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ORIGINAL ARTICLE

Tobacco phytochelatin synthase (NtPCS1) plays important roles in cadmium and arsenic tolerance and in early plant development in tobacco

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Abstract Phytochelatin synthase (PCS) catalyzes the synthesis of phytochelatins, which are involved in heavy metal detoxification in plants and other living organisms. Previously, we cloned a *PCS1* gene from tobacco (*Nicotiana tabacum*) and showed that its expression in yeast (*Saccharomyces cerevisiae*) resulted in increased cadmium (Cd) tolerance and Cd accumulation (Kim et al., J Plant Biol 48:440–447, 2005). To examine the role of NtPCS1 in tobacco, we generated transgenic tobacco lines over-expressing *NtPCS1* in the sense or antisense direction. Compared with other *PCS1*-expressing plants, *NtPCS1*-expressing tobacco exhibited a unique phenotype: increased tolerance to cadmium and arsenite, but no change in cadmium and arsenic accumulation. In addition, the *antisense-NtPCS1* tobacco lines showed growth retardation in the early stage, suggesting that phytochelatin also plays a role in plant development. These results demonstrate that NtPCS1 plays important roles in metal(loid) tolerance as well as in growth and development in tobacco.

Keywords Phytochelatin synthase · Cadmium · Arsenic · Development · *Nicotiana tabacum*

Introduction

Toxic heavy metals such as cadmium (Cd) and arsenic (As) can be detoxified by binding to three classes of peptides: glutathione (GSH), metallothioneins (MTs), and phytochelatins (PCs). All of these peptides have been implicated in heavy-metal homeostasis in plants (Vatamaniuk et al. 1999). Among them, PCs constitute a family of peptides with the general structure $(\gamma\text{-Glu-Cys})_n$-Gly, where $n = 2$–11. The PCs contain a high percentage of Cys sulfhydryl residues, which bind to heavy-metal ions such as Cd^{2+} with high affinity, and sequester them in stable complexes. The PCs localize together with Cd^{2+} in the vacuole of intact cells and strongly contribute to Cd^{2+} detoxification in plants (Vogeli-Lange et al. 1990). Both arsenate and arsenite have been reported to trigger the accumulation of PCs in plants (Grill et al. 1987; Maitani et al. 1996). The presence of As induces PCs and results in the accumulation of As–PC complexes, suggesting that PCs also play a role in detoxifying As in plants (Schmöger et al. 2000).

There are structural similarities between PCs and GSH. Induction of PCs in the presence of Cd coincided with a transient decrease in the levels of GSH. In plants, GSH is synthesized from its constituent amino acids in two sequential, ATP-dependent enzymatic reactions catalyzed by γ-glutamyl-Cys synthetase and glutathione synthetase (GS), respectively. Phytochelatin synthase (γ-glutamyl-cysteine dipeptidyl transpeptidase; EC 2.3.2.15) subsequently catalyzes the elongation of $(\gamma\text{-Glu-Cys})_n$ by transferring a γ-Glu-Cys group to GSH or to PCs (Zenk 1996; Chen et al. 1997).

Several previous studies have focused on the role of PCS1 in metal tolerance in plants. *PCS1* genes from eight plant species have been expressed in five different plant species. Five different *PCS1* genes have been expressed in *Nicotiana tabacum*; *AtPCS1* from *Arabidopsis thaliana* (Pomponi et al. 2006; Wojas et al. 2010a, b; Brunetti et al. 2011), *SpPCS1* from *Schizosaccharomyces pombe* (Wawrzynski et al. 2006), *CePCS* from *Caenorhabditis*

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elegans (Wojas et al. 2010a, b), *TcPCS1* from *Thlaspi caerulescens* (Liu et al. 2011), and *CdPCS1* from *Ceratophyllum demersum* (Shukla et al. 2012). Four different *PCS1* genes have been expressed in *A. thaliana*: *AtPCS1* (Lee et al. 2003; Li et al. 2004; Brunetti et al. 2011), *AsPCS1* from garlic (*Allium sativum*) (Guo et al. 2008), *NnPCS1* from sacred lotus (*Nelumbo nucifera*) (Liu et al. 2012), and *CdPCS1* from *C. demersum* (Shukla et al. 2013). *AtPCS1* was expressed in *Brassica juncea* (Gasic and Korban 2007), *TaPCS1* from wheat (*Triticum aestivum*) was expressed in *Nicotiana glauca* (Gisbert et al. 2003), and *TaPCS1* was expressed in *Oryza sativa* (Wang et al. 2012). To date, 16 types of transgenic plants expressing *PCS1* have been reported (Table 1). In this study, to explore the role of endogenous *NtPCS1* in metal detoxification in tobacco, we generated transgenic tobacco over-expressing or under-expressing the *NtPCS1* gene and investigated their tolerance to, and accumulation of, Cd and As.

Materials and methods

Plasmid construction

NtPCS1 was subcloned into the *Not*I-digested vector pFL1 and then digested with *Xba*I and *Eco*RI. The resulting 1.5-kb *Xba*I–*Eco*RI fragment was subcloned into pBI121.

Plant materials and transformation

Leaves of tobacco (SR1) plants grown in sterile agar medium were used for leaf disc transformation. The binary vector pBI121 containing *NtPCS1* under the control of the CaMV 35S promoter was transformed into *Agrobacterium tumefaciens* strain LBA4404 by the freeze–thaw method. Tobacco leaf discs were transformed with *A. tumefaciens* as described by Horsch et al. (1986). Shoots and roots were induced on solid MS medium containing 50 μg/mL kanamycin sulfate (Duchefa Co., Haarlem, The Netherlands), transferred to soil, and grown to maturity in growth chambers under a 16L/8D photoperiod at 23 °C.

RT-PCR analysis

mRNA was purified from total RNA using a PolyATtract mRNA isolation kit (Promega, Madison, WI, USA), and RT-PCR was performed with an RT-PCR kit (Takara, Dalian, China) (Kim et al. 2006). The *NtACT* gene encoding *N. tabacum* actin was used as the loading control. Specific primers used in RT-PCR were as follows: *NtACT*, 5′-TATTGTGTTGGACTCTGG-3′ (forward) and 5′-CTG CTGGAATGTGCTAAG-3′ (reverse); *NtPCS1*, 5′-GCGA GGATCCATGGCGATGGCGGGTTT-3′ (forward) and 5′-GAAACTCGAGCTAGAAGGGAGGTGCAG-3′ (reverse). The ORF region of each gene was amplified in the RT-PCR analyses.

Table 1 Comparison of metal tolerance and level in various *PCS1*-expressing plants

Plants	*PCS*	Cd Tol	Cd level	As Tol	As level	Ref.
N. tabacum	*NtPCS1*	+	0	+	0	Lee (submitted)
	CdPCS1	+	+	ND	+	Shukla (2012)
	AtPCS1	+	ND	ND	ND	Brunetti (2011)
	TcPCS1	+	+	ND	ND	Liu (2011)
	AtPCSl	−	0	ND	ND	Wojas (2010a)
	CePCS	+	− (L)	ND	ND	Wojas (2010b)
	AtPCS1	ND	ND	+	+	Wojas (2010a)
	CePCS	ND	ND	+	+	Wojas (2010a)
	SpPCS1	0	0 (L)	ND	ND	Wawrzynski (2006)
	AtPCS1	+	+	ND	ND	Pomponi (2006)
A. thaliana	*CdPCS1*	0	+	0	+	Shukla (2013)
	NnPCS1	ND	+	ND	ND	Liu (2012)
	AtPCS1	+(h), −(l)	ND	ND	ND	Brunetti (2011)
	AsPCS1	+	+	+	+	Guo (2008)
	AtPCS1	−	0	+	0	Li (2004)
	AtPCSl	−	ND	ND	ND	Lee (2003)
B. juncea	*AtPCS1*	+	−	+	0	Gasic (2007)
O. sativa	*TaPCS1*	−	+	ND	ND	Wang (2012)
N. glauca	*TaPCS1*	+	ND	ND	ND	Gisbert (2003)

+ Increase, − decrease, *ND* no data, (*L*) leaf, (*h*) high concentration, (*l*) low concentration. Ref (reference) indicates the first author of the articles

Western blot analysis

Transgenic plants were harvested and homogenized in extraction buffer (20 mM Tris–HCl, pH 8.0, 1 mM DTT, 0.3 mM EDTA, and protease inhibitor cocktail). Total protein (20 μg) was separated on SDS-PAGE gels, transferred to nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences, Little Chalfont, UK) and hybridized with an NtPCS1 antibody (1/1000 dilution with 1 % v/v Tween 20) followed by goat anti-rabbit horseradish peroxidase-conjugated IgG (Amersham Biosciences) as a secondary antibody. Enhanced chemiluminescence (Amersham Biosciences) was used for detection. The Coomassie blue-stained Rubisco large subunit was used as the loading control. The NtPCS1 antibody was produced and supplied by Peptron Co. (Daejun, Korea). The specific peptide sequence CIDPGRKWKGPWRW-NH_2 of NtPCS1 was designed and synthesized considering hydrophobicity, antigenicity, and cross-reactivity, and used to produce the antibody.

Assessment of Cd and As tolerance

Seeds of transgenic tobacco lines (homozygous T_3) were surface sterilized, and then germinated and grown on ½ MS agar medium containing 50 μM cadmium sulfate or 25 μM sodium arsenite for 3 weeks. This concentration was designed to give an approximately 50 % reduction in the fresh weight of wild-type seedlings. Individual seedlings were harvested from each plate, washed three times with ice-cold 5 mM $CaCl_2$ and distilled water, and the attached agar and water were removed. The fresh weight of each seedling from three plates was measured ($n = 90$). Metal tolerance is expressed as relative fresh weight (fresh weight in the presence of the metal divided by fresh weight in its absence). The tobacco seedlings were photographed after 3 weeks. These experiments were performed three times.

Measurement of cadmium and arsenic concentrations in tobacco plants

Seeds of transgenic tobacco homozygous lines (homozygous T_3) were surface sterilized and then germinated and grown on ½ MS agar medium containing 50 μM cadmium sulfate or 25 μM sodium arsenite for 3 weeks. All the seedlings on each plate were harvested, washed three times with ice-cold 5 mM $CaCl_2$, and dried for 3 days at 60 °C. Dried samples (1.0 g) were digested with concentrated HNO_3 and $HClO_4$ in a Teflon digestion vessel (Savillex, Minnetonka, MN, USA). The Cd and As contents were measured in triplicate by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Perkin Elmer Optima 4300 DV, USA) at a wavelength of 228.802 nm (for Cd) and 188.979 nm (for As) at the Korea Basic Science Institute. Three independent cultures were used to calculate average metal concentrations in tobacco tissues.

Statistical analysis

Data were analyzed by analysis of variance using SAS (version 9.1), and mean values were compared using the Tukey's multiple comparisons with the family confidence coefficient 0.95.

Results and discussion

Generation of transgenic tobacco over-expressing or under-expressing *NtPCS1*

To explore the roles of *NtPCS1* (Kim et al. 2005) in metal detoxification in plants, we generated transgenic tobacco expressing *NtPCS1* in the sense or antisense direction. Thirteen *NtPCS1-sense* plants and three *NtPCS1-antisense* plants were obtained, and three *NtPCS1-sense* plants (s-11, 13, and 17) and two *NtPCS1-antisense* plants (as-2 and 3) were selected for further analyses. RT-PCR analyses confirmed that the transcript level of *NtPCS1* in *NtPCS1-sense* plants was higher than that in the control plant harboring the empty vector pBI121 and that the mRNA level of *NtPCS1* in *NtPCS1-antisense* plants was suppressed (Fig. 1a). To compare protein levels of NtPCS1 among transgenic tobacco plants, soluble proteins extracted from 3-week-old plants were subjected to a western blot analysis. Using the NtPCS1-antibody, we identified that the protein level of NtPCS1 was higher in *NtPCS1-sense* plants than in the control plants and that the NtPCS1 protein was not detectable in *NtPCS1-antisense* plants (Fig. 1b).

NtPCS1-overexpressing tobacco plants showed enhanced Cd tolerance

To examine the effects of *NtPCS1* over-expression on Cd tolerance and accumulation, we measured changes in fresh weight and Cd concentrations in transgenic tobacco lines grown on media containing 50 μM Cd(II) for 3 weeks. As shown in Fig. 2a, b, the tolerance rates of *NtPCS1*-expressing tobacco lines s-11, s-13, and s-17 were 80.6, 70.7, and 71.5 %, respectively, whereas that of the control plant was 43.9 %. However, the Cd concentration in *NtPCS1*-tobacco (10.7–10.8 μmol/g DW) was similar to that in control plants (12.2 μmol/g DW) (Fig. 2c). These results suggested that NtPCS1 plays an important function in Cd tolerance, but probably not in Cd accumulation in tobacco.

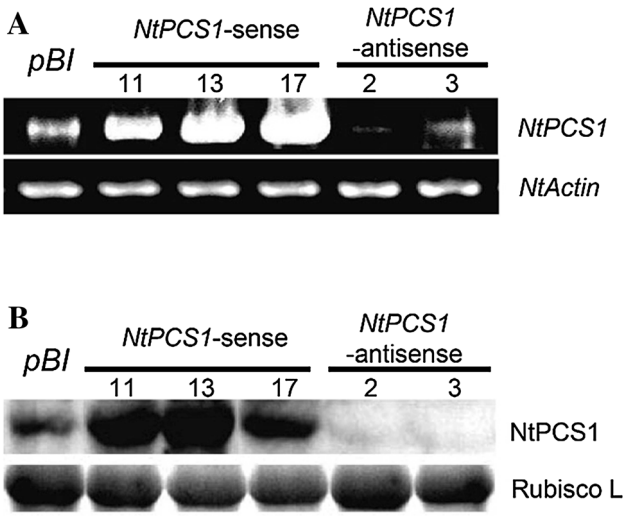

Fig. 1 Expression level of *NtPCS1* in transgenic *Nicotiana tabacum*. **a** RT-PCR analysis showing transcript levels of *NtPCS1* in transgenic plants. *Actin* transcript level was used as loading control. **b** Western blot analysis showing amount of NtPCS1 protein in transgenic plants. Rubisco L (large subunit) was used as loading control

Among the 17 types of *PCS1*-expressing plants, including that described in the present study, 10 showed increased tolerance to Cd (Table 1). In four instances, the plants showed decreased Cd tolerance: over-expression of *AtPCS1* in *Arabidopsis* (Lee et al. 2003; Li et al. 2004) and *N. tabacum* (Wojas et al. 2010b), and expression of *TaPCS1* in *Oryza sativa* (Wang et al. 2012). Cadmium tolerance was unchanged in two instances.

Our result that *NtPCS1* expression increased Cd tolerance but did not affect Cd accumulation is new, compared with results previously reported for *N. tabacum* expressing *PCS1*. As shown in Table 1, among transgenic *N. tabacum* lines expressing *PCS1* genes, there were three instances of increased Cd tolerance and increased Cd accumulation [tobacco plants expressing *AtPCS1* (Pomponi et al. 2006), *TcPCS1* (Liu et al. 2011), and *CdPCS1* (Shukla et al. 2013)]. *SpPCS1*-expressing tobacco did not show changes either in Cd tolerance or Cd accumulation (Wawrzynski et al. 2006). Interestingly, Cd tolerance was decreased in *AtPCS1*-expressing *N. tabacum*, but its Cd concentration was unchanged (Wojas et al. 2010b).

Expression of *NtPCS1* increased arsenite tolerance in transgenic tobacco

Next, we determined the effects of *NtPCS1* expression on As(III) tolerance and accumulation in plants. The tolerance rates of *NtPCS1*-expressing tobacco lines s-11, 13, and 17 were higher (45.1, 36.9, and 37.5 %, respectively) than that

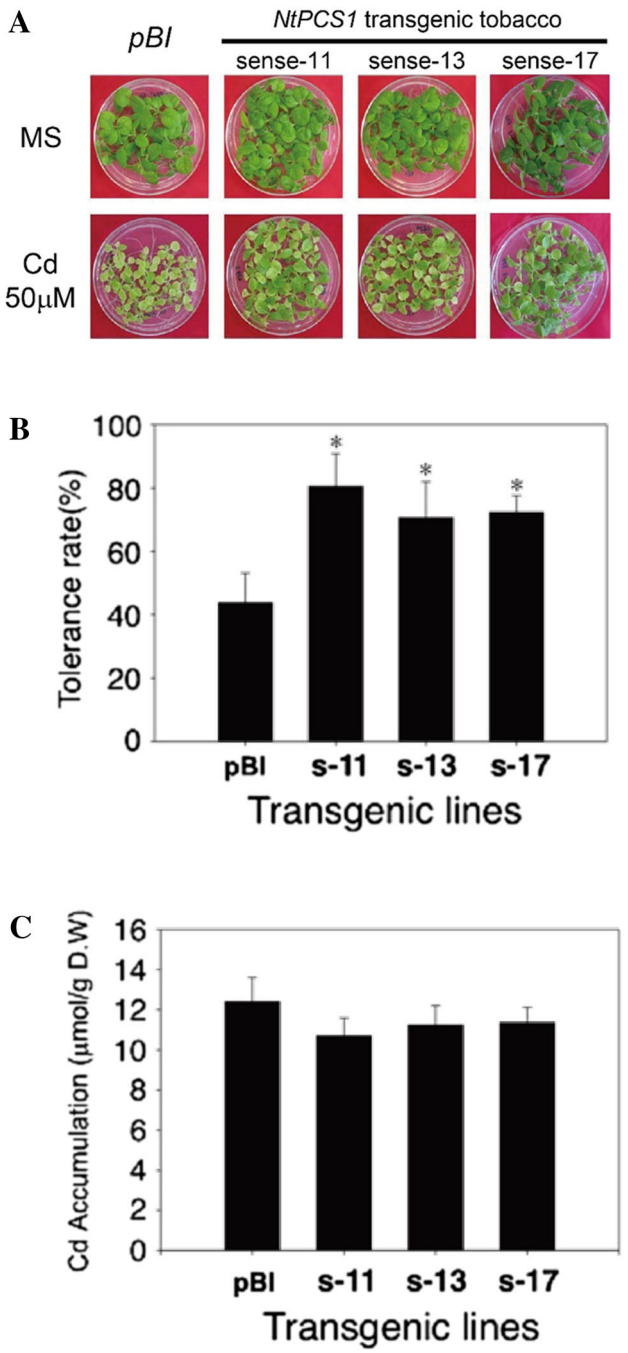

Fig. 2 Over-expression of *NtPCS1* promotes cadmium tolerance in transgenic plants. **a** Photographs show different Cd tolerance phenotypes of transgenic tobacco germinated and grown for 3 weeks on ½ MS agar media. **b** Relative Cd tolerance rates of transgenic tobacco. Metal tolerance is expressed as relative fresh weight (fresh weight in the presence of 50 μM Cd divided by fresh weight in its absence). Fresh weight of each plant (3 weeks old) was measured ($n = 90$). **c** Cadmium concentrations in transgenic tobacco; Cd concentrations were not significantly different between *NtPCS1-sense* tobacco and control plants. Each value corresponds to mean ± SE ($n = 3$); asterisk indicates significant difference ($P < 0.05$) from control plant (pBI)

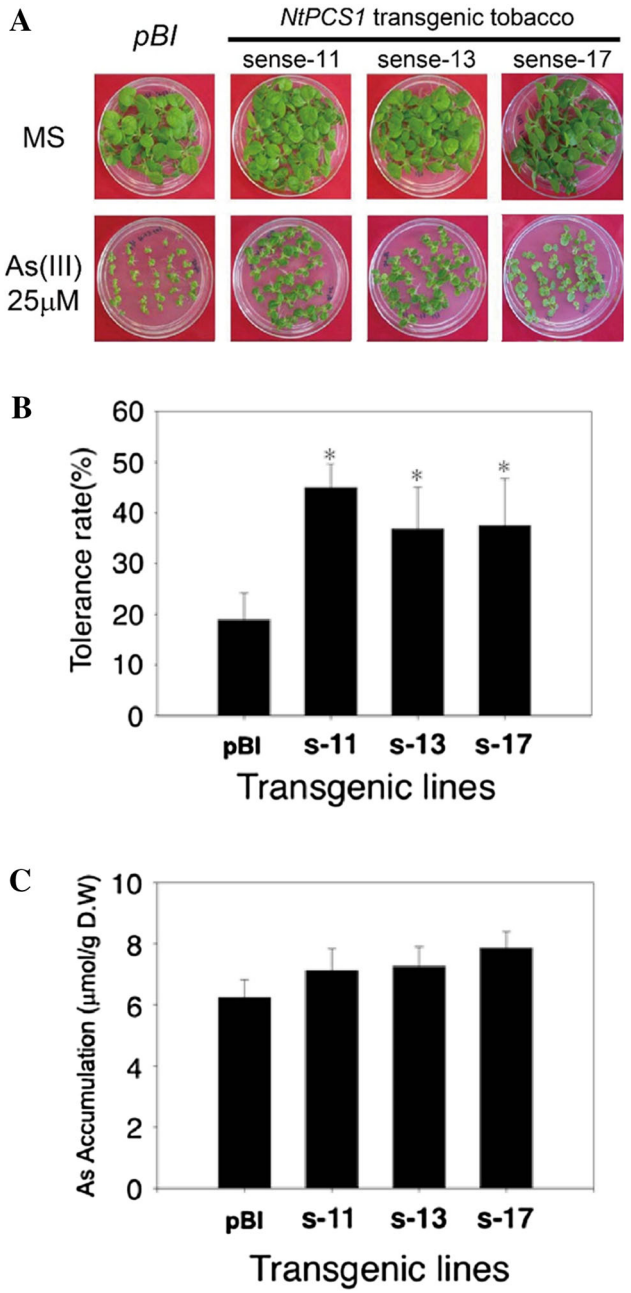

Fig. 3 *NtPCS1*-expressing transgenic tobacco shows increased arsenic tolerance. **a** Photograph of transgenic tobacco germinated and grown for 3 weeks on media with or without 25 μM arsenite. **b** Arsenic tolerance is expressed as relative fresh weight (fresh weight in the presence of 25 μM arsenite divided by fresh weight in its absence). Fresh weight of each plant (3 weeks old) on three plates was measured ($n = 90$). **c** Arsenic level in *NtPCS1*-expressing tobacco was not significantly different from that in the control plant. Each value corresponds to mean ± SE ($n = 3$); *asterisk* indicates significant difference ($P < 0.05$) from the control plant (pBI)

of the control plant (19.0 %) (Fig. 3a, b). However, the As concentration in transgenic lines was not significantly different from that in the control plant (Fig. 3c). This is the first report showing that expression of its own *PCS1* enhances As tolerance in tobacco although a previous study showed that expression of *AtPCS1* and *CePCS1* also increased As tolerance in tobacco (Wojas et al. 2010a).

Previous studies have shown that PCS1 is involved in As tolerance; for example, As tolerance was enhanced in *S. cerevisiae* expressing *AtPCS1* (Vatamaniuk et al. 1999), *TaPCS1* from wheat (Wysocki et al. 2003), and *NtPCS1* from tobacco (Kim et al. 2005). Additionally, the As concentration was increased in *AtPCS1*-expressing *Escherichia coli* grown on medium containing 20 μM arsenate (Sauge-Merle et al. 2003) and in *NtPCS1*-expressing *S. cerevisiae* DTY167 (Kim et al. 2005). Several previous studies have analyzed tolerance to, and accumulation of, As in higher plants expressing *PCS1* genes. As shown in Table 1, transgenic tobacco expressing *AtPCS1* or *CePCS1* and *Arabidopsis* expressing *AsPCS1* from garlic (*Allium sativum*) showed increased As tolerance and greater As accumulation (Wojas et al. 2010a; Guo et al. 2008). In contrast, *Arabidopsis* and *Brassica juncea* expressing *AtPCS1* showed increased As tolerance, but no change in As accumulation (Table 1; Li et al. 2004; Gasic and Korban 2007). Those results are similar to our findings, in that As tolerance was enhanced but the As concentration was unchanged in *NtPCS1*-expressing tobacco (Figs. 3, 4). To summarize all of the results from previous relevant studies, 10 out of 17 PCS1-expressing plants (58.8 %) showed increased Cd tolerance, and six out of seven PCS1-expressing plants (85.7 %) showed increased As tolerance.

Suppression of *NtPCS1* inhibits plant growth at an early stage

Antisense-NtPCS1 tobacco plants had very low levels of *NtPCS1* mRNA and protein, compared with those in the control plant (Fig. 1). Interestingly, *antisense-NtPCS1* plants showed growth-retarded phenotypes at an early stage up to 3–4 weeks after germination. Compared with the control, the *antisense-NtPCS1* plants showed a 3- to 4-day delay in germination, slow growth, and elongated leaves (Fig. 4a, b). The fresh weight of *antisense-NtPCS1* plants was 73 % lower than that of control plants, but they recovered to the normal growth rate after 3–4 weeks. This growth-retarded phenotype is unique, because *Cad1*, an *Arabidopsis* mutant deficient in PC synthase activity, was sensitive to Cd but showed normal growth rate and phenotype (Howden et al. 1995). Our results imply that NtPCS1 may be involved in plant development as well as in metal detoxification. To date, PC synthase has been implicated in metal detoxification and tolerance to ultraviolet-B radiation. However, our study is the only one to date that suggests it also plays roles in plant development.

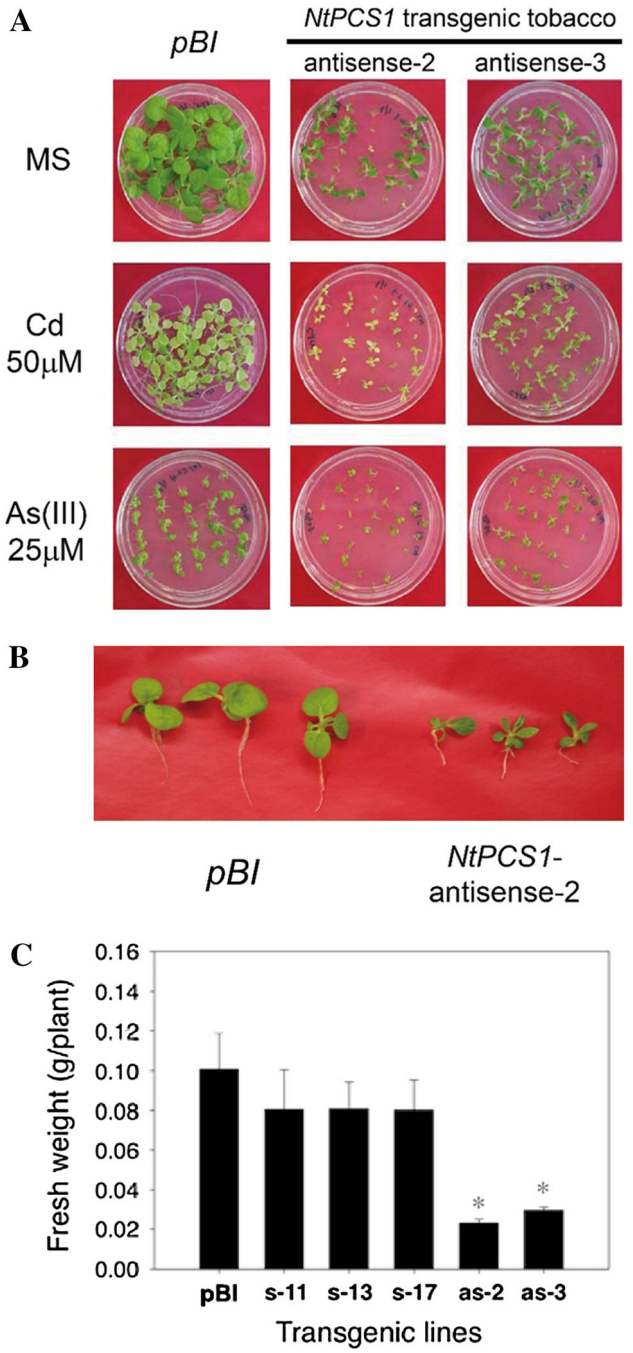

Fig. 4 Phenotypes of *NtPCS1-antisense* transgenic plants. **a** Control (pBI) and transgenic plants (*NtPCS1-antisense* plants; as-2 and 3) were germinated and grown on ½ MS media with or without heavy metals (50 μM Cd or 25 μM As) for 3 weeks. **b** Growth-retarded phenotype of *NtPCS1-antisense* plants. Figure shows 2-week-old control plants and *NtPCS1-antisense* plants grown on ½ MS medium. **c** Fresh weights of control, *NtPCS1-sense*, and *NtPCS1-antisense* plants germinated and grown for 3 weeks on ½ MS media ($n = 90$). Each value corresponds to mean ± SE ($n = 3$); *asterisk* indicates significant difference ($P < 0.05$) from control plants (pBI, s-11, 13, 17)

Table 2 Relationship between metal tolerance and metal level

Cd Tol	[Cd]	As Tol	[As]	% (Plant #)
+	+			33 (4/12)
+	−			17 (2/12)
−	+			8 (1/12)
+	0			8 (1/12)
0	+			8 (1/12)
0	0			8 (1/12)
–	0			17 (2/12)
		+	+	43 (3/7)
		+	0	43 (3/7)
		0	+	14 (1/7)

Cd Tol Cadmium tolerance, *[Cd]* Cd level, *As Tol* arsenic tolerance and *[As]* As level

Relationship between metal tolerance and accumulation in *PCS1*-expressing plants

Based on published data on *PCS1*-expressing transgenic plants (Table 1), we investigated the relationship between metal tolerance and metal concentration (Table 2). First, out of the 12 plants in which Cd tolerance and Cd accumulation were examined, four showed increases in both Cd tolerance and accumulation, two showed increased Cd tolerance and decreased Cd accumulation, and one showed decreased Cd tolerance and increased Cd accumulation. Thus, among *PCS1*-expressing transgenic plants, 33.3 % showed a positive relationship between Cd tolerance and Cd accumulation, and 25 % showed a negative relationship. Similarly, a negative relationship has been observed between As tolerance and As accumulation; over-expression of *Ntcyc07* increased arsenite tolerance and decreased arsenic accumulation in yeast (Mok et al. 2008) and tobacco (Lee and Hwang 2012). It is assumed that the negative relationship between metal tolerance and metal concentration is partly caused by metal toxicity, which increases with higher metal concentrations and decreases with lower metal concentrations. Second, out of the seven plants in which As tolerance and As accumulation were measured, three showed increased As tolerance and increased As accumulation, and three showed increased As tolerance but unchanged As accumulation, consistent with our results (Fig. 3; Tables 1, 2). To summarize the results from all of the relevant previous studies, 43 % of *PCS1*-expressing plants showed a positive relationship between As tolerance and As accumulation, while 43 % of *PCS1*-expressing plants showed increased As tolerance and unchanged As accumulation. It is interesting that a negative relationship has not been observed between As tolerance

and As accumulation, while 25 % of *PCS1*-expressing plants showed a negative relationship between Cd tolerance and Cd accumulation. Taken together, the results show that *PCS1*-expressing plants often show a positive relationship between metal tolerance and accumulation for both Cd (33 %) and As (43 %), probably because of increased levels of PCs.

In conclusion, over-expression of *NtPCS1* in transgenic tobacco increased Cd tolerance but did not affect Cd accumulation. This result is unique among plants expressing *PCS1*. In addition, As tolerance was increased, but As content was unchanged in *NtPCS1*-expressing tobacco, a result that has been reported for 43 % of *PCS1*-expressing plants in previous studies. In further research, to understand the mechanisms of increased tolerance to Cd and As in *NtPCS1*-tobacco, and to explain the growth retardation in *NtPCS1-antisense* plants, the levels of PCs, GSH, and oxidative stress should be examined.

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