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Cadmium tolerance and phytochelatin content of *Arabidopsis* seedlings over-expressing the phytochelatin synthase gene *AtPCS1*

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Abstract

Previous studies demonstrated that expression of the *Arabidopsis* phytochelatin (PC) biosynthetic gene *AtPCS1* in *Nicotiana tabacum* plants increases the Cd tolerance in the presence of exogenous glutathione (GSH). In this paper, the Cd tolerance of *Arabidopsis* plants over-expressing *AtPCS1* (AtPCSox lines) has been analysed and the differences between *Arabidopsis* and tobacco are shown. Based on the analysis of seedling fresh weight, primary root length, and alterations in root anatomy, evidence is provided that, at relatively low Cd concentrations, the Cd tolerance of AtPCSox lines is lower than the wild type, while *AtPCS1* over-expressing tobacco is more tolerant to Cd than the wild type. At higher Cd concentrations, *Arabidopsis* AtPCSox seedlings are more tolerant to Cd than the wild type, while tobacco *AtPCS1* seedlings are as sensitive as the wild type. Exogenous GSH, in contrast to what was observed in tobacco, did not increase the Cd tolerance of AtPCSox lines. The PC content in wild-type *Arabidopsis* at low Cd concentrations is more than three times higher than in tobacco and substantial differences were also found in the PC chain lengths. These data indicate that the differences in Cd tolerance and in its dependence on exogenous GSH between *Arabidopsis* and tobacco are due to species-specific differences in the endogenous content of PCs and GSH and may be in the relative abundance of PCs of different length.

Key words: *Arabidopsis*, Cd tolerance, PCS1 over-expression, phytochelatins, seedlings, tobacco.

Introduction

Phytochelatins (PCs) are small, heavy metal-binding peptides with the general structure (γ-Glu-Cys)$_n$-Gly found in some fungi, in plants and in other organisms (Cobbett and Goldsbrough, 2002). PCs are synthesized, as a mixture of peptides of different length, from reduced glutathione (GSH) by phytochelatin synthase (PCS), which is activated by a range of heavy metals (Grill *et al.*, 1989; Clemens and Peršoh, 2009). PCs form stable heavy metal complexes that

are subsequently transported into the vacuoles. Longer chain lengths bind metals more tightly *in vitro* (Loeffler *et al.*, 1989), but their cellular roles are unknown. PCs have a major role in Cd detoxification, as mutant lines of *Arabidopsis thaliana, Schizosaccharomyces pombe,* and *Caenorhabditis elegans* defective in PC synthesis are sensitive to Cd. PCs are induced in plants and cultured cells exposed to metal ions, Cd in particular. To increase the level of these metal-binding

peptides and, consequently, to enhance heavy metal tolerance, *PCS* genes from *Arabidopsis thaliana* (Ha *et al.*, 1999; Vatamaniuk *et al.*, 1999) *Triticum aestivum* (Clemens *et al.*, 1999), *Caenorhabditis elegans, Schizosaccharomyces pombe* (Ha *et al.*, 1999; Clemens *et al.*, 2001), *Allium sativum* (Zhang *et al.*, 2005), and *Brassica juncea* (Heiss *et al.*, 2003), have been over-expressed in different plant species, as well as in yeast and *Escherichia coli*. Over-expression of the *Arabidopsis AtPCS1* gene in *E. coli* (Sauge-Merle *et al.*, 2003) and in *Saccharomyces cerevisiae* (Vatamaniuk *et al.*, 1999) resulted in enhanced Cd tolerance and accumulation. Analogously, over-expression of wheat *TaPCS1* in *Nicotiana glauca* greatly enhanced Cd and Pb tolerance and Pb accumulation (Gisbert *et al.*, 2003; Martinez *et al.*, 2006). *Nicotiana tabacum* expressing *AtPCS1* also displayed enhanced Cd tolerance and accumulation, mainly when the plants were cultivated in culture medium supplied with GSH (Pomponi *et al.*, 2006), and expression of the same gene in *Brassica juncea* led to higher Cd and Zn tolerance, but a lower accumulation of these metals in both root and shoot tissues (Gasic and Korban, 2007). By contrast, over-expression of *AtPCS1* in *Arabidopsis* led to hypersensitivity to Cd despite enhanced PC production (Lee *et al.*, 2003*a, b*; Li *et al.*, 2004). Similarly, it has been reported recently that *Nicotiana tabacum* lines over-expressing *AtPCS1*, but not those over-expressing the *Caenorhabditis CePCS1* gene, were hypersensitive to Cd in the absence of exogenous GSH, whereas no changes in Cd accumulation were observed relative to controls (Wojas *et al.*, 2008). On the other hand, simultaneous over-expression in *Arabidopsis* of *Allium sativum PCS1* and *Saccharomyces cerevisiae GSH1* (which encodes a GSH biosynthetic enzyme) increases the tolerance and accumulation of Cd (Guo *et al.*, 2008). Thus, while in most species an increase in PC production results in an enhanced metal tolerance, apparently conflicting results were obtained for *Nicotiana tabacum* and *Arabidopsis thaliana*. However, these results were obtained by different authors under widely different experimental conditions.

In this work, the tolerance to Cd of *Arabidopsis* seedlings over-expressing *AtPCS1* in relation to the levels of PCs is analysed in the presence and absence of GSH, in much the same way as the response of tobacco plants was previously analysed. The results obtained with *Arabidopsis* are compared with new and published data obtained on tobacco.

Materials and methods

Plant expression constructs, transformation, and selection

Agrobacterium tumefaciens strain GV3101 carrying the binary plasmid *pCAMBIA::35S-PCS1* (Pomponi *et al.*, 2006) was used to transform *Arabidopsis thaliana* wild-type plants (ecotype Columbia) by standard dip floral transformation (Clough and Bent, 1998). Transformed plants were analysed by PCR with the following primers: 35S 5′-ACGCACAATCCCACTATCCTTC-3′; PCS1rev: 5′-GAACTAATAGGCAGGAGCG-3′ and by real-time RT-PCR (see below). Homozygous T$_2$ generations were obtained by self-fertilization of primary transformants and the seeds were grown as described below.

RNA extraction and real-time RT-PCR

Total RNA was extracted from seedlings grown for 5 d or 9 d in the presence of 30 μM CdSO$_4$, using the TRIZOL method (Invitrogen) and treated with RNase-free Dnase (DNase I Amplification Grade, Carlsbad, CA, USA). RT reactions were performed by using SUPERSCRIPT™ II First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions.

A SYBR-Green based quantitative assay was performed as previously described by Cecchetti *et al.* (2004). The primers used for PCS1 were 5′-TGCGTGATGGGAATGAACAA-3′; 5′-TTTGCG-TCGATGGCACTAAC-3′ and *ACTIN2* gene 5′-CCGATCCAGA-CACTGTACTTCCTT-3′; 5′-CTTGCACCAAGCAGCATGAA-3′ and were designed by using Primer Express 2 (ABI PRISM).

Plant growth conditions and metal treatments

Wild-type and AtPCSox seedlings were germinated on half-strength MS basal agar medium (pH 5.8) (Murashige and Skoog, 1962) in a growth chamber in a 16/8 h light/dark cycle at 22 °C. After 7 d, 10 seedlings were transferred to a half-strength MS basal medium supplemented with sucrose at the indicated percentages, in the absence or at different concentrations of CdSO$_4$ (15, 30, 60, 90, or 180 μM) on vertical plates. Seedlings were weighed and the root length was measured after 5 d or 9 d of further growth. Experiments were performed with or without 100 or 250 μM GSH. The experiments were performed in triplicate.

PC and GSH analysis

Seedlings were grown as described above, on half-strength MS basal medium supplemented with 0.5% sucrose, in the absence or presence of 30 or 90 μM CdSO$_4$, with or without 250 μM GSH. After 3 d or 9 d of Cd exposure, seedlings were immediately frozen in liquid nitrogen and 200 mg of seedlings were used for HPLC analyses according to Pomponi *et al.* (2006). The experiments were performed in triplicate.

Statistical analysis

Student's *t* test was used to evaluate the statistical significance probability levels. All experiments were repeated at least three times.

Histological analysis

The morphological and histological analyses were carried out on wild-type and AtPCSox roots from seedlings grown for 9 d on half-strength MS basal medium supplemented with 0.5% sucrose, in the absence or presence of 30, 60 or 90 μM CdSO$_4$. Roots were treated with chloral hydrate solution (chloral hydrate:distilled water:-glycerol, 8:1:2 w/v/v) for observation with Nomarski optics applied to a DAS Leica DMRB microscope (Leica). The evaluation of the percentage of damaged roots and anomalous lateral branching was carried out on 30 plants for each genotype and treatment.

Alternatively, roots were fixed in 70% ethanol, dehydrated, embedded in Technovit 7100 (Heraeus Kulzer), sectioned at 4 μm with an automatic microtome (Microm HM 350 SV), stained with 0.05% (w/v) toluidine blue, and examined under the same microscope. All the histological and morphological images were acquired with a DC500 video camera applied to the DMRB microscope and then analysed with a personal computer (Opti-Xex GX 240 MT) using the Leica IM1000 image-analysis software (Leica).

Results

Production of Arabidopsis *lines over-expressing* AtPCS1

Arabidopsis plants were transformed with the construct *pCAMBIA::35S-PCS1* harbouring the coding region of *AtPCS1* under the control of the CaMV-35S promoter,

previously used to transform tobacco plants (Pomponi *et al.*, 2006): ten hygromycin-resistant plants were selected and the over-expression of *AtPCS1* was determined in seedlings after 7 d of germination in the absence of Cd by real-time RT-PCR (not shown). Four homozygous lines AtPCSox-1, AtPCSox-3, AtPCSox-4, and AtPCSox-5 were analysed for transcript levels of *AtPCS1* and PC content under experimental conditions used to test Cd sensitivity (see below). After 7 d of germination, seedlings from wild-type and AtPCSox lines were transferred to a medium containing 30 µM $CdSO_4$ and after 5 d and 9 d of growth *AtPCS1* transcript levels were measured by real-time RT-PCR. As shown in Fig. 1A, all over-expressing lines exhibited higher levels of *AtPCS1* transcripts, compared with the wild type, after 9 d of growth. To determine the content of PCs in AtPCSox lines, seedlings grown for 9 d in the presence of 30 µM $CdSO_4$ were collected and PC levels measured by HPLC. As shown in Fig. 1B, the PC content in AtPCSox-4 and AtPCSox-5 seedlings was 2.78 and 2.54 times higher, respectively, than in the wild-type seedlings. A slightly smaller increase was observed in AtPCSox-3 and AtPCSox-1 seedlings that have PC contents of 2.32 and 2.24 times higher, respectively, than in the wild-type seedlings. All four lines were used for subsequent analysis.

Cd tolerance of AtPCSox lines relative to the wild type varies with Cd concentration

To test the Cd sensitivity, the growth of wild-type and AtPCSox seedlings was analysed through two different parameters: fresh weight and primary root length. After 7 d of germination, wild-type and AtPCSox seedlings were grown in the absence or presence of 15, 30, 60, 90, and 180 µM $CdSO_4$. Fresh weight and root length were scored after 9 d. As shown in Fig. 2A–D, the growth of wild-type seedlings was unaffected at 15 µM $CdSO_4$, slightly reduced at 30 and 60 µM $CdSO_4$, and severely inhibited at 90 µM and 180 µM Cd $CdSO_4$. The growth of AtPCSox seedlings was comparable with that of the wild type in the absence of Cd, whereas it was more inhibited than in the wild type from 15–30 µM $CdSO_4$, mainly in terms of root length (Fig. 2A, B, E). At 60 µM $CdSO_4$, the growth of AtPCSox seedlings was comparable with the wild type with the exception of AtPCSox-1 seedlings that showed a reduction in fresh weight and root length.

By contrast, at 90 µM $CdSO_4$ the fresh weight of AtPCSox seedlings was significantly higher than that of the wild type, and foliar chlorosis and necrosis were less severe (Fig. 2C, E), while root growth was comparable with that of wild-type seedlings, with the exception of a slight enhancement in the case of AtPCSox-4 at 90 µM Cd (Fig. 2D). After 9 d at 180 µM $CdSO_4$, AtPCSox and wild-type seedling growth was seriously affected, with the exception of a slight increase in fresh weight of AtPCSox-5 seedlings. Interestingly, a slight but significantly enhanced root growth was observed at 180 µM Cd in the case of AtPCSox-5, AtPCSox-3, and AtPCSox-4 seedlings after 5 d of culture (Fig. 2F).

Since tobacco plants over-expressing *AtPCS1* had a much higher Cd tolerance when GSH was added to the growth medium (Pomponi *et al.*, 2006), the effects of GSH on the sensitivity to Cd of *Arabidopsis* AtPCSox seedlings was assessed.

At low Cd concentrations, root growth was mainly affected and so the primary root length assay was used to analyse the effect of different GSH concentrations (100 and 250 µM) on seedlings exposed to 0, 15, and 30 µM $CdSO_4$. As shown in Fig. 3A, in the absence of Cd, 100 µM of GSH resulted in a slight root growth inhibition both in wild-type and AtPCSox seedlings, while 250 µM GSH caused a strong inhibition of growth in all types of roots (Fig. 3A, C), that was more severe in AtPCSox roots. At both 15 and 30 µM $CdSO_4$, 100 µM GSH caused a considerable decrease in the growth of both wild-type and AtPCSox roots, with the exception of AtPCSox-5 roots whose growth was more inhibited than in the wild type (Fig. 3A, only results with 15 µM $CdSO_4$ are reported) whereas at 250 µM GSH root growth was more inhibited in all AtPCSox seedlings compared with the wild-type seedlings. To assess whether exogenous GSH has an inhibitory effect on the expression of GSH biosynthetic genes that are responsible for the
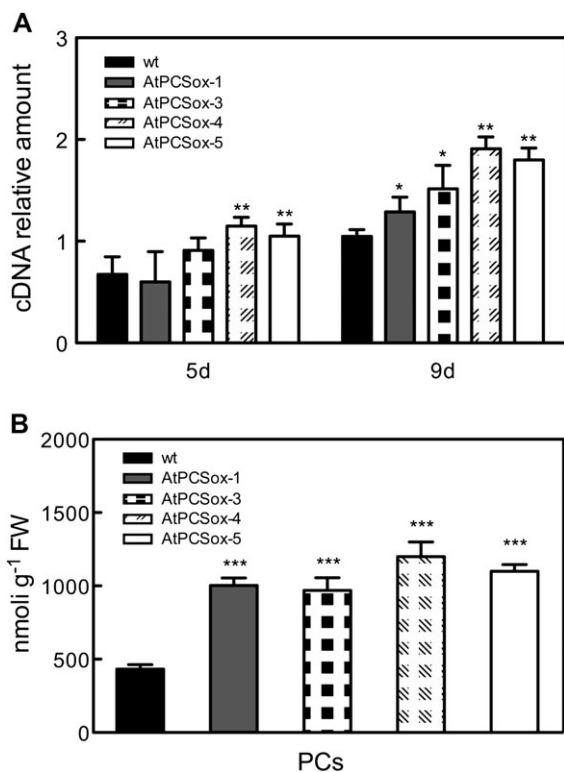
Fig. 1. Quantitative analysis of *AtPCS1* transcript and PC content in AtPCSox seedlings. Wild-type, AtPCSox-1, AtPCSox-3, AtPCSox-4, and AtPCSox-5 seedlings were grown in the presence of 30 µM Cd. (A) Real-time RT-PCR of mRNA extracted from wild-type and AtPCSox seedlings after 5 d and 9 d of growth. Data are expressed as a mean value (±SEM) of *AtPCS1* cDNA levels relative to actin cDNA. (B) The content of total PCs was analysed, by means of HPLC, after 9 d of growth. Values correspond to means ±SE (n=3). Significant differences of AtPCSox from the wild type are indicated (*P <0.05, **P <0.01, ***P <0.001)
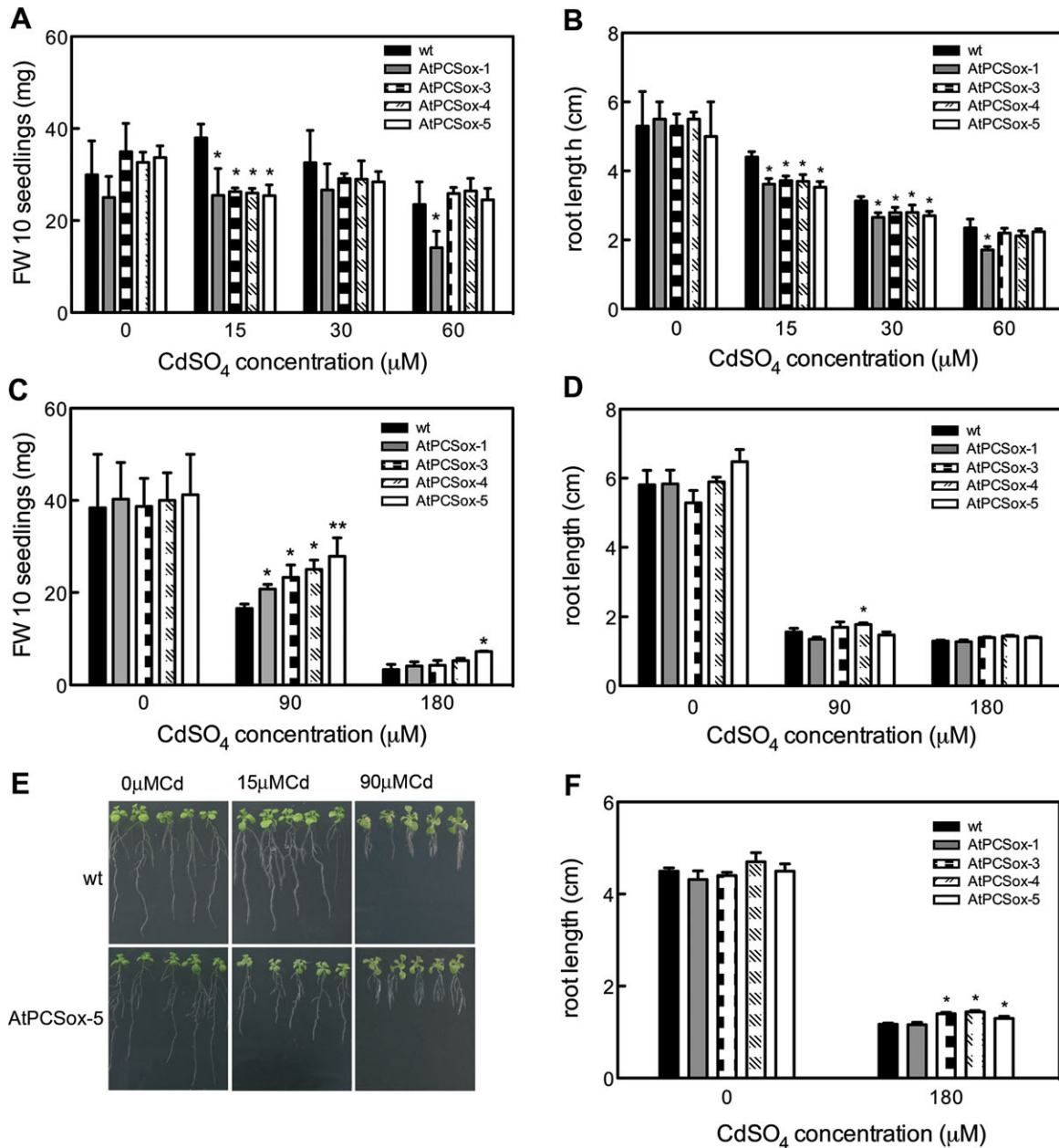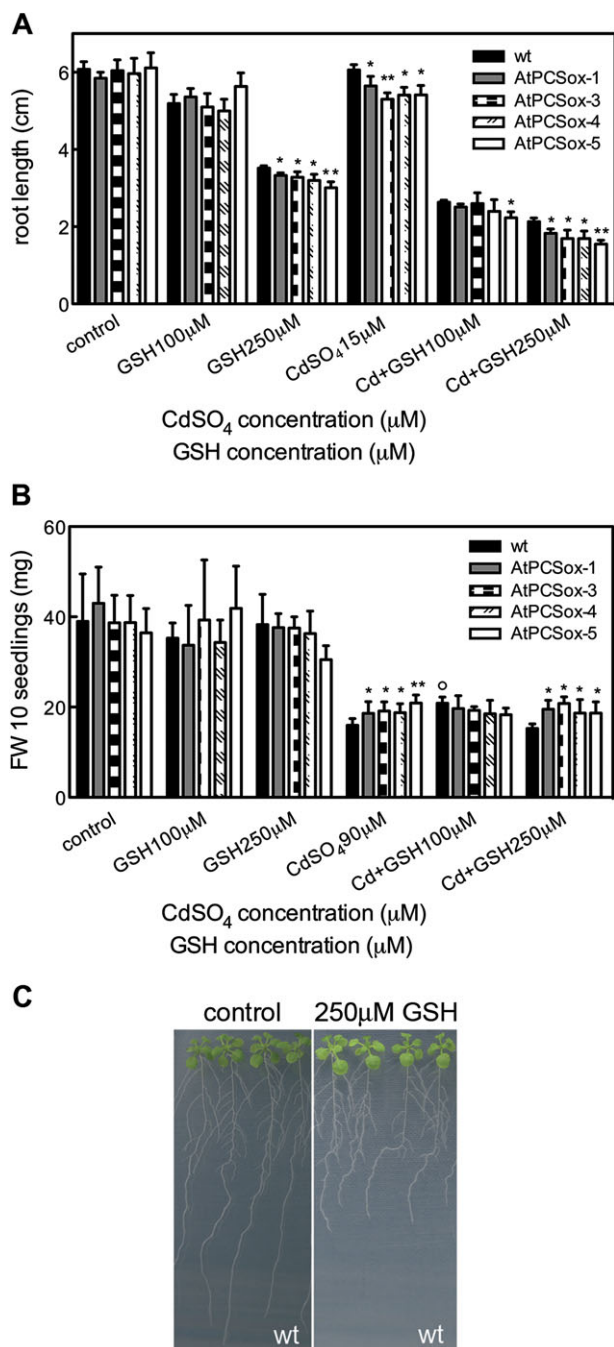
Fig. 2. Cd tolerance of wild-type and AtPCSox seedlings. Wild-type, AtPCSox-1, AtPCSox-3, AtPCSox-4, and AtPCSox-5 seedlings were incubated on medium containing 0, 15, 30 or 60 μM $CdSO_4$ (A, B) or 0, 90 or 180 μM $CdSO_4$ (C, D), or 0 or 180 μM $CdSO_4$ (F). Fresh weight was measured after 9 d (A, C). Root length was measured after 9 d (B, D) or 5 d (F). (E) Wild-type and AtPCSox-5 seedlings at 0 μM $CdSO_4$ (left), 15 μM $CdSO_4$ (middle), 90 μM $CdSO_4$ (right). Wild-type seedlings show no root growth inhibition at 15 μM $CdSO_4$, whereas AtPCSox-5 root growth is inhibited. Wild-type seedlings show severe root growth inhibition and foliar chlorosis at 90 μM $CdSO_4$. AtPCSox-5 root growth inhibition is comparable to the wild type, but foliar chlorosis is not observed. (F) AtPCSox-3, AtPCSox-4, and AtPCSox-5 root growth is less inhibited than the wild type after 5 d at 180 μM $CdSO_4$. Fresh weight values correspond to means $\pm$SE (n=6). Root length values correspond to means $\pm$SE (n=6). Significant differences from wild type are indicated (*P <0.05, **P <0.01).

reduction in growth of wild-type and AtPCSox seedlings, the transcript levels of *AtGSH1*, the controlling gene in GSH synthesis, were analysed. Wild-type and AtPCSox seedlings were grown in the presence or absence of exogenous GSH and, after 9 d, seedlings were collected and *AtGSH1* mRNA levels were analysed by real time RT-PCR. As shown in Supplementary Fig. S1 at *JXB* online, the

transcript levels of *AtGSH1* in wild-type and AtPCSox seedlings, grown in the presence or absence of exogenous GSH, was comparable in all genotypes.

The effect of 100 and 250 μM GSH on the fresh weight of seedlings growth was analysed in the presence of 0, 90 or 180 μM $CdSO_4$, as, at high Cd concentrations, this growth parameter is mainly affected (see above). In the absence of

Fig. 3. Cd tolerance of wild-type and AtPCSox seedlings grown in the presence of different concentrations of GSH. Wild-type, AtPCSox-1, AtPCSox-3, AtPCSox-4, and AtPCSox-5 seedlings were incubated on medium containing 15 µM $CdSO_4$ in the presence of 0, 100 or 250 µM GSH (A) or containing 90 µM $CdSO_4$ in the presence of 0, 100 or 250 µM GSH (B). Root length (A) and fresh weight (B) were measured after 9 d. (C) Wild-type seedlings at 0 µM $CdSO_4$, 0 µM GSH (left), 0 $CdSO_4$, 250 µM GSH (right). Wild-type seedlings show root growth inhibition and a slight increase in the growth of the aerial part, at 250 µM GSH. Fresh weight values correspond to means ±SE ($n=6$). Root length values correspond to means ±SE ($n=30$). Significant differences of AtPCSox seedlings from the wild type are indicated (*$P <0.05$, **$P <0.01$). Significant differences of wild-type seedlings at different GSH and Cd concentrations are indicated (°$P <0.01$)

Cd, both 100 and 250 µM GSH had no effect on wild-type and AtPCSox seedlings (Fig. 3B). At 90 or 180 µM $CdSO_4$, 100 µM GSH resulted in a slight increase in Cd tolerance of wild-type, but not of AtPCSox, seedlings and 250 µM GSH had no appreciable effect on both types of plants (Fig. 3B, only the results with 90 µM $CdSO_4$ are reported). In summary, AtPCSox plants are more sensitive than the wild type to low Cd concentrations, while they show a higher tolerance than the wild type to higher Cd concentrations. In addition, in contrast with what observed in tobacco, exogenous GSH does not affect the Cd tolerance of *Arabidopsis* seedlings over-expressing *AtPCS1*. These four lines all behaved similarly when exposed to different Cd concentrations in the presence and in the absence of GSH. Thus AtPCSox-1 and AtPCSox-5 lines were used for subsequent analysis.

Cd induces histo-anatomical alterations in roots

To assess the possible damage to the root system of plants exposed to Cd, a morphological and histological analysis of primary and lateral roots of wild-type and AtPCSox seedlings was performed after 9 d of growth under the conditions described above.

As shown in Fig. 4A, no morphological alterations were observed in wild-type roots up to 30 µM $CdSO_4$. At 60 µM $CdSO_4$, about 40% of roots showed alterations in the elongation zone characterized by the anomalous presence of root hairs and premature xylem differentiation (Fig. 4B). By contrast, the apex of the elongated primary root, as well as of lateral roots, was regular in shape and size and so was the apical dome of the lateral primordia (not shown). At 90 µM $CdSO_4$, about 75% of roots in wild-type seedlings were very hairy and stunted, and 16% showed irregular lateral branching (Fig. 4C).

In AtPCSox seedlings, no morphological alterations were observed in the absence of Cd, whereas at 30 µM $CdSO_4$, 4.0% and 4.5% of AtPCSox-1 and AtPCSox-5 seedlings, respectively, exhibited damaged roots showing hair formation and xylem differentiation in the elongation zone in primary roots (Fig. 4D). At 60 µM $CdSO_4$, these alterations were more severe compared with the wild type in AtPCSox-1 seedlings (Fig. 4E), showing a significantly higher percentage of altered roots compared with the wild type (54%, $P <0.05$), whereas in AtPCSox-5 seedlings, the percentage was slightly but not significantly higher than the wild type (47%). The apical dome of the lateral primordial in AtPCSox lines showed irregular expansion in the cortex (Fig. 4F). At 90 µM $CdSO_4$, the damage to the roots was macroscopically comparable with the wild type (about 75%) and the percentage of lateral branching was significantly higher for AtPCSox-5 (32%, $P <0.01$) (Fig. 4G) and only slightly, but not significantly, higher in AtPCSox-1 (22.5%). At 180 µM $CdSO_4$, the damage was less severe compared with wild-type roots after 5 d of exposure to Cd, but comparable to the wild type after 9 d (data not shown).

Histological analysis confirmed the absence of anomalies in the root apex (Fig. 4H), elongation zone, and primary structure zone (Fig. 4I) of wild-type seedlings at 30 µM
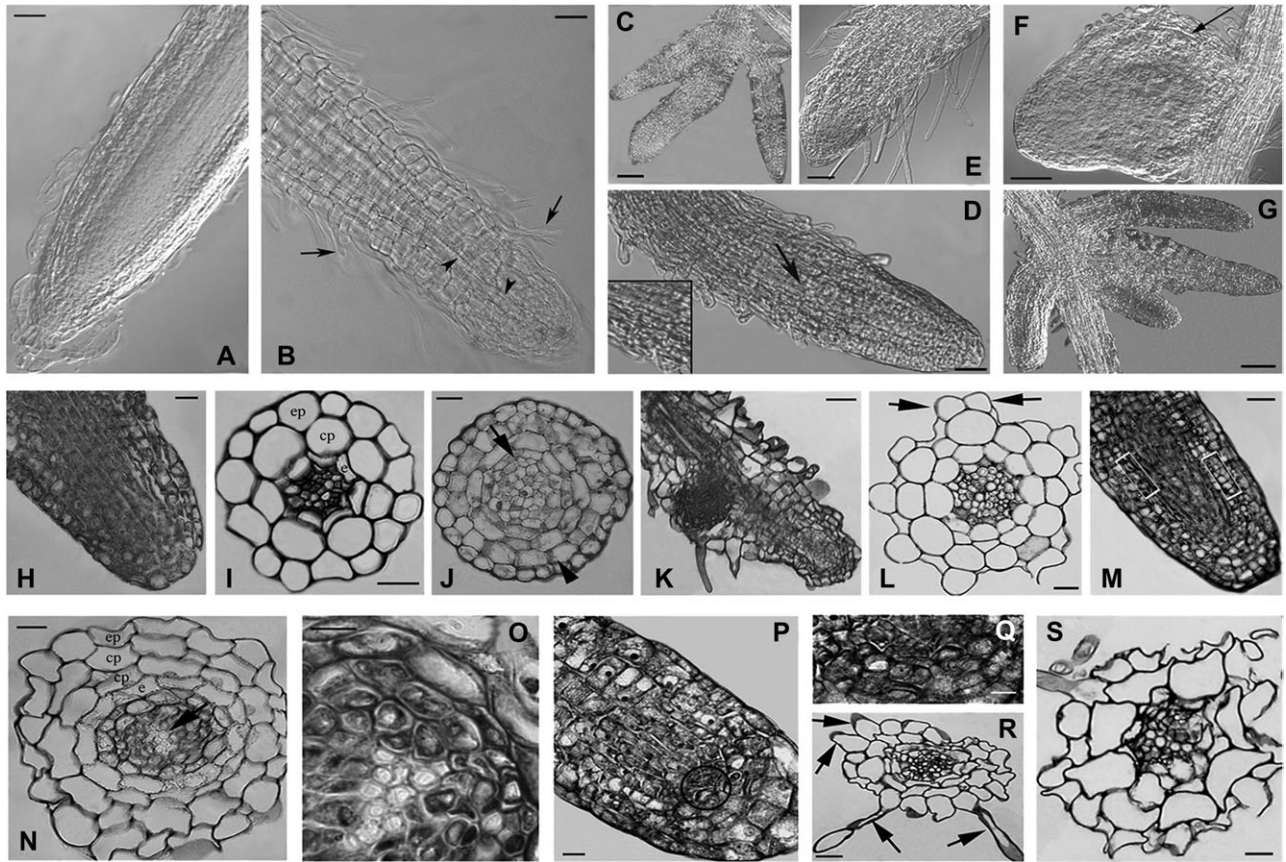
Fig. 4. The effects of Cd treatments on root anatomy of wild-type, AtPCSox-5, and AtPCSox-1 seedlings. Nomarski DIC images (A–G), and histological images (H–S) of wild-type and AtPCSox roots from seedlings treated for 9 d with different concentrations of $CdSO_4$. (A) Regular morphology of wild-type roots in seedlings treated with 30 µM $CdSO_4$. (B) Root showing precocious xylem differentiation in the elongation zone (arrowheads) and abundant hair formation (arrows) in wild-type seedlings treated with 60 µM $CdSO_4$. (C) Irregular lateral root branching in wild-type seedlings treated with 90 µM $CdSO_4$. (D) Abundant hair formation and xylem differentiation up to the apex (arrow and inset) in the root of AtPCSox-1 seedlings treated with 30 µM $CdSO_4$. (E) Very high production of root hairs up to the apex, and a reduction in cap extension in AtPCSox-1 seedlings treated with 60 µM $CdSO_4$. (F) Anomalous shape of a lateral root primordium due to irregular proliferation of the cortical cells (arrow) in AtPCSox-5 seedlings treated with 60 µM $CdSO_4$. (G) Irregular lateral root branching of AtPCSox-5 seedlings treated with 90 µM $CdSO_4$. (H) Normal histological structure of a wild-type root apex (longitudinal section, 30 µM $CdSO_4$ treatment). (I) Normal histological structure of a wild-type root apex (transection, 30 µM $CdSO_4$ treatment). (J) Hypertrophy in the cortical parenchyma (arrowhead) and pericycle proliferation (arrow) of a wild-type root near the apex (transection, 60 µM $CdSO_4$ treatment). (K) Anomalous position of a lateral root primordium in a wild-type primary root (longitudinal section, 60 µM $CdSO_4$ treatment). (L) Anomalous trichoblast differentiation (arrows) in a wild-type root (transection, 60 µM $CdSO_4$ treatment). (M) Anomalous proliferation and expansion of cells committed to differentiate cortical parenchyma, endodermis, and pericycle in an AtPCSox-1 root apical meristem (rectangles) (longitudinal section, 30 µM $CdSO_4$ treatment). (N) Doubling of the cortical parenchyma and abundant xylem differentiation (arrow) in an AtPCSox-1 root (transection, 60 µM $CdSO_4$ treatment). (O) Detail of the stelar region in a primary root, showing xylem overproduction at higher magnification. (P) AtPCSox-5 primary root apex showing the proliferation of initial cells around the quiescent centre (circle) (longitudinal section, 60 µM $CdSO_4$ treatment). (Q) The proliferating zone at higher magnification. (R) Anomalous root hairs (arrows) differentiated in an AtPCSox-5 root (transection, 60 µM $CdSO_4$ treatment). (S) Epidermal and cortical cells anomalous in shape in an AtPCSox-5 root (transection, 90 µM $CdSO_4$ treatment). Sections from (H) to (S) were stained with toluidine blue. Bars: 10 µm (I, J, L, N–Q), 20 µm (H, R, S), 25 µm (M), 50 µm (A, B, E, F, D, K), 100 µm (C, G); e, endodermis; cp, cortical parenchyma; ep, epidermis.

$CdSO_4$. At 60 µM $CdSO_4$, the proliferation of randomly located cells of the pericycle, and the expansion of the cortical parenchyma cells were observed in wild-type primary roots (Fig. 4J); lateral root primordia very near the apex of the primary root (Fig. 4K) and the anomalous position of root hairs in the epidermis in primary roots were also observed (Fig. 4L).

The same anomalies were observed in AtPCSox roots at 30 µM $CdSO_4$. In addition, in AtPCSox roots, an anomalous proliferation and expansion of the derivative cells committed to differentiate cortical parenchyma, endodermis, and pericycle occurred in the root apical meristem (Fig. 4M, rectangles). At 60 µM $CdSO_4$ in AtPCSox roots, together with xylem overproduction, quiescent centre cell

proliferation and cortex doubling were also observed (Fig. 4N, O, P, Q compared with Fig. 4L), anomalies, which were not observed in wild-type roots at any Cd concentration. At 90 μM $CdSO_4$, most of the root cells were collapsed in both wild-type and AtPCSox seedlings (Fig. 4S).

Sucrose affects Cd sensitivity of AtPCSox seedlings

Cd tolerance of *Arabidopsis* seedlings has been analysed at different sucrose concentrations by different authors (Lee *et al.*, 2003a, b; Li *et al.*, 2004; Guo *et al.*, 2008), while the possible effects of sucrose on Cd tolerance have not yet been verified. To assess whether sucrose affects the Cd tolerance of AtPCSox lines at low $CdSO_4$ (15–30 μM), the fresh weight and the primary root length of wild-type AtPCSox-5 and AtPCSox-1 seedlings were measured after 9 d of culture in the absence and in the presence of $CdSO_4$, and in the absence and in the presence of 0.5% and 2% sucrose. In the absence of Cd (Fig. 5A, B), fresh weight and primary root length increased with sucrose concentration in both wild-type and AtPCSox seedlings. At 30 μM $CdSO_4$, the root growth of AtPCSox and the fresh weight of AtPCSox-5 seedlings were more inhibited at 2% sucrose than at 0.5% or in the absence of sucrose. At 90 μM $CdSO_4$ (Fig. 5C, D) the fresh weight, but not root growth, of AtPCSox seedlings was significantly higher than that of the wild type but only in the presence of 0.5% sucrose.

PC and GSH levels in wild-type and AtPCSox plants are related to Cd concentrations

To establish a correlation between Cd tolerance, PC content, and GSH level, the concentration of PC and GSH was measured in wild-type and AtPCSox seedlings cultured in the presence of different concentrations of Cd. At 30 μM $CdSO_4$ (Fig. 6A), PC and GSH levels in AtPCSox seedling were about the double that in the wild type. Differences in PC chain length between the wild type and AtPCSox were modest, PC_2 and PC_3 being the most abundant oligomers in both types of seedlings.

At 90 μM $CdSO_4$, the PC level increased 4.6-, 2.2-, and 3-fold, respectively, in wild-type, AtPCSox-1, and AtPCSox-5 seedlings compared with 30 μM $CdSO_4$, thus being significantly higher in AtPCSox-5, but not in AtPCSox-1, compared with the wild type (Fig. 6B). At this Cd concentration, PC_3 but not PC_2 is the most abundant oligomer in wild-type and AtPCSox lines (Fig. 6B), suggesting that quantitative as well as qualitative changes in PC content may account for the different sensitivity of over-expressers at different Cd concentrations. As of GSH, at 90 μM $CdSO_4$, a 1.9-, 1.1-, and 1.35-fold increase was observed, respectively, in wild-type, AtPC-Sox-5, and AtPCSox-1 seedlings compared with 30 μM $CdSO_4$.

PC and GSH levels are considerably higher in
Arabidopsis *than in tobacco*

To assess whether differences in the content of PCs (and GSH) between *Arabidopsis* and tobacco seedlings would account for the different effects of *AtPCS1* over-expression on Cd tolerance observed in the two species, PC and GSH content was measured in wild-type seedlings grown for 3 d at 30 μM $CdSO_4$, in the presence or absence of 250 μM GSH—the optimal GSH concentration for tobacco (Pomponi *et al.*, 2006).

In the absence of exogenous GSH (Fig. 7), PC and GSH contents were 3.2 and 2.77 higher, respectively, in untransformed *Arabidopsis* than in tobacco. In addition, PC_2 was the most abundant oligomer in *Arabidopsis* while PC_3 and PC_4 were the more abundant oligomers in tobacco.

In the presence of exogenous GSH, PC (and GSH) content increased in both *Arabidopsis* and tobacco seedlings, and was about four times higher in the former than in the latter. PC_3 was the most abundant oligomer in *Arabidopsis*, followed by PC_4, whereas, in tobacco, the PC_4 content was slightly higher than PC_3.

Discussion

Conflicting results have been reported on the Cd tolerance of *Arabidopsis thaliana* and *Nicotiana tabacum* over-expressing the PC biosynthetic gene (Lee *et al.*, 2003a, b; Li *et al.*, 2004; Pomponi *et al.*, 2006; Wojas *et al.*, 2008; Guo *et al.*, 2008), possibly due to different experimental conditions and the use of different constructs. In this paper, the effects of over-expression of *AtPCS1* in *Arabidopsis* seedlings were analysed under the experimental conditions used previously for tobacco. AtPCSox lines were obtained by transforming *Arabidopsis* with the same construct used for tobacco, containing the *AtPCS1* coding region driven by the CaMV-35S promoter (Pomponi *et al.*, 2006). To assess whether growth inhibition was due to defects in root elongation or in the overall growth of the aerial part of the seedlings, Cd tolerance was assayed using two different parameters, primary root length (as previously used for tobacco) and seedling fresh weight. Other authors have used these parameters to evaluate the Cd tolerance of *Arabidopsis* seedlings (Lee *et al.*, 2003a, b; Li *et al.*, 2004). Histological analysis of roots was also performed to compare the Cd damage of wild-type and over-expressing seedlings.

This work demonstrated that, at $CdSO_4$ concentrations up to 60 μM that have no or only slightly toxic effects on the growth of wild-type *Arabidopsis* seedlings, *AtPCS1* over-expression results in a decrease in Cd tolerance compared with the wild type, as mainly revealed by a reduced root growth. By contrast, at higher Cd concentrations (90–180 μM $CdSO_4$)—toxic to wild-type seedlings as manifested by a significant decrease in fresh weight and root growth as well as by foliar chlorosis—*AtPCS1* over-expression conferred an increase in Cd tolerance: AtPCSox seedlings showed a significantly less severe reduction of fresh weight and a less pronounced foliar chlorosis than the wild type. Root growth was slightly enhanced in AtPCSox seedlings. This effect was observed after 9 d at 90 μM $CdSO_4$ only in the AtPCSox-4 line (showing the highest level of PCs) and in most of the other lines after 5 d of
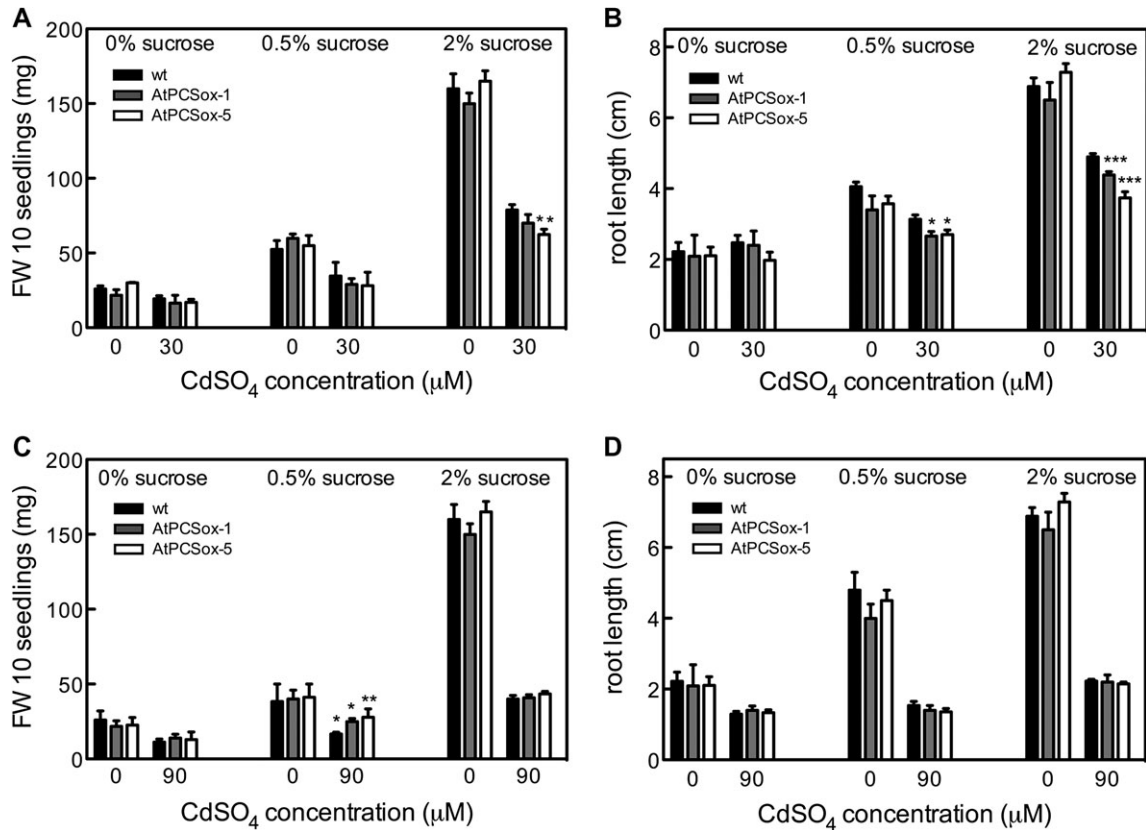
Fig. 5. Cd tolerance of wild-type, AtPCSox-1, and AtPCSox-5 seedlings grown at difference sucrose percentages. Wild-type, AtPCSox-1, and AtPCSox-5 seedlings were incubated on medium containing 0 or 30 μM $CdSO_4$ at 0, 0.5 or 2% sucrose (A, B) or 0 or 90 μM $CdSO_4$ at 0, 0.5 or 2% sucrose (C). Fresh weight (A, B) and root length (C, D) were measured after 9 d. Fresh weight values correspond to means ±SE (*n*=6). Root length values correspond to means ±SE (*n*=30). Significant differences from the wild type are indicated (*P <0.05, **P <0.01, ***P <0.001).
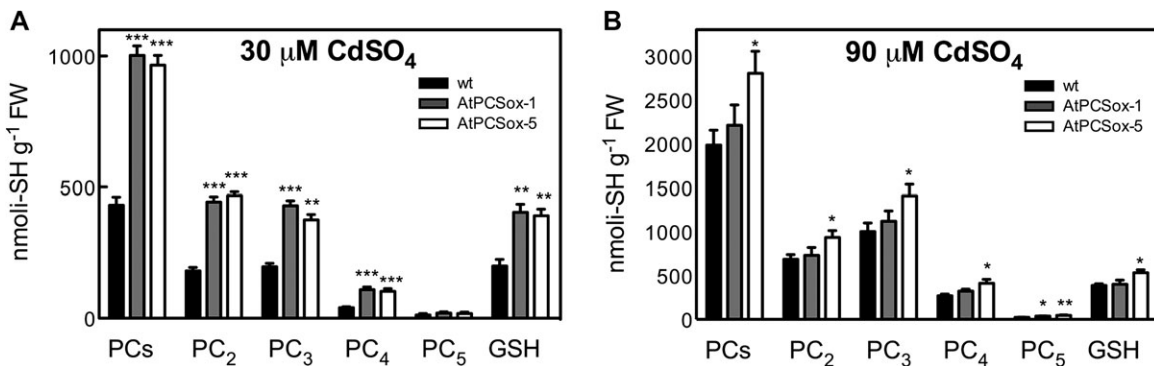

Fig. 6. PC and GSH content of wild-type, AtPCSox-1, and AtPCSox-5 seedlings. Wild-type, AtPCSox-1, and AtPCSox-5 seedlings were incubated on medium containing 30 μM $CdSO_4$ (A) or 90 $CdSO_4$ (B). After 9 d, the content of GSH, total PCs, PC_2, PC_3, PC_4, and PC_5 fractions was analysed by means of HPLC. Values correspond to means ±SE (*n*=3). Significant differences from the wild type are indicated (*P <0.05, **P <0.01, ***P <0.001).

exposure to 180 μM $CdSO_4$, suggesting a transient effect on root growth due to the increased PC content (see below).

Cd tolerance was correlated with the level of PCs by comparing PC content in wild-type and AtPCSox lines at different Cd concentrations. By means of HPLC analysis, evidence was obtained that the PC level increases in both types of plants with the increase of Cd in the medium, and that it is significantly higher in AtPCSox than in the wild type. At low

Cd concentrations (30 μM $CdSO_4$), the PC level of AtPCSox seedlings is approximately 2.5 times higher than that of the wild type, while it is approximately 1.5 times higher at high Cd (90 μM $CdSO_4$). In addition, differences in the content of individual PC oligomers at low and high Cd concentrations were observed in both the wild type and over-expressers: the PC_2 and PC_3 oligomers are highest at low Cd, whereas PC_3 is the most abundant oligomer at high Cd.
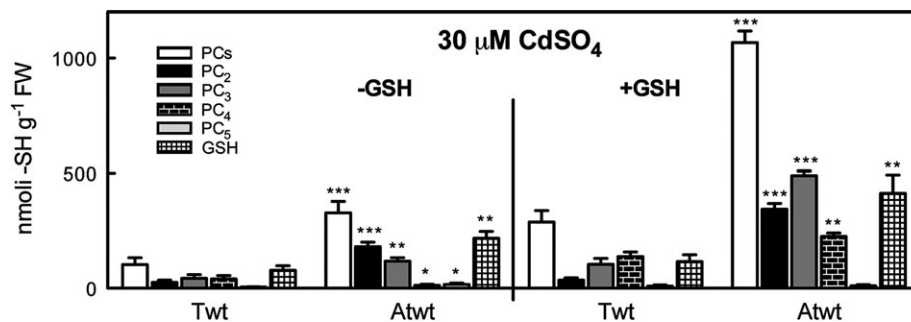
Fig. 7. Comparison of PC and GSH content between tobacco wild-type and *Arabidopsis* wild-type seedlings. Tobacco wild-type and *Arabidopsis* wild-type seedlings were incubated on medium containing 30 µM CdSO$_4$ in the presence or absence of 250 µM GSH. After 3 d, the content of GSH, total PCs, PC$_2$, PC$_3$, PC$_4$, and PC$_5$ fractions was analysed by means of HPLC. Values correspond to means ±SE (n=3). Significant differences from wild type are indicated (*P <0.05, **P <0.01, ***P <0.001).

These data suggest that PC overproduction may be advantageous for *Arabidopsis* in tolerating high Cd concentrations when the initial level of PCs may possibly be below the concentration necessary to bind all the Cd. The reduced tolerance of AtPCSox lines to low Cd may thus be due to a supraoptimal PC content. In addition, since the the stability of PC–Cd complexes increases with the number of γ-Glu-Cys dipeptide repeats (Satofuka *et al.*, 2000), the observed selective increase of PC$_3$ oligomers in AtPCSox lines at high Cd may contribute to the increased tolerance to the metal as PC$_3$ are able to displace PC$_2$ in the complexes with Cd (Gusmão *et al.*, 2010).

A supraoptimal PC content may also explain the Cd hypersensitivity previously reported for *Arabidopsis* lines over-expressing *AtPCS1* under the control of the constitutive A2 actin promoter (Li *et al.*, 2004) and of the *AtPCS1* promoter (Lee *et al.*, 2003b). These authors did not report any positive effect on Cd tolerance of over-expressers at high Cd concentration. This discrepancy with our results could be a consequence of the different promoters utilized and of the different increases of PC content achieved in over-expressers compared with the wild type. In the case of the A2 promoter, Li *et al.* (2004) utilized for their analysis over-expressing lines with very high relative levels of PCs (about 6 times those of the wild type) compared with the ones reported here for AtPCS1ox lines. In the case of the PCS1 promoter, the overall PC level in over-expressers (1.6–2 times higher than in the wild type) was comparable with that of our AtPCSox lines. However, as the PCS1 promoter, in contrast to the 35S promoter utilized in the present work, is not expressed in all the tissues (Blum *et al.*, 2010; P Brunetti *et al.*, unpublished data), the levels of PCs in the specific cells over-expressing PCS1 can be much higher. In agreement with this hypothesis, Lee *et al.* (2003a) showed that Cd hypersensitivity was exhibited only by lines with the highest levels of PC production, and suggest that is a consequence of a supraoptimal PC concentration. Furthermore, in this work, it has been demonstrated that Cd tolerance of *Arabidopsis* seedlings also depends on sucrose, as the increase in Cd tolerance of AtPCSox seedlings is not observed at the high sucrose (2%) concentration such as those used by Li *et al.*

(2004). This agrees with the observed stimulation of growth of *Arabidopsis* roots by 2% sucrose (Carrie *et al.*, 2009).

The morphological and histological damage caused by Cd was analysed in the roots of *Arabidopsis* wild-type and AtPCSox seedlings. This analysis confirmed that, in wild-type seedlings at non-toxic CdSO$_4$ concentrations (30 µM), there is no anatomical damage in the root, whereas Cd-sensitive *AtPCS1* over-expressing seedlings show alterations. Part of these, such as root hair and abnormal xylem formation—pointing to premature maturation causing a reduction in root length—have already been described in pine (Schützendübel *et al.*, 2001) and barley (Ďurčeková *et al.*, 2007), and were also observed in wild-type roots at higher CdSO$_4$ concentrations (60–90 µM). AtPCSox roots also showed quiescent centre cell proliferation and cortex doubling, which may be associated with repairing of the root apical meristems. This effect may be interpreted as a developmental defence reaction to limit the stress effect and damage induced by toxic Cd on root growth.

This work shows that significant differences exist between *Arabidopsis* and tobacco with respect to Cd tolerance. In a previous paper it was observed that at low Cd (30 µM CdSO$_4$) root growth (elongation) of wild-type tobacco seedlings was severely inhibited, while *AtPCS1* over-expressers were more tolerant to Cd than the wild type (Pomponi *et al.*, 2006), in particular upon the addition of GSH. The present work shows that at 30 µM CdSO$_4$, *Arabidopsis* AtPCSox lines are less tolerant to Cd than the wild type, and that their tolerance is not enhanced by exogenous GSH. It is also shown that *Arabidopsis* seedlings have PC and GSH contents approximately 3.5 times and 3 times higher, respectively, than in tobacco and that the abundance of individual PC oligomers of the two plants is different: PC$_3$ and PC$_4$ being the most abundant oligomers (the PC$_2$ level is negligible) in wild-type tobacco, whereas PC$_2$ and PC$_3$ are the most abundant in *Arabidopsis*. These differences in GSH and PC levels (and PC chain length) between *Arabidopsis* and tobacco may account for the observed differences in Cd tolerance and GSH dependence. Our data suggest that Cd tolerance is mainly related to PC content. Accordingly, Wojas *et al.* (2010) found that

AtPCS1-expressing tobacco plants, showing Cd hypersensitivity, had a significant decrease in both the cytosolic and vacuolar pool of PCs, whereas enhanced Cd tolerance of *CePCS* plants was accompanied by an increased cytosolic and vacuolar PC/Cd ratio.

Our data, although obtained in part from the comparison with previous experiments on tobacco *AtPCS1* overexpressing plants, indicate that Cd tolerance is related to PC content and not on any particular characteristic of the species. This allows us to hypothesize a model on the effects of PCs on Cd tolerance.

In summary, it is proposed that PC overproduction increases Cd tolerance only when, in a given plant, overproduction results in an optimal ratio between PC level (and/or PC chain length) and Cd, which depends not only on PC overproduction but also on the endogenous levels of PCs.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementatry Fig. S1. Quantitative analysis of *AtGSH1* transcript in AtPCSox seedlings.

Acknowledgements

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