Journal of Plant Biology, April 2007, 50(2) : 220-223

SHORT COMMUNICATION

Overexpression of *Arabidopsis* Phytochelatin Synthase (*AtPCS1*) Does Not Change the Maximum Capacity for Non-Protein Thiol Production Induced by Cadmium

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Phytochelatins (PCs) play an important role in heavy-metal homeostasis and detoxification. However, we previously reported that the overexpression of PC synthase in *Arabidopsis* does not lead to increased tolerance of cadmium but, rather, plants show higher Cd sensitivity. Here, we compared the maximum capacity for non-protein thiol (NPT) production at various concentrations of Cd in order to estimate PC synthesis indirectly for both transgenic (pcs9) and wild-type plants. The pcs9 line produced the highest level of NPT when treated with 200 μM Cd for 3 d. In comparison, the maximum productivity by the wild type was in response to 500 μM Cd. Nevertheless, the absolute amounts of NPT produced did not differ significantly between those two genotypes. Furthermore, exogenous application of 1 mM GSH did not dramatically change the capacity for either pcs9 or wild-type plants. These results suggest that Cd hypersensitivity in the transgenic pcs9 may not be caused by supraoptimal intracellular concentrations of PC, but may, instead, be due to overexpressed PC synthase itself because that enzyme can bind metals. This action, therefore, may lead to some unknown disruption in cellular metal homeostasis under Cd stress.

Keywords: Arabidopsis, cadmium, phytochelatin, phytochelatin synthase

Phytoremediation is a technology whereby green plants are used to either reduce the toxicity of pollutants or remove them from a contaminated environment. Its implementation has enabled rapid progress in the clean-up of heavy metal-polluted areas in a cost-effective and environmentally-friendly manner (Salt et al., 1995, 1998; Raskin et al., 1997). One promising approach to phytoremediation is the utilization of naturally selected "hyperaccumulators". These plant species deposit extremely high levels of heavy metals in their biomass. However, most known hyperaccumulators typically store only a specific element, grow slowly, and produce relatively small amounts of biomass (Cunningham et al., 1995). Because of these disadvantages, genetic and molecular biological studies of plant defense mechanisms for heavy-metal stress have been initiated to optimize and improve the efficiency of phytoremediation (Cobbett, 2000).

Plants have several mechanisms for dealing with this type of stress. One involves the production of cysteine-rich peptides, such as phytochelatins (PCs) and metallothioneins (MTs), for the detoxification or homeostasis of heavy metals (Rauser, 1999; Cobbett, 2000). PCs comprise a family of enzymatically synthesized peptides with a general structure of $(\gamma\text{-Glu-Cys})_n$-Gly, where n equals 2 to 11 (Rauser, 1990). These peptides are rapidly synthesized by plants in response to exposure to toxic levels of heavy metals (Zenk, 1996; Cobbett, 1999). The role of a PC is to form stable complexes with heavy metals in the cytosol; those complexes are subsequently sequestered into the vacuole (Zenk, 1996; Cobbett, 2000). PC synthase catalyzes the synthesis of PCs by transferring the g-Glu-Cys moiety of GSH either to another GSH molecule or to a growing PC (Zenk, 1996). Genes encoding PC synthase have been cloned from *Arabidopsis thaliana* (*AtPCS1*), wheat (*TaPCS1*), *Schizosaccharomyces pombe* (*SpPCS1*), *Caenorhabditis elegans* (*CePCS1*), and other species (Clemens et al., 1999, 2001; Ha et al., 1999; Vatamaniuk et al., 1999). The identification of PC synthase genes has facilitated molecular and biochemical studies (Lee and Kang, 2005a, b), with their foci including mechanisms for enzyme activation (Vatamaniuk et al., 2000), tissue-specific expression (Lee et al., 2002), and transcriptional regulation (Clemens et al., 1999; Lee and Korban, 2002).

In a previous study, we over-expressed an *Arabidopsis* PC synthase gene (*AtPCS1*) in transgenic *Arabidopsis* with the goal of increasing their PC synthesis, metal accumulation, and metal tolerance (Lee et al., 2003a, b). However, although our transgenic plants showed a relatively high level of expression for the *35S::AtPCS1* transgene, they did not have enhanced Cd tolerance but, rather, manifested greater sensitivity under some conditions. Therefore, our objective in the current study was to determine whether higher levels of PCs are associated with sensitivity to Cd. Here, we tested transgenic and wild-type plants to compare their induced levels of non-protein thiols (NPTs) at various concentrations of Cd.

MATERIALS AND METHODS

Plant Materials, Growing Conditions, and Cadmium Treatments

Seeds of *A. thaliana* ecotype Columbia [wild-type and transgenic lines (pcs1, pcs3, and pcs9) (Lee et al., 2003a)] were germinated and grown for 14 d on an agar medium containing half-strength MS (Murashige and Skoog, 1962) salts and 2% (w/v) sucrose (pH 5.8) in 100 × 100 × 15 mm square plates. The seedlings were then transferred to fresh media containing various concentrations of $CdCl_2$ and grown for another 3 d. Plates were maintained in a growth

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chamber at 23°C under a 12-h photoperiod provided by cool-white fluorescent tubes at a light intensity of approximately 80 μE m^{-2} s^{-1}.

Glutathione (GSH) and Non-Protein Thiol (NPT) Analysis

Seedlings were harvested and 100-mg samples were frozen in liquid nitrogen and ground with a mortar and pestle. Afterward, 300 mL of a solution containing 1 M NaOH and 1 mg L^{-1} $NaBH_4$ was added. The homogenates were centrifuged at 13000*g* for 5 min at 4°C before the 300 mL supernatant was acidified by adding 50 mL of 37% (w/v) HCl. This solution was used for spectrophotometric measurements of the GSH and NPT contents. For our analysis of total NPT, a 10 mL solution was added to 500 mL of Ellman's (1959) reagent [5,5′-dithiobis(2-nitrobenzoic acid)], and incubated at 30°C for 2 min. Absorbance at 412 nm was measured with a UV-VIS Shimadzu spectrophotometer. Analysis of total GSH was performed using the glutathione reductase recycling assay, as described by Anderson (1985).

RESULTS AND DISCUSSION

We have previously reported that, compared with wild-type plants, those from the Cd-sensitive pcs9 transgenic line show approximately two-fold higher total PC production and a 50% increase in NPTs under stress from 85 μM Cd (Lee et al., 2003a). In that study, we selected that particular level of Cd treatment for our PC analysis because it resulted in the largest difference in growth rates between transgenic and wild-type plants. However, we did not ascertain the optimum concentration of cadmium that could cause the greatest contrast in PC production capacity for those two genotypes. Therefore, in this current research, we treated plants with various concentration of Cd, and then estimated PCs production indirectly by measuring intracellular levels of both NPT and GSH. The transgenics produced the maximum amount of NPT when exposed to 200 μM Cd, while the wild-type plants showed it in response to 500 μM Cd. Despite these data, however, our results demonstrated that the maximum capacity for NPT production was almost the same for pcs9 and wild-type plants (Fig. 1A). Moreover, the amount of GSH was not a main factor in determining the increase in NPT level because, when we compared 500 μM Cd-treated transgenic and wild-type plants with an untreated control, we observed a simultaneous rise in NPT and a decline in GSH (Fig. 1B). Similar pcs9-related levels of NPT production were recorded when pcs1 and pcs3 lines were tested with either 100 or 500 μM Cd (data not shown).

To determine whether the pattern of maximum NPT pro-

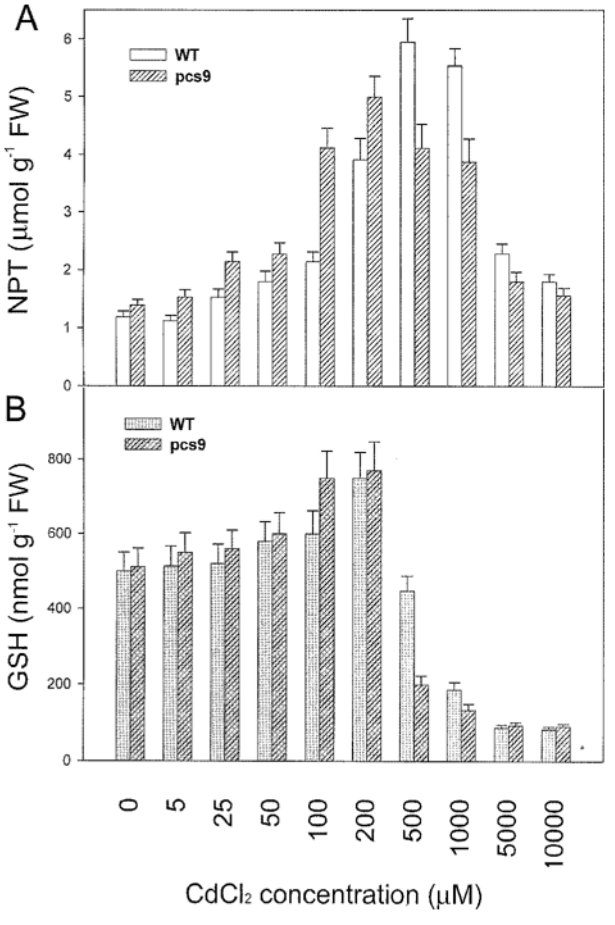

Figure 1. Effect of cadmium on intracellular levels of NPT and GSH. Fourteen-day-old *Arabidopsis* seedlings (pcs9 and wild-type) grown on MS media were transferred to fresh media containing various concentrations of Cd, incubated 3 d further, then analyzed for levels of NPT (**A**) and GSH (**B**). Values are mean ± SE of three replicates.

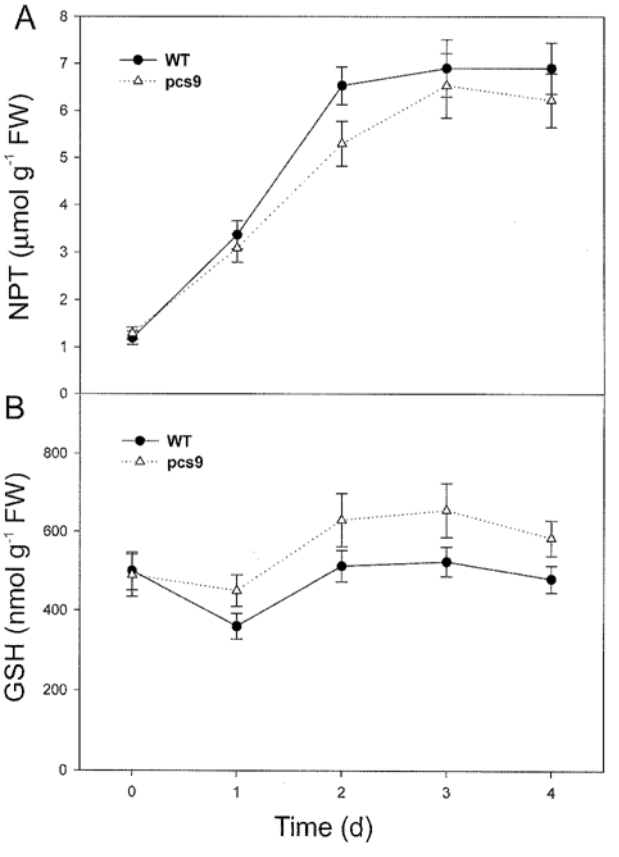

Figure 2. Time-dependent intracellular levels of both NPT and GSH in Cd-treated *Arabidopsis*. Fourteen-day-old seedlings grown on MS media were transferred to fresh media containing Cd (100 μM for pcs9 or 500 μM for wild-type), incubated further, then analyzed for NPT (**A**) and GSH (**B**) at designated times. Values are mean ± SE of three replicates.

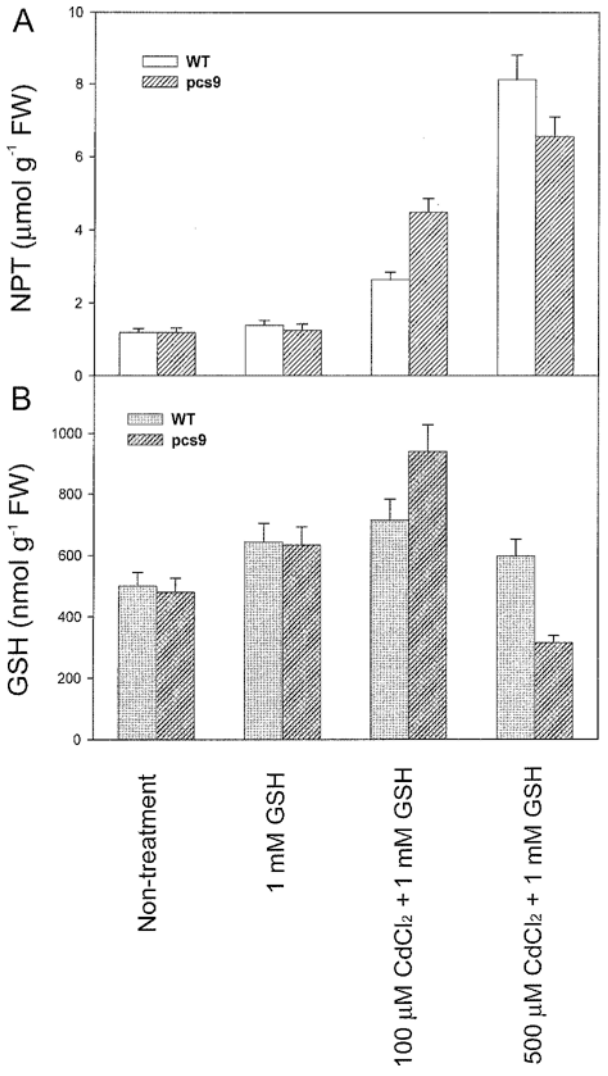

Figure 3. Effect of GSH on intracellular levels of both NPT and GSH. Fourteen-day-old *Arabidopsis* seedlings grown on MS media were transferred to fresh media containing either GSH or GSH plus Cd, incubated 3 d further, then analyzed for levels of NPT (**A**) and GSH (**B**). Values are mean ± SE of three replicates.

duction was time-dependent, we applied cadmium to pcs9 plants (100 μM) and wild-type plants (500 μM) for 4 d. Total NPT production was saturated at 3 d for both genotypes (Fig. 2A). However, their levels of GSH did not increase in a manner similar to that of NPT (Fig. 2B). Therefore, we can conclude that trends in maximum Cd-induced NPT production were similar over time for pcs9 and wild-type plants.

To investigate whether NPT production could be limited by the supply of intracellular GSH, we applied 1 mM GSH to plants exogenously. Although this treatment induced a slight rise in the intracellular GSH level, no significant increase was recorded in the NPT content (Fig. 3). When GSH and Cd were applied simultaneously, NPT levels rose significantly compared with either non-treatment or exposure to 1 mM GSH alone. Nevertheless, these levels were similar to, or slightly higher than, those measured in response to cadmium treatment (Fig. 1, 3). Therefore, our results indicate that the amount of intracellular GSH is not a factor critical to the Cd-induced production of NPT under our experimental conditions. This conclusion is confirmed because the approximately 2-fold increase in GSH did not lead to a rise in the NPT amount that would be equivalent to that produced by exposure to 100 μM Cd and 1 mM GSH (Fig. 3).

We have previously demonstrated that PC is toxic to plants when present at supraoptimal concentrations, as is the case with Cys and GSH (Lee et al., 2003a). We have also reported that, with one exception, the overexpression of *AtPCS1* can complement the Cd-sensitive *Arabidopsis* mutant *cad1-3* (Lee et al., 2003a). The cad1pcs6 line, a transgenic *cad1-3* with the highest ectopic expression of *AtPCS1* among all lines, does not recover its Cd sensitivity to the degree seen in wild-type plants. Even though we indirectly estimated the pattern of PC production by measuring NPT here, we now propose, based on our previous results and those from this study, that the reason for Cd hypersensitivity in pcs9 is not the supraoptimal intracellular concentration of PC but because of the overexpressed PC synthase itself, which can bind metals. That property can lead to some unknown disruption in cellular metal homeostasis under cadmium stress. In further experiments, we will generate and examine transgenic plants expressing mutated *AtPCS1* that cannot produce PC but do retain this metal-binding trait.

ACKNOWLEDGEMENT

This work was supported by a grant from the Korea Research Foundation (KRF-2004-042-F00006).

Received October 20, 2006; Revised February 1, 2007; accepted March 20, 2007.

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