Azuki Bean Cells Are Hypersensitive to Cadmium and Do Not Synthesize Phytochelatins[1]

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Suspension-cultured cells of azuki bean (*Vigna angularis*) as well as the original root tissues were hypersensitive to Cd (<10 μM). Repeated subculturings with a sublethal level of Cd (1–10 μM) did not affect the subsequent response of cells to inhibitory levels of Cd (10–100 μM). The azuki bean cells challenged to Cd did not contain phytochelatin (PC) peptides, unlike tomato (*Lycopersicon esculentum*) cells that have a substantial tolerance to Cd (>100 μM). Both of the cell suspensions contained a similar level of reduced glutathione (GSH) when grown in the absence of Cd. Externally applied GSH to azuki bean cells recovered neither Cd tolerance nor PC synthesis of the cells. Furthermore, enzyme assays in vitro revealed that the protein extracts of azuki bean cells had no activity converting GSH to PCs, unlike tomato. These results suggest that azuki bean cells are lacking in the PC synthase activity per se, hence being Cd hypersensitive. We concluded that the PC synthase has an important role in Cd tolerance of suspension-cultured cells.

Hayashi and his group (Murasugi et al., 1981; Kondo et al., 1984) first discovered the Cd-binding peptides "cadystins" in a fission yeast (*Schizosaccharomyces pombe*) exposed to Cd ions, and chemically identified the structures to $(\gamma EC)_nG$ ($n = 2, 3$). Similar $(\gamma EC)_nG$ peptides ($n = 2$–11) were then found in a Cd-binding complex produced by higher plants and named "phytochelatins" (PCs) (Grill et al., 1985). These Cd-binding peptides have been reported to contribute to Cd tolerance in many higher plants as well as in the fission yeast (for reviews, see Rauser, 1995; Zenk, 1996).

Possible roles of reduced glutathione (GSH) in PC synthesis and Cd tolerance have been suggested using an inhibitor and some mutants. Buthionine sulfoximine (BSO), a specific inhibitor of γEC synthetase (EC 6.3.2.2) (Griffith and Meister, 1979), decreases the levels of GSH and PCs, and reduces the Cd tolerance in many plant cells (Steffens et al., 1986; Grill et al., 1987; Scheller et al., 1987). Similarly, mutants lacking either γEC synthetase or GSH synthetase (EC 6.3.2.3) of fission yeast (Mutoh and Hayashi, 1988) and Arabidopsis (Howden et al., 1995a; Cobbett et al., 1998) are PC deficient and Cd hypersensitive. These results suggest that the GSH biosynthesis is required for PC synthesis and Cd tolerance of the organisms.

PC synthase that mediates the synthesis of PCs from GSH has been reported in suspension cultures of *Silene cucubalus* (Grill et al., 1989). It was characterized as the γEC dipeptidyl transpeptidase (EC 2.3.2.15) that sequentially adds γEC-unit of GSH to another GSH or PCs in vitro (Grill et al., 1989; Loeffler et al., 1989). This enzyme is constitutively expressed but requires Cd ion as the most efficient activator of metal ions (Grill et al., 1989). Similar enzyme activities have been reported in the other plants (Howden et al., 1995a, 1995b; Klapheck et al., 1995; Chen et al., 1997). More recently, PC synthase genes were isolated in Arabidopsis (Ha et al., 1999; Vatamaniuk et al., 1999) and wheat (*Triticum aestivum*; Clemens et al., 1999). Homologous genes are found in fission yeast and *Caenorhabditis elegans*. These findings suggest that the PC synthase (gene) may be more widespread and have more general functions in organisms.

Studies on the Cd-sensitive phenotypes are also very important to understand the roles of PCs and PC synthase in many organisms. So far, some mutants (*cad1*) of Arabidopsis that lack PC synthase have been obtained after an artificial mutagenesis (Howden et al., 1995b). However, little evidence has been presented for the plants that are naturally lacking in PC synthase. Previously we found that the azuki bean (*Vigna angularis*) roots were very sensitive to Cd (<10 μM) and might not produce a Cd-binding PC complex (Inouhe et al., 1994). In the present study, we examined the effects of Cd on growth and PC synthesis in suspension-cultured cells derived from azuki bean roots, to understand the biochemical basis for the Cd-sensitive phenotype. We found that the azuki bean cells are Cd hypersensitve, PC deficient, and lacking PC synthase activity.

RESULTS

Effects of Cd on Growth and Viability of Cells

In the previous study, we found that azuki bean and tomato (*Lycopersicon esculentum*) seedlings exhib-

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ited the lesser tolerance to Cd (10–30 μM) than did some monocotyledonous plants (100 μM), where 10 μM Cd inhibited the root growth by 85% and 40% in azuki bean and tomato, respectively (Inouhe et al., 1994). Here, we examined the effects of Cd on growth of suspension cells originated from their root tissues. Cd at various concentrations was added to the Murashige-Skoog growth media just before cell inoculation (time 0). Cd at 50 to 100 μM did not inhibit the growth of tomato cells for 12 d (Fig. 1), suggesting that the suspension cells have the greater Cd tolerance than root tissues in tomato, as previously reported (Inouhe et al., 1991, 1994). Cd at 150 and 200 μM strongly inhibited the initial growth of tomato cells but the reduced growth rates restored after d 4 and 8 (Fig. 1). In contrast, Cd even at 10 μM strongly inhibited growth of azuki bean cells and the inhibited growth rates did not recover (Fig. 2). These results suggest that azuki bean cells are much more sensitive to Cd than tomato cells, and may not adapt to inhibitory levels of Cd further, unlike tomato cells (Inouhe et al., 1991). Effects of Cd on growth and viability of the both plants were further compared with the larger population of cells, which had been grown for 3 d in the absence of Cd. Tomato cells at this stage are known to have the highest PC synthase activity (Chen et al., 1997). Under these conditions, growth of azuki bean cells was strongly inhibited by 10 to 20 μM Cd, whereas that of tomato cells was not inhibited at 100 μM Cd (Fig. 3). Viability of cells was determined by the method of 2,3,5-triphenyltetrazolium chloride (TTC) reduction (Fig. 4). Cd at 10 to 20 μM caused an approximately 50% decrease in the cell viability in azuki bean but viability in tomato cell cultures was little affected at 100 μM Cd. These results together suggest that azuki bean cells are hypersensitive to Cd throughout the growth stage as compared with tomato cells. Figure 4 also shows that the treatment with 1 mM GSH had no apparent effect on the cell viability of azuki bean in the presence of 10 to 100 μM Cd.

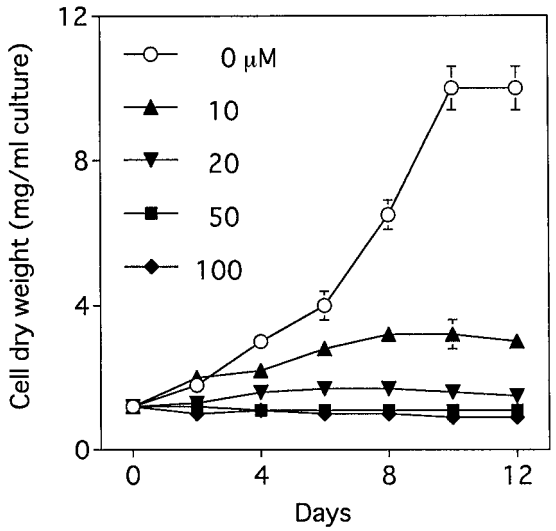

Figure 2. Effect of Cd on growth of suspension-cultured cells of azuki bean. Azuki bean cells (100 mg) were inoculated in 30 mL of liquid Murashige-Skoog medium and treated with 0 to 100 μM $CdSO_4$ for 12 d in the same medium. Cells were collected at a 2-d interval and the dry weights were determined. Data are expressed as means ± SE (n = 3).

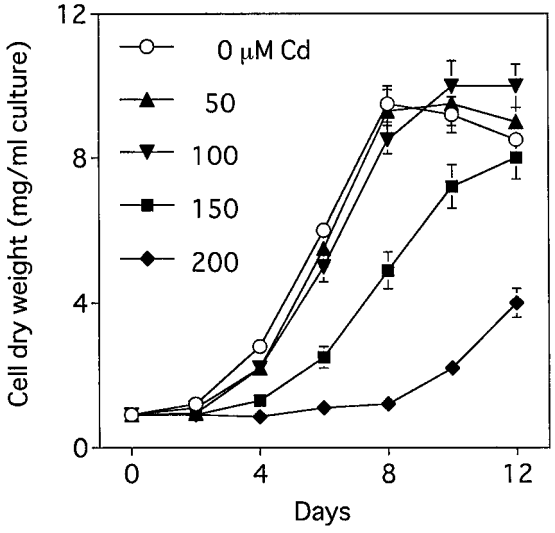

Figure 1. Effect of Cd on growth of suspension-cultured cells of tomato. Tomato cells (100 mg) were inoculated in 30 mL of liquid Murashige-Skoog medium and treated with 0 to 200 μM $CdSO_4$ for 12 d in the same medium. Cells were collected at a 2-d interval and the dry weights were determined. Data are expressed as means ± SE (n = 3).

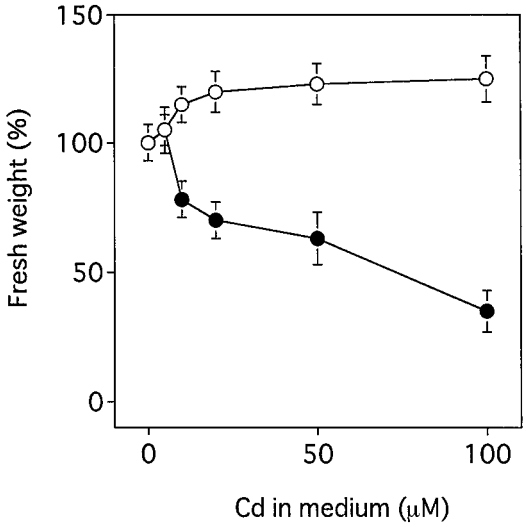

Figure 3. Effects of Cd on growth of suspension-cultured cells of tomato and azuki bean. The suspension cells were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium and then treated with various concentrations of $CdSO_4$ for 3 d in the same medium. Data are expressed as percentage of control (means ± SE, n = 3). ○, Tomato; ●, azuki bean.

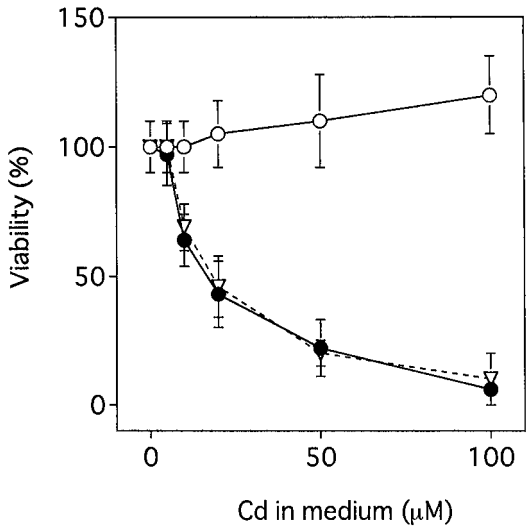

Figure 4. Effects of Cd on cell viability in tomato and azuki bean. The suspension cells were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium and then treated with various concentrations of $CdSO_4$ for 3 d in the same medium. Viability was determined by the TTC reduction method. Data are expressed as percentage of control (means ± SE, $n = 3$). ○, Tomato; ●, azuki bean; ▽, azuki bean pretreated with 1 mM GSH for 3 d.

Production of PC Peptides in Cells

The heavy metal-binding PC peptides have been reported to participate in the metal detoxification and thus heavy metal tolerance in many plant cells (Scheller et al., 1987; Mendum et al., 1990; Inouhe et al., 1991; Reese et al., 1992; Chen and Goldsbrough, 1994). A typical example for tomato is shown in Figure 5, where substantial levels of PCs are synthesized in the RCd400 cells that have been accommodated to 400 μM Cd (Inouhe et al., 1991). Therefore, we determined the levels of PC peptide in tomato and azuki bean cells grown under the same conditions. The cells were precultured for 3 d in the absence of Cd, then exposed to 0 to 100 μM Cd for 3 d. Under these conditions, tomato cells produced PCs (n = 2–4) proportional to the Cd concentration in medium, however azuki bean cells were not capable of producing PC peptides in response to Cd (Fig. 6). These cells had been stored at −30°C for 1 to 5 d and the extractions were carried out in the absence of reducing agents. To ascertain that the lack of PCs in azuki bean cells is not an artifact, we further analyzed PC peptides using more freshly prepared samples (frozen at −80°C in the presence of 100 mM ascorbate), which had been exposed to 10 μM Cd for 3 d. The PC peptides (n = 2–4) were sharply detected in tomato (Fig. 7A) but not in azuki (Fig. 7B). We also determined the PC contents in azuki bean roots that had been exposed to 0 to 20 μM Cd for 3 d, as described above. None of these roots contained detectable levels of PC peptides (Fig. 7C; M. Inouhe and S. Ninomiya, unpublished data), suggesting that the PC deficiencies are not an artifact arising from the generation of suspension cells. An alternative HPLC assay (the precolumn method) also demonstrated that azuki bean cells or root tissues are lacking in PC peptides (data not shown). These results suggest that the Cd-sensitive phenotype of azuki bean may be due to a deficiency in PC synthesis.

GSH Levels in Cells

GSH is the substrate for PC synthesis. To confirm whether an effect on GSH biosynthesis is involved in the defective PC synthesis in azuki bean cells, we determined the total GSH contents in the azuki bean and tomato cells (Fig. 8). These cells grown in the absence of Cd contained equivalent levels of GSH, suggesting that the GSH biosynthesis in azuki bean cells is not affected. The level of GSH in azuki bean cells was slightly higher in the presence of 10 to 20 μM Cd than its absence. This might be due to an enhanced synthesis of GSH by Cd, as reported previously (Chen and Goldsbrough, 1994). The decreased levels of GSH in azuki bean at 50 to 100 μM Cd might be the result of cell death. In tomato cells, Cd has been known to cause binary effects in GSH metabolism, i.e. an increase of GSH level by enhanced GSH synthesis and a decrease by enhanced

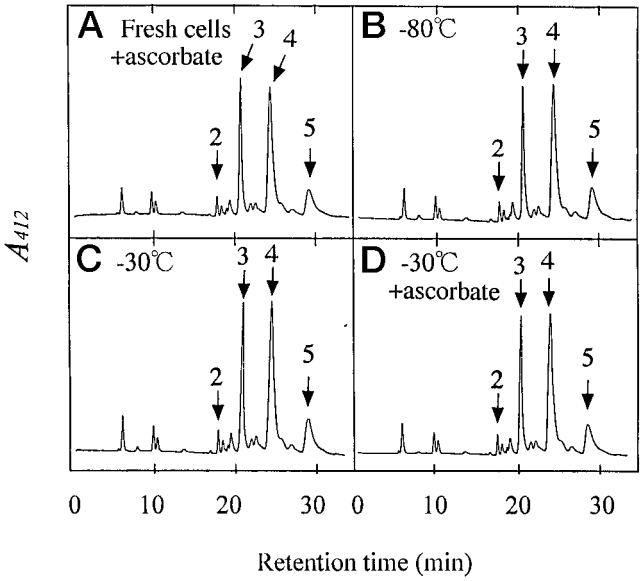

Figure 5. Effect of storage temperatures and ascorbate on the PC peptide contents in tomato cells. The RCd400 cells of tomato that had been obtained by the subculturings with 400 μM $CdSO_4$ in Murashige-Skoog medium were harvested at a stationary stage (10 d after cell inoculation). The cells (200 mg fresh weight) were frozen at −80°C in the presence of 100 mM sodium ascorbate and immediately extracted with 10% (w/v) SSA (A). The other cell samples (200 mg each) were stored for 7 d at −80°C or −30°C in the absence of ascorbate (B and C), or at −30°C in the presence of 100 mM ascorbate (D), and then extracted with 10% (w/v) SSA. The extracted PCs were analyzed by the post-column HPLC method (Mendum et al., 1990). Arrows with numbers (2–5) represent the corresponding peaks of $(\gamma EC)_nG$ peptides (n = 2–5).

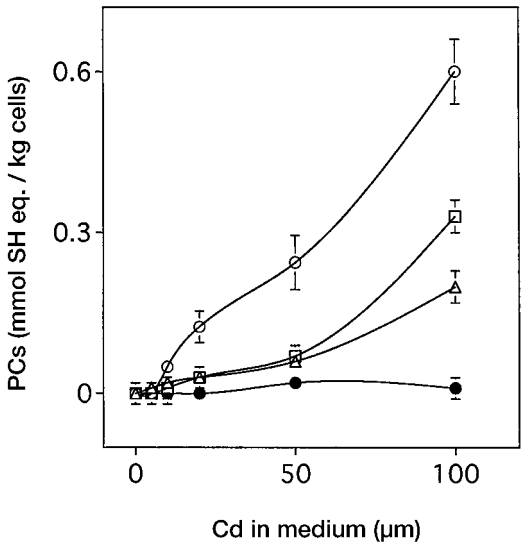

Figure 6. Effects of Cd on the levels of PC peptides in tomato and azuki bean cells. The suspension cells were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium and then treated with various concentrations of $CdSO_4$ for 3 d in the same medium. PC peptides (n = 2–4) were determined by the post-column HPLC method (Mendum et al., 1990). Data are expressed as means ± SE (n = 3). ●, Total PCs in azuki bean cells; △, $(\gamma EC)_2G$; ○, $(\gamma EC)_3G$; □, $(\gamma EC)_4G$ in tomato cells.

GSH consumption for PC synthesis (Scheller et al., 1987; Mendum et al., 1990; Chen and Goldsbrough, 1994). However such an effect of Cd in tomato cells was neither apparent in the present experiment.

Effects of GSH and BSO on Cell Growth

Exogenous GSH (50–1,000 μM) restores Cd tolerance in tomato cells affected by 30 to 200 μM BSO (Scheller et al., 1987; Mendum et al., 1990). These results were explained by an enhanced PC synthesis due to the increased levels of intracellular GSH (approximately 3- to 5-fold increase after 500 μM GSH application, even in the absence of BSO; Mendum et al., 1990). We confirmed that 1 mM GSH enhanced the tolerance of tomato cells to 150 to 400 μM Cd (data not shown), but did not influence the sensitivity of azuki bean cells to 10 to 100 μM Cd (Fig. 4), whereas it significantly increased internal GSH concentrations in both cells (data not shown). These results implied that the azuki bean cells might be lacking in some step(s) of PC synthesis after GSH biosynthesis.

Table I shows a typical example of the effects of BSO on the cell growth of azuki bean cells as affected by lower concentrations of Cd. BSO (50 μM) caused an approximately 50% inhibition of growth of azuki bean cells only in the presence of 5 μM Cd. This inhibition was completely recovered with the addition of 1 mM GSH (data not shown). GSH may therefore be essential for the Cd response of the cells, even if it cannot be convertible to PCs and hence does not contribute to further Cd tolerance of the cells.

PC Synthase Activities

PC synthase activities in the protein extracts obtained from tomato and azuki bean cells were determined under same assay conditions (Fig. 9). Tomato had a PC synthase activity, mainly producing $(\gamma EC)_2G$, as reported previously (Chen et al., 1997), however, azuki bean lacked such an enzyme activity in vitro. The PC synthase activities were further determined at different Cd concentrations (10 and 100 μM) in vitro, using protein extracts from freshly prepared cells and roots (flash-frozen in liquid N_2) (Fig. 10). Even under these conditions, PC synthase activities were detectable neither in suspension cells nor root tissues of azuki bean. Tomato cells produced $(\gamma EC)_2G$ peptides in response to Cd concentrations in the reaction mixtures (Fig. 10) and also in the absence of Cd to lesser extent (data not shown). We conclude that the lack of PC synthase may be the cause for the PC-deficient and Cd-sensitive characteristics of azuki bean cells.

DISCUSSION

The Cd-tolerance characteristics of plant cells have been well described in the liquid suspension cultures

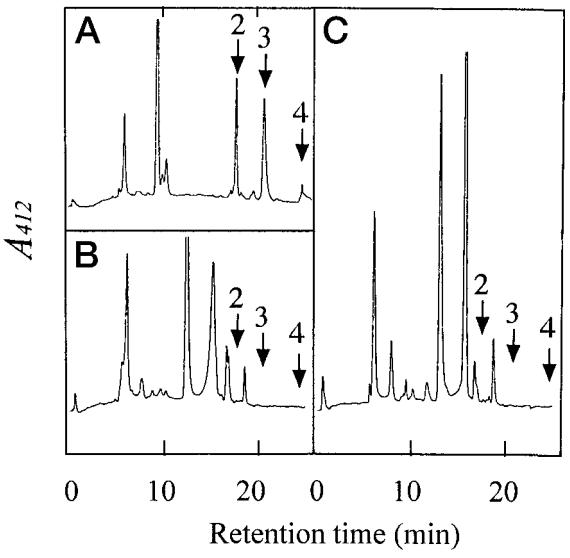

Figure 7. Formations of PC peptides in suspension cells of tomato and azuki bean and roots of azuki bean. The suspension cells of tomato (A) and azuki bean (B) were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium and then treated with 10 μM $CdSO_4$ for 3 d in the same medium. Azuki bean roots grown for 7 d (C) were exposed to 10 μM $CdSO_4$ for 3 d, as described in "Materials and Methods." The cells and root tissues were frozen at −80°C in the presence of 100 mM sodium ascorbate and immediately extracted with 10% (w/v) SSA. The extracts were analyzed by the post-column HPLC method. Arrows with numbers (n = 2–4) represent the elution times of $(\gamma EC)_nG$ peptides (n = 2–4).

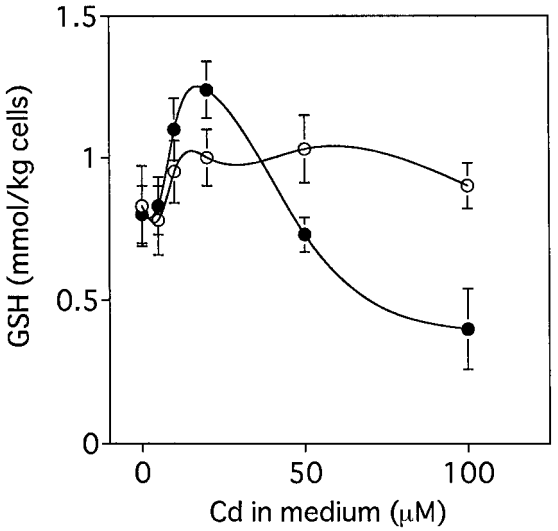

Figure 8. Effects of Cd on the total glutathione contents in tomato and azuki bean cells. The suspension cells were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium and then treated with various concentrations of $CdSO_4$ for 3 d in the same medium. The total GSH contents in the cells were determined as described in "Materials and Methods." Data are expressed as means ± SE ($n = 3$). ○, Tomato; ●, azuki bean.

as well as in roots of intact whole plants. Suspension cells originated from tomato root in this study had a substantial tolerance to Cd (Figs. 1 and 3) as reported previously in tomato (Scheller et al., 1987; Inouhe et al., 1991) and other plants (Jackson et al., 1984; Reese and Wagner, 1987; Delhaize et al., 1989). These cells exhibit tolerance to 100 μM Cd and can increase the Cd tolerance after repeated subculturings with Cd, in general. In the present study, we found that the suspension-cultured cells of azuki bean were very sensitive to Cd (Figs. 2–4), as observed in their original root tissues (Inouhe et al., 1994). The cells were not able to increase the Cd tolerance after repeated subculturing with Cd (Fig. 2; N. Sasaola and M. Inouhe, unpublished results). From these results, we conclude that the plant is naturally Cd hypersensitive. Such a Cd-sensitive response of suspension cells has not been reported for other species or cultivars of useful plants.

Biochemical and genetic bases of the Cd-tolerance/resistance phenotypes of plants may involve both the PC-dependent and PC-independent processes (Krotz et al., 1989; Wagner and Krotz, 1989; Mehra and Winge, 1990). The former also involves several different processes: the activation of PC synthase (Grill et al., 1987; Chen and Goldsbrough, 1994), GSH biosynthesis (Ruegsegger and Brunold, 1992; Chen and Goldsbrough, 1994), the accumulation of acid-labile sulfides (Verkleij et al., 1990; Reese et al., 1992), and sulfur assimilation (Nussbaum et al., 1988). All of these would be required for the formation of the more stable and functional Cd-binding complexes in plants (Rauser, 1990; Steffens, 1990; Zenk, 1996). In the present studies, we first demonstrated that azuki bean cells are deficient in PC synthase activity (Figs. 6, 7, 9, and 10). This result strongly supports the idea that the PC synthase is essentially required for Cd-tolerance phenotype of plant cells (Howden et al., 1995b).

The PC-deficient *cad1* mutants of Arabidopsis are also supersensitive to Cd (Howden et al., 1995b; Ha et al., 1999). The lowest concentration of Cd to which the *cad1* mutants (*cad1-3*) were sensitive was 0.3 μM, whereas suspension cells and roots of azuki bean were not apparently sensitive to Cd at 5 and at 1 μM, respectively (Figs. 3 and 4; M. Inouhe, unpublished data). These data suggest that some PC-independent processes might be involved in such a difference in Cd responses of plants. Although the genetic background for the lacking of PC synthase is not yet established, azuki bean can be used to study the PC-dependent mechanisms as a negative control and also to disclose the novel PC-independent mechanisms in plants. Furthermore, such a naturally PC-deficient cultivar or species is potentially important for the biological assessment of Cd contamination and for the agronomic productions of Cd-free crops and vegetables in the fields. However, behaviors of mature plants against lower levels of Cd but for longer exposure should be examined in future, as a model system for field experiments.

There remains an important question to what extent GSH is involved in Cd tolerance of plants when the PC synthesis is suppressed, since GSH itself can protect against metal toxicity as a major antioxidant or a metal acceptor in plants (Rennenberg, 1982; Noctor and Foyer, 1998). In the present study, we found that the endogenous level of total GSH in azuki bean cells was identical to that in tomato cells (Fig. 8) and that GSH treatment had no effect on the Cd response of azuki bean cells (Fig. 4). These results simply suggest that GSH cannot substitute for PCs in Cd tolerance. However, BSO significantly decreased the growth rate and viability of azuki bean cells in the

Table I. *Effect of Cd on the growth of azuki bean cells as affected by BSO*

Azuki bean cells were grown in Murashige-Skoog medium in the presence or absence of 50 μM BSO for 3 d, and they were treated with 5 to 10 μM Cd for 3 d in the same media. Dry wt of the cells were measured and percent inhibitions were calculated. Data represent means of three experiments ± SE.

Treatment	Dry Wt of Cells	Inhibition
	mg/mL culture	*%*
0 μM BSO		
+ 0 μM Cd	6.5 ± 0.3	0
+ 5 μM Cd	5.9 ± 0.4	9
+10 μM Cd	3.7 ± 0.2	43
50 μM BSO		
+ 0 μM Cd	6.2 ± 0.2	5
+ 5 μM Cd	3.3 ± 0.3	49
+10 μM Cd	2.1 ± 0.3	68

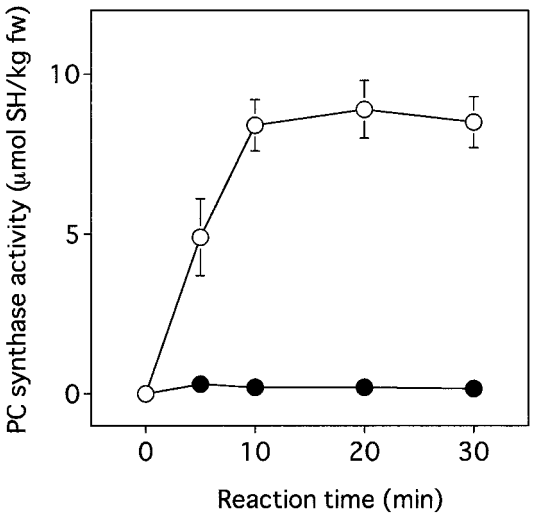

Figure 9. PC synthase activities in the extracted protein fractions of tomato and azuki bean cells. The suspension cells were precultured for 3 d in 30 mL of liquid Murashige-Skoog medium. The PC synthase activities in the cell extracts were determined for 5- to 30-min reaction periods as described in the "Materials and Methods." Data are expressed as μmol sulfhydryl residue (SH) of GSH equivalents (means ± SE, $n = 3$). Only $(\gamma EC)_2G$ was formed in this assay. ○, Tomato; ●, azuki bean.

presence of 5 to 10 μM Cd (Table I). This suggests that GSH itself may have a role in Cd tolerance and detoxification in such a Cd-sensitive type of plant cells. Cd at 10 to 20 μM caused a significant increase in the level of total GSH in azuki bean cells (Fig. 8). This result suggests that the biosynthesis might be stimulated by Cd in azuki bean cells, as reported in the other plants (Ruegsegger and Brunold, 1992; Chen and Goldsbrough, 1994; Xiang and Oliver, 1998). However, there remains another possibility that GSH is binding Cd, resulting in an increase in total GSH levels but not through any regulatory effect in the PC-deficient plants and cells. It is to be examined in future whether the over-expression of genes for GSH biosynthesis can remedy the level of Cd tolerance in azuki bean cells (Zhu et al., 1999).

MATERIALS AND METHODS

Plant Materials

Suspension-cultured cells of tomato (*Lycopersicon esculentum* cv Palace) and azuki bean (*Vigna angularis* Owhi et Ohashi cv Takara-Wase) were originated from callus tissues of the roots, according to the standard protocols (Dodds and Roberts, 1985). The suspension cells were maintained by subculturings in 90 mL of liquid Murashige-Skoog medium (Murashige and Skoog, 1962) in a 300-mL flask at 10-d intervals. Usually, one-tenth of the volume of original cultures was inoculated into new medium and the suspension cells were grown with shaking at 80 rpm under the weak white-light condition at 27°C. Seedlings of azuki bean were grown for 7 d at 25°C and the roots were exposed to 0 to 20 μM $CdSO_4$ for 3 d, as described previously (Inouhe et al., 1994).

Growth Experiments

Sterilized CdSO4 solutions (1.0–10.0 mM) were added to 30 mL of liquid Murashige-Skoog media in 100-mL flasks just before cell inoculations. Suspension cells (approximately 100 mg in fresh weight) were inoculated to each of these media and grown for 10 d at 27°C. In some experiments, the $CdSO_4$ solutions were applied to the suspension cells, which had been precultured in Cd-free Murashige-Skoog media for 3 d after initial cell inoculation. Cell growth was determined by changes in the fresh or dry weights. Cell viability was determined by the method of TTC reduction (Dodds and Roberts, 1985). Also cells were collected, frozen in liquid N_2, and stored at −30°C for the following biochemical analyses. Storage of cells at −30°C for at least 7 d caused no apparent degradation or oxida-

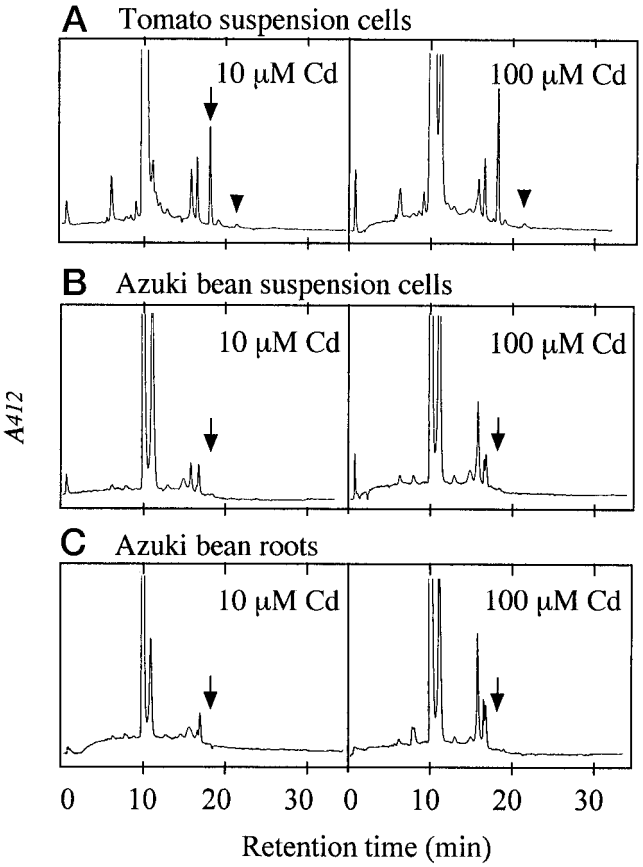

Figure 10. PC synthase activities in the extracted protein fractions of suspension cells and azuki bean roots. The suspension cells grown for 3 d in Murashige-Skoog medium or roots tissues excised from 7-d-old azuki bean seedlings were frozen at −80°C and then immediately extracted in the presence of 100 mM sodium ascorbate. The protein extracts were reacted with 10 mM GSH in the presence of 10 or 100 μM Cd for 30 min at 27°C. The reaction products were analyzed by the post-column HPLC method. Arrow and arrowhead represent the elution times for $(\gamma EC)_2G$ and $(\gamma EC)_3G$, respectively. PCs are synthesized only in the tomato protein extracts.

tion of PC peptides in tomato (Fig. 5). However, to minimize such an artificial effect, some samples of suspension cells and root tissues were frozen at −80°C in the presence of 100 mM sodium-ascorbate (pH 7.0), then immediately subjected to the following assays.

HPLC Analysis of PC Peptides and Assay for Glutathione Contents

Suspension cells were extracted with an equal volume (1 mL g^{-1} fresh weight) of 10% (w/v) 5-sulfosalicylic acid (SSA) at 0°C, as described previously (Mendum et al., 1990). The extracts were centrifuged at 10,000*g* for 1 min and the supernatants were kept at 0°C for 30 min just before HPLC analysis. The separation of PCs was carried out by the post-column method of Mendum et al. (1990) with some modification. In brief, 20-μL samples were injected to a reverse-phase column (Hibar Lichrosorb RP-18, Cica-Merck, Darmstadt, Germany) and connected to an HPLC pump (L-7110, Hitachi, Tokyo), and the column was eluted with a linear gradient of acetonitrile in 0.1% (w/v) trifluoroacetic acid at flow rate of 0.5 mL min^{-1}. The gradient program of acetonitrile was 0% for 4 min, 0% to 10% in 4 min, and then 10% to 20% (v/v) in 40 min. The column eluant was derivatized with 75 μM 5,5′-dithiobis (2-nitrobenzoic acid) in 50 mM potassium phosphate (pH 7.6) at flow rate of 1 mL min^{-1} and monitored at 412 nm (Grill et al., 1987; Mendum et al., 1990), using a UV-visible detector (L-7420, Hitachi). The retention times of PC peptides were identified with corresponding authentic (γEC) nG peptides (n = 2–5) (Matsumoto et al., 1990). The PCs contents were expressed as millimoles of sulfhydryl equivalent per kilogram fresh weight of cells, using GSH as standard. A precolumn method introduced by Scheller et al. (1987) was also adopted as an alternative method to detect PC peptides produced by cells (Inouhe et al., 1996). The total glutathione contents (glutathione + glutathione disulfide) in cell extracts were measured by the glutathione reductase recycling assay (Anderson, 1985).

Assay for PC Synthase Activity

Frozen cells were packed into two-layered nylon mesh and thawed at below 4°C. The cytoplasmic solution of the materials was pressed out into the test tube with gloved fingers and a pair of nose pliers. The insoluble materials in the solution were removed by membrane filtration (pore size, 0.45 μm). Cytoplasmic proteins in the filtrates were concentrated by ultrafiltration (USY-1, Advantec, Tokyo) under N_2 and used for enzyme assays. Assays for PC synthase activities were carried out according to the method of Grill et al. (1987): Approximately 50 μg of the total proteins prepared from tomato or azuki bean cells were reacted in a 300-μL mixture solution containing 10 mM GSH, 0.1 mM $Cd(NO_3)_2$, and 90 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)-HCl (pH 8.0) for 30 min at 27°C. After each 10-min interval, a part of the reaction mixture (50 μL) was taken and treated with 50 μL of 10% (w/v) SSA at 0°C to cease reaction. PC peptides in the reaction product (20 μL) were determined by HPLC as described above. Some cells (or root tissues) were frozen at −80°C in the presence of 100 mM sodium ascorbate and the protein extracts were directly subjected to assays for PC synthase as described above.

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