

RESEARCH PAPER

# Overexpression of phytochelatin synthase in tobacco: distinctive effects of *AtPCS1* and *CePCS* genes on plant response to cadmium

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## Abstract

Phytochelatin synthase (PCS) (EC 2.3.2.15) is a heavy-metal-binding polypeptide, synthesized by phytochelatin synthase (PCS) (EC 2.3.2.15). Previous studies on plants overexpressing PCS genes yielded contrasting phenotypes, ranging from enhanced cadmium tolerance and accumulation to cadmium hypersensitivity. This paper compares the effects of overexpression of *AtPCS1* and *CePCS* in tobacco (*Nicotiana tabacum* var. Xanthi), and demonstrates how the introduction of single homologous genes affects to a different extent cellular metabolic pathways leading to the opposite of the desired effect. In contrast to WT and *CePCS* transformants, plants overexpressing *AtPCS1* were Cd-hypersensitive although there was no substantial difference in cadmium accumulation between studied lines. Plants exposed to cadmium (5 and 25  $\mu\text{M}$  CdCl<sub>2</sub>) differed, however, in the concentration of non-protein thiols (NPT). In addition, PCS activity in *AtPCS1* transformants was around 5-fold higher than in *CePCS* and WT plants. *AtPCS1* expressing plants displayed a dramatic accumulation of  $\gamma$ -glutamylcysteine and concomitant strong depletion of glutathione. By contrast, in *CePCS* transformants, a smaller reduction of the level of glutathione was noticed, and a less pronounced change in  $\gamma$ -glutamylcysteine concentration. There was only a moderate and temporary increase in phytochelatin levels

due to *AtPCS1* and *CePCS* expression. Marked changes in NPT composition due to *AtPCS1* expression led to moderately decreased Cd-detoxification capacity reflected by lower SH:Cd ratios, and to higher oxidative stress (assessed by DAB staining), which possibly explains the increase in Cd-sensitivity. The results indicate that contrasting responses to cadmium of plants overexpressing PCS genes might result from species-dependent differences in the activity of phytochelatin synthase produced by the transgenes.

Key words: Cadmium,  $\gamma$ -glutamylcysteine, glutathione, phytochelatin, tobacco.

## Introduction

Phytochelatin synthase (PCS) is a heavy metal-binding, cysteine-rich polypeptide with the general structure of (( $\gamma$ -Glu-Cys)<sub>n</sub>Gly ( $n=2-11$ )), present in plants, fungi, and other organisms (Grill *et al.*, 1985; Gekeler *et al.*, 1988; Piechalak *et al.*, 2002). They are synthesized from glutathione (GSH) in the presence of heavy metals by the enzyme phytochelatin synthase (PCS) (Grill *et al.*, 1989; Tomaszewska *et al.*, 1996; Vatamaniuk *et al.*, 2000) and form complexes with some of those ions, subsequently transported from the cytosol into the vacuole (Salt and Rauser, 1995).

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Abbreviations: PC, phytochelatin; PCS, phytochelatin synthase; GSH, glutathione;  $\gamma$ -EC,  $\gamma$ -glutamylcysteine; NPT, non-protein thiols; DAB, 3,3'-diaminobenzidine; WT, wild type.

PCs are necessary for cadmium tolerance in plants and all the mutants reported to lack the ability to synthesize those peptides are Cd hypersensitive (Howden *et al.*, 1995). Thus, it was reasonable to expect that overexpression of PCS in plants, with the aim of overproducing these metal-chelating peptides, may result in higher Cd tolerance and accumulation. It was considered that such transgenic plants could be used in the phytoremediation of cadmium-contaminated soils. The results on overexpression of PCS reported in the last few years, have been contradictory, however. For example, as expected, overexpression of the *AtPCS1* gene in *Escherichia coli* (Sauge-Merle *et al.*, 2003) and *Saccharomyces cerevisiae* (Vatamaniuk *et al.*, 1999), enhanced cadmium tolerance and accumulation. Surprisingly, its overexpression in *Arabidopsis thaliana* led to Cd-hypersensitivity despite the enhanced phytochelatin production (Lee *et al.*, 2003a, b; Li *et al.*, 2004). By contrast, expression of the wheat phytochelatin synthase gene *TaPCS1* in tobacco (*Nicotiana glauca*) resulted in increased Cd and Pb tolerance and accumulation (Gisbert *et al.*, 2003; Martinez *et al.*, 2006). However, as recently reported by Pomponi *et al.* (2006), *Nicotiana tabacum* expressing *AtPCS1* displayed enhanced cadmium tolerance and accumulation, but only when plants were cultivated in culture medium supplied with GSH. Interestingly, expression of the same gene in *Brassica juncea* led to higher Cd and Zn tolerance, but significantly lower accumulation of those elements in both root and shoot tissues (Gasic and Korban, 2007). Despite the vast literature on the role of phytochelatin synthase genes, it is still impossible to explain such differences among the reported phenotypes. Researchers used different PCS genes and plant species for transformation, which could have contributed to the observed disparities. Therefore, overexpression of PCS genes from different species in one model organism could yield important insights into their functions and answer some of the questions raised by the researchers.

This work addresses the mechanisms underlying the variation in response to cadmium reported for PCS overexpressing plants. It attempts to determine if the plant phenotype resulting from PCS overexpression could depend on the gene used for transformation. Two phytochelatin synthase genes: *AtPCS1* from *A. thaliana* (Ha *et al.*, 1999) and *CePCS* from *Caenorhabditis elegans* (Clemens *et al.*, 2001; Vatamaniuk *et al.*, 2001) were introduced into one model species, tobacco (*N. tabacum* var. Xanthi). This is the first study comparing the effects of the overexpression of two different PCS genes in the same organism.

## Materials and methods

### Plant expression constructs, transformation and selection

The *AtPCS1* and *CePCS* gene constructs used for transformation derived from constructs used for functional expression in

*Schizosaccharomyces pombe* (Cazalé and Clemens, 2001; Clemens *et al.*, 2001, respectively). The expression cassettes were subcloned into pRT100 between the CaMV 35S promoter and the CaMV polyadenylation signal. The resulting cassettes were transferred as *Hind*III fragments into the binary plasmid vector pCB302 (Xiang *et al.*, 1999). The constructs were introduced into *Agrobacterium* GV3101 strain. Tobacco (*N. tabacum* var. Xanthi) plants (seeds from stock of the Institute of Biochemistry and Biophysics PAS, Warszawa, Poland) were transformed by the standard leaf disc method (Horsh *et al.*, 1985). F<sub>1</sub> seeds were collected from generation F<sub>0</sub> with confirmed gene presence (PCR) and expression (RT-PCR). The primer sequences used for the PCR reaction were: *AtPCS1* forward 5'-GGTGGCTGAGATCCGAATTA-3'; reverse 5'-TGAGCTGCTTCATTTTCATGC-3'; *CePCS* forward 5'-GCAA-AATGTAATCGTCTAAAATCTACAG-3'; reverse 5'-TTCTAAT-GGATCACATAATAAGAATTGA-3'.

### Expression analysis (RT-PCR)

Total RNA was extracted from around 100 mg of frozen tissue using an RNeasy Plant Mini-Kit (Qiagen). Following DNase digestion (Invitrogen), 1 µg of RNA from each sample was used for 20 µl RT-reaction (as recommended by the manufacturer, Fermentas). The RT-PCR product for the *Tac9* actin gene was used as a control to confirm that equal amounts of RNA were used in each reaction. PCR was carried out with 2 µl of the RT reaction product using the specific primers for *AtPCS1* and *CePCS* as described above and for *Tac9* as: *Tac9* forward 5'-CCTCCCA-CATGCTATTCTCC-3'; reverse 5'-AGAGCCTCCAATCCAGACAC-3'.

### Experimental plant material and hydroponic conditions

Tobacco seeds were sterilized and germinated on agar plates, positioned vertically, on quarter-strength Knop's medium, 1% (w/v) agar, and 2% (w/v) sucrose. For the selection of transgenic plants and to determine the segregation ratio of Basta-resistant:Basta-sensitive seedlings, the herbicide Basta was added to the medium at a concentration of 10 µg ml<sup>-1</sup>. Seedlings were grown for 3 weeks in a climate chamber; temperature 24 °C, photoperiod 16/8 h day/night; quantum flux density (PAR) 250 µmol m<sup>-2</sup> s<sup>-1</sup> fluorescent Flora tubes, and subsequently transferred to 2.0 l pots containing quarter-strength Knop's liquid medium. In addition, to check the efficiency of the selection, after 2 d of growth in hydroponics the seedlings were sprayed with Basta (100 µg ml<sup>-1</sup>) for three consecutive days, and grown under control conditions for the next 8–10 d. The presence of the transgene was then checked by PCR.

All experiments were performed on Basta-resistant plants grown in hydroponics on quarter-strength Knop's medium under the conditions described above. The aerated nutrient solution was changed weekly. Initially, experiments were conducted on seven and 10 independent lines representing *AtPCS1* and *CePCS* tobacco transformants, respectively. For further detailed studies the following lines (with 3:1 segregation ratio of Basta-resistant:Basta-sensitive T<sub>1</sub> seedlings) were selected: four lines expressing *AtPCS1* (PaII3, PaII4, PaII8, PaII12), five lines expressing *CePCS* (Pc30, PcII1, PcII3, PcII4, PcII5), and wild-type Xanthi.

### Cadmium treatments

For each cadmium treatment, 5-week-old plants were placed in pots containing nutrient solution as described above, with five replicate pots per treatment. After 1 week of growth the nutrient solution was replaced by a fresh one of the same composition and cadmium chloride was added to achieve a final concentration of 5–35 µM. Untreated plants were grown in parallel under the same conditions.

At the end of the incubation period (2–6 d) shoots and roots were used for further analysis.

#### *Cadmium tolerance determination: hydroponic experiments*

**Appearance assessment and growth assay:** After 6 d of growth at 0, 25  $\mu\text{M}$   $\text{CdCl}_2$  or 35  $\mu\text{M}$   $\text{CdCl}_2$ , the condition of the plants was assessed (leaf colour, presence of necrosis). They were photographed, then shoots and roots were separated and dried for 4 d at 60 °C in an oven and their dry matter was determined. Based on dry weight, the tolerance index was calculated separately for shoots and roots of cadmium exposed plants as the percentage of dry weight of untreated (control) plants of the same line.

**Pigment analysis:** One of the first effects of cadmium toxicity on plants is chlorosis. Thus, the determination of photosynthetic pigment concentrations could be used to assess cadmium tolerance. After 2 d of growth at 0 or 25  $\mu\text{M}$   $\text{CdCl}_2$ , the fourth leaf (counting from the top) was collected, frozen, and stored at –80 °C. Chlorophyll *a* and *b* and carotenoid contents were measured spectrophotometrically in acetone extracts according to Lichtenthaler and Wellburn (1983).

#### *Cadmium tolerance determination: growth of plants on contaminated soil*

To assess the tolerance of plants to the presence of Cd, Zn, and Pb in industrially contaminated soil, 4-week-old hydroponically grown tobacco seedlings were planted in pots containing 2.5 kg of contaminated industrial soil (Cd  $18.6 \pm 1.2$  mg  $\text{kg}^{-1}$  DW, Zn  $2374 \pm 142$  mg  $\text{kg}^{-1}$  DW, Pb  $363 \pm 142$  mg  $\text{kg}^{-1}$  DW, pH  $\sim 7.0$ ) and uncontaminated control soil (commercially obtained). They were grown for 6 weeks in a greenhouse, 24–27/18–20 °C day/night, photoperiod 16/8 h day/night, 65–75% humidity. Plant appearance was assessed and height and number of leaves were measured each week. Total dry weight of above-ground plant parts, expressed as a percentage of dry weight of plants grown on control soil, served as an indicator of plant tolerance to the industrial soil. This experiment was planned to demonstrate, in addition to tolerance level, the suitability of transformation with *PCS* genes for phytoremediation.

#### *Determination of cadmium accumulation: plants grown in hydroponic culture*

After 2–6 d of 5 or 25  $\mu\text{M}$   $\text{CdCl}_2$  treatment roots were washed briefly in distilled water, then for 15 min in ice-cold 5 mM  $\text{CaCl}_2$ , then again twice in water. Roots and shoots were dried for 4 d at 60 °C in an oven and their dry matter was determined. Dried plant material was used for the determination of cadmium concentration by AAS.

#### *Determination of cadmium accumulation: plants grown on contaminated soil*

At the end of the growth period on contaminated soil (details described above) upper leaves, lower leaves, and a stem were collected, dried in 60 °C for 4 d and weighed. Cadmium contents were determined by AAS.

#### *Determination of cadmium concentration*

Dried plant samples were digested in 65%  $\text{HNO}_3$  and 39%  $\text{H}_2\text{O}_2$  (9:1, v:v) in a closed system microwave mineralizer (Milestone Ethos). Cadmium was measured using a flame atomic absorption spectrophotometer (TJA Solution Solar M). Certified reference material (Virginia tobacco leaves CTA-VTL-2) was included in the analysis.

#### *Non-protein thiol analysis*

Several modifications of the method for non-protein thiol (NPT) analysis by Sneller *et al.* (2000) were tested. The method described below resulted from many trials and was used for NPT analysis of all tobacco samples.

At the end of the cadmium treatment, the fourth leaf (counting from the top) and roots were harvested for NPT analysis and processed immediately. NPT were extracted by homogenizing 200–300 mg of fresh material in a mortar, using a pestle and quartz sand in 1.78 ml of 6.3 mM ice-cold diethylenetriaminepenta-acetic acid (DTPA), 100  $\mu\text{l}$  of 1 N NaOH, and 100  $\mu\text{l}$  of 6 M  $\text{NaBH}_4$  (in 0.1 N NaOH). *N*-acetyl-L-cysteine was added during homogenization as an internal standard to a final concentration of 10  $\mu\text{M}$ . The homogenates were centrifuged for 5 min at 10 000 *g*. 250  $\mu\text{l}$  of plant extract were mixed with 10  $\mu\text{l}$  of 20 mM monobromobimane and 450  $\mu\text{l}$  of 200 mM HEPES buffer, pH 8.2, containing 6.3 mM DTPA. Derivatization was carried out for 30 min in a water bath at 45 °C, in the dark. The reaction was stopped by the addition of 300  $\mu\text{l}$  of 1 M methanesulphonic acid. The sample was filtered through a Costar Spin-X centrifuge tube with a nylon filter (0.22  $\mu\text{m}$ ). The samples were stored in the dark at 4 °C prior to the HPLC analysis. NPT were separated on a Nova-Pak  $\text{C}_{18}$  analytical column (60 Å, 4  $\mu\text{m}$ , 3.9 $\times$ 300 mm, Waters) at 37 °C and were eluted with a slightly concave gradient of methanol and water, both with 0.1% (v/v) TFA, using fluorescence detection (Waters 464). The injection volume was 10  $\mu\text{l}$  and the total analysis time was 70 min. The analytical data were integrated using Waters Millennium Software. Phytochelatin concentrations were corrected for derivatization efficiency according to Sneller *et al.* (2000). Calibration was based on GSH concentrations ranging from 5–20  $\mu\text{M}$ .

#### *Phytochelatin synthase activity assay*

Phytochelatin synthase activity was determined in crude extracts according to a modified protocol by Finkemeier *et al.* (2003). Plant tissue (200 mg) was extracted in 2 ml of buffer containing 20 mM HEPES-NaOH, pH 7.5, 10 mM  $\beta$ -mercaptoethanol, 100  $\mu\text{M}$   $\text{CdSO}_4$ , 20% (w/v) glycerol, and 100 mg  $\text{ml}^{-1}$  polyvinylpyrrolidone by homogenization in a mortar, using some quartz sand. Following centrifugation at 13 000 *g* for 10 min at 4 °C, the assay contained 400  $\mu\text{l}$  extract and 100  $\mu\text{l}$  reaction buffer (25 mM glutathione, 100  $\mu\text{M}$   $\text{CdSO}_4$ , 10% (w/v) glycerol, and 250 mM HEPES-NaOH, pH 8.0) and protease inhibitor mix 'Complete' as recommended by the manufacturer (Sigma). The incubation was carried out at 35 °C for 90 min and terminated by addition of 125  $\mu\text{l}$  20% (w/v) trichloroacetic acid. SH-groups were derivatized using monobromobimane, and HPLC analysis was performed as described above.

#### *H<sub>2</sub>O<sub>2</sub> histochemical staining as an indication of oxidative stress level*

Hydrogen peroxide accumulation was visualized with 3,3'-diaminobenzidine (DAB) according to Thordal-Christensen *et al.* (1997). Five-week-old tobacco plants grown in hydroponic culture were used for the experiment. Briefly, fourth leaves (counting from the top) excised from cadmium treated plants (2 d at 25  $\mu\text{M}$   $\text{CdCl}_2$ ) or from untreated plants were placed in Petri dishes containing DAB solution (1 mg  $\text{ml}^{-1}$ ). Plates were left in a climate chamber at 24 °C in darkness and DAB staining was assessed visually 8 h later.

#### *Determination of ascorbate and dehydroascorbate content*

Ascorbate and dehydroascorbate contents in fourth leaves and roots of 6-week-old cadmium treated (2 d and 3 d at 25  $\mu\text{M}$   $\text{CdCl}_2$ ) and untreated plants were determined according to the protocol for tobacco by Kampfenkel *et al.* (1994).

### Determination of GSH/GSSG level

GSH and GSSG levels were determined according to the modified methods of Zhang and Kirkham (1996) and Somparn *et al.* (2007). The fourth leaves of 6-week-old tobacco plants were homogenized in 5 ml of ice-cold 5% meta-phosphoric acid. The homogenate was centrifuged for 20 min at 10 000 *g* at 4 °C. The supernatant was collected and neutralized with 5 M KOH. One ml of the neutralized supernatant was mixed with 100 µl of water and used for the enzymatic assay of total glutathione. In addition, another 1 ml of the neutralized extract was mixed with 100 µl of 33 mM 1-methyl-2-vinylpyridine (M2VP), a GSH scavenger, incubated for 2 min at room temperature and used for the assay of GSSG. For the enzymatic assay of glutathione, 330 µl of 0.3 mM DTNB in 150 mM NaPO<sub>4</sub> (pH 7.2) containing 15 mM EDTA and 0.04% BSA were mixed with 50 µl of the sample and 290 µl of 150 mM NaPO<sub>4</sub> (pH 7.2). The reaction was started by adding 330 µl of 150 mM NaPO<sub>4</sub> (pH 7.2) containing 1 mM EDTA, 0.02% BSA, 0.6 mM NADPH, and glutathione reductase (1.2 U ml<sup>-1</sup>) and the absorbance was recorded at 412 nm for 3 min. Glutathione levels were determined against a standard curve. GSH content was calculated as the difference between total glutathione and GSSG.

### Statistical analysis

Statistical significance was evaluated at the 0.05 probability levels using Student's *t* test. All experiments were repeated at least three times.

## Results

### Selection of transgenic lines overexpressing AtPCS1 and CePCS

After transformation and regeneration, the presence of the transgene was confirmed by PCR for 14 and 19 lines of tobacco transformed with *AtPCS1* or *CePCS* construct, respectively. RT-PCR analysis of PCR-positive plants confirmed the expression of introduced genes in eight *AtPCS1* and 11 *CePCS* harbouring lines (data not shown). The seeds of RT-PCR positive plants were collected and used for further tests.

All seedlings able to develop on agar-plates containing Basta and subsequently transferred to the hydroponic culture, also survived spraying three times with this agent, which indicated 100% efficiency of the selection. PCR examination showed that all Basta-resistant plants contained the transgene (data not shown). Transgenic lines (of both F<sub>0</sub> and F<sub>1</sub> generations) and the wild type grown under control conditions did not differ from each other in their development, appearance, and reproduction (data not shown).

### Overexpression of AtPCS1, but not CePCS results in increased sensitivity to cadmium

In the first experiment, 3-d exposure to 25 µM CdCl<sub>2</sub> in hydroponic cultures resulted in a substantial difference in the plant appearance (Fig. 1). In contrast to the green, healthy-looking leaves of *CePCS* transformants and wild-type Xanthi plants (Fig. 1D, F), all tobacco lines expressing *AtPCS1* had leaves with necrotic spots and

wilted areas (Fig. 1E). The difference in the level of tolerance to cadmium between Xanthi and *CePCS* did not become evident until exposure to a higher concentration of this element, 35 µM Cd<sup>2+</sup> (Fig. 1G, I). These results demonstrated that expression of *AtPCS1*, but not *CePCS*, led to enhanced cadmium sensitivity. It is important to note that both types of transformants did not show any phenotypic differences from WT when grown under control conditions without Cd<sup>2+</sup> (Fig. 1A–C). To test whether the necrotic spots on leaves developing in *AtPCS1*-expressing plants were preceded by a loss of photosynthetic pigments, the concentrations of chlorophyll and carotenoids were determined. After 2 d of exposure to 25 µM CdCl<sub>2</sub>, however, both *AtPCS1* and *CePCS*-expressing plants had slightly higher chlorophyll (Fig. 2A) and carotenoid (Fig. 2B) contents compared with WT.

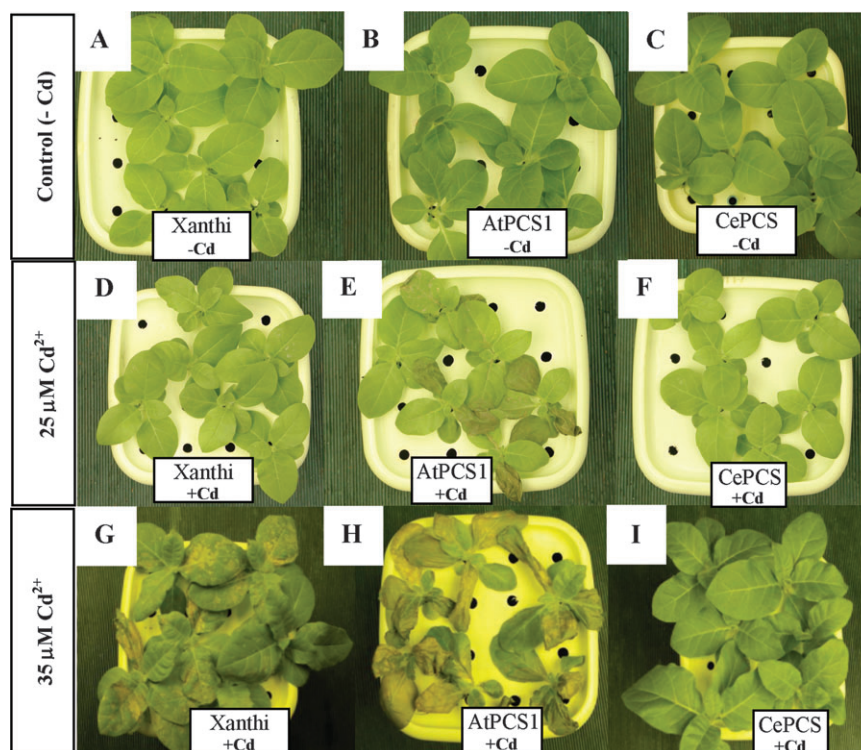
When tested for the response to cadmium in industrially contaminated soil (accompanied by elevated Pb and Zn levels), *AtPCS1* transformants produced a slightly lower (decreased by ~20%) shoot biomass relative to *CePCS* plants and the wild type (data not shown). The slower growth of *AtPCS1*-expressing plants might have partially resulted from their increased sensitivity to cadmium.

### Overexpression of PCS did not lead to higher cadmium accumulation

The determination of cadmium content in *AtPCS1* and *CePCS* plants after 6 d of exposure to 25 µM CdCl<sub>2</sub> in liquid medium demonstrated that the overexpression of both genes in tobacco did not cause dramatic differences in metal accumulation (Fig. 3A–D). The moderate decrease in the cadmium concentration in shoots (by 15–25%) was detected in some *AtPCS1* transformed lines (Fig. 3A). Similar to the hydroponic experiments, no significant difference between the lines studied was found in cadmium concentrations in above-ground parts of plants grown for 6 weeks on cadmium-contaminated industrial soil (data not shown). Therefore, transformation with *AtPCS1* and *CePCS* does not seem to improve the suitability of plants for phytoremediation of cadmium-contaminated soil. For that purpose, an increase in metal accumulation by at least 100–200% is required.

### Expression analysis and selection of transgenic lines used for further experiments

Semi-quantitative expression analysis was performed for selected T<sub>1</sub> lines. The results shown in Fig. 4 demonstrate different levels of *PCS* expression. In order to correlate the level of phytochelatin synthase expression with the detected phenotype, two lines of both *AtPCS1* (PaII4, PaII12) and *CePCS* (PcII3, PcII4) expressing plants, one with a high, the other with a low expression level, were chosen for further analysis.



**Fig. 1.** Appearance of 6-week-old *AtPCS1* (line PaII4; E, H) and *CePCS* (line PcII4; F, I) transformed, and wild type var. Xanthi (D, G) plants cultivated for 3 d in hydroponic culture containing 25  $\mu\text{M}$  (D–F) or 35  $\mu\text{M}$  (G–I)  $\text{CdCl}_2$ . Untreated control (–Cd) plants: wild type Xanthi (A); *AtPCS1* plants (B); *CePCS* plants (C).

#### Overexpression of *AtPCS1* and *CePCS* resulted in only a moderate increase of phytochelatin levels

To investigate the effects of *PCS* overexpression on PC accumulation, plants were exposed to 5  $\mu\text{M}$   $\text{CdCl}_2$  and to a more toxic concentration (25  $\mu\text{M}$ ), and harvested after 2 d and 3 d of exposure. The total PC levels are shown in Fig. 5, and the concentrations of individual PC species (PC2, PC3, and PC4) synthesized at 25  $\mu\text{M}$  cadmium are given in Fig. 6. In general, the contents of phytochelatins were much higher in leaves than in roots and increased with Cd concentration and the duration of metal treatments. PCs were not detected in the absence of cadmium from the growth medium (data not shown).

There was a moderate difference in the total PC concentration between the WT, *AtPCS1* and *CePCS* transformants. As shown in Fig. 5A and B, after 2 d of 5  $\mu\text{M}$   $\text{CdCl}_2$  exposure, an increase by  $\sim 30$ – $40\%$  in PC production in leaves, and to a lesser extent in roots of both types of transformants was observed. However, on the third day of Cd treatment, the PC level in *AtPCS1* expressing plants, in contrast to *CePCS* transformants, began to decrease both in roots and leaves (Fig. 5A, B). This tendency was more pronounced in roots of plants grown at 25  $\mu\text{M}$  cadmium (Fig. 5C). However, in leaves the level of total PC was lower in all transgenic lines relative to WT (Fig. 5C, D).

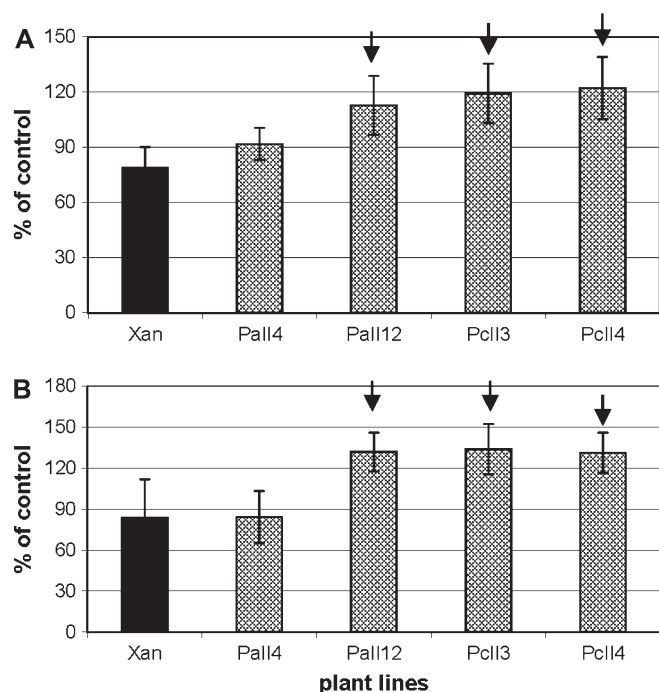
Figure 6 shows levels of the major PC species synthesized in response to a 3 d exposure to cadmium. In

general, at 5  $\mu\text{M}$   $\text{Cd}^{2+}$  the prevalent PC species in roots was PC2, and in leaves PC4 (Fig. 6A, B), whereas at 25  $\mu\text{M}$   $\text{Cd}^{2+}$  it was PC4 both in roots and leaves (Fig. 6C, D). The difference between both types of transformants and the WT is in the composition of synthesized PCs. Thus, at 5  $\mu\text{M}$   $\text{Cd}^{2+}$  the concentration of PC3 and PC4 in roots and leaves of *AtPCS1* plants was lower compared to Xanthi, whereas in *CePCS* plants the concentration has not changed or has slightly increased (Fig. 6A, B). A similar tendency was detected at more toxic 25  $\mu\text{M}$   $\text{Cd}^{2+}$  in roots (Fig. 6C). In turn, in leaves the concentrations of almost all forms of PCs were reduced in *AtPCS1* transformants (Fig. 6C), while in *CePCS* ones the reduction of only PC3 was observed (Fig. 6D).

The analysis of the content of cysteine, the substrate for GSH used subsequently for PC synthesis, showed that this amino acid was not the limiting factor for phytochelatin production. The cysteine level was similar in all plant lines tested and even slightly higher under cadmium exposure than in control conditions (data not shown).

#### Overexpression of *AtPCS1* results in dramatic $\gamma$ -glutamylcysteine accumulation and glutathione depletion in leaves

Overexpression of *PCS* did not influence GSH and  $\gamma$ -glutamylcysteine ( $\gamma$ -EC) concentrations in plants grown under control conditions without cadmium. There was no



**Fig. 2.** Total chlorophyll (A) and carotenoid (B) content in the fourth leaf of 6-week-old tobacco plants after 2 d of 25  $\mu\text{M}$   $\text{CdCl}_2$  treatment shown as a percentage of untreated (control) plants of the same line. Values correspond to means  $\pm$ SD ( $n=4$ ); those significantly different from wild type var. Xanthi are indicated with the arrow ( $P < 0.05$ ). Concentration of chlorophyll ( $\mu\text{g g}^{-1}$  FW) in untreated (control) plants: Xanthi ( $609 \pm 147$ ); PaII4 ( $572 \pm 57$ ); PaII12 ( $586 \pm 77$ ); PcII3 ( $590 \pm 61$ ); PcII4 ( $791 \pm 77$ ). Concentration of carotenoids ( $\mu\text{g g}^{-1}$  FW) in untreated (control) plants: Xanthi ( $94 \pm 23$ ); PaII4 ( $89 \pm 9$ ); PaII12 ( $80 \pm 10$ ); PcII3 ( $88 \pm 6$ ); PcII4 ( $113 \pm 11$ ).

significant difference in their levels in either leaves or roots between the wild type and transgenic plants. The content of GSH was generally higher in leaves ( $200\text{--}320$  nmol  $\text{g}^{-1}$  FW) compared with roots ( $140\text{--}180$  nmol  $\text{g}^{-1}$  FW). In turn, in all plant lines studied  $\gamma$ -EC level in leaves was very low, ranging from  $5\text{--}10$  nmol  $\text{g}^{-1}$  FW, whereas in roots it was close to the detection limit, which stays in accordance with the data reported in the literature (Arisi *et al.*, 1997; Creissen *et al.*, 1999).

The concentration of  $\gamma$ -EC and GSH after 3 d of exposure to  $5$   $\mu\text{M}$  and  $25$   $\mu\text{M}$  cadmium is shown in Fig. 7. No significant difference was detected between the root GSH levels of two types of transformants and WT at the less toxic  $5$   $\mu\text{M}$  cadmium (Fig. 7A); however, at  $25$   $\mu\text{M}$  cadmium the level of  $\gamma$ -EC in roots of *AtPCS1* and *CePCS* expressing plants increased markedly (Fig. 7C). In leaves, the overexpression of *AtPCS1*, in contrast to *CePCS*, resulted in a substantial decrease in glutathione content and in a dramatic increase in  $\gamma$ -EC after 3 d of exposure to both  $5$   $\mu\text{M}$  and  $25$   $\mu\text{M}$  cadmium (Fig. 7B, D). *CePCS*-expressing plants, in contrast to *AtPCS1* ones, showed leaf GSH levels similar to WT on both cadmium treatments (Fig. 7B, D) whereas the  $\gamma$ -EC concentration

was increased relative to WT only at  $25$   $\mu\text{M}$   $\text{CdCl}_2$  (Fig. 7D).

#### *Overexpression of AtPCS1 leads to a moderate decrease in cadmium detoxification capacity*

In order to correlate the concentration of cadmium and NPTs in *AtPCS1*, *CePCS* and WT plants after heavy metal exposure of different duration, the cadmium content was determined in the fourth leaf and roots of plants also used for NPT analyses following 3 d of exposure to  $5$   $\mu\text{M}$   $\text{CdCl}_2$ . There were no substantial differences between the lines (data not shown), thus the observed Cd hypersensitive phenotype of the *AtPCS1* plants did not result from higher Cd accumulation in leaves.

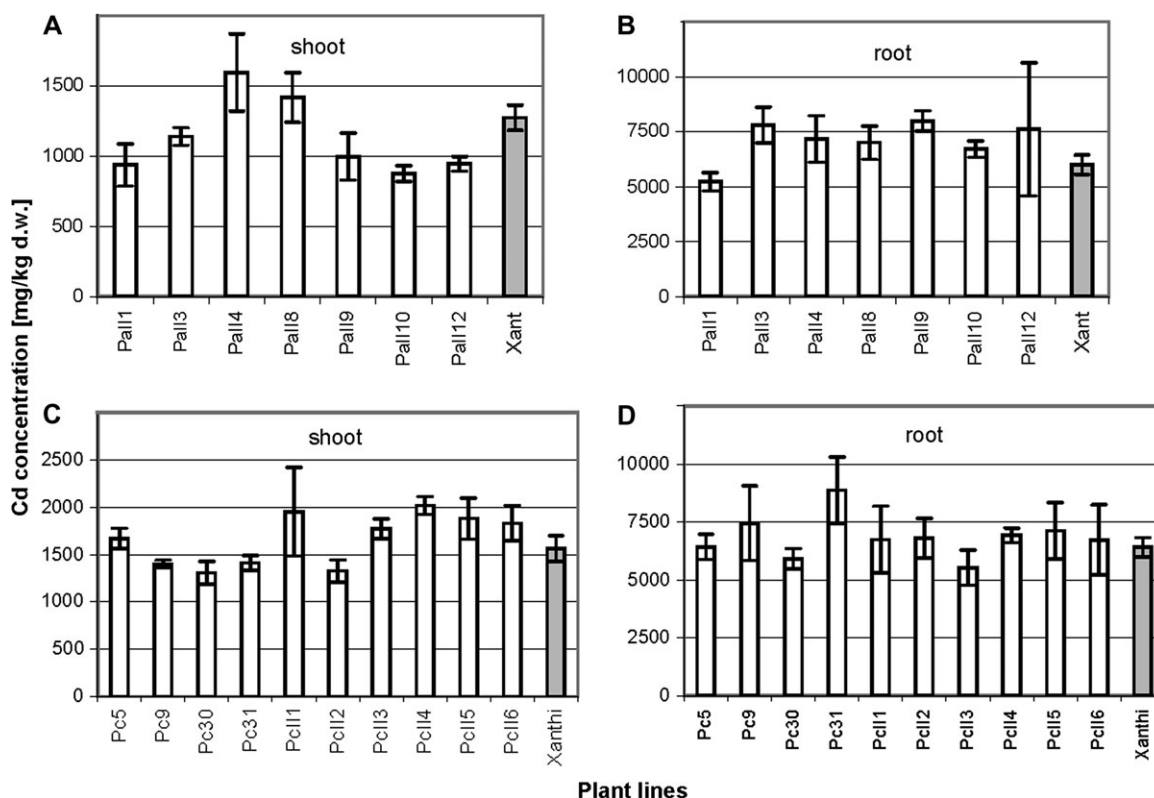
The stoichiometric relationship between the molar concentrations of sulphhydryl groups in PCs and the Cd concentration after 3 d at  $5$   $\mu\text{M}$   $\text{CdCl}_2$  is shown in Fig. 8. The PC-SH:Cd ratio was lower in *AtPCS1* plants relative to *CePCS* and WT plants only in roots. In contrast, the PC-SH:Cd ratio in leaves of *CePCS* transformants was higher than in *AtPCS1* ones and Xanthi. The lower ratio of PC sulphhydryl groups to cadmium indicated a lower Cd detoxification capacity of those plants and may be partially responsible for their reported hypersensitive phenotype. *CePCS* overexpressing plants seem to have an increased cadmium detoxification capacity compared with the *AtPCS1* transformants and WT.

#### *PCS activity is considerably higher in AtPCS1-expressing plants compared with WT and CePCS plants*

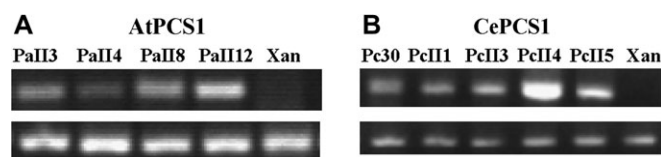
To verify if the observed changes in thiol composition due to *AtPCS1* and *CePCS* overexpression resulted from the difference in phytochelatin synthase activity, a PCS assay was performed using leaf protein extracts. It demonstrated that PCS activity in *AtPCS1* plants from both studied lines was dramatically higher than in WT, whereas *CePCS1* expression resulted in a much smaller, although significant, increase in PCS activity (Fig. 9).

#### *Overexpression of AtPCS1 leads to increased oxidative stress in the presence of cadmium*

It is known that GSH plays a major role in the control of the cell redox state. Therefore, it was assumed that the Cd-hypersensitivity of *AtPCS1* plants reported in this paper, manifested by necrosis on leaves, may result from the decrease in GSH levels (found in *AtPCS1* plants; Fig. 7B, D), which probably contributes to the generation of oxidative stress. To investigate the level of oxidative stress in leaves in the presence of cadmium, hydrogen peroxide formed in leaf tissues was visualized using histochemical staining with DAB. After 2 d of  $25$   $\mu\text{M}$   $\text{CdCl}_2$  exposure, the staining intensity was higher in leaves of *AtPCS1* transformants than in those of *CePCS*



**Fig. 3.** Cadmium accumulation in the shoot and the root of 6-week-old tobacco plants transformed with *AtPCS1* (A, B) and *CePCS* (C, D) and wild type (var. Xanthi) cultivated for 6 d in hydroponic culture containing 25  $\mu\text{M}$   $\text{CdCl}_2$ . Error bars represent SD at  $P \leq 0.05$  ( $n=3$ ).



**Fig. 4.** The expression level of *AtPCS1* (A) and *CePCS* (B) in independent plant lines tested by RT-PCR. Lower lines show the RT-PCR product for actin used as a control for equal amounts of RNA used in each reaction.

or WT plants (Fig. 10). Thus, the oxidative stress generated in the presence of cadmium was higher in Cd-hypersensitive *AtPCS1* plants.

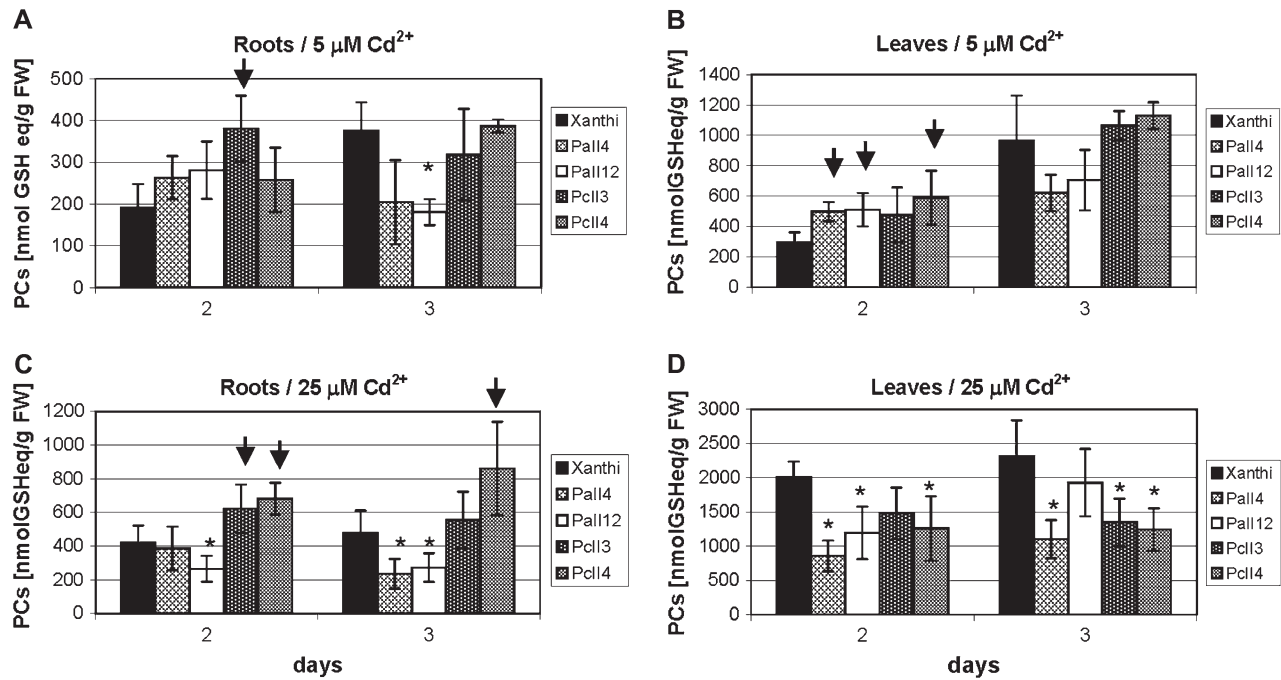
*In the presence of cadmium, AtPCS1 overexpression affects GSH/GSSG level and ascorbate contents*

It is well known that the ascorbate–glutathione cycle is crucial for the removal of  $\text{H}_2\text{O}_2$  from the cell metabolism (Noctor and Foyer, 1998; Kanwischer *et al.*, 2005; Semane *et al.*, 2007), therefore both components of the cellular antioxidative system were examined in control (–Cd) plants and after exposure to 25  $\mu\text{M}$   $\text{CdCl}_2$ .

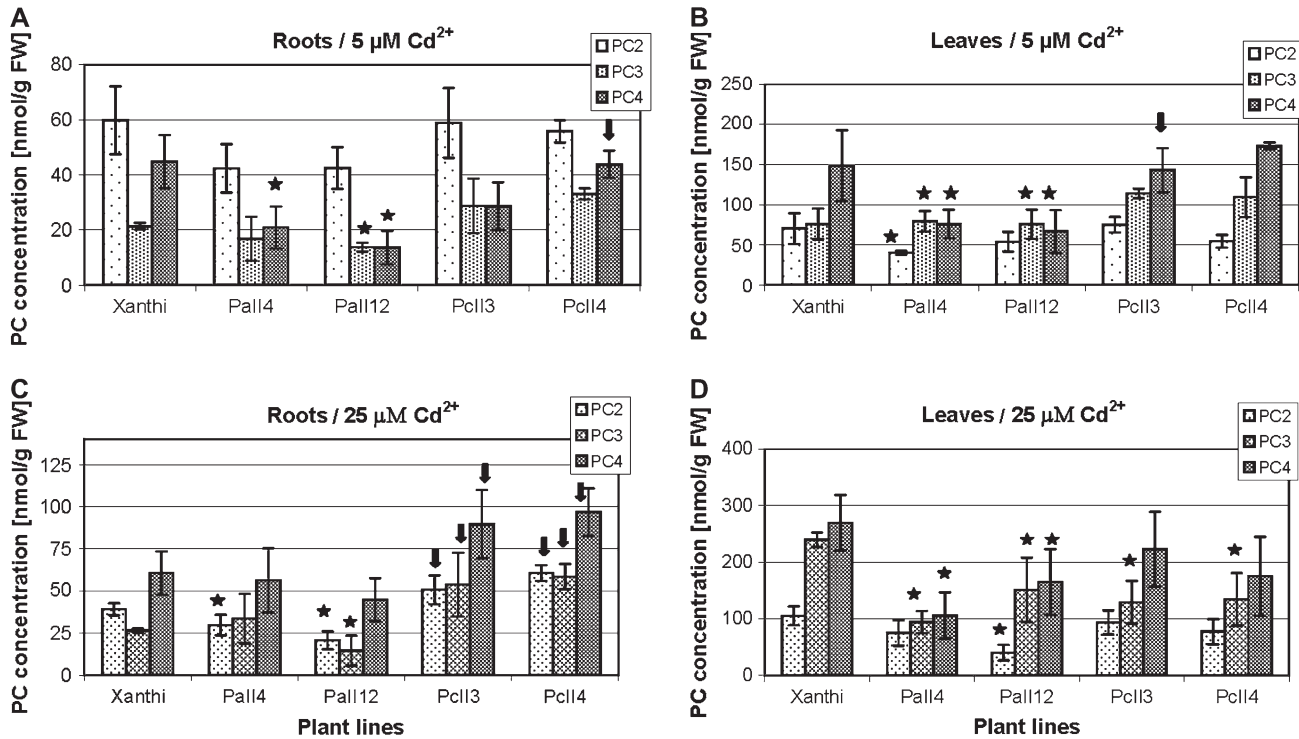
The results of glutathione quantification are shown in Fig. 11. The increased sensitivity to cadmium of *AtPCS1* expressing tobacco was accompanied not only by higher  $\text{H}_2\text{O}_2$  level (Fig. 10) and reduced concentration of

glutathione (Fig. 7) but also by a change in the GSH redox status. A decreased relative content of reduced GSH in leaves of *AtPCS1* plants relative to Xanthi and *CePCS* ones (Fig. 11B) confirms an elevated level of oxidative stress generated by cadmium in these plants (as detected by DAB staining; Fig. 10). Total GSH concentration and the reduced GSH relative content were not statistically different between lines not exposed to cadmium (Fig. 11A, C).

As demonstrated in Fig. 11A and B, no difference was found in total root and leaf ascorbate concentration, nor in the relative content of its reduced form (AsA) (Fig. 12C, D) between both types of transformants and WT plants when grown under control conditions (without cadmium). However, 2 d and 3 d treatment with 25  $\mu\text{M}$   $\text{CdCl}_2$  resulted in a significant increase in root ascorbate pool of WT and *CePCS1* transformants, whereas in *AtPCS1* its concentration remained unchanged and similar to that of untreated plants (Fig. 12A). On the other hand, *AtPCS1* expressing plants showed a significant increase in leaf total ascorbate level after a 2 d cadmium treatment, whereas in *CePCS1* and WT leaf AsA content did not change relative to plants grown on medium without the metal (Fig. 12B). An increased pool of ascorbate in leaves of *AtPCS1* transformants could be related to higher levels of oxidative stress. Interestingly, cadmium treatment did

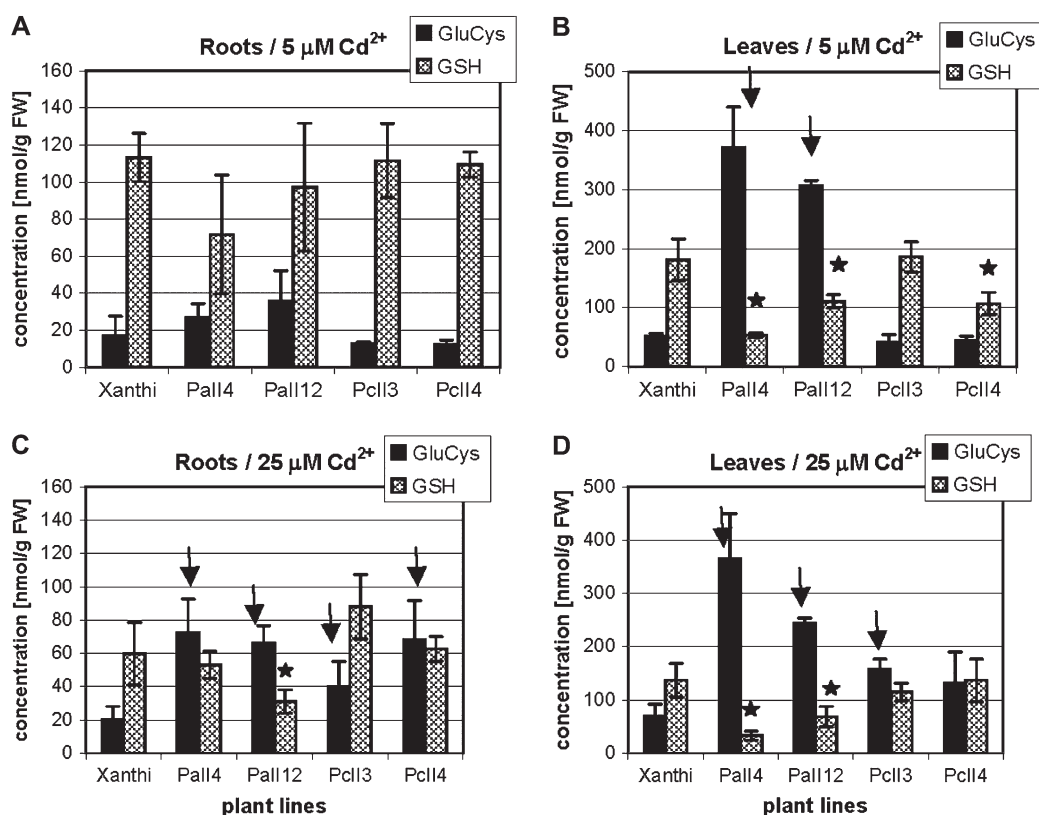


**Fig. 5.** Total PC levels in roots (A, C) and leaves (B, D) of 6-week-old tobacco plants (wild type var. Xanthi, *AtPCS1*-expressing lines, Pall4, Pall12; *CePCS*-expressing lines, Pcll3, Pcll4) after 2 d or 3 d of 5  $\mu\text{M}$  (A, B) or 25  $\mu\text{M}$  (C, D)  $\text{CdCl}_2$  exposure. PCs were not detected in plants grown on medium without cadmium. Values correspond to means  $\pm$ SD ( $n=5$ ); those significantly higher from wild type var. Xanthi are indicated with the arrow, significantly lower are marked with the asterisk ( $P < 0.05$ ).



**Fig. 6.** Concentration of major PC species in roots (A, C) and leaves (B, D) of 6-week-old tobacco plants (wild type var. Xanthi; *AtPCS1*-expressing lines, Pall4 and Pall12; *CePCS*-expressing lines, Pcll3 and Pcll4) after 3 d of 5  $\mu\text{M}$  (A, B) and 25  $\mu\text{M}$  (C, D)  $\text{CdCl}_2$  treatment. Values correspond to means  $\pm$ SD ( $n=5$ ); those significantly higher from wild type var. Xanthi are indicated with the arrow, significantly lower are marked with the asterisk ( $P < 0.05$ ).





**Fig. 7.**  $\gamma$ -Glutamylcysteine and glutathione levels in roots and leaves of 6-week-old tobacco plants (wild type var. Xanthi; *AtPCS1*-expressing lines, PaII4 and PaII12; *CePCS*-expressing lines, PcII3 and PcII4) after 3 d of 5  $\mu$ M (A, B) or 25  $\mu$ M CdCl<sub>2</sub> (C, D) cadmium treatment. Values correspond to means  $\pm$ SD ( $n=5$ ); those significantly higher from wild type var. Xanthi are indicated with the arrow, significantly lower are marked with the asterisk ( $P < 0.05$ ).

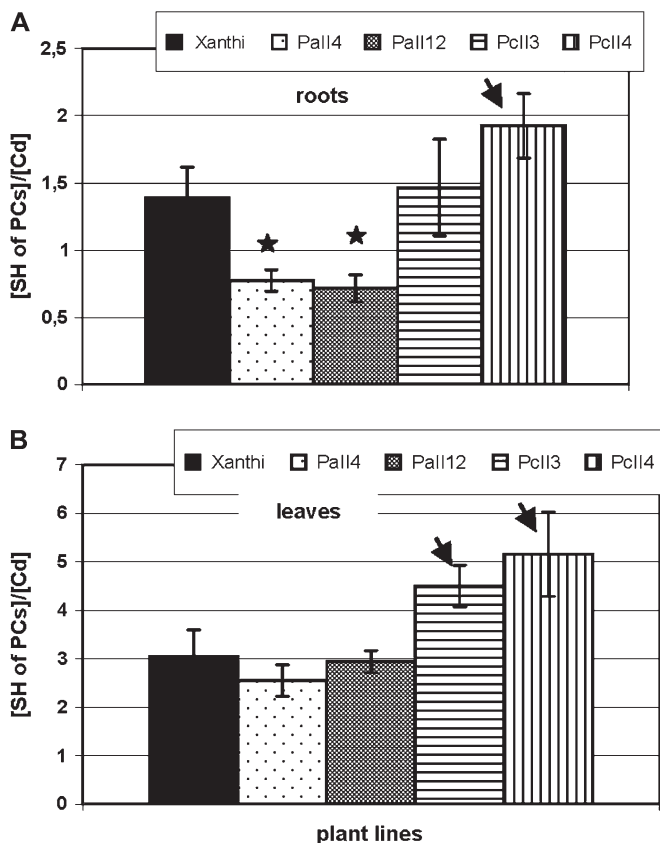
not affect the relative content of the reduced ascorbate form in roots and leaves of all tested plant lines (Fig. 12C, D) which indicates that the cellular system maintaining the balance between the reduced and oxidized form of this compound has not been affected.

## Discussion

Phytochelatin synthase play a major role in constitutive cadmium (Clemens *et al.*, 1999) and arsenate (Bleeker *et al.*, 2006) tolerance. Following the isolation of the first genes encoding phytochelatin synthase from different organisms by three independent groups (Clemens *et al.*, 1999, 2001; Ha *et al.*, 1999; Vatamaniuk *et al.*, 1999, 2001), many researchers tried to overexpress *PCS* to enhance plant heavy metal tolerance and accumulation. The results on overexpression of different *PCS* genes in various plant species published in the last few years have, however, been contradictory thus far, with plant responses reported to range from increased cadmium tolerance and accumulation (Lee *et al.*, 2003a; Gisbert *et al.*, 2003; Martinez *et al.*, 2006; Pomponi *et al.*, 2006), through increased cadmium tolerance accompanied by its decreased

accumulation (Gasic and Korban, 2007), to cadmium hypersensitivity without increased accumulation (Lee *et al.*, 2003a, b; Li *et al.*, 2004).

To date only papers describing overexpression of *AtPCS1* (in *A. thaliana*, *N. tabacum*, and *B. juncea*), *TaPCS1* (in *N. glauca* and *cad1-3 Arabidopsis* mutant), and *SpPCS1* (in *N. tabacum*) are available, and each focused on one donor *PCS* gene and one target species. Cd-hypersensitivity was reported so far only as a result of *AtPCS1* overexpression (Lee *et al.*, 2003a,b; Li *et al.*, 2004) but not in all the target species (Pomponi *et al.*, 2006; Gasic and Korban, 2007) or lines tested (Lee *et al.*, 2003a, b). A plausible explanation for the observed disparities in response to cadmium has not yet been found. It has been suggested that different *PCS* expression levels and genetic differences between target plant species (in particular with regard to the regulation of the glutathione synthetic pathway or the downstream processing of PC–Cd complexes) may contribute to the diverse phenotypes of *PCS* transformants. In this study, the experimental system was simplified by introducing two different phytochelatin synthase genes *AtPCS1* and *CePCS* into one model organism—tobacco. This approach allowed the interspecific variability to be eliminated and

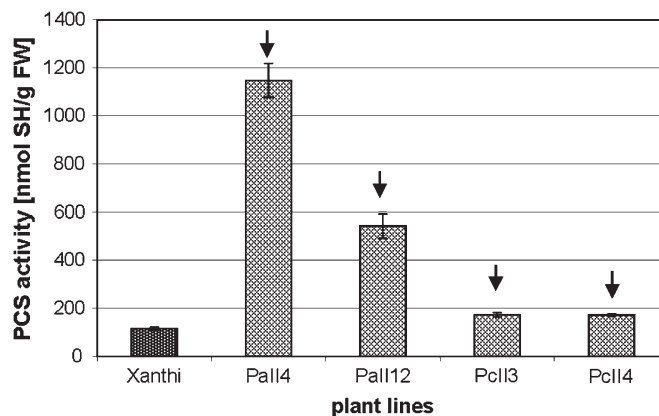


**Fig. 8.** Stoichiometry of SH of PCs to cadmium (nmol SH/nmol Cd) in roots (A), and leaves (B) of 6-week-old tobacco plants (wild type var. Xanthi; *AtPCS1*-expressing lines, PaII4, PaII12; *CePCS*-expressing lines, PcII3, PcII4) after 3 d at 5  $\mu$ M CdCl<sub>2</sub>. Values correspond to means  $\pm$ SD ( $n=5$ ); those significantly higher from wild type var. Xanthi are indicated with the arrow, significantly lower are marked with the asterisk ( $P < 0.05$ ).

to focus only on the possible functional differences between PCS enzymes as a reason for the distinct phenotypes observed.

This work demonstrated that the effects of overexpression of *AtPCS1* and *CePCS* in the same species were different. Overexpression of *AtPCS1* led to increased cadmium sensitivity (Fig. 1), similar to the majority of literature reports (Lee *et al.*, 2003a, b; Li *et al.*, 2004). On the other hand, tobacco plants transformed with *CePCS* were more tolerant to cadmium relative to WT (which became evident only after the exposure to 35  $\mu$ M Cd<sup>2+</sup>). In addition, both types of transformants did not show substantially increased cadmium accumulation (Fig. 3), which is consistent with a number of papers describing *AtPCS1* overexpression (Lee *et al.*, 2003a, b; Li *et al.*, 2004; Gasic and Korban, 2007).

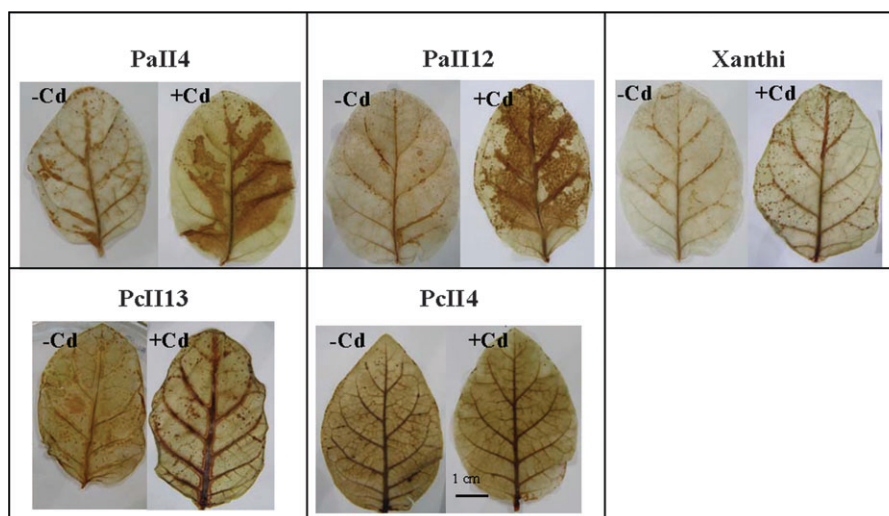
This study showed that increased sensitivity to Cd of *AtPCS1*-expressing tobacco plants (relative to WT) was accompanied by dramatic  $\gamma$ -EC accumulation and glutathione depletion (Fig. 7B, D). Similar results, though not as pronounced, were reported by Li *et al.* (2004) for



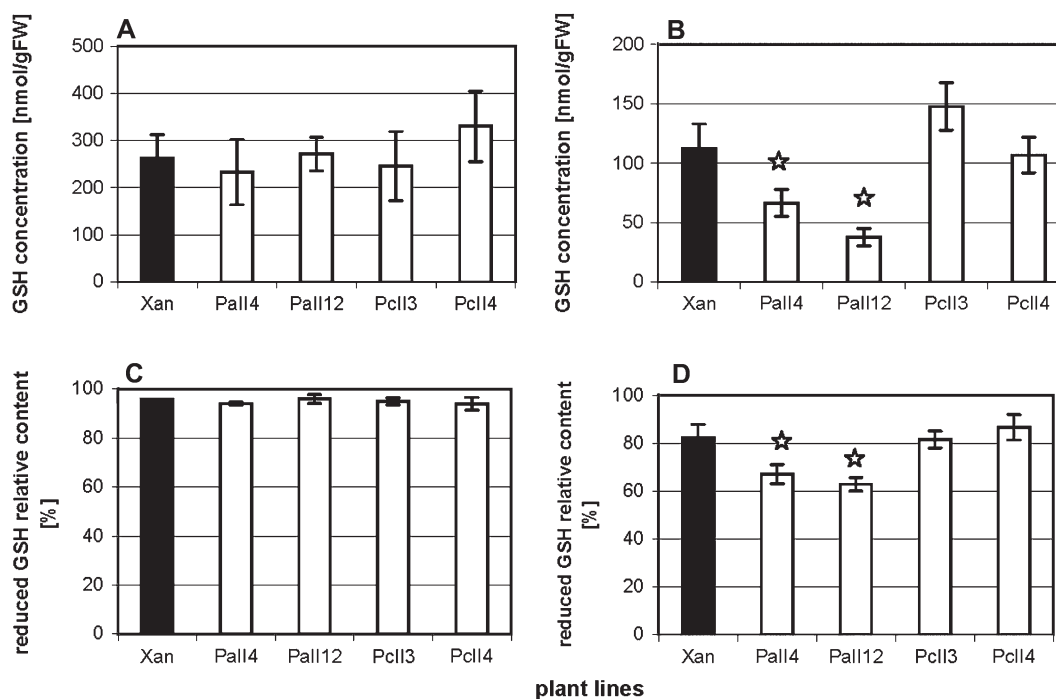
**Fig. 9.** Activity of phytochelatin synthase in extracts from tobacco plants (wild type var. Xanthi; *AtPCS1*-expressing lines, PaII4 and PaII12; *CePCS*-expressing lines, PcII3 and PcII4). Values correspond to means  $\pm$ SD ( $n=3$ ); those significantly higher from wild type var. Xanthi are indicated with the arrow ( $P < 0.05$ ).

*AtPCS1*-overexpressing, Cd-hypersensitive *A. thaliana* plants. On the other hand, *CePCS*-overexpressing plants had GSH levels similar to WT, but increased  $\gamma$ -EC concentrations (Fig. 7D). The transformants differed also with respect to PC accumulation. There was only a moderate and temporary increase in their level due to *AtPCS1* expression after only 2 d of exposure to low 5  $\mu$ M cadmium followed by a decrease later on, whereas in *CePCS* plants the PC level was generally higher (Fig. 5A–C). As demonstrated by other researchers, overexpression of *PCS* in tobacco did not result in an increase of PC levels, unless GSH was added to the medium (Pomponi *et al.*, 2006) or additional genes from the GSH biosynthetic pathways were introduced (Wawrzyński *et al.*, 2006). The moderate increase in phytochelatin levels in *AtPCS1* transformants reported in this study suggested that Cd-hypersensitivity of *AtPCS1*-overexpressing plants did not result from the accumulation of phytochelatin at the supra-optimal levels as initially proposed by Lee *et al.* (2003b). However, in the recent communication of Kim and Lee (2007) it was shown that Cd-hypersensitivity of *A. thaliana* overexpressing *AtPCS1* did not result from the accumulation of NPT at supra-optimal levels. Instead, an unknown disruption in cellular metal homeostasis under Cd-stress due to the properties of the enzyme itself (e.g. binding metal ions by the enzyme) was proposed as the mechanism for the observed increase in Cd sensitivity of transgenic plants.

In this study, *PCS* genes of different origin were introduced into the same species, and therefore the difference in Cd-sensitivity and NPT concentrations in the lines studied might result from PCS protein specificity. Indeed, it was found that *AtPCS1*-expressing plants had  $\sim$ 5-fold higher PCS enzymatic activity than *CePCS*-transformed and WT plants (Fig. 9). Knowing this, it is tempting to hypothesize that a higher rate of PCS activity



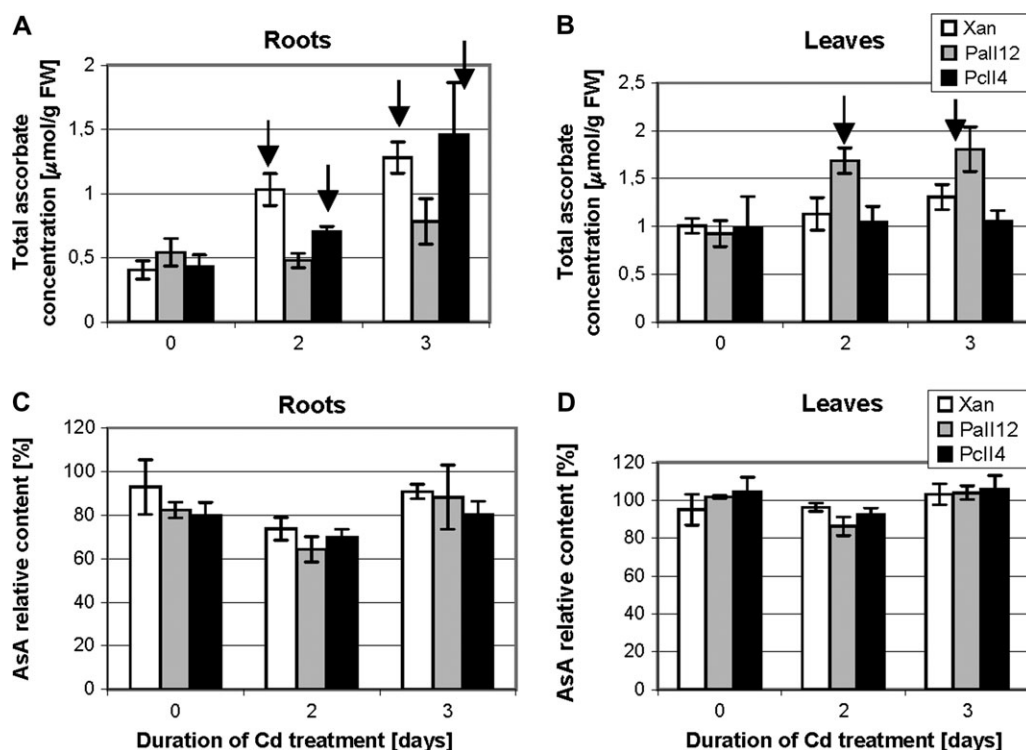
**Fig. 10.** Hydrogen peroxide accumulation in leaves of 5-week-old hydroponically grown tobacco plants wild type var. Xanthi and transformed with *AtPCS1* (lines PaII4 and PaII12) and *CePCS* (lines PcII3 and PcII4). Fourth leaves (counting from the top) excised from 5-week old plants after 2 d of 25  $\mu\text{M}$   $\text{CdCl}_2$  exposure (+Cd) or growth in the absence of cadmium (-Cd) were placed in Petri dishes containing DAB solution (1  $\text{mg ml}^{-1}$ ). Plates were left in a climate chamber at 24  $^\circ\text{C}$  in darkness and DAB staining was assessed visually 8 h later.



**Fig. 11.** Total GSH concentration (A, B) and relative content of its reduced form (C, D) in leaves of 6-week-old tobacco plants: wild type var. Xanthi and transformed with *AtPCS1* (lines PaII4 and PaII12) and *CePCS* (lines PcII3 and PcII4). Plants were grown under control conditions without cadmium (A, C) or exposed for 3 d to 25  $\mu\text{M}$   $\text{CdCl}_2$  (B, D). The redox status of glutathione is indicated by the ratio of (total glutathione-2 $\times$ GSSG) to total glutathione. Values correspond to means  $\pm$ SD ( $n=4$ ); those significantly lower from wild type var. Xanthi are indicated with the asterisk ( $P < 0.05$ ).

in *AtPCS1*-expressing plants and a concomitantly higher rate of PC formation would result in faster depletion of GSH in those plants and a concomitant increase of  $\gamma$ -glutamylcysteine synthetase activity due to alleviation of the feedback inhibition of this enzyme by GSH. The high

level of newly-synthesized  $\gamma$ -EC may not have been used for the glutathione and subsequent PC synthesis because, in the presence of Cd ions, the activity of glutathione synthetase, the second enzyme in the PC synthesis pathway, seems to be limiting (Rausser *et al.*, 1991). As



**Fig. 12.** Total ascorbate concentration (A, B) and relative content of its reduced form AsA (C, D) in roots (A, C) and leaves (B, D) of 6-week-old tobacco plants: wild type var. Xanthi and transformed with *AtPCS1* (lines Pall12 and Pall14) and *CePCS* (lines PclI3 and PclI4). Plants were grown under control conditions (–Cd) or exposed for 2 d and 3 d to 25  $\mu\text{M}$   $\text{CdCl}_2$ . Values correspond to means  $\pm$ SD ( $n=4$ ). Significant differences between control (not exposed to cadmium) and Cd-treated plants of the same line are indicated with the arrow ( $P < 0.05$ ).

well as changes in cellular thiol concentrations, disturbances in the downstream processing of PC–Cd complexes due to *PCS* overexpression might appear. If the formation rate of PC–Cd complexes in *AtPCS1* expressing plants, initially higher than in *CePCS* ones and WT plants, would exceed the rate of their ATP-dependent transport into the vacuole, PC–Cd would accumulate in the cytosol of those plants and probably be degraded (most likely to  $\gamma$ -EC, but at present the products of PC degradation are unknown). In addition, it cannot be excluded that the enzyme itself may take part in PC cleavage under certain conditions. It has been reported recently that *AtPCS1* can catalyse the cleavage of GSH-bimane conjugates to  $\gamma$ -EC-bimane, particularly under cadmium exposure (Grzam *et al.*, 2006; Blum *et al.*, 2007). Moreover, as shown by Tsuji *et al.* (2005), this enzyme may cleave not only GSH but also PCs to supply the  $\gamma$ -EC unit for elongation of the PC chain. As a result of possible degradation, one can assume that both glutathione and PC concentrations would be decreased and the  $\gamma$ -glutamylcysteine level elevated. The decrease in PC levels in roots and leaves of *AtPCS1* plants relative to WT after longer treatments with lower Cd concentration (5  $\mu\text{M}$ ), or in roots after higher 25  $\mu\text{M}$ , could be taken to suggest degradation of PCs depending on the intracellular conditions. As a result, despite the higher *PCS* activity, the total PC level might not

significantly increase in the *AtPCS1* transformants. It seems possible that due to PC–Cd degradation in the cytosol, the amount of free Cd ions could be elevated, leading to more pronounced toxic effects, and in particular that Cd-detoxification capacity decreased, as reflected by the PC-SH:Cd ratio, (Fig. 8).

However, elevated  $\gamma$ -glutamylcysteine formation in the *PCS* activity assay, that could indicate a peptidase activity of *PCS*, was not observed. It seems possible that either peptidase or PC formation activity of the enzyme might be regulated in intact cells by factors not present in the extract. This could be dependent, for example, on the efficiency of the transport of PC–Cd complexes into the vacuole. Presently however, it can be only speculated about the disturbances in the PC pathway and on the regulation of PC transport and the products of their degradation. The vacuolar transporters of PC–Cd, probably members of MRP family (Rea, 1999), still remain unknown. Interestingly, the decrease in cadmium tolerance was not observed when *AtPCS1* was targeted to *A. thaliana* chloroplasts (Picault *et al.*, 2006), which is consistent with the above hypothesis about the role of vacuolar transport in the generation of the Cd hypersensitivity phenomenon due to *AtPCS1* overexpression.

Cadmium induces oxidative stress, although it is not a Fenton reaction type metal (Jonak *et al.*, 2004; Semane

*et al.*, 2007). Glutathione plays a major role in the control of the cell redox state (Noctor *et al.*, 1998) and the inability to maintain sufficient GSH levels under cadmium exposure may lead to ROS production. Enhanced concentration of  $\gamma$ -EC, as shown by Creissen *et al.* (1999) might be an additional factor in oxidative stress generation. Thus Cd-hypersensitivity of *AtPCS1*-expressing tobacco could result, among others, from higher oxidative stress due to leaf GSH depletion together with  $\gamma$ -EC accumulation (Fig. 7D). As expected,  $H_2O_2$  production in the presence of the metal was higher in leaves of *AtPCS1* transformants relative to *CePCS* and WT (Fig. 10). In parallel, the decrease in GSH level (Fig. 7), a lower relative content of reduced GSH (Fig. 11) and an increase in leaf ascorbate level (Fig. 12B) were detected. The importance of AsA accumulation could probably be explained by its involvement in non-enzymatic antioxidative activity in *AtPCS1* plants with a higher level of  $H_2O_2$  (Fig. 10) (Noctor and Foyer, 1998; Noctor, 2006; Semane *et al.*, 2007). It is known, that the total ascorbate pool is not stable and changes, among others, depending on the developmental stage as well as in response to the level of oxidative stress (Noctor, 2006). As shown by Franceschi and Tarlyn (2002), AsA is synthesized in photosynthetic organs and subsequently transported to non-photosynthetic tissues like roots, although the mechanism of the regulation of the transport has not been elucidated yet. Therefore, it cannot be excluded that its regulation is part of a plant's antioxidative defence system. An observed contrasting pattern of AsA accumulation in leaves and roots of *AtPCS1* plants, compared to *CePCS* and Xanthi ones, could be the manifestation of the compensation mechanism under the diversified level of oxidative stress in tested plant lines. A similar mechanism was previously demonstrated by Kanwischer *et al.* (2005) for GSH-deficient *cad2* mutants. Furthermore, the maintenance of the AsA relative content in all studied plants exposed to cadmium at the level of control plants grown without the metal in the medium (Fig. 12C, D), indicates that the efficiency of the AsA/DHA antioxidative system has not been affected in cadmium-treated plants. To summarize, cadmium hypersensitivity of *AtPCS1* plants relative to *CePCS* ones could be linked to the generation of a higher level of oxidative stress. However, to elucidate the underlying mechanism it will be necessary to examine in detail the ascorbate–glutathione antioxidative network. Moreover, to supplement the short-term experiments, long-term ones will need to be conducted for further understanding of these processes. Recently, it has been clearly demonstrated, that acclimation to cadmium stress is also crucial for good performance in contaminated environments of hyperaccumulators like *T. caerulescens* (Küpper *et al.*, 2007).

The monitoring of cysteine, the substrate for GSH biosynthesis, revealed that its concentration was similar in

all plant lines tested and not decreased by cadmium treatments (data not shown). This result is in accordance with the literature suggesting that the level of cysteine is not a major factor deciding about the rate of glutathione biosynthesis under cadmium exposure (Noctor *et al.*, 1998). According to Noctor *et al.* (1998), the other substrates from the GSH/PC biosynthesis pathway, glutamic acid and glycine, are not rate-limiting; therefore, their level was not checked in this study.

Lee *et al.* (2003a) characterizing the effects of *AtPCS1* overexpression in *A. thaliana* reported that the decrease of Cd-tolerance was only noticed in lines with the highest *AtPCS1* expression level. In the present study, two lines of *AtPCS1* and *CePCS* transformants chosen for detailed investigation (PaII4, PaII12, and PcII3; PcII4, respectively) differed in the expression level (Fig. 4). However, no correlation was detected between the expression level (mRNA) and the monitored phenotype.

In conclusion, by expressing *AtPCS1* and *CePCS* in the same species (thus eliminating the interspecific differences), the first evidence has been obtained that the diverse effects of overexpression may result from the functional differences between the enzymes from diverse organisms. The interrelationship between the PCS enzymatic activity/the rate of PCs biosynthesis, and their transport to vacuole/degradation, is probably different in *AtPCS1* and *CePCS* plants and possibly one of the factors contributing to the observed distinct sensitivity to cadmium. The disturbances of thiol homeostasis, due to increased PCS activity over a certain threshold, and probably the lack of the synchronization between PC–Cd formation and their transport to the vacuole, may increase the oxidative stress level and decrease the cadmium detoxification capacity (as in the case of *AtPCS1*-expressing plants) leading to Cd-hypersensitivity. This study demonstrates how much the overexpression of a single gene can interfere in related metabolic processes occurring in the cell, leading to results that are opposite from those expected. Thus not all *PCS* genes would be suitable for the transformation of all plant species for the phytoremediation purposes.

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