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Phytochelatin Is Not a Primary Factor in Determining Copper Tolerance

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Phytochelatin (PC) is involved in the detoxification of harmful, non-essential heavy metals and the homeostasis of essential heavy metals in plants. Its synthesis can be induced by either cadmium (Cd) or copper (Cu), and can form stable complexes with either element. This might suggest that PC has an important role in determining plant tolerance to both. However, this is not clearly apparent, as evidenced by a PC-deficient and Cd-sensitive *Arabidopsis* mutant (*cad1-3*) that shows no significant increase in its sensitivity to copper. Therefore, we investigated whether the mechanism for Cu tolerance differed from that for Cd by analyzing copper sensitivity in Cd-tolerant transgenics and Cd-sensitive mutants of *Arabidopsis*. Cadmium-tolerant transgenic plants that over-expressed *A. thaliana* phytochelatin synthase 1 (*AtPCS1*) were not tolerant of copper stress, thereby supporting the hypothesis that PC is not primarily involved in this tolerance mechanism. We also investigated Cu tolerance in *cad2-1*, a Cd-sensitive and glutathione (GSH)-deficient *Arabidopsis* mutant. Paradoxically, *cad2-1* was more resistant to copper stress than were wild-type plants. This was likely due to the high level of cysteine present in that mutant. However, when the growth medium was supplemented with cysteine, the wild types also exhibited copper tolerance. Moreover, *Saccharomyces cerevisiae* that expressed *AtPCS1* showed tolerance to Cd but hypersensitivity to Cu. All these results indicate that PC is not a major factor in determining copper tolerance in plants.

Keywords: Arabidopsis, cad1, cad2, cadmium, copper, phytochelatin

Plants have several defense mechanisms for dealing with heavy-metal stress (Rauser, 1999; Clemens, 2001; Cobbett and Goldsbrough, 2002). Metals such as copper (Cu) and zinc (Zn) are required for plant growth and development, but are toxic at supra-optimal concentrations. Therefore, plants must have a way to ensure appropriate contents of essential heavy metals in their cells. Until now, this action was not well understood.

Cadmium (Cd) is a non-essential heavy metal (i.e., not required for growth and development). When present in high quantities, it can cause inhibited growth or even death. One important means for Cd detoxification in plants is the production of phytochelatins (PCs). PCs are a family of small, enzymatically synthesized peptides with a general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n = 2 to 11 (Rauser, 1990). These peptides are rapidly produced in response to toxic levels of heavy metals in all tested species (Zenk, 1996). PCs are synthesized from GSH or GSH-related compounds through transpeptidation of the γ-Glu-Cys moiety of GSH, via γ-Glu-Cys dipeptidyl transpeptidase, either to another GSH or to a growing PC peptide (Grill et al., 1989; Cobbett, 2000a, b). The role of PCs in heavy-metal tolerance has been well-characterized in two Cd-sensitive mutants of *Arabidopsis*, *cad1* and *cad2* (Howden and Cobbett, 1992; Howden et al., 1995; Cobbett et al., 1998). Both are deficient in PC production due to mutations either in γ-glutamylcysteine synthetase, as with the *cad2* mutant, or in PC synthase (PCS), as with the *cad1* mutant. In the cytosol, PCs form stable complexes with heavy metals, which are subsequently sequestered in the vacuole (Grill et al., 1985; Zenk, 1996; Cobbett, 2000a, b).

Genes that encode PCS have been cloned from *Arabidopsis thaliana* (*AtPCS1*), wheat (*TaPCS1*), and other species (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999). The identification of PCS genes from these various organisms now facilitates molecular and biochemical studies, and provides additional information on the mechanism of PCS activation by cadmium (Vatamaniuk et al., 2000; Oven et al., 2002). Furthermore, it allows researchers to investigate tissue-specific expression and regulation of those genes (Clemens et al., 1999; Lee and Korban, 2002; Lee et al., 2002).

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Copper stress also can induce PC synthesis (Grill et al., 1987; de Vos et al., 1992; Hartley-Whitaker et al., 2001), and can produce a stable Cu-PC complex (Scarano and Morelli, 1998). These activities imply that PC may be involved either in homeostasis or detoxification of copper. However, there is no conclusive evidence to support the functioning of PC in plant responses. In fact, its role in Cu stress is rather doubtful as a Cd-sensitive *cad1* mutant has been reported that is slightly sensitive to copper (Howden and Cobbett, 1992). In this study, we were interested in determining whether PC was important in a plant's response to Cu stress. We also compared responses to both copper and cadmium stresses by using mutant and transgenic *Arabidopsis* plants with known resistance and sensitivity to Cd.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds from mutant, transgenic, and wild-type *A. thaliana* (L.) Heynh. (ecotype Columbia) were germinated and grown in a vertical orientation on $100 \times 100 \times 15$ mm square plates containing half-strength MS (Murashige and Skoog, 1962) agar media (pH 5.8) and 2% (w/v) sucrose. All plates were maintained in a growth chamber at 23°C under a 12-h photoperiod provided by cool-white fluorescent tubes at a photon flux density of approximately 80 μmol m^{-2} s^{-1}. The two mutants are deficient in their PC production due to a mutation either in γ-glutamylcysteine synthetase (*cad2-1*) or in PCS (*cad1-3*) (Howden and Cobbett, 1992; Howden et al., 1995; Cobbett et al., 1998). The transgenic *PCS* (+) *23*, as described previously by Lee et al. (2003b), is a Cd-tolerant line that over-expresses the *Arabidopsis* PCS, *AtPCS1*.

Treatments with Heavy Metals and Cysteine

Approximately 10 seeds each of the wild type, two mutants, and transgenic *PCS* (+) *23* were germinated and grown for 7 d on MS agar media containing various concentrations of either cysteine (L- or D-form) or one of the following heavy metals: $ZnCl_2$, $CuCl_2$, $CdCl_2$, or $HgCl_2$. The plates were placed vertically on shelves, and all treatments were repeated on two separate occasions. Root length measurements were used as an index of heavy-metal sensitivity.

Growth Assays and Yeast Transformation

The *Saccharomyces cerevisiae* strain INVsc1 (*his3* △ *1/his3* △ *1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52*) (Invitrogen, USA) was used for growth assays as well as for transformation via the lithium acetate method (Sambrook et al., 1989). Transformed yeast cells were grown in a yeast nitrogen base supplemented with appropriate amino acids and 2% galactose. To assay for sensitivity, cells were grown to the log phase and then incubated for 20 h at 30°C in the presence of various concentrations of heavy metals. Afterward, cell density was measured by a spectrophotometer at 600 nm.

DNA Manipulation

Escherichia coli strain DH5α was used for DNA manipulation. The FLAG-tagged *A. thaliana* PC synthase (*AtPCS1*) cDNA of the PCS (+) vector (Lee et al., 2003b) was subcloned into *Bam*HI/*Sac*I sites of the pYES2 vector (Invitrogen), resulting in pYES2-AtPCS1. This vector was used in the yeast transformation.

RESULTS AND DISCUSSION

We previously reported that transgenic *A. thaliana* over-expressing *AtPCS1* was, paradoxically, hypersensitive to Cd stress but not to Cu stress (Lee et al., 2003a). This implied that the tolerance mechanism for cadmium differed from that for Cu. Recently, we also developed transgenic *A. thaliana PCS* (+) lines that were rendered cadmium-resistant via over-expression of *AtPCS1* (Lee et al., 2003b).

To test whether the Cd-resistant transgenic *PCS* (+) *23* line was also resistant to Cu stress, its seeds were grown on MS agar media containing various concentrations of $CuCl_2$ in order to compare its copper sensitivity with wild-type plants. No significant differences were seen between the two genotypes (Fig. 1). This finding supports the theory that the detoxification mechanism for Cu varies from that for Cd in *Arabidopsis*. Moreover, we again demonstrated here that it is unlikely that critical expression of *AtPCS1* could lead to increased copper tolerance. We previously had reported that a 10- or 20-fold increase in *AtPCS1* expression was not closely correlated with the development of Cu tolerance (Lee et al., 2003a).

To further determine whether PC is related to Cu homeostasis, we examined the Cu-response in two Cd-sensitive mutants, *cad1-3* and *cad2-1*. The former

is known to be slightly more sensitive to copper stress when compared with wild-type plants (Howden and Cobbett, 1992). However, because no data had been reported for whether the latter showed a degree of sensitivity similar to that of *cad1-3*, we investigated this possibility in *cad2-1*. We also included Zn and mercury (Hg) to ensure that our experimental conditions were the same as those used in earlier research by Howden and Cobbett (1992).

In the current study, the sensitivity to Zn stress was higher in both *cad2-1* and *cad1-3* than in the wild type (Fig. 2A). Thus, PC seems to be involved in the detoxification of zinc, just as it is with cadmium. For copper, however, the response of *cad1-3* did not differ significantly from that of the WT, whereas *cad2-1* plants exhibited significantly higher resistance to Cu stress than either of the other two genotypes (Fig. 2B). This latter result was unexpected because *cad2-1* is

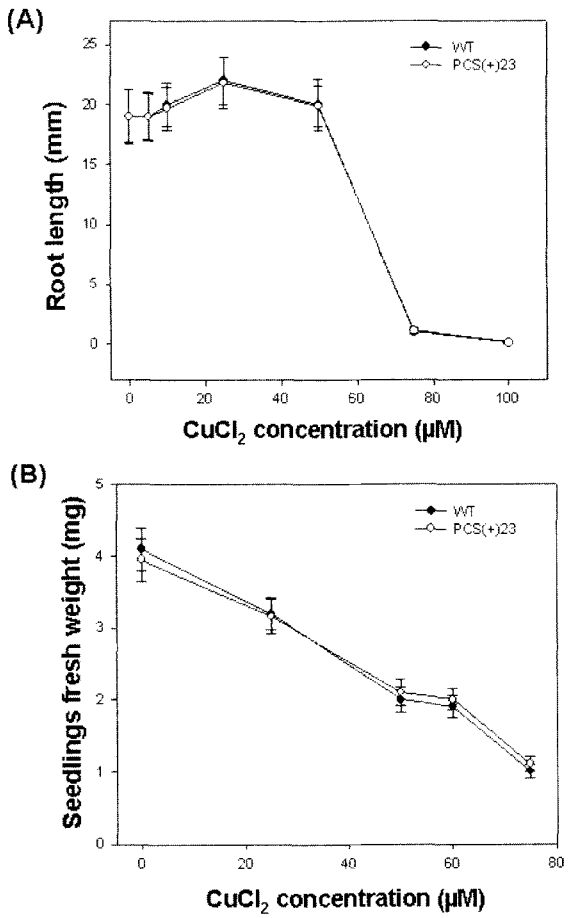

Figure 1. Sensitivity to copper in wild-type (WT) plants and transgenic *Arabidopsis PCS* (+) *23* line over-expressing phytochelatin synthase, *AtPCS1*. Seeds from both genotypes were germinated, then grown for 7 d on MS agar media containing different concentrations of $CuCl_2$. Both root lengths and seedling fresh weights were measured. Bars correspond to means ± S.E. of 10 plants of each transgenic line or wild-type *Arabidopsis* per treatment.

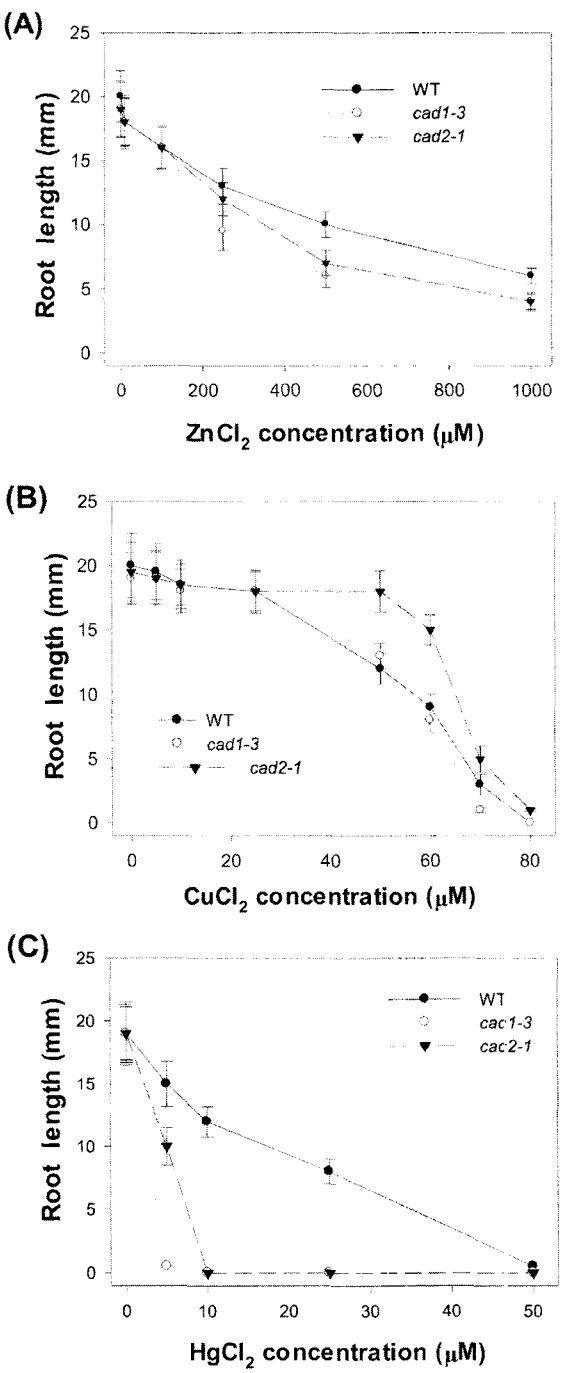

Figure 2. Sensitivity to copper, zinc, and mercury in cadmium-sensitive *Arabidopsis* mutants (*cad1-3* and *cad2-1*) and wild-type (WT) plants. Seeds from all 3 genotypes were germinated, then grown for 7 d on MS agar media containing different concentrations of **(A)** $ZnCl_2$, **(B)** CuC_2, or **(C)** $HgCl_2$. Root lengths were measured afterward. Bars correspond to means ± S.E. of 10 plants of each transgenic line or wild-type *Arabidopsis* per treatment.

generally known to be sensitive to Cd stress. Therefore, we further supported the hypothesis that Cu detoxification differs from the process for Cd.

The *cad2-1* response to mercury, one of the most toxic heavy metals, was investigated to determine whether this mutant would exhibit a similar response to copper. Here, both *cad2-1* and *cad1-3* plants showed significantly higher sensitivities to Hg stress than did the wild types (Fig. 2C). Those two mutant lines also appeared to be more sensitive to mercury than to either zinc or copper (Fig. 2A-C). Thus, it is presumed that *cad2-1* tolerance is restricted to Cu stress.

The tolerance to copper by *cad2-1* is interesting because the reduced capacity of PC biosynthesis can lead to such a response. This tolerance may also have been due to higher cysteine levels in that mutant. Cobbett et al. (1998) have reported that *cad2-1* shows a 2-fold increase in cysteine content compared with the wild type. This effect on copper tolerance may be related either to the cysteine itself (i.e., direct binding of Cu to the thiol group of cysteine) or to greater availability of the cysteine pool required for producing a cysteine-rich small protein, metallothionein (MT). Plant MT may be involved in plant responses to Cu stress (de Miranda et al., 1990; Murphy and Taiz, 1995; van Vliet et al., 1995; van Hoof et al., 2001). However, no conclusive evidence exists to support any role for that protein in copper homeostasis.

In a preliminary experiment, we measured root growth in wild-type, *cad1-3*, and *cad2-1 Arabidopsis* plants as it was affected by supplemental cysteine in the growth media. In this current study, we also investigated the effect of D-cysteine as a standard because it could not be metabolized in those cells to achieve a physiological function. L-cysteine slightly inhibited root development in both wild-type and mutant plants (Fig. 3A). Although D-cysteine also caused this inhibition in all genotypes, it was to a higher degree (Fig. 3B). Furthermore, in the presence of 60 μM $CdCl_2$, the toxic effect of cadmium on root growth was significantly reduced in all genotypes when the media were supplemented with either 0.5 or 1.0 mM L-cysteine (Fig. 3C); cf. the only slightly inhibited root development by L-cysteine alone (Fig. 3A). Finally, root growth in all three genotypes was significantly inhib-

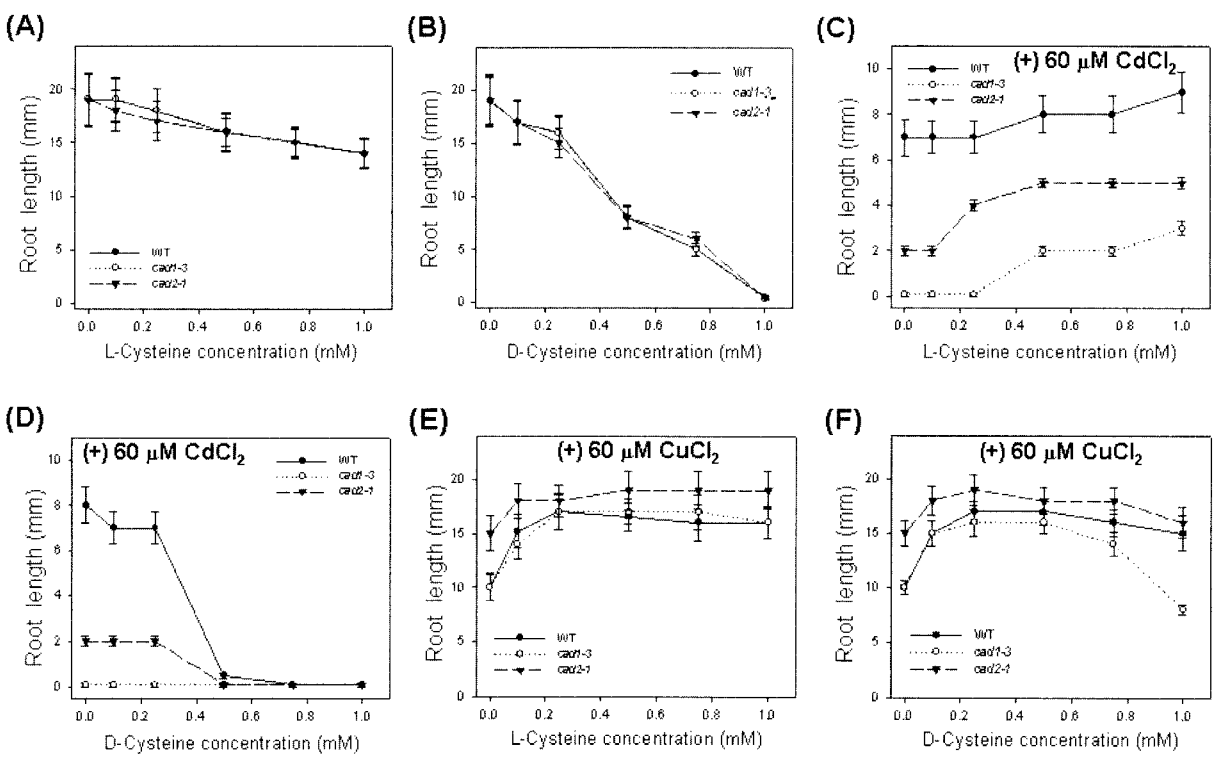

Figure 3. Effects of L- and D-cysteine on sensitivity to cadmium and copper in two Ca-sensitive *Arabidopsis* mutants -- *cad1-3* and *cad2-1* -- and in wild-type (WT) plants. Seeds from all 3 genotypes were germinated, then grown for 7 d on MS agar media containing different concentrations of **(A)** L-cysteine or **(B)** D-cysteine; 60 μM $CdCl_2$ plus **(C)** L-cysteine or **(D)** D-cysteine; and 60 μM $CuCl_2$ plus **(E)** L-cysteine or **(F)** D-cysteine. Bars correspond to means ± S.E. of 10 plants of each transgenic line or wild-type *Arabidopsis* per treatment.

ited at high concentrations of either 0.5 or 1.0 mM D-cysteine (Fig. 3D).

In the presence of 60 μM $CuCl_2$, the toxic effect of copper on root growth was decreased when the media were supplemented with L-cysteine (Fig. 3E). Likewise, with D-cysteine, all genotypes showed decreased inhibition of root development in the presence of Cu, although root growth in the *cad1-3* mutants was slightly reduced in response to 1.0 mM D-cysteine (Fig. 3F). These results suggest that the thiol group of cysteine (both L- and D-forms) probably develops strong complexes with Cu, but less so with Cd. This was confirmed because the toxic effect of D-cysteine was either undetectable or significantly diminished in the presence of copper (Fig. 3F). The lower degree of Cd toxicity in the presence of L-cysteine may have been due to increased levels of GSH and PC, rather than because of any formation of a Cd/L-cysteine complex. This can be attributed to the fact that no decrease in Cd toxicity was observed in plants supplemented with D-cysteine (Fig. 3C and D). Therefore, the copper resistance observed in *cad2-1* plants probably resulted from the formation of a cysteine/Cu complex. We also cannot exclude the likelihood that a higher cysteine level would facilitate MT biosynthesis to form a complex with Cu. For example, the ability of *Silene cucubalus* plants to prevent the GSH depletion that results from copper-induced PC production is related to copper tolerance (de Vos et al., 1992). However, the level of GSH, a substrate of PC synthase, that we measured in *cad2-1* was approximately 20% of that present in the wild-type *Arabidopsis* (Howden et al., 1995). Therefore, our results contradict those reported by de Vos et al. (1992) because it appears that low GSH levels can indeed lead to copper tolerance in *cad2-1*.

We have identified transgenic *Arabidopsis* lines that show a~20-fold increase in the expression of *A. thaliana* phytochelatin synthase, *AtPCS1*, and which demonstrate hypersensitivity to cadmium (Lee et al., 2003a). These transgenic lines, which carry a genomic clone of *AtPCS1* under the control of a 2.0-kb *AtPCS1* promoter, exhibit a 2-fold increase in both non-protein thiol (NPT) and PC production compared with the wild type when plants are exposed to 85 μM Cd for 3 d. Such hypersensitivity to cadmium in those plants might be attributed to the toxicity of PC at supra-optimal concentrations. In addition, those plants show hypersensitivity to zinc, but not copper.

Either elevated or novel production of PCs can increase resistance to Cd stress by yeast and bacteria (Clemens et al., 1999; Sandrine et al., 2003). However, no research has shown the same response with regard to copper. Therefore, to determine whether

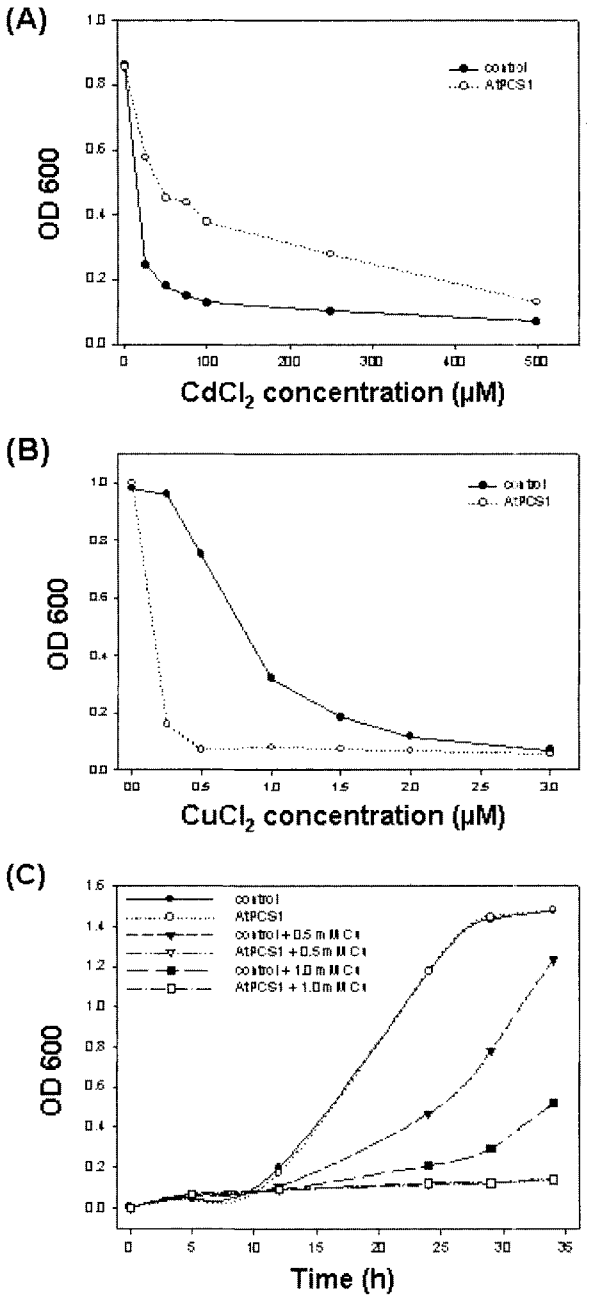

Figure 4. Sensitivity to cadmium and copper in *S. cerevisiae* cells expressing *AtPCS1*. Yeast cells containing either empty pYES2 (control) or pYES2-AtPCS1 (AtPCS1) were grown in media containing different concentrations of cadmium **(A)** and copper **(B)** for 20 h at 30°C. Afterward, cell density was measured spectrophotometrically at 600 nm. Values are means of two replicates. **(C)** Growth of yeast cells (control and AtPCS1) in media containing 0.0, 0.5, or 1.0 mM $CuCl_2$ at several time points. Growth was measured as indicated above.

PCs are involved with counteracting Cu stress, we compared sensitivities to cadmium and copper by expressing *AtPCS1* in *S. cerevisiae* under the control of a strong inducible promoter. Transgenic yeast cells showed higher resistance to Cd stress (Fig. 4A) than did the control, which contained the empty pYES vector (Clemens et al., 1999). However, *AtPCS1* expression paradoxically led to increased Cu-stress sensitivity (Fig. 4B and C). Although this result was unexpected, we had previously reported that such over-expression was associated with increased sensitivity to Cd stress in transgenic *Arabidopsis* (Lee et al., 2003a). One reason for this greater sensitivity in our transgenic yeast expressing *AtPCS1* might have been the disruption of homeostasis for Cu when PCs existed at supra-optimal concentrations, as we had proposed earlier (Lee et al., 2003a). Therefore, all our current results indicate that, although PC is not a main factor in copper detoxification, it is likely that the level of cysteine does play a role in that process.

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