine biosynthesis rather than the production of PCs may be more important for phytoremediation purposes. It should be noted that line 1-2, with only 20% of the wild-type total OASTL activity, behaved phenotypically just as the wild-type and was not hypersensitive to Cd. This result indicates that a complex regulatory network of cysteine biosynthesis operates in *Arabidopsis* to ensure that appropriate levels of cysteine are maintained. However, constitutively expressed OASTL in line 10-10 acted independently from the regulatory control, and therefore was able to support a higher cysteine synthesis under heavy metal stress. Similar to our system, when rice OASTL was expressed in transgenic tobacco plants, high levels of sulphur-containing compounds were produced to detoxify Cd (Harada *et al*., 2001). However, in *B. juncea*, the limiting step for Cd tolerance seems to be GSH biosynthesis, rather than cysteine availability, as overexpression of γ-glutamylcysteine synthetase enhanced the tolerance to a level similar to that observed here (Zhu *et al*., 1999).

The enhanced Cd tolerance of line 10-10 seems to be due to increased Cd accumulation in the leaves. Employing a transgenic approach, we were able to obtain a plant line that accumulated 670 p.p.m. Cd in the leaves without phytotoxicity. Foliar Cd levels above 1 p.p.m. are usually considered to be toxic (Pence *et al*., 2000). Thus, the increased availability of cysteine enhances the ability of plants for metal sequestration, mainly in the trichomes. In nature, there are metal hyperaccumulator plants that can grow in metal-rich soils, such as the well-known *Thlaspi caerulescens*. These plants can accumulate and tolerate high levels of heavy metals in the shoot. *T. caerulescens* is a member of the Brassicaceae family, and certain ecotypes are able to accumulate up to 1000 p.p.m. Cd in their shoots without exhibiting toxic symptoms (Salt *et al*., 1998). Clearly, we have been able to obtain a non-accumulator plant that can accumulate Cd to similar levels as a hyperaccumulator, demonstrating the potential of genetic engineering in phytoremediation. In an effort to further increase Cd sequestration, we used Ca as a soil amendment, based on a previous report that showed a positive effect of Ca on Cd tolerance in tobacco plants (Choi *et al*., 2001). Our results show an increased tolerance to the heavy metal that is not correlated with enhanced Cd accumulation. It has been shown that Cd permeates through Ca channels (Perfus-Barbeoch *et al*., 2002) and, consequently, Ca should compete with Cd for uptake. Plants exhibit higher tolerance because less Cd is taken up.

A general mechanism used by plants for heavy metal detoxification involves chelation of the metal and subsequent compartmentalization of the complex in vacuoles (Cobbett, 2000). The second aspect is also crucial for plant tolerance to heavy metals. We analysed the distribution of Cd in the epidermal leaf surface, and clearly demonstrated that trichomes represent the main locations of heavy metal accumulation. Cd accumulates preferentially in the base of trichomes, where the vacuolar space is present. Considering that the volume of the trichome is 200-fold higher than that of any other epidermal cell, and that the vacuole occupies 90%–95% of this volume, each trichome possesses substantial storage capacity (Gutiérrez-Alcalá *et al*., 2000). In addition, strong GUS activity, driven by the promoter of PC synthase, has been detected in the same area of the *Arabidopsis* trichomes, supporting them as main points of sequestration of PC–Cd complexes (Lee *et al*., 2002). Similar distributions of zinc and Cd inside trichomes have been observed in the hyperaccumulator *Arabidopsis halleri* (Küpper *et al*., 2000).

The chemical forms of Cd in *A. thaliana* trichomes are at present unknown, although phosphorus and/or sulphur may be involved, as suggested by their increased levels following Cd treatment. Numerous evidence exists highlighting the importance of sulphide ions in the detoxification of Cd through the stabilization of PC complexes (Cobbett, 2000). Ca has also been implicated in the detoxification of Cd in tobacco plants, which actively exclude the heavy metal by forming and excreting Cd/Ca-containing crystals through the head cells of trichomes (Choi *et al*., 2001). *A. thaliana* also contains a large amount of Ca distributed throughout the trichomes; however, similar crystals have not been found. It is important to emphasize that *A. thaliana* trichomes are unicellular and morphologically different from tobacco trichomes.

If *Arabidopsis* trichomes are involved in the sequestration of PC–Cd complexes, a high capacity for the synthesis of the precursor thiol molecules (cysteine and GSH) should be required in this cell type. We have demonstrated that the GSH biosynthetic pathway is highly active in trichomes, highlighting the physiological importance of this cell type in relation to the specific function of detoxification (Gutiérrez-Alcalá *et al*., 2000). To date, the unicellular trichomes in *Arabidopsis* have been considered dispensable, as mutants lacking trichomes (*gl1* mutants) grow normally (Marks, 1997). From our work, we can conclude that trichomes most likely function efficiently in the detoxification of heavy metals through GSH-dependent mechanisms. Furthermore, this function can be improved through genetic modification, as we were able to quantify increased Cd accumulation in the trichomes of transgenic line 10-10.

Experimental procedures

Plant material, growth and treatment

A. thaliana plants were grown on moist perlite supplemented with Hoagland medium at 20 °C in the light and 18 °C in the dark, under a 16 h white light/8 h dark photoperiod. Cadmium chloride treatment was performed by the addition of $CdCl_2$ to the Hoagland medium at the indicated concentrations. For the *in vivo* growth measurements, seeds were germinated on solid Murashige–Skoog (MS) medium for 5 days on vertical Petri dishes, and then transferred on to medium containing various concentrations of $CdCl_2$. Seedlings were weighed and roots measured following 6 days of further growth.

Construction of the pBIOAS plasmid and plant transformation

The full-length *Atcys-3A* cDNA (Barroso *et al*., 1995) replaced the β-glucuronidase gene in the binary vector pBI121

(Clontech, Palo Alto, CA, USA) and was under the control of the cauliflower mosaic virus 35S promoter. A fragment containing the polyadenylation signal from the nopaline synthase gene was placed downstream of the gene. The orientation and reading frame of the *Atcys-3A* cDNA insert were confirmed by sequencing. The resulting plasmid, named pBIOAS, was transformed into *Agrobacterium tumefaciens* strain CV50, and *A. thaliana* (ecotype Columbia) was transformed using the vacuum infiltration method. Transformed plants were selected on kanamycin-containing solid MS medium. Ten independent homozygous transformed lines were obtained and, after initial characterization, two of these were selected for the study presented here.

RNA isolation and Northern blot analysis

Total RNA was isolated from leaves and Northern blot analysis was performed following previously reported methods (Barroso *et al*., 1995). Blots were hybridized with ^{32}P-labelled *Atcys-3A* cDNA.

Determination of SAT and OASTL activities

Plant material was homogenized in 50 mM phosphate buffer (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 mM phenylmethylsulphonylfluoride (PMSF) using a mortar and pestle with liquid nitrogen. After the homogenate had been centrifuged at 15 000 ***g*** for 15 min at 4 °C, the resulting supernatant was used as the crude extract and assayed for enzyme activity.

SAT activity was determined by measuring the amount of OAS formed after derivatization with o-phthalaldehyde, followed by reverse phase-high performance liquid chromatography (RP-HPLC) (Droux *et al*., 1998). The reaction was carried out at 30 °C in a final volume of 275 μL containing 33.3 mM phosphate buffer (pH 7.5), 20 mM serine, 1.66 mM acetyl-CoA and the appropriate amount of enzyme. The reaction was initiated by the addition of acetyl-CoA and stopped after 10 min by the addition of 25 μL of 20% (p/v) trichloroacetic acid. To the resulting mixture was added the same volume of derivatization reagent that contained 75% (v/v) of 10 mM *o*-phthalaldehyde and 25% (v/v) of 0.4 M borate buffer (pH 10.5) with 21.5 mM 2-mercaptoethanol. Following 2 min of incubation, a 60 μL aliquot was injected on to an RP-18 column (5 μm particle size, LiChrospher 100, ECOM S.R.O., Praha, Czech Republic) connected to the HPLC system. The fluorescence of the *o*-phthalaldehyde derivatives was monitored and OAS was quantified by measuring the corresponding peak identified using standards. One unit of activity corresponded to the amount of enzyme that catalysed the formation of one micromole of OAS per minute.

OASTL activity was measured in crude extracts as described previously (Domínguez-Solís *et al*., 2001).

The total amount of protein was determined by the method of Bradford (1976).

Analytical determination of cysteine and GSH

Cysteine and GSH were extracted and subsequently quantified by RP-HPLC after derivatization with monobromobimane (Molecular Probes, Eugene, OR, USA), according to previously described methods (Domínguez-Solís *et al*., 2001).

Cadmium quantification in whole plant leaves

Three-week-old plants were irrigated for 14 days with Hoagland medium supplemented with a final $CdCl_2$ concentration of 250 μM. The leaves were collected, dried and prepared for analysis by inductively coupled plasma-atomic emission spectrometry (ICP-AES), as described previously (Domínguez-Solís *et al*., 2001).

EDX analysis of tissues

Sections of leaves from plants treated with 250 μM $CdCl_2$ for 14 days were excised and mounted in a stainless steel stub, rapidly frozen in liquid nitrogen and then freeze-dried for 24 h. Samples were evaporatively coated with carbon to produce an electrically conductive surface. EDX analysis was performed in a JEOL (Peabody, MA, USA) JSM-5400 scanning electron microscope using an acceleration voltage of 20 kV and a working distance of 18–20 mm. A two-dimensional distribution pattern was recorded by scanning an area of the specimen repeatedly for up to 2 h and integrating the counts for Cd, Ca and K within their respective spectrum windows into dot maps.

PIXE microanalysis

Leaves from plants treated with 250 μM $CdCl_2$ for 14 days were frozen and freeze-dried for 48 h at –50 °C at a pressure of 10^{-3} mbar. Leaves were then mounted on carbon tape on a standard aluminium frame and placed on the sample holder inside the microprobe target chamber. PIXE microanalysis was performed following previously described methods (Ager *et al*., 2002).

Acknowledgements

This work was funded by Dirección General de Investigación Grants BIO2000-0270 and BOS2001-1084 (Spain) and Junta de Andalucía (Spain) Grant CVI712.

References

Ager, F.J., Ynsa, M.D., Domínguez-Solís, J.R., Gotor, C., Respaldiza, M.A. and Romero, L.C. (2002) Cadmium localization and quantification in the plant *Arabidopsis thaliana* using micro-PIXE. *Nucl. Instrum. Methods Phys. Res. B*, **189**, 494–498.

Barroso, C., Vega, J.M. and Gotor, C. (1995) A new member of the cytosolic O-acetylserine (thiol) lyase gene family in *Arabidopsis thaliana*. *FEBS Lett*. **363**, 1–5.

Bradford, N.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem*. **72**, 248–254.

Choi, Y.-E., Harada, E., Wada, M., Tsuboi, H., Morita, Y., Kusano, T. and Sano, H. (2001) Detoxification of cadmium in tobacco plants: formation and active excretion of crystals containing cadmium and calcium through trichomes. *Planta*, **213**, 45–50.

Cobbett, C.S. (2000) Phytochelatins and their roles in heavy metal detoxification. *Plant Physiol*. **123**, 825–832.

Domínguez-Solís, J.R., Gutiérrez-Alcalá, G., Vega, J.M., Romero, L.C. and Gotor, C. (2001) The cytosolic O-acetylserine (thiol) lyase gene is regulated by heavy metals and can function in cadmium tolerance. *J. Biol. Chem*. **276**, 9297–9302.

Droux, M., Ruffet, M.-L., Douce, R. and Job, D. (1998) Interactions between serine acetyltransferase and O-acetylserine (thiol) lyase in higher plants. Structural and kinetic properties of the free and bound enzymes. *Eur. J. Biochem*. **255**, 235–245.

Gutiérrez-Alcalá, G., Gotor, C., Meyer, A.J., Fricker, M., Vega, J.M. and Romero, L.C. (2000) Glutathione biosynthesis in Arabidopsis trichome cells. *Proc. Natl. Acad. Sci. USA*, **97**, 11 108–11 113.

Harada, E., Choi, Y.-E., Tsuchisaka, A., Obata, H. and Sano, H. (2001) Transgenic tobacco plants expressing a rice cysteine synthase gene are tolerant to toxic levels of cadmium. *J. Plant Physiol*. **158**, 655–661.

Harada, E., Yamaguchi, Y., Koizumi, N. and Sano, H. (2002) Cadmium stress induces production of thiol compounds and transcripts for enzymes involved in sulfur assimilation pathways in *Arabidopsis*. *J. Plant Physiol*. **159**, 445–448.

Hatzfeld, Y., Maruyama, A., Schmidt, A., Noji, M., Ishizawa, K. and Saito, K. (2000) β-Cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and *Arabidopsis*. *Plant Physiol*. **123**, 1163–1171.

Heiss, S., Schäfer, H.J., Haag-Kerwer, A. and Rausch, T. (1999) Cloning sulfur assimilation genes of *Brassica juncea* L.: cadmium differentially affects the expression of a putative low affinity sulfate transporter and isoforms of ATP sulfurylase and APS reductase. *Plant Mol. Biol*. **39**, 847–857.

Hell, R., Jost, R., Berkowitz, O. and Wirtz, M. (2002) Molecular and biochemical analysis of the enzymes of cysteine biosynthesis in the plant *Arabidopsis thaliana*. *Amino Acids*, **22**, 245–257.

Howarth, J.R., Domínguez-Solís, J.R., Gutiérrez-Alcalá, G., Wray, J.L., Romero, L.C. and Gotor, C. (2003) The serine acetyltransferase gene family in *Arabidopsis thaliana* and the regulation of its expression by cadmium. *Plant Mol. Biol*. **51**, 589–598.

Jost, R., Berkowitz, O., Wirtz, M., Hopkins, L., Hawkesford, M.J. and Hell, R. (2000) Genomic and functional characterization of the *oas* gene family encoding O-acetylsereine (thiol) lyases, enzymes catalyzing the final step in cysteine biosynthesis in *Arabidopsis thaliana*. *Gene*, **253**, 237–247.

Küpper, H., Lombi, E., Zhao, F.-J. and McGrath, S.P. (2000) Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta*, **212**, 75–84.

Lee, S., Moon, J.S., Domier, L.L. and Korban, S.S. (2002) Molecular characterization of phytochelatin synthase expression in transgenic *Arabidopsis*. *Plant Physiol. Biochem*. **40**, 727–733.

Lee, S., Moon, J.S., Ko, T.-S., Petros, D., Goldsbrough, P.B. and Korban, S.S. (2003) Overexpression of Arabidopsis phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. *Plant Physiol*. **131**, 656–663.

Marks, M.D. (1997) Molecular genetic analysis of trichome development in *Arabidopsis*. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **48**, 137–163.

Noji, M., Inoue, K., Kimura, N., Gouda, A. and Saito, K. (1998) Isoform-dependent differences in feedback regulation and subcellular localization of serine acetyltransferase involved in cysteine biosynthesis from *Arabidopsis thaliana*. *J. Biol. Chem*. **273**, 32 739–32 745.

Pence, N.S., Larsen, P.B., Ebbs, S.D., Letham, D.L.D., Lasat, M.M., Garvin, D.F., Eide, D. and Kochian, L.V. (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proc. Natl. Acad. Sci. USA*, **97**, 4956–4960.

Perfus-Barbeoch, L., Leonhardt, N., Vavasseur, A. and Forestier, C. (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *Plant J*. **32**, 539–548.

Salt, D.E., Prince, R.C., Pickering, I.J. and Raskin, I. (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiol*. **109**, 1427–1433.

Salt, D.E., Smith, R.D. and Raskin, I. (1998) Phytoremediation. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **49**, 643–668.

Xiang, C. and Oliver, D.J. (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. *Plant Cell*, **10**, 1539–1550.

Yamaguchi, Y., Nakamura, T., Kusano, T. and Sano, H. (2000) Three *Arabidopsis* genes encoding proteins with differential activities for cysteine synthase and β-cyanoalanine synthase. *Plant Cell Physiol*. **41**, 465–476.

Zhu, Y.L., Pilon-Smits, E.A.H., Tarun, A.S., Weber, S.U., Jouanin, L. and Terry, N. (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. *Plant Physiol*. **121**, 1169–1177.