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ORIGINAL PAPER

Overexpression of *PtPCS* enhances cadmium tolerance and cadmium accumulation in tobacco

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Abstract Phytochelatins chelate heavy metal ions to decrease their toxicity. The chelates are then transferred to, and stored in, the vacuole. Phytochelatin synthase (PCS), which is involved in phytochelatin synthesis, is thought to be a key enzyme for phytoremediation. In this study, a *PCS* gene encoding phytochelatin synthase was cloned from poplar (*Populus tomentosa* Carr.), a widely grown model woody plant that accumulates high levels of heavy metals, especially cadmium. Poplar is considered to have potential applications in phytoremediation. The full-length *PtPCS* cDNA (1512-bp) encoded a polypeptide of 503 amino acid residues. The *PtPCS* cDNA was transferred into tobacco by *Agrobacterium*-mediated leaf disk transformation. The transgenic and wild-type (WT) lines of tobacco were subjected to a one time Cd treatment (90 μmol Cd^{2+}) for 30 days, and then evaluated to determine their Cd tolerance. We evaluated morphological and physiological indices including leaf relative electrolyte leakage, malondialdehyde content, total superoxide dismutase activity, chlorophyll content and root activity. Compared with WT plants, the transgenic plants expressing *PtPCS* grew better in the Cd treatment and showed significantly higher Cd tolerance. Compared with WT plants, the transgenic lines accumulated higher concentrations of Cd (1.7 to 3.0-fold higher Cd concentration in roots; 1.24 to 2.28-fold higher Cd concentration in leaves). However, the transfer coefficient was lower in the transgenic lines than in wild type. We concluded that *PtPCS* encodes a functional PCS that may be involved in Cd tolerance and accumulation, but not in Cd transport.

Keywords Phytochelatin synthase · Cadmium accumulation · Cadmium tolerance · Physiological index · Cadmium content

Introduction

With increasing industrialization, there is greater pollution of soils with heavy metals, which negatively affect human health. Phytoremediation refers to the process in which heavy metals are absorbed by plants, and are consequently removed from the soil. This process is regarded as a healthy, environmentally friendly, and low-cost method to control soil heavy metal pollution (Shukla et al. 2013). Several genes and gene families have been identified to play roles in heavy metal enrichment in plants. These genes/gene families include those encoding cation diffusion facilitator (CDF) family proteins (Clemens et al. 2002), natural resistance macrophage associated proteins (Nramp) (Thomine et al. 2000), heavy metal-transporting ATPases (Bernarda et al. 2004; Lee et al. 2007), phytochelatin synthases (PCS) (Clemens et al. 1999), metallothioneins (Hasegawa et al. 1997; Vrbová et al. 2013), the yellow stripe-like (YSL) protein family (Curie et al. 2009), Zn^{2+} transporters (ZIP) (Pence et al. 2000), ATP-binding cassette (ABC) transporter (Rea 2007; Bhuiyan et al. 2011a, b), and some key enzymes involved in glutathione (GSH) biosynthesis of GSH *S*-transferases (GSTs) (Polle et al. 2013) and γ-glutamylcysteine synthetase (γ-ECS) (He et al. 2015).

Phytochelatins (PCs) are a family of thiol-rich peptides, with the general structure (γ-Glu-Cys)n-Gly (n = 2–11).

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These compounds were first identified from *Rauvolfia serpentine* by Grill et al. (1985). PCs can bind heavy metal ions, especially cadmium ions, forming almost non-toxic heavy metal-protein complexes. This decreases the concentration of free heavy metal ions and decreases their toxicity in plant cells. Also, heavy metal-PC complexes can be transferred to, and stored in, the vacuole, where they are separated from enzymes and other active substances in the cytoplasm (Yadav 2010). This heavy metal response mechanism is ubiquitous in plants (DalCorso et al. 2008; Meyer et al. 2011). The role of PCs in heavy metal detoxification was confirmed in glutathione-deficient mutants (gsh^{-}) of *Schizosaccharomyces pombe* (Glaeser et al. 1991) and in phytochelatin-deficient mutants (PC^{-}) of *Arabidopsis thaliana* (Cobbett 2000).

Phytochelatins are not gene-encoded proteins, but are produced from GSH via the activity of phytochelatin synthase (PCS). A transgenic line expressing a PCS showed increased contents of PCs (Gasic and Korban 2007a; Brunetti et al. 2011) and non-protein thiols (NPTs) in cadmium-treated shoots (Gasic and Korban 2007a). Several studies have shown that the *PCS* gene encoding PCS is constitutively expressed, but is further activated in the presence of heavy metal ions (Vatamaniuk et al. 2004; Rea 2012; Shukla et al. 2012). Several *PCS* genes have been cloned from various species, such as *Arabidopsis* (*AtPCS1*) (Vatamaniuk et al. 1999), *Triticum aestivum* (*TaPCS1*) (Clemens et al. 1999; Martínez et al. 2006; Wang et al. 2012), *Brassica juncea* (*BjPCS1*) (Heiss et al. 2003), *Cynodon dactylon* (*CdPCS1*) (Li et al. 2006), *Thlaspi caerulescens* (*TcPCS1*) (Meyer et al. 2011; Liu et al. 2011), *Phragmites australis* (*PaPCS*) (Zhao et al. 2014), and *Ciona intestinalis* (*CiPCS*) (Franchi et al. 2014). Functional analyses have confirmed their roles in heavy metal resistance and accumulation.

Populus tomentosa Carr. is a model woody plant that plays important roles in ecological and environmental protection. It has been demonstrated that poplar plants can accumulate high concentrations of heavy metals (Zacchini et al. 2009; Polle et al. 2013), especially Cd and zinc (Robinson et al. 2000; Lee et al. 2003). The Cd contents of dry leaves can be as high at 209 μg g^{-1} when the Cd concentration in soil is >100 μg g^{-1} (Robinson et al. 2000). The cadmium concentrations in leaf, bark and root of *Populus* × *canescens* were about 200, 200 and 700 μg g^{-1}, respectively, when the poplar plant were exposed to 200 μM $CdSO_4$ in sand culture (He et al. 2013). Therefore, poplar has potential applications in the phytoremediation of Cd-polluted soil. The aim of this study was to clone the *PCS* gene from *P. tomentosa* Carr., and evaluate whether it could increase Cd-tolerance and Cd accumulation in transgenic lines of *Nicotiana tabacum* L. The results of this study contribute to our understanding of the molecular mechanism of Cd resistance in woody plants, and provide an important genetic resource for generating phytoremediation via genetic engineering.

Materials and methods

Plant materials and expression vector

The *PtPCS* gene was cloned from the *P. tomentosa* cultivar TC1521. Aseptic plantlets of *N. tabacum* (tobacco) were used for gene transformation. We used the plant expression vector pEZR(K)-LC, which contains two expression units; 35S promoter-*GFP* gene and 35S promoter-neomycin phosphotransferase II (*npt II*) gene. The latter confers kanamycin resistance for host cells. Both the expression vector and *Agrobacterium tumefaciens* strain LBA4404 were stored in our laboratory. We used cadmium chloride hemi-pentahydrate ($CdCl_2$ 99.0 %) solution for the Cd treatment.

Cloning of *PtPCS* gene and construction of expression vector

Total RNA was isolated from the Cd^{2+}-treated (100 mL 0.9 mM $CdCl_2$) poplar leaves by the TRIzol method (Invitrogen, Carlsbad, CA, USA). Based on the *PtPCS* sequence reported by Liu et al. (2012), the following pair of adaptor primers was designed: PCSLCF: 5′-CGG AAGCTTATGGCGATGGCGGGGTTGTAC-3′ (*Hin*d III site underlined) and PCSLCR: 5′-CGGGAATTCCTAGGA AAGAGGTGCGCCGAG-3′ (*Eco*R I site underlined). The full-length *PtPCS* gene was cloned from *P. tomentosa* samples and inserted into the pEASY-Blunt vector. After digestion with *Hin*d III and *Eco*R I, the positive gene clone and the plant expression vector pEZR(K)-LC were ligated to form the recombinant expression vector pEZR(K)-LC-PtPCS, which was introduced into *A. tumefaciens* LBA4404 by electroporation.

Gene transformation of tobacco plants

The *Agrobacterium*-mediated leaf disk method was used for the genetic transformation of tobacco. Cells of *A. tumefaciens* LBA4404 harboring the recombinant expression vector pEZR(K)-LC-PtPCS were grown overnight at 28 °C in YEB medium (pH 7.0) containing 50 μg/mL kanamycin. The activated bacteria were transferred to antibiotic-free LB liquid medium and cultivated to an OD_{600} of 0.4. Young tobacco leaves were cut into small pieces (0.5 × 0.5 cm). The pieces were immersed in the bacterial solution for 5 min, transferred to differentiation medium [MS + 3 mg/L 6-benzyladenine (BA) + 0.2 mg/L naphthaleneacetic acid

(NAA) + 3 % (w/v) sucrose + 0.8 % (w/v) agar], and kept in the dark at 28 °C for 3 d. Then, the tobacco pieces were transferred onto MS differentiation medium containing kanamycin (300 mg/L) and sodium cefotaxime (250 mg/L). Regenerated shoots were transferred onto MS rooting medium [MS + 0.1 mg/L NAA + 3 % (w/v) sucrose + 0.8 % (w/v) agar] containing kanamycin (300 mg/L) and sodium cefotaxime (250 mg/L). RNA were extracted from the regenerated tobacco leaves. Successful transformation was confirmed by RT-PCR analyses with the gene-specific primers.

Evaluation of transgenic tobacco lines

The transgenic and wild-type (WT) tobacco plants were transplanted into plastic pots (15 × 20 cm) containing perlite and vermiculite (1:1 v/v). Six replicates for each line were grown in a greenhouse at 25 °C for 3 weeks. For the Cd treatment, 100 mL 0.9 mM $CdCl_2$ was added to each pot. After 1 month, the plants were analyzed to determine their Cd content and Cd resistance based on phenotypic and physiological indices.

To measure electrolyte leakage, 10 leaf discs (1 cm in diameter) were collected from leaves of each tobacco line, immersed in 40 mL deionized water, and shaken overnight. The electrical conductivity of the solution was measured (R1). The solutions containing the leaves were then boiled for 15 min, shaken overnight, and then the total conductivity was determined (R2; maximum conductivity of the tissue). Relative electrolyte leakage (REL) was calculated using the formula REL = R1/R2 × 100 %.

Malondialdehyde (MDA) levels were estimated by the thiobarbituric acid (TBA) method. Briefly, 0.2 g leaf tissue was homogenized in 1 mL of 10 % trichloroacetic acid (TCA). The mixture was centrifuged, and then the supernatant was collected and mixed with 0.6 % TBA. The mixture was boiled for 10 min, immediately cooled on ice, and then centrifuged. Absorbance of the supernatant at 450, 532, and 600 nm was measured. The MDA content was calculated as follows: $(6.45(A_{532}-A_{600}) - 0.56A_{450})$ × total extract volume (mL)/total fresh weight of sample (g).

To estimate chlorophyll content, 0.1 g leaf tissue was immersed in 10 mL dimethylsulfoxide (DMSO) in the dark for 2 days. The absorbance of the solution at 645 and 663 nm was measured. Chlorophyll *a* and chlorophyll *b* contents were calculated using the following formulae: $Chla = (0.0127 \times A_{663} - 0.00269 \times A_{645})$ × total extract volume (mL)/total fresh weight of sample (g); $Chlb = (0.0029 \times A_{645} - 0.00468 \times A_{663})$ × total extract volume (mL)/total fresh weight of sample (g).

Root activity was determined by TTC (2,3,5-triphenyltetrazolium chloride) method. 0.1 g cleansed roots was incubated in 2.5 mL of 0.4 % TTC for 24 h in the dark. After rinsing with distilled water and drying out with filter paper, the samples were putting into 5 mL 95 % ethanol at 60 °C for 4 h. Absorbance at 490 nm was measured. The tripheny formazan (TTF) content was inferred with the standard curve and computed for the root vitality by TTF content/root dry weight (g).

Total superoxide dismutase activity was estimated with a Total Superoxide Dismutase (SOD) Kit (Jiancheng, Nanjing, China) following the manufacturer's instructions.

Detection of cadmium content in plant tissues

The Cd content in transgenic and WT tobacco was measured after the 1 month $CdCl_2$ treatment. Leaf or root samples were washed in distilled water three times, and then dried at 105 °C for 30 min and then at 80 °C for 48 h. The dry tissues were ground and digested in $HNO_3/HClO_4$ (4:1, v/v) at 100 °C for 20 min, and then heated at 190 °C for 60 min until the liquid evaporated. The digest was dissolved in deionized water and the Cd^{2+} content was measured using an Agilent 7500 ICP-MS instrument (Agilent Technologies Inc, USA). The following formula was used to calculate Cd content: (C × 0.1L)/M, where C is the Cd concentration detected by the instrument and M is sample dry weight (g). The Cd transfer coefficient was calculated as follows: Cd content in leaves/Cd content in roots.

Statistical analysis

Data were statistically analyzed by single-factor ANOVA using SAS software (SAS Institute, Cary, NC, USA; PROC

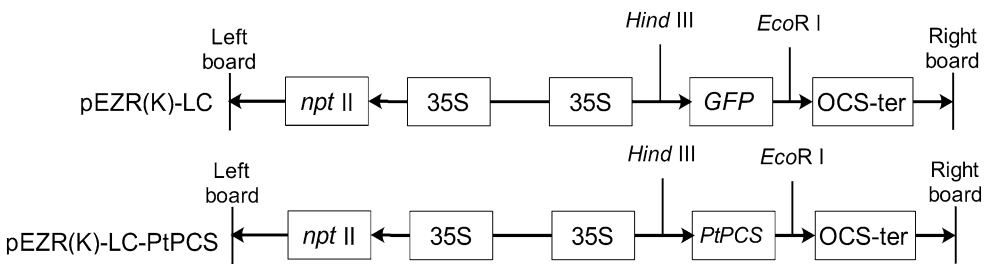


Fig. 1 Construction of recombinant expression vector pEZR(K)-LC-PtPCS. The *upper* is the original expression vector and the *lower* is the recombinant vector where the *GFP* gene was replaced by *PtPCS* gene

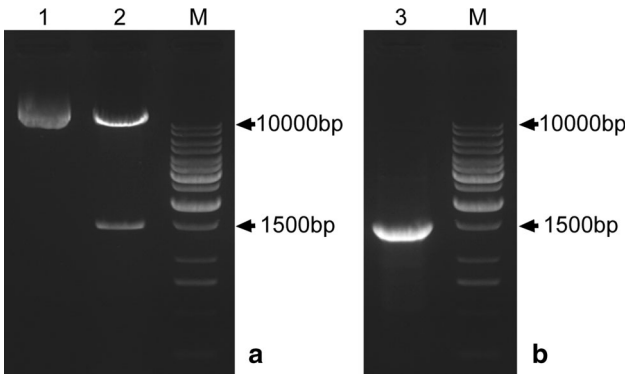


Fig. 2 Identification of the recombinant expression plasmid pEZR(K)-LC-PtPCS. *Lane 1* in **a** undigested plasmid of pEZR(K)-LC-PtPCS; *Lane 2* in **a** double digested plasmid of pEZR(K)-LC-PtPCS with H*ind* III and E*co*R I; *Lane 3* in **b** PCR amplification of the plasmid pEZR(K)-LC-PtPCS with *PtPCS* specific primer; M, 1 kb DNA Marker (Thermo Fisher Scientific Inc. USA)

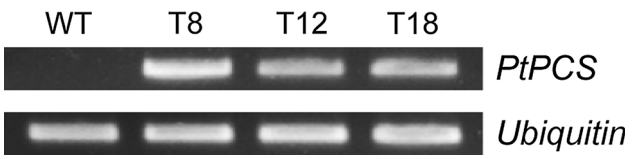


Fig. 3 Semi-quantitative RT-PCR analysis of *PtPCS* gene expression in transgenic tobacco lines.The PCR was conducted with the cDNA template from mature tobacco leaves of wild type (WT) and transgenic plants (T8, T12 and T18) by using the specific primers for *PtPCS* gene or reference *Ubiquitin* gene

ANOVA). The gene effect on tested parameters were evaluated by *t* test and least significant difference (LSD) at 0.05 and 0.01 probability levels.

Results

Cloning of *PtPCS* and construction of expression vector

The full-length 1512-bp *PtPCS* cDNA was amplified from poplar (*P. tomentosa*) cDNA using a pair of adaptor primers incorporating H*ind* III and E*co*R I sites. The cloned fragment was confirmed by sequencing. The pEZR(K)-LC expression plasmid was double-digested with restriction enzymes, and then ligated as the recombinant expression vector pEZR(K)-LC-PtPCS in which the *GFP* fragment of pEZR(K)-LC was replaced by *PtPCS* (Fig. 1). A transformed *A. tumefaciens* clone was identified using the plasmid double-digestion test (Fig. 2a).

Screening of transgenic plants

The leaves of tobacco were cut and infected with *A. tumefaciens* strain LBA4404 containing the recombinant plasmid pEZR(K)-LC-PtPCS. After initial culturing in the dark, differentiation, and regeneration, three regenerated healthy seedlings were obtained and were confirmed to contain *PtPCS* transcripts by semi-quantitative RT-PCR analysis (Fig. 3).

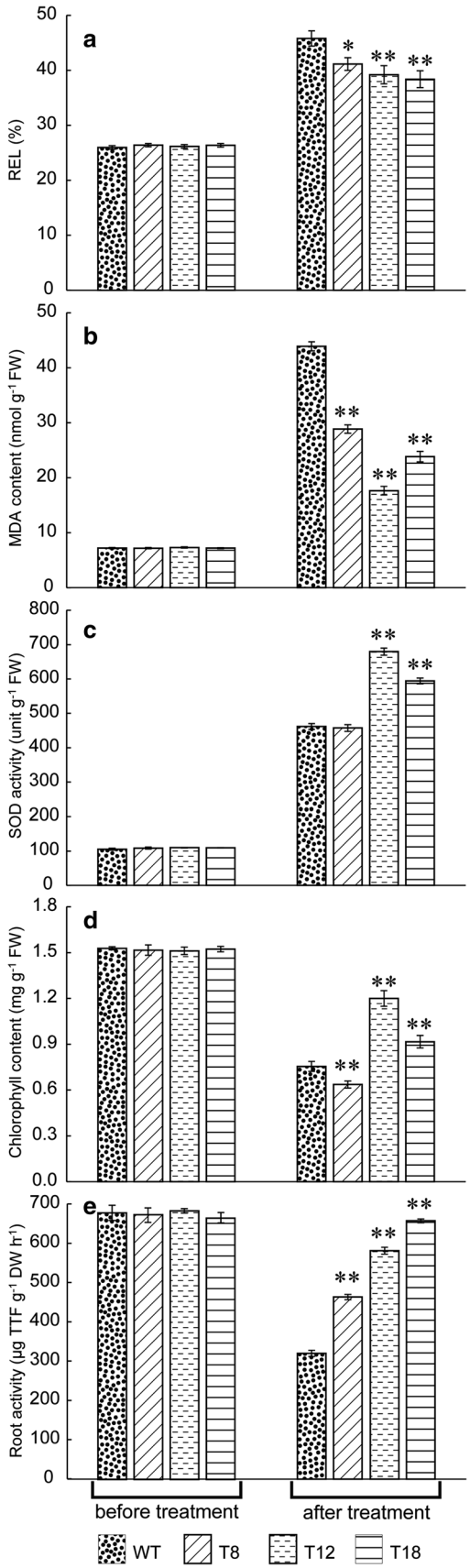
Fig. 4 The shoot **a** and relative root **b** performance of the wild type (WT) and transgenic tobacco plants (T8, T12, T18) under Cd stress of 90 μmol per pot for 1 month

Phenotyping of transgenic tobacco lines under cadmium stress

After a 1 month Cd treatment, WT plants showed slower growth and wilting of lower leaves, while the transgenic plants showed better growth performance (Fig. 4a). The WT plants also showed poor root performance, embodied in much more brown senile roots, one-third to a half of the biomass when compared to the transgenic plants. The 124-mm root length of WT was just two-thirds that of T12 and T18 (Fig. 4b). Among the transgenic lines, T8 was more sensitive to Cd than were the other two lines, and showed some symptoms of Cd toxicity. All of the transgenic lines were more Cd tolerant than were WT plants.

Physiology of transgenic plants in response of cadmium stress

The physiological and biochemical parameters of the transgenic and WT plants were measured before and after



◀**Fig. 5** Physiological analyses of transgenic (T8, T12, T18) and wild-type tobacco plants before and after a 1 month cadmium treatment. The physiological parameters were measured with the leaf or root tissues sampled before or after cadmium treatment (90 μmol $CdCl_2$ per pot) for 1 month. Data are expressed as mean ± SD of six independent experiments. *Asterisk* and *double asterisk* indicates values that differ significantly from WT at $P < 0.05$ and $P < 0.01$ according to LSD *t* test (SAS PROC ANOVA). **a** The leaf Relative electrolyte leakage (REL); **b** The leaf MDA content; **c** The leaf SOD content; **d** The leaf Chlorophyll content; **e** The root activity

the 1 month Cd treatment. A variance analysis showed that there were no significant differences between transgenic and WT lines before the Cd treatment, while the transgenic lines showed significant differences from WT plants after the Cd treatment in four leaf parameters and root activity tested (Fig. 5). After the Cd treatment, the REL, MDA content, and SOD activity of leaves in transgenic line T12 were 39.19 %, 17.63 nmol g^{-1}, and 679.64 unit g^{-1}, respectively, compared with 45.80 %, 43.90 nmol g^{-1}, and 461.64 unit g^{-1} in WT (Fig. 5a–d). Compared with root activity after Cd stress treatment, the range of value in transgenic plants was from 463.6 to 657.7 μg TTF g^{-1} DW h^{-1}, which had a significant difference with 320.2 μg TTF g^{-1} DW h^{-1} in WT (Fig. 5e).The results of the physiological analyses indicated that expression of *PtPCS* strongly enhanced the Cd-tolerance of the transgenic lines, consistent with their morphological appearance.

Cadmium content in transgenic lines and wild type

After a 1 month Cd treatment, the Cd content was higher in transgenic lines than in WT (Table 1). This result suggested that expression of *PtPCS* resulted in enhanced Cd accumulation in tobacco plants. The Cd content in leaves of transgenic lines was 1.24- to 2.28-fold than that in leaves of WT, and the Cd content in roots of transgenic lines was 1.7- to 3-fold than that in roots of WT. In both WT and transgenic plants, the Cd concentration was higher in roots than in leaves. The Cd transfer coefficient was markedly lower in transgenic lines than in WT plants..

Discussion

Phytochelatins can chelate heavy metal ions in the cytoplasm, thereby reducing their toxic effects and protecting normal cellular metabolism (Shukla et al. 2012). Some PC-heavy metal complexes accumulate in the vacuole through an ATP-dependent process (Park et al. 2012; Lin et al. 2012). This effectively stores heavy metals away from sensitive cellular targets (Vögeli-Lange and Wagner 1990; Salt and Rauser 1995; Li et al. 1997; Benavides et al. 2005;

Table 1 Cadmium content and transfer coefficient of wild type tobacco (WT) and transgenic lines (T8, T12, T18) under Cd^{2+} stress (90 μmol $CdCl_2$ per pot for 1 month)

Tobacco line	Cadmium content		Transfer coefficient[a]
	Root (μg/g dry weight)	Leaf (μg/g dry weight)	
WT	5.22 ± 0.38	4.32 ± 0.03	0.83 ± 0.06
T8	15.62 ± 0.27**	9.87 ± 0.13**	0.63 ± 0.01**
T12	9.48 ± 0.29**	5.37 ± 0.27**	0.57 ± 0.02**
T18	8.95 ± 0.17**	6.36 ± 0.24**	0.71 ± 0.02*

Data are expressed as mean ± SD of six independent experiments

* and ** Significance level at $P < 0.05$ or $P < 0.01$ based on t test (SAS PROC TTEST) between the transgenic and WT line

[a] The transfer coefficient was calculated by leaf cadmium contents/root cadmium content for each line

Migocka et al. 2011). Consequently, *PCS*, which encodes the key PC biosynthesis enzyme, is thought to be a key gene in tolerance and accumulation of heavy metal ions in plant tissues. In the present study, transgenic plants overexpressing *PtPCS* from poplar showed enhanced growth performance under Cd stress in comparison with WT tobacco plants, or higher Cd tolerance based on the physiological analysis. The transgenic plants also showed the higher accumulation of Cd, up to 2.28-fold or 3-fold than that in leaves or roots of WT. Some other reports also provided the evidences for the PCS function. For example, the transgenic tobacco plants expressing the *AtPCS* gene from *Arabidopsis* were with 2.2–2.75 times longer roots and double Cd content than those of WT after cadmium treatment (Pomponi et al. 2006). The transgenic tobacco expressing *CdPCS1* from *Ceratophyllum demersum* accumulated Cd to a level 6-fold higher than that in WT when grown medium with Cd (300 μM) (Shukla et al. 2012, 2013). The differences in heavy metal tolerance and accumulation level among various transgenic plants might be due to the sequence variation of *PCS* genes from various sources (Zhao et al. 2014), and/or the differences in downstream Cd-PC complexes processing (Gasic and Korban 2007b). Recently, a study on *Pid3* orthologs in rice revealed that the sequence variation caused the different resistance responses to *Magnaporthe oryzae* (Xu et al. 2014). It suggested that the extensive mining of *PCS* orthologs would be valuable for genetic engineering programs aimed at producing effective phytoremediation. Also, the research here mentioned that the transfer coefficient of Cd from underground to ground was markedly lower in transgenic lines than in WT. The similar results were also reported by Pomponi et al. (2006). These findings suggest that PtPCS functions were limited in Cd tolerance and accumulation, but not in Cd transport.

Even all the transgenic lines showed better cadmium tolerance and accumulation in comparison with wild type plants, a little difference was revealed between T8 and the transgenic line. Obviously, T8 accumulated the higher Cd concentration in leaf (about 1.75 folds) and root (about 1.84 folds). It suggested that there might be an extra copy of the gene in the T8 line. RT-PCR results also provide the evidence of that (Fig. 3). The high level of Cd accumulation in the tissues may have negatively affected growth. This could explain why the T8 line was more sensitive to Cd than other transgenic lines, as reflected by its poor growth and physiological appearance. These results suggested a threshold Cd concentration existed in plant cells. Excess value would be adversely harmful for plant growth. Therefore, Cd tolerance and accumulation, as well as Cd transport, should be considered as a whole in genetic engineering programs aimed at producing effective phytoremediation.

Conclusions

The present work showed that the overexpression of *PtPCS* gene in tobacco not only improves the tolerance but also increases Cd accumulation in plants relative to wild type plants, even excess accumulation of the cadmium might be harmful to plant growth. It suggested *PtPCS* as a candidate gene for heavy metal bioremediation or phytoremediation.

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