Original Paper

An improved tolerance to cadmium by overexpression of two genes for cysteine synthesis in tobacco

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Abstract   Contamination of soil by heavy metals such as Cd causes a serious negative impact on agricultural production and human health. Thus, improvement of tolerance to Cd is one of the major challenges in plant biotechnology. In the present study, we have generated transgenic *Nicotiana tabacum* (tobacco) plants overexpressing both serine acetyltransferase (SAT) and cysteine synthase (CS) [*O*-acetylserine (thiol)-lyase], which are committed in the last two steps of cysteine (Cys) biosynthesis, by crossing the respective single-gene transgenic plants. Two enzymatic activities were high in these two-gene overexpressing plants, and these plants exhibited more resistance to Cd stress than wild-type and single-gene transgenic plants. The two-gene transgenic plants also exhibited a higher production of phytochelatins (PCs) in an inducible manner by the Cd stress. The levels of free non-chelated Cd were lower in the two-gene transgenic plants than the wild-type and single-gene transformants. The levels of Cys and γ-glutamylcysteine (γ-EC) were also increased in the dual transgenic plants, presumably enhancing the metabolic flow of Cys biosynthesis leading to the ultimate synthesis of PCs which detoxify Cd by chelating. These results suggested that the overexpression of two genes, SAT and CS, could be a promising strategy for engineering Cd resistant plants.

Key words: Cadmium, cysteine synthase, *Nicotiana tabacum*, phytochelatin, serine acetyltransferase.

Sulfur-containing metabolites play critical roles for the plant life cycle (Saito 2004). Cysteine (Cys) is the key sulfur-containing organic metabolite in the sulfur assimilation pathway from inorganic sulfate ion taken up from the soil by sulfate transporters (Takahashi et al. 2011). Sulfate is reduced to sulfide by the reductive sulfate assimilation pathway. Sulfide is then coupled with *O*-acetylserine (OAS), which is formed from serine, yielding Cys. Serine acetyltransferase (SAT) (EC 2.3.1.30) and Cys synthase (CS) [*O*-acetylserine (thiol)-lyase] (EC 4.2.99.8) catalyze these two sequential reactions leading to Cys production (Figure 1). These two enzymes were demonstrated to localize in three major plant cell compartments, i.e., cytosol, chloroplasts and mitochondria (Hell and Wirtz 2011; Saito 2004; Takahashi et al. 2011). Cys is afterwards used for biosynthesis of protein, methionine, glutathione (GSH), phytochelatin (PC) and other sulfur-containing metabolites. Cys, GSH and PC, are particularly important in terms of response and mitigation to abiotic stresses such as heavy metal and oxidation.

Heavy metals such as Cd in the soil cause serious worldwide environmental and human health problems (Clemens 2001; Hall 2002). Plants developed mechanisms that detoxify heavy metals (Hall 2002). PCs, of the basic structure (γ-Glu-Cys)n-Gly ($n=2$–11) (Grill et al. 1985), play an important role in detoxification of toxic metals such as Cd and As (Cobbett 2000). PCs are synthesized from GSH by an inductive manner when exposed to heavy metals. The detoxification mechanism by PCs involves the formation of chelating complex of PCs with the heavy metals. GSH is synthesized from Cys by a two-step enzymatic reaction catalyzed by γ-glutamylcysteine (γ-EC) synthetase and GSH synthetase. Furthermore, GSH biosynthesis is presumed to be limited mostly by the Cys availability (Noctor et
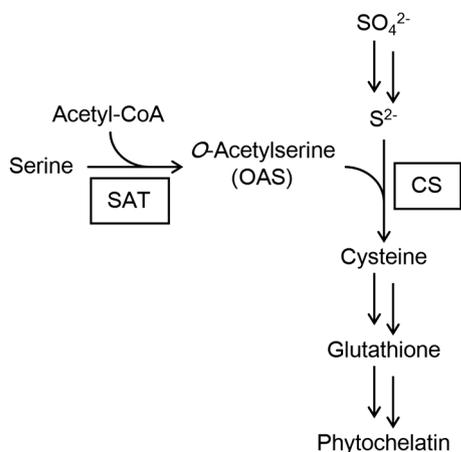

Figure 1.   Simplified pathway of sulfur assimilation, biosynthesis of cysteine, and conversion to glutathione and phytochelatins. The single arrow indicates a single biochemical reaction is involved, whereas the double arrow does multiple reactions involved.

al. 1996). As a logical consequence, several trials have been reported to overexpress genes involved in Cys biosynthesis to confer the tolerance to heavy metals in plants. The transgenic plants of *Arabidopsis thaliana* (Domínguez-Solís et al. 2001) and tobacco (*Nicotiana tabacum*) (Harada et al. 2001) overexpressing CS in cytosol showed the tolerance upon Cd treatment. Over-accumulation of CS both in cytosol and chloroplasts of tobacco confers the tolerance to Cd, Se and Ni with the increased levels of GSH and Cys in tobacco (Kawashima et al. 2004). Enhanced Cd-tolerance (Moontongchoon et al. 2008) was observed in transgenic *Ipomoea aquatica* expressing genes encoding SAT from *A. thaliana* and CS from rice (Meerak et al. 2006). These results suggest that the enhanced formation of Cys may lead to resistance to heavy metals (Domínguez-Solís et al. 2004). However, the more detailed investigation on the effects of over-accumulation of SAT and CS in different subcellular compartments and changes in the levels of different thiol metabolites (Cys, GSH and PC) in the transgenic plants are required for further understanding and biotechnological application.

In the present study, we have generated the transgenic tobacco plants over-accumulating both SAT and CS in cytosol and chloroplasts and evaluated the tolerance to Cd. We demonstrate that the double over-expression of genes for SAT and CS confers the increased tolerance to Cd, which is most likely ascribed to the reduced level of free non-chelated toxic Cd caused by the increased thiol metabolites, mostly PCs.

Materials and methods

Transgenic plants

We previously constructed transgenic tobacco (*N. tabacum cv*. SR1) carrying the spinach cytosolic CS cDNA (CSc) or

the chimeric CS cDNA (CSp) fused with the sequence for chloroplast-targeting transit peptide driven by cauliflower mosaic virus 35S promoter (Saito et al. 1994). Similarly we constructed transgenic tobacco carrying watermelon cytosolic SAT cDNA mutated at Gly277 to Cys (SATc) or chimeric mutated SAT cDNA (SATp) fused with the sequence for chloroplast-targeting transit peptide driven by cauliflower mosaic virus 35S promoter. The mutation at Gly277 to Cys diminished the regulatory property of SAT for feedback inhibition by Cys (Inoue et al. 1999; Noji and Saito 2002). The binary plasmids harboring SATc and SATp, respectively, for the plant transformation were constructed as follows. The coding region of mutated watermelon SAT cDNA (Inoue et al. 1999) was amplified by PCR using oligonucleotide primers XbaI-pSAT2-F (5′-CAT GCT CTA GAA TGC CAG TTG GTG AG-3′) and pSAT2-BamHI-R (5′-GCA TGG GAT CCC TAT CAA ATT ATG TAA TC-3′), and the resulting PCR product that corresponded to SATc was cloned into pBluescript II SK- (Stratagene) and fully sequenced. SATp chimeric gene was created by crossover PCR. For first-round PCRs, the cDNA fragments carrying transit peptide of ribulose-1,5-bisphosphate carboxylase small subunit of *Arabidopsis thaliana* (Krebbers et al. 1988) was amplified using the primers XbaI-R-SAT2-F (5′-CAT GCT CTA GAA TGG CTT CTA TGA TAT CC-3′) and SAT2-R-R (5′-ACC AAC TGG CAT GCA CTT TAC TCT TCC-3′), and the cDNA fragment encoding the coding region of the mutated watermelon SAT (Inoue et al. 1999) was amplified using the primers R-SAT2-F (5′-AGA GTA AAG TGC ATG CCA GTT GGT GAG-3′) and pSAT2-BamHI-R. The resulting DNA fragments amplified by the first-round PCRs were fused by the crossover PCR using the primers XbaI-R-SAT2-F and pSAT2-BamHI-R. The final PCR product that corresponded to SATp was cloned into pBluescript II SK- and fully sequenced. The *Xba*I–*Bam*HI fragments of SATc and SATp were cloned between *Xba*I site and *Bam*HI site of the binary vector, pBE2113Not (Liu et al. 1998). The resulting binary plasmids were transferred to *Agrobacterium tumefaciens* LBA4404 (Hoekema et al. 1983) by electroporation. Leaf disks of *N. tabacum cv*. SR1 were infected with *A. tumefaciens* harboring binary plasmids by the method previously described (De Block et al. 1987). Transgenic plants (T_0) were selected on the medium containing $100\,mg\,l^{-1}$ kanamycin, and regenerated as described (De Block et al. 1987). The homozygous lines of CSc, CSp, SATc and SATp were generated by self-fertilization and further crossed to yield the heterologous lines of CSc×SATc, CSc×SATp, CSp×SATc and CSp×SATp. Plants were maintained on A1 agar medium [one-half-strength Murashige and Skoog salts (Murashige and Skoog 1962), 1% (w/v) sucrose, 1% (w/v) agar, pH 5.7] by vegetative propagation.

Plant growth and exposure to Cd

Wild-type and transgenic tobacco plants were grown on A1 agar medium under 16 h light and 8 h dark cycles at 25°C. Top of the plant with two matured leaves was cut from the vegetative-propagated plant, and was incubated for 2 weeks

on A1 agar medium. The Cd treatment was performed by the addition of CdCl₂ to the A1 medium to a final concentration of 1.5 mM. Three ml of 25 mM CdCl₂ was added to the 50 ml of culture medium and uniformly-spread on the gel surface without touching to the plant. The plant tops with matured young leaves at the same position and developmental stage were harvested after 3, 6, 9 and 12 days of Cd exposure, frozen in liquid N_2 and powdered by ball milling using Multi Beads Shocker (Yasui Kikai, Osaka, Japan). The resulting tissue powder was used for the determination of thiol and Cd contents. The growth of each plant on Cd-containing medium was analyzed by measuring the digital image area of the top leaf of each plant using the open-source software, 'Image J' 〈http://rsb.info.nih.gov/ij/〉.

RT-PCR analysis

Top of the vegetative-propagated plant with two matured leaves was cut and incubated for two weeks on A1 agar medium. Total RNA was extracted from the top leaf of each plant using the RNeasy plant mini kit (Qiagen), and was treated with DNase I to avoid the contamination of genomic DNA. Each total RNA was reverse-transcribed with SuperScript II RNase H⁻ reverse transcriptase (Invitrogen). First-strand cDNA that derives from 2.5 ng total RNA was used for the amplification. PCR was carried out with *ExTaq* DNA polymerase (Takara, Tokyo, Japan) and the following oligonucleotide primer pairs; SAT-F (5′-ACT TTG CTC CTC CAC GCT TC-3′) and SAT-R (5′-GCC TGT CTC CAC ACA TCT TTC C-3′) for watermelon *SAT*, CS-F (5′-GAA GGC TGA GGA GAT CCG TG-3′) and CS-R (5′-CTG GGA AAG ACA GCG ACG AT-3′) for spinach *CS*. For the internal control, tobacco *actin* gene was amplified using the oligonucleotide primer pair previously reported (Chen et al. 2001). PCR products were separated by electrophoresis using 1% agarose gel, and stained with ethidium bromide and visualized by UV light.

Determination of SAT and CS activities

Plants were grown for three weeks on GM (Valvekens et al. 1988) agar medium. Leaves were homogenized in 20 volumes of extraction buffer containing 250 mM potassium phosphate (pH 8.0), 0.5 mM EDTA, and 10 mM 2-mercaptoethanol, on a fresh weight basis. The enzymatic activity of SAT was determined in the reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 0.1 mM acetyl-CoA, and 1 mM Ser. The reaction was performed at 37°C and the decrease in A_{232} due to the loss of thioester bond of acetyl-CoA was monitored. The enzymatic activity of CS was determined in the reaction mixtures containing 50 mM potassium phosphate (pH 8.0), 5 mM Na_2S, 12.5 mM OAS. The reaction was performed at 30°C for 10 min and terminated by the addition of 1/5 volumes of 7.5% (w/v) trichloroacetic acid. The Cys produced was quantified by spectrophotometry using the acid-ninhydrin method at 560 nm (Gaitonde 1967). Protein concentrations were determined according to the method previously described (Bradford 1976).

Determination of thiol metabolites

Quantitative analysis of reduced forms of thiol metabolites was performed by a combination of monobromobimane-fluorescent labeling and HPLC (Anderson 1985; Fahey and Newton 1987). Plant materials were homogenized in 2 volumes of ice-cold 0.1 M HCl on a fresh weight basis. A mixture of 20 μl of plant extract and 40 μl of 5% (w/v) 5-sulfosalicylic acid solution containing 25 μM N-acetylcysteine as the internal standard was allowed to react with 5 μl of 30 mM monobromobimane in acetonitrile and 10 μl of 5 M N-ethylmorpholine for 20 min at 37°C in the dark. The labeling reaction was terminated by the addition of 8 μl of acetic acid, and then resulting solution was subjected to HPLC analysis. HPLC was performed as previously described with a modification for gradient program (Saito et al. 1994). PC-1, PC-2 and PC-3 standards were synthesized by Sigma-Aldrich.

Cd quantification

The concentration of total Cd in plants was determined after wet-ashing of the powdered plants with mixed acid (60% HNO_3/60% $HClO_4 = 4:1$, v/v) by inductively coupled argon plasma-mass spectrometry (ICP-MS) (Agilent7500cs; Yokogawa Analytical Systems) as described previously (Suzuki et al. 1994).

For the determination of free unchelated Cd^{2+} in plants, plant materials were homogenized in 4 volumes of ice-cold 0.1 M HCl on a fresh weight basis. Plant extracts were centrifuged for 10 min at 20,950 g, and the supernatants were mixed with 1 volume of 1.6 M N-ethylmorpholine and 48 volumes of water. The concentration of free Cd^{2+} in each mixture was measured colorimetrically using Spectroquant Cadmium Cell Test (Merck Millipore), according to the manufacturer's protocol.

Relative Cd^{2+}-chelating activities of thiol metabolites were evaluated by a standard method for evaluation of chelating activity (Day and Underwood 1967; Reilley et al. 1959). The test solutions containing 10 μM CdCl₂, 32 mM N-ethylmorpholine and thiol metabolite at the concentration ranging from 0 to 25 μM were prepared, and the amounts of unchelated Cd^{2+} remained in each solution were measured using Spectroquant Cadmium Cell Test (Merck Millipore). The chelating activity of each thiol was evaluated by using the data obtained at the concentration of thiol in the pre-determined linear range of the analysis (25 μM for Cys and GSH, 10 μM for PC-1, 5 μM for PC-2 and PC-3), and was calculated as the ratio of the concentration of chelated Cd^{2+} to that of total Cd.

Results

Construction of transgenic tobacco plants overexpressing two genes for CS and SAT

Transgenic tobacco plants over-accumulating spinach CS in cytosol (CSc) and plastid (CSp) were obtained previously (Saito et al. 1994). In the present study, we have newly obtained transgenic tobacco plants over-accumulating the mutated version of watermelon SAT
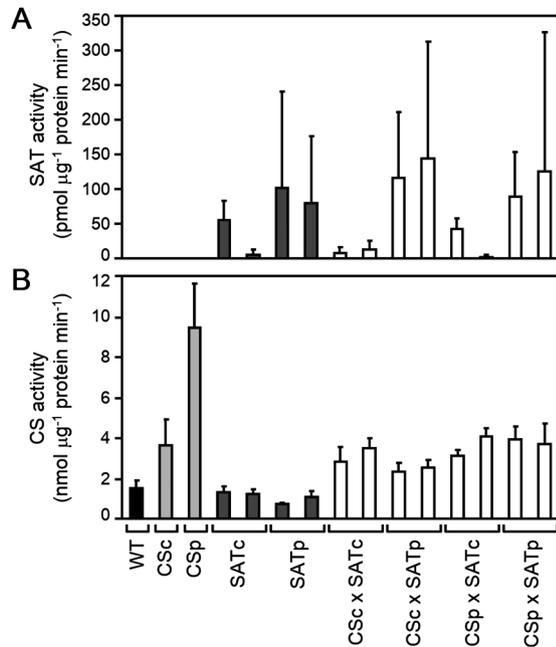
Figure 2. The enzymatic activities of SAT and CS. (A) SAT activity in the cell-free extracts of plants. (B) CS activity in the cell-free extracts of plants. Each enzyme activity was determined in the plant extracts from fully expanded leaves. For only-SAT overexpressing plants and double-overexpressing plants, two independent transgenic lines were analyzed. The data are the means of duplicate determinations. The error bars represent the standard deviations.

in cytosol (SATc) and plastid (SATp). The site-directed mutation was incorporated on the watermelon SAT (Saito et al. 1995) at Gly277 to Cys, which renders desensitization of feedback inhibition by Cys (Inoue et al. 1999). The levels of OAS, Cys and GSH in the transgenic Arabidopsis plants expressing the mutated SAT were higher than those of the plants expressing the wild-type SAT as the effects of this site-directed mutation (Noji and Saito 2002). Homozygous lines of these four transgenic tobacco plants were crossed to generate four different double-overexpressing lines of CSc×SATc, CSc×SATp, CSp×SATc and CSp×SATp, in which two respective proteins were accumulated in cytosol and/or plastid.

Enhanced activities of CS and SAT in double-overexpressing plants

The enzymatic activities of CS and SAT were determined in transgenic plants (Figure 2). As expected, the enhanced SAT activities were detected in the plants carrying SAT gene (Figure 2A). Only trace SAT activities were detected in the wild-type and only-CS overexpressing plants. Regarding CS activity, the transgenic plants carrying CS gene exhibited several-fold higher activities over the wild-type and only-SAT overexpressing plants (Figure 2B). For further analysis, the plants exhibiting higher activity of SAT from two independent lines for each construct were used for exposure to Cd. The accumulation of mRNA for SAT and
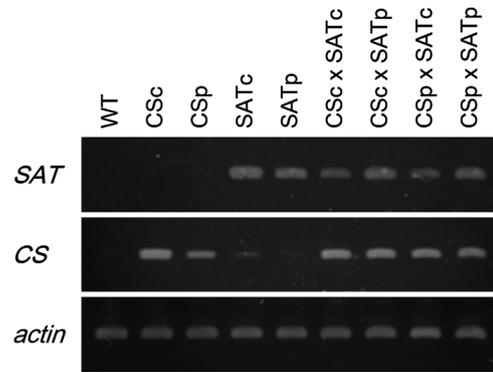

Figure 3. Accumulation of transcripts for SAT and CS in transgenic plants. Total RNA was extracted from fully expanded leaves of wild-type and transgenic plants. RT-PCR was performed with the gene-specific primers for SAT, CS, or actin.
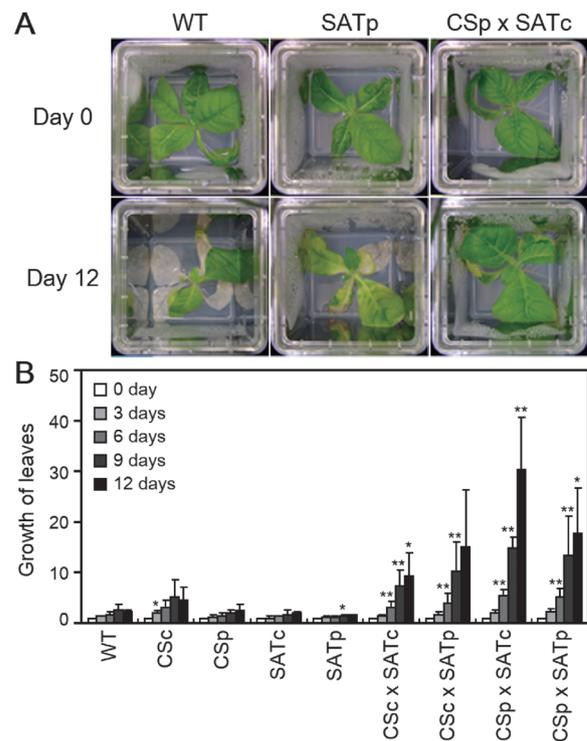

Figure 4. The tolerance of transgenic plants to Cd. Wild-type and transgenic plants were cultivated on the agar medium containing 1.5 mM $CdCl_2$ for up to 12 days. (A) Photographs of wild-type and transgenic plants before and after the exposure to Cd. (B) The time-course of plant growth under Cd stress. The area of top leaf of each plant at day 0 is set to 1. Values and bars represent means±SD ($n=3$–5). Asterisks indicate significant difference from the wild-type plants at the same time point of Cd exposure (* $p<0.05$; ** $p<0.005$).

CS was observed for the transgenic plants carrying SAT and CS transgenes, respectively (Figure 3).

Tolerance of transgenic plants to Cd stress

Figure 4A shows the visible phenotype of plants exposed to $CdCl_2$ for 12 days. The time-course of plant growth under Cd stress is presented in Figure 4B. These data clearly indicated that the four kinds of crossed plants

overexpressing both CS and SAT genes exhibited higher tolerance than the wild-type plants and the single gene transgenic plants.

Enhanced levels of the thiol metabolites and their chelating activity to Cd^{2+}

Six thiol metabolites (Cys, γ-EC, GSH, PC-1 ($n=2$), PC-2 ($n=3$) and PC-3 ($n=4$)) were determined in transgenic plants exposed to CdCl$_2$ for up to 12 days (Figure 5). The levels of PCs were dramatically increased along with the prolonged exposing time to Cd; whereas the levels of GSH were even a little decreased by the

exposing time. As a general trend, the levels of all six thiol metabolites were highly elevated in the dual transgenic plants compared with the wild-type and single transgenic plants; in particular, increase of PCs in the dual transgenic plants was remarkable. However, the level of GSH was slightly higher in the SAT transgenic plants than in the dual transgenic plants.

Since the thiol metabolites contribute, presumably by their chelating activity, to the detoxification of Cd^{2+}, the chelating abilities of these thiol metabolites were determined compared with that of EDTA (Table 1). PC-3 exhibited the highest chelating activity followed by PC-2 and PC-1.

These data all together suggested that the Cd-chelating activity by thiol metabolites represented mostly by PCs in the dual transgenic plants was highly induced by Cd stress, and this increased activity has been enforced by dual overexpression of SAT and CS genes.

Cadmium accumulation

The concentrations of total and free (non-chelated) Cd were determined in transgenic plants after the exposure to CdCl$_2$ (Figure 6). As expected, the levels of total Cd increased with time of exposure in all plants (Figure 6A). However, the elevation of free Cd^{2+} levels in the
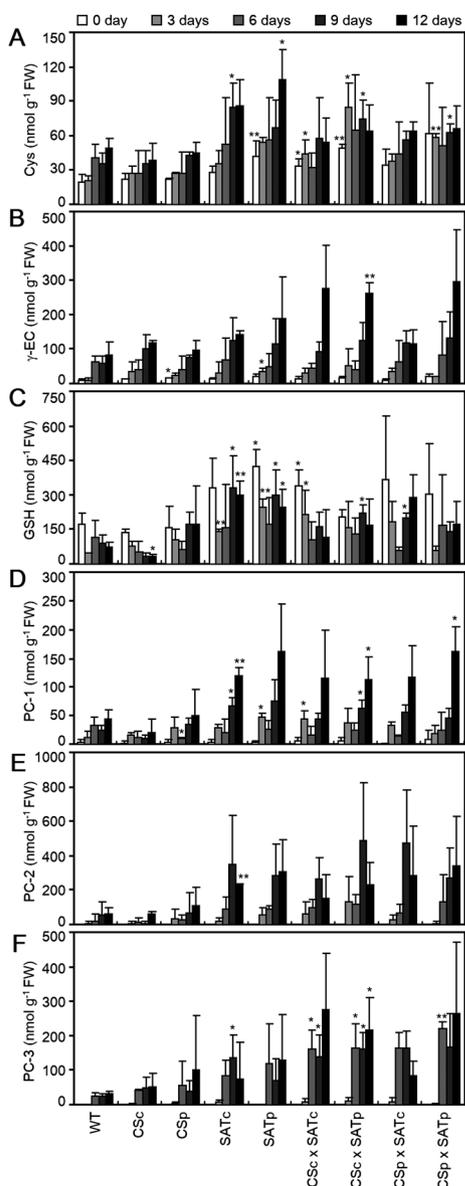

Figure 5. The levels of thiol metabolites under the Cd stress. Wild-type and transgenic plants were cultivated on the agar medium containing 1.5 mM CdCl$_2$ for up to 12 days. The levels of Cys (A), γ-EC (B), GSH (C), PC-1 (D), PC-2 (E) and PC-3 (F) in the leaves were determined. Values and bars represent means±SD ($n=3$). Asterisks indicate significant difference from the wild-type plants at the same time point of Cd exposure (* $p<0.05$; ** $p<0.005$).

Table 1. Chelating activities of thiol metabolites to Cd^{2+}.

	Cys	GSH	PC-1	PC-2	PC-3
Chelating activity*	0.27±0.00	0.17±0.01	0.49±0.01	0.77±0.04	0.93±0.05

*Chelating activity is expressed as the relative activity to that of EDTA (means±SD, $n=3$).
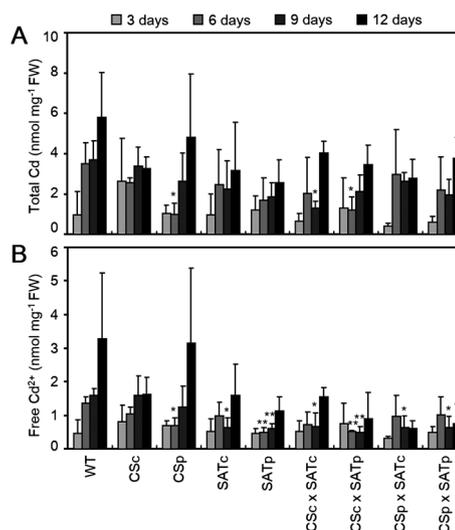

Figure 6. The concentration of Cd in the plants. Wild-type and transgenic plants were cultivated on the agar medium containing 1.5 mM CdCl$_2$ for up to 12 days. (A) The concentration of total Cd in plants. (B) The concentration of free non-chelated Cd^{2+} in plants. Values and bars represent means±SD ($n=3$). Asterisks indicate significant difference from the wild-type plants at the same time point of Cd exposure (* $p<0.05$; ** $p<0.005$).

dual transgenic plants was less than that of the wild-type plant (Figure 6B). On the later days (9 and 12 days after onset of exposure), in particular, although the total Cd were nearly in the same level as in the wild-type and the transgenic plants, the levels of free non-chelated Cd^{2+} in the dual transgenic plants were remarkably low.

Discussion

In the present study, we have generated transgenic tobacco plants overexpressing both SAT and CS genes by crossing the respective single-gene transgenic plants. As expected, two enzymatic activities, SAT and CS, were high in those dual transgenic plants, which also exhibited more resistance to Cd stress than wild-type and single transgenic plants (Figures 2, 4). The characteristic feature of the dual transgenic plants was a highly-inducible production of PCs by the Cd stress (Figure 5). This elevated level of PCs presumably contributes as chelating agents for free Cd^{2+} efficiently, resulting in lowering the level of free Cd^{2+} and consequently detoxifying Cd (Figure 6). The levels of Cys and γ-EC were also increased in the dual transgenic plants (Figure 5). Since these two thiol metabolites are the precursors of PCs, the double overexpression of SAT and CS enhanced the metabolic flow of Cys biosynthesis leading to the ultimate synthesis of PCs. Importance of PC synthesis for Cd resistance was suggested also by the increased tolerance in the transgenic Indian mustard over-expressing γ-EC synthetase (Zhu et al. 1999), and in the transgenic tobacco and Indian mustard plants over-expressing PC synthase (Gasic and Korban 2007; Pomponi et al. 2006). In addition, the steady-state mRNA levels of the genes involved in Cys biosynthesis were induced by Cd stress leading to the enhanced production of thiol metabolites (Harada et al. 2002).

In terms of different subcellular localization of over-accumulated proteins of CS and SAT, there is no apparent difference among the dual transgenic plants. Since there is a complicated compartmentation of Cys biosynthetic enzymes (Hell and Wirtz 2011; Watanabe et al. 2008), the mixed effects of compartment-specific accumulation of each enzyme could be combined; hence no clear trend was observed regarding different subcellular localization of over-accumulating proteins. However, it could be safely concluded that dual over-accumulation of CS and SAT in either cytosol or plastid could positively effect on the metabolic flow of Cys synthesis in vivo. Leaf trichomes are suggested to be the site of heavy metal accumulation in plants (Choi et al. 2004; Domínguez-Solís et al. 2004). Thus, it might be interesting to investigate the levels of Cd in the trichomes of our dual transgenic plants whether the Cd levels in this specific organ increase or not.

Together with several previous publications, which described conferring tolerance to heavy metals including Cd by the manipulation of Cys biosynthetic enzymes, our present study further provides the detailed data on the mechanistic insight how tolerance has been conferred. Those studies would be the strong basis for engineering the heavy metal-resistant crops and bioenergy plants in the future. The more detailed molecular mechanisms regulating the synthesis of PCs and Cd tolerance responding to enhancement of Cys biosynthesis still remained to be investigated.

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