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Cadmium-Sensitive, *cad1* Mutants of *Arabidopsis thaliana* Are Phytochelatin Deficient[1]

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An allelic series of *cad1*, cadmium-sensitive mutants of *Arabidopsis thaliana*, was isolated. These mutants were sensitive to cadmium to different extents and were deficient in their ability to form cadmium-peptide complexes as detected by gel-filtration chromatography. Each mutant was deficient in its ability to accumulate phytochelatins (PCs) as detected by high-performance liquid chromatography and the amount of PCs accumulated by each mutant correlated with its degree of sensitivity to cadmium. The mutants had wild-type levels of glutathione, the substrate for PC biosynthesis, and in vitro assays demonstrated that each of the mutants was deficient in PC synthase activity. These results demonstrate conclusively the importance of PCs for cadmium tolerance in plants.

Plants respond to heavy-metal stress with the induction of a class of peptides consisting of repeating units of γ-glutamylcysteine followed by a C-terminal Gly. These have been named PCs (Grill et al., 1985), cadystins (Kondo et al., 1983), poly-γ(EC)$_n$G peptides (Jackson et al., 1987), and Cd-binding peptides (Reese and Wagner, 1987a) in various studies and will be referred to here as PCs. PCs appear to be ubiquitous in higher plants (Gekeler et al., 1989) and have also been found in algal species (Gekeler et al., 1988) and in some yeasts, particularly *Schizosaccharomyces pombe*, in which they were first discovered (Murasugi et al., 1981, 1983). The structure of PCs suggested that GSH (γ-glutamylcysteinylglycine) is the substrate for PC biosynthesis. Plant cell cultures that are treated with BSO, an inhibitor of γ-glutamylcysteine synthetase, do not accumulate PCs because of the block in GSH biosynthesis. BSO also renders cells hypersensitive to Cd (Mendum et al., 1990). In addition, genetic studies using *S. pombe* have shown that GSH-deficient mutants are also PC deficient and Cd hypersensitive (Mutoh and Hayashi, 1988; Glaeser et al., 1991). The role of GSH as a substrate for PC biosynthesis was confirmed by the purification of a plant enzyme PC synthase (γ-glutamylcysteine dipeptidyl transpeptidase), which transfers a γ-glutamycysteine moiety from GSH to a second molecule of GSH or a previously formed PC molecule (Grill et al., 1989). The enzyme appears to be expressed constitutively in *Silene cucubalis*, but its activity is absolutely dependent on the presence of heavy metal ions. This provides an elegant feedback control mechanism for PC biosynthesis in which "free" heavy metal ions that activate the biosynthesis of PCs are then bound and no longer available to activate the PC synthase (Loeffler et al., 1989).

Recently, a second pathway of PC biosynthesis was demonstrated in vitro for *S. pombe*. Crude cell extracts were found to form γ-Glu-Cys polymers derived from GSH with the subsequent addition of the terminal Gly in a reaction catalyzed by GSH synthetase (Hayashi et al., 1991). This pathway was not dependent on the presence of heavy metal ions. The relative contribution of these two pathways to PC biosynthesis in vivo has not been determined.

That PCs play an important role in the detoxification of heavy metals has been inferred from the experiments referred to above using BSO and the genetic studies of various mutants of *S. pombe*, in which a deficiency in the biosynthesis of PCs or in the formation of Cd-PC complexes in vivo confers heavy metal hypersensitivity on an organism or cell culture. In contrast, studies of naturally evolved heavy-metal-tolerant varieties of plants or laboratory-selected tolerant cell lines have not demonstrated a clear correlation between increased resistance and increased PC production. In Cd-tolerant tomato cells, PCs accumulated at higher levels than in the nontolerant line (Steffens et al., 1986; Gupta and Goldsbrough, 1991). Cd-tolerant and nontolerant cell-suspension cultures of *Datura innoxia* accumulated PCs at similar rates but the rate of metal-complex formation was higher in the tolerant line (Delhaize et al., 1989). Similarly, Cu tolerance in a naturally selected line of *Mimulus guttatus* appears to be attributable to PC formation (Salt et al., 1989), whereas tolerance to Cd and Zn in different lines of *Silene vulgaris* is not due to increased PC production (Harmens et al., 1993). Thus, it seems that accumulation of PCs is a major component of heavy-metal detoxification processes but increased tolerance to metals may involve either other aspects of PC function or other mechanisms for heavy-metal detoxification.

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Abbreviations: BSO, L-buthionine sulfoximine; DEB, 1,2:3,4-diepoxybutane; HMW, high molecular weight; LMW, low molecular weight; MT, metallothionein; PC, phytochelatin [(γ-Glu-Cys)$_n$-Gly]; PC$_2$, (γ-Glu-Cys)$_2$-Gly.

In animal cells and some fungi, MTs appear to play the major role in heavy-metal detoxification. MTs are small Cys-rich proteins that are also induced by heavy metals but, in contrast to PCs, are gene products (Hamer, 1986). In plants, studies of heavy-metal-binding complexes have generally revealed the presence of PCs but not MTs and it was thought that PCs were the plant kingdom's equivalent of MTs (Grill et al., 1987). However, more recently, MT genes have been isolated from many plant species, including *Arabidopsis* (de Miranda et al., 1990; Evans et al., 1990; de Framond, 1991; Kawashima et al., 1991; Zhou and Goldsbrough, 1994). The importance of these MT proteins in vivo has, in most cases, not been demonstrated, although the *Arabidopsis* MT genes have been shown to restore metal tolerance to an MT-deficient mutant of *Saccharomyces cerevisiae* (Zhou and Goldsbrough, 1994). Both PCs and MTs appear to have roles in heavy-metal detoxification in different organisms; however, it has not yet been determined to what extent their individual roles are complementary or redundant in plants.

As a part of a systematic genetic analysis of the mechanism(s) for heavy-metal detoxification in plants, we previously described the isolation of a Cd-sensitive mutant, *cad1*, of *Arabidopsis thaliana* that appeared to be unable to accumulate or sequester Cd (Howden and Cobbett, 1992). Here we describe the isolation and biochemical characterization of an allelic series of *cad1* mutants and demonstrate that these mutants are deficient both in their ability to accumulate PCs when exposed to Cd and in PC synthase activity measured in vitro.

MATERIALS AND METHODS

Plant Materials

Methods for the surface sterilization of seeds and the growth and genetic analysis of plants were as described previously (Howden and Cobbett, 1992). All mutants were isolated in the Columbia ecotype. Mutagenesis using ethyl methanesulfonate was as described by Haughn and Somerville (1986). For mutagenesis using DEB seeds were soaked overnight in water and then shaken gently in water containing 11 mM DEB for 4 h. They were then washed repeatedly before sowing directly onto soil. M_2 populations were screened for new *cad1* mutants by germinating approximately 10,000 surface-sterilized seeds on nutrient medium containing 0.8% agar and 3 μM $CdSO_4$ and allowing them to grow for 7 to 10 d. The roots embedded in the medium were then examined for the presence of a brown coloration (see below) by observing the inverted plates against a white background under a dissecting microscope. Growth measurements of seedlings in the presence of Cd were as described previously (Howden and Cobbett, 1992) except that seedlings were transferred to the test medium 8 d after imbibition and the seedling weights were measured after an additional 8 d. The Cd-sensitive phenotype was routinely scored on medium containing 3 or 6 μM $CdSO_4$.

Gel-Filtration Chromatography of Cd-Binding Complexes

Seedlings were grown in 25 mL of liquid nutrient medium containing 2% Glc for 10 d in the absence of Cd and then exposed for an additional 3 d to 6 or 30 μM Cd containing 0.3 μCi $^{109}CdCl_2$. Preparation of extracts of plant tissue and their subsequent fractionation by chromatography on a 1.6- × 92-cm column of Sephadex G-50 was according to the procedure described by Speiser et al. (1992a). Two-milliliter fractions were collected and assayed directly for radioactivity by using a γ-radiation counter.

Glutathione Assays

Plant material was grown as described above in the absence of Cd. Extracts were prepared and assayed for total glutathione using the glutathione reductase recycling assay described by Anderson (1985).

Quantitation of GSH and PCs by HPLC

Seedlings were grown in 25 mL of half-strength Murashige-Skoog medium containing 1% Glc for 10 or 11 d in the absence of Cd and then exposed for an additional 1 d to 30 μM Cd. Approximately 0.5 g of seedling tissue was placed in a microfuge tube and frozen in liquid N_2, 10% (w/v) 5-sulfosalicylic acid was added (1 mL/g tissue), and the mixture was ground with a pestle. After the sample was vortexed and incubated on ice for 10 min, insoluble material was removed by centrifugation. Extract (220 μL) was mixed with 60 μL of 2 mM *N*-acetyl-Cys (included as a standard) and passed through a 0.2-μm filter. This mixture (250 μL) was analyzed by HPLC using postcolumn derivatization to detect GSH and PCs (Gupta and Goldsbrough, 1991). The detection limit of the HPLC assay for PC_2 was 0.2 nmol.

PC Synthase Assay

Seedlings were grown as described above without exposure to Cd. Approximately 2 g of seedling tissue was frozen in liquid N_2 and then ground in a chilled mortar in extraction buffer (0.05 M Tris-HCl [pH 8.0], 0.01 M β-mercaptoethanol, 10% [v/v] glycerol; 1 mL of buffer per g of tissue). The homogenate was centrifuged at 10,000*g* for 10 min and the supernatant was used to assay for PC synthase. The assays contained 0.2 M Tris-HCl (pH 8.0), 0.01 M β-mercaptoethanol, 3.3 mM GSH, 0.5 mM $CdCl_2$ and were incubated at 37°C. Samples were taken after 0 and 40 min and the reaction was stopped with the addition of 5-sulfosalicylic acid to a final concentration of 5% (w/v). Denatured protein was removed by centrifugation and the supernatants were assayed for PCs by HPLC as described above.

RESULTS

Isolation of New *cad1* Mutant Alleles

When the original *cad1-1* mutant was grown on agar medium in the presence of inhibitory levels of Cd, growth was inhibited, the leaves became progressively chlorotic, and the roots developed a distinct brown pigment (not

shown). This was clearly not due to death and tissue necrosis, because the roots continued to grow extensively, although at a reduced rate compared with wild type. This phenotype was not observed for the wild type even at higher, equally inhibitory, concentrations of Cd. Similarly, when *cad1* seedlings were grown in liquid culture and exposed to Cd the tissue and the growth medium became brown. Although the nature of the brown pigment or the mechanism by which it is formed has not been determined, it provides a simple visible phenotype for identifying *cad1* mutants.

Three additional independent mutants were isolated using this phenotype. Each was crossed to the wild type, and in the F_1 the mutant phenotype was recessive to the wild type and in the F_2 the mutant phenotype segregated as a recessive Mendelian character (data not shown). Each mutant was also crossed to the *cad1-1* line and at least 30 F_1 individuals from each cross were tested. All of the F_1 individuals tested were Cd sensitive, indicating that the new mutations were alleles of *cad1*. One of these, *cad1-3*, was derived from DEB mutagenesis of a line homozygous for the *ara1* mutation (Dolezal and Cobbett, 1991). From the backcross to the wild type, two lines that were homozygous wild type at the *ARA1* locus were isolated. The Cd sensitivity of both lines was indistinguishable from that of the original mutant (data not shown). The alleles *cad1-4* and *cad1-5* were isolated from ethyl methanesulfonate-mutagenized wild-type M_2 seeds. These, too, have each been backcrossed once to the wild type.

The sensitivity of the new *cad1* mutants to Cd was compared with the wild type and the *cad1-1* mutant previously studied. The data comparing the growth of wild type with that of the *cad1-1* and *cad1-3* mutants are shown in Table I and the growth of each mutant in the presence of various concentrations of Cd is summarized Figure 1. The lowest concentration of Cd to which the *cad1-3* and *cad1-4* mutants were sensitive was 0.3 μM, whereas *cad1-1* and *cad1-5* were sensitive to 0.6 and 1.5 μM, respectively. As shown in Figure 1 growth of *cad1-1* in the presence of 0.3 μM Cd was reduced slightly compared with the wild type, but the characteristic chlorosis observed with the sensitive phenotype was not apparent. The roots of the *cad1-5* mutant were less brown than the other mutants even in the presence of 3 μM Cd, a concentration at which all of the mutants appeared equally sensitive.

Table I. *Sensitivity of cad1 mutants to Cd*

Seedlings were treated as in Figure 1 and were visually scored for the sensitive phenotype: reduced growth, chlorosis, and brown roots. R, Insensitive; S, sensitive. Root color refers to the color of the roots of seedlings grown in the presence of 3 μM Cd at the time the sensitive phenotype was scored: dark, dark brown; pale, pale brown.

Strain	$CdSO_4$ (μM)					Root Color
	0.15	0.3	0.6	1.5	3.0	
Wild type	R	R	R	R	R	White
cad1-1	R	R	S	S	S	Dark
cad1-3	R	S	S	S	S	Dark
cad1-4	R	S	S	S	S	Dark
cad1-5	R	R	R	S	S	Pale

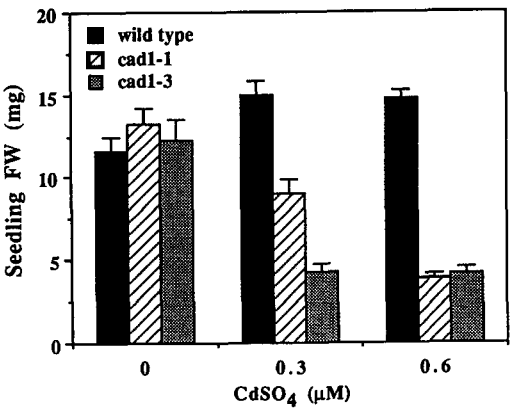

Figure 1. Sensitivity of *cad1* mutants to Cd. Eight-day-old seedlings were transferred (10 plants per 9-cm Petri dish) to medium containing added $CdSO_4$. Total leaf fresh weights (means ± SE, n = 5) were determined after an additional 8 d of growth. FW, Fresh weight.

Of these new alleles *cad1-3* has been studied most extensively. Since this mutant is more sensitive than the original *cad1-1* mutant, it was important to confirm that the sensitive phenotype was conferred by only a single mutation. First, the F_2 population generated after crossing to the wild type segregated in a 3:1 ratio of 661 wild type:237 sensitive (χ^2 = 0.89) individuals, indicating that only a single genetic locus was involved. Second, two homozygous mutant lines tested after the first backcross both exhibited the more sensitive phenotype. Third, the isolation of the *cad1-4* mutant, which is as sensitive as the *cad1-3* mutant, supports the hypothesis that this phenotype is conferred solely by the mutation at the *cad1* locus. Furthermore, because DEB is known to generate deletion mutations (Reardon et al., 1987), it is possible that *cad1-3* is a null allele.

Gel-Filtration Chromatography of Cd-Binding Complexes

One explanation for the Cd-sensitive phenotype of the *cad1* mutants is that they are deficient, to different extents, in their ability to form Cd-binding complexes. To test this, extracts from seedlings exposed to Cd and labeled with ^{109}Cd were chromatographed on a Sephadex G-50 gel-filtration column. Figure 2A shows the column profiles obtained for wild-type seedlings exposed to 6 and 30 μM $CdSO_4$. Two peaks, referred to as HMW and LMW, of Cd-binding material were observed. In extracts from seedlings exposed to 6 μM $CdSO_4$ the LMW peak was predominant, whereas at 30 μM the LMW peak was visible only as a shoulder on the larger HMW peak. In contrast, in extracts from *cad1-1* mutant seedlings exposed to 30 μM Cd only a small peak of LMW material was observed (Fig. 2B), and for the more sensitive *cad1-3* mutant no Cd-binding complexes were detected (Fig. 2C). Thus, the *cad1* mutants are deficient in their ability to form Cd-binding complexes.

Accumulation of PCs in the Wild Type and Mutants

To determine whether the inability of *cad1* mutants to form Cd-binding complexes reflected an inability to accumulate PCs, extracts obtained from seedlings unexposed or

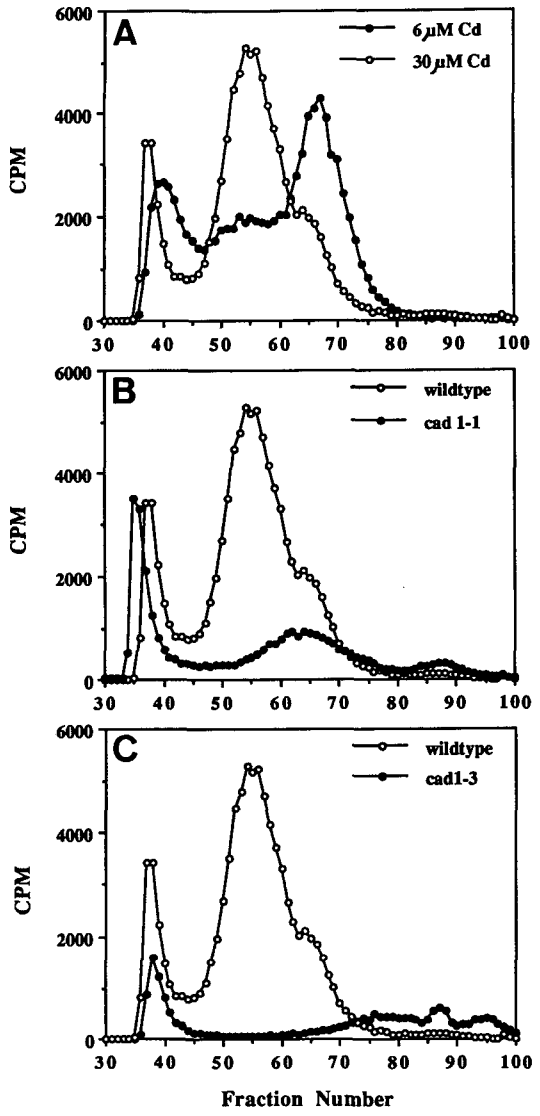

Figure 2. Cd-binding complexes of wild type and *cad1* mutants detected by gel-filtration chromatography. Seedlings were exposed to Cd in liquid culture in the presence of ^{109}Cd. Extracts were chromatographed on a Sephadex G-50 column. ^{109}Cd in the indicated fractions was measured by using a γ-counter. A void volume peak probably due to nonspecific binding of Cd to cellular components is centered at fraction 38; peaks for the HMW and LMW Cd-binding complexes are centered around fractions 54 and 67, respectively; and the free Cd peak is centered around fraction 94. A, Extracts of wild type exposed to 6 and 30 μM Cd; B, extracts of wild type and *cad1-1* exposed to 30 μM; C, extracts of wild type and *cad1-3* exposed to 30 μM Cd.

exposed to Cd were assayed for total PC content by reversed-phase HPLC as described in "Materials and Methods." In both the wild type and all but one of the *cad1* mutants only a low level of PC_2 was detected in the absence of Cd. No PCs were detected in extracts prepared from *cad1-3* seedlings. Exposure of wild-type seedlings to 30 μM Cd for 1 d resulted in a significant accumulation of PCs (Table II). Over a range of Cd concentrations from 10 to 100 μM, the levels of PCs in wild-type seedlings were approximately proportional to the Cd concentration in the medium (data not shown). In contrast, PC accumulation was reduced in each of the *cad1* mutants exposed to 30 μM Cd compared to the wild type. HPLC profiles of extracts from wild type, *cad1-1*, and *cad1-3* seedlings are shown in Figure 3 and the data for all mutants are shown in Table II. In the *cad1-3* mutant, which is the most sensitive to Cd, no PCs were detected even after 3 d of exposure to Cd. In *cad1-4*, which is as sensitive as *cad1-3*, and in the *cad1-1* and *cad1-5* mutants, which are less sensitive than *cad1-3*, PC levels were 8, 25, and 33%, respectively, of wild-type levels. The accumulation of PCs correlated with the Cd sensitivity of the different mutants.

GSH Levels in the Wild Type and Mutants

It was possible that the *cad1* mutants failed to accumulate PCs because of an inability to synthesize GSH. Thus, GSH levels were also determined for the wild type and mutants by HPLC assay (Fig. 4). In the absence of Cd the concentrations of GSH in the wild type and mutants were similar, indicating that the mutants were not deficient in GSH biosynthesis. In some of the *cad1* mutants the GSH level consistently decreased about 30% compared with the wild type after further growth for 1 d in the absence of Cd. The cause of this effect is unknown. Treatment of wild-type seedlings with 30 μM Cd for 1 d also resulted in a small decrease in GSH, whereas Cd exposure produced a dramatic increase in GSH concentration in each of the mutants.

The wild type and *cad1-1* and *cad1-3* mutants were also assayed for GSH in the absence of exposure to Cd using the glutathione reductase recycling assay.The level for the wild type was about 2-fold that obtained for the HPLC assay. The *cad1-1* mutant had a level of GSH approximately equal to that in the wild type; however, in *cad1-3* the GSH concentration was about 2-fold that in the wild type (data not shown; Howden et al., 1995). These seedlings were grown under different conditions from those prepared for the HPLC assays and this may account for the differences in GSH levels obtained using the two assays. In addition, the HPLC assay measures only GSH and not GSSG.

Table II. *PC accumulation by wild-type and mutant seedlings*

Total PCs (measured as GSH equivalents) were assayed in extracts of seedlings exposed to 30 μM Cd for 1 d (mean ± SE, n = 11 for 0 Cd treatment, n = 5 for 30 μM Cd treatment).

Cd	Total PCs				
	Wild type	*cad1-1*	*cad1-3*	*cad1-4*	*cad1-5*
μM	*μmol GSH equivalents/kg fresh wt (% wild type)*				
0	5 ± 1	5 ± 0	<0.2	2 ± 0	1 ± 1
30	150 ± 13 (100)	37 ± 5 (25)	<0.2 (<0.2)	12 ± 1 (8)	50 ± 6 (33)

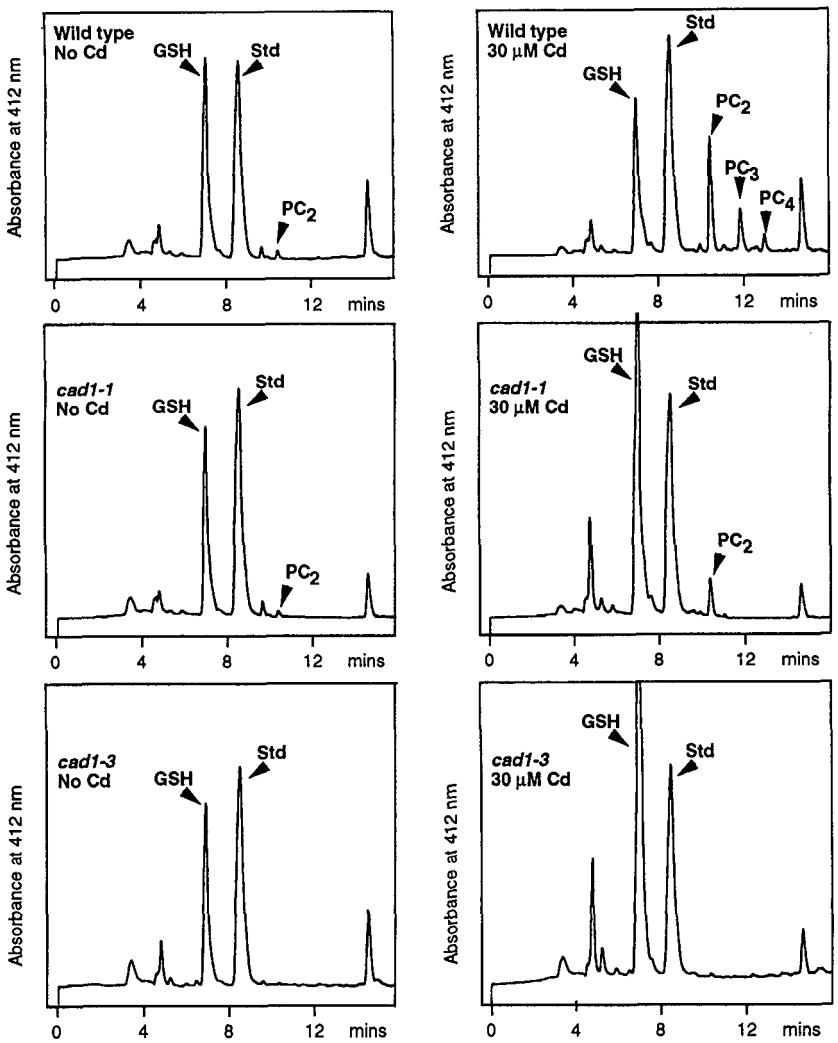

Figure 3. PC accumulation by wild-type and mutant seedlings. Extracts from wild-type, *cad1–1*, and *cad1–3* seedlings grown under control conditions or after exposure to 30 μM Cd for 1 d were analyzed by HPLC as described in "Materials and Methods." The peaks that represent GSH, *N*-acetyl-Cys (Std), PC_2, PC_3, and PC_4 are identified.

PC Synthase Activity in Wild Type and Mutants

Because PC accumulation is deficient in the *cad1* mutants but GSH biosynthesis is unaffected, it seemed likely that the PC synthase activity in these mutants was deficient. PC synthase has been reported to be constitutively expressed and is activated by heavy metals, particularly Cd. Thus, extracts prepared from seedlings grown in the absence of Cd were assayed for PC synthase activity as described previously. Extracts from both wild type and a new Cd-sensitive mutant, *cad2–1*, described in the accompanying paper (Howden et al., 1995), were able to synthesize significant amounts of PC_2 in vitro (Table III). However, this activity could not be detected reproducibly in extracts of any of the *cad1* mutants. In some experiments small amounts of PC_2 were synthesized in extracts from *cad1–1*, but this was barely above the limit of detection for this compound using the HPLC assay. We conclude that the level of PC synthase activity in each of the *cad1* mutants is less than 1% of that observed in either the wild type or *cad2–1*. Given that some PCs are detected in most of the *cad1* mutants, these are likely to contain a low level of PC synthase activity. The method currently used to detect PCs

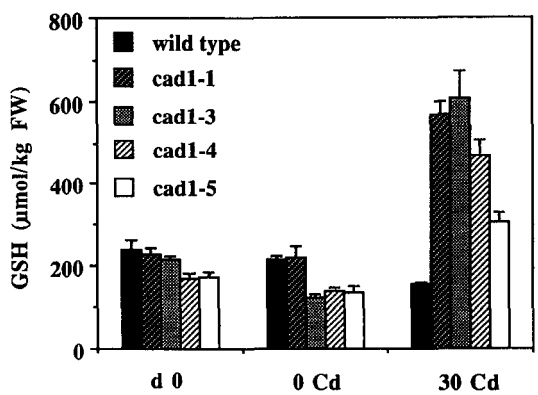

Figure 4. GSH levels in wild type and mutants before and after exposure to Cd. Extracts from wild-type and mutant seedlings were assayed for GSH (μmol GSH/kg fresh weight [FW] ± SE) by HPLC as described in "Materials and Methods." Samples of tissue were obtained immediately before exposure (d 0) and after 1 d in the absence (0 Cd) or presence (30 Cd) of 30 μM Cd. The number of replicates ranged from two to six.

Table III. *PC synthase activity in wild type and cad1 mutants*

PC synthase was assayed in extracts prepared from 10-d-old seedlings grown in liquid medium containing no added Cd. Results are the means (± SE) of five independent assays for each genotype. PC synthase activity is reported as nmol of GSH equivalents formed min^{-1} mg^{-1} of protein. Only PC_2 was formed in this assay.

Genotype	PC Synthase Activity
Wild type	3.04 (0.55)
cad1-1	0.01 (0.01)
cad1-3	<0.01
cad1-4	<0.01
cad1-5	<0.01
cad2-1	2.17 (0.49)

is not sufficiently sensitive to measure this activity in the *cad1* mutants.

Effect of BSO on *cad1-3*

It has been proposed that GSH itself plays a role in Cd detoxification in mammals (Freedman et al., 1989), and in the yeast *Candida glabrata*, CdS crystallites coated with GSH have been isolated (Dameron et al., 1989). In contrast, PCs are 1000-fold more efficient than GSH at reactivating metal-poisoned enzymes in vitro (Kneer and Zenk, 1992), indicating that GSH might be expected to play only a minor role, if any, in Cd detoxification in comparison with PCs. We previously examined the effect of BSO on the *cad1-1* mutant (Howden and Cobbett, 1992). The above data demonstrate that *cad1-1* is able to accumulate small quantities of PCs and, therefore, the effect of BSO on GSH biosynthesis cannot be distinguished from its additional effect on PC biosynthesis. This difficulty can be avoided by using *cad1-3*, in which no PCs were detectable. Figure 5 shows the growth of *cad1-3* in the presence and absence of BSO at various concentrations of Cd. BSO significantly increased the sensitivity of wild type to Cd but in contrast did not have a significant effect on either the growth or appearance (not shown) of *cad1-3* plants. Slight differences between the wild type and mutant in the presence of BSO were observed on 0.3 and 0.6 μM Cd; however, on 0.15 μM Cd the wild type and mutant were indistinguishable. This suggests that GSH itself does not play a significant role in Cd detoxification.

DISCUSSION

The observation that the roots of the *cad1-1* mutant develop a distinct brown pigment in the presence of Cd has provided a new approach to the isolation of Cd-sensitive mutants. Scoring M_2 seedlings for the brown root phenotype is a much less tedious procedure than the original root growth assay used to identify Cd-sensitive mutants and has been used to identify the three new alleles of *cad1* described here and a new mutant, *cad2*, described in the accompanying paper (Howden et al., 1995). The basis of this phenotype is not yet understood. Preliminary data (R. Howden, C.R. Andersen, and C.S. Cobbett, unpublished observations) suggest that the brown pigment is probably a deposit of various metal-sulfide complexes. The production of high levels of sulfide may be an adaptive response to the presence of Cd in plants unable to form sufficient amounts of PCs.

The experiments described above demonstrate that the *cad1* mutants are deficient in PC synthase activity. Four independent *cad1* mutants with three differing degrees of Cd sensitivity have been analyzed. The gel-filtration assays of the *cad1-1* and *cad1-3* mutants demonstrate that they are deficient in their ability to form Cd-binding complexes in vivo. In the wild type two peaks of Cd-binding complexes were observed. This is similar to that observed in *S. pombe*. The HMW complexes from *S. pombe* have a higher S^{-2}:Cd ratio and are more stable than the LMW complexes (Murasugi et al., 1983). Formation of the HMW complex is essential for Cd tolerance in *S. pombe* as demonstrated by the isolation of various Cd-sensitive mutants that are deficient in formation of the HMW complex (Ortiz et al., 1992; Speiser et al., 1992b).

Two peaks of Cd-binding complexes have not generally been observed in extracts from plants and have only been described for tomato (Reese et al., 1992) and a Se-tolerant variety of *S. vulgaris* (Speiser et al., 1992a). In the assays described here, the proportion of bound Cd appearing in each peak depended on the level of Cd to which the plants were exposed and at the higher concentration (30 μM Cd) the LMW peak was almost undetectable. It may be that in other studies the concentration of Cd has been sufficiently high that only a single peak of Cd-binding complexes was observed. Likewise, the duration of exposure may influence the relative size of the two peaks; however, this was not investigated here. Neither of the peaks in the wild type was directly assayed for PCs using HPLC. However, the observations that the two peaks appear to be interconvertible and that both are absent in the *cad1-3* mutant suggest that they both consist of essentially the same material. Furthermore, since no PCs were detected in the *cad1-3* mutant, it is reasonable to assume that these peaks consist of PC-Cd complexes. In the *cad1-1* mutant a residual peak of Cd-binding material was observed and this correlated with the observations that *cad1-1* was less sensitive than

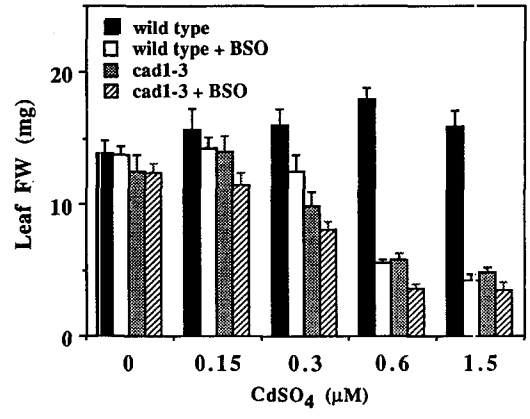

Figure 5. The effect of BSO on Cd sensitivity of wild type and *cad1-3*. Seedlings were treated as in Figure 1 and grown in the presence of Cd with or without 0.5 mM BSO. FW, Fresh weight.

cad1–3 and that it accumulated some PCs when exposed to Cd.

All of the *cad1* mutants were deficient in PC accumulation when exposed to Cd and the level of PCs observed correlated with the level of sensitivity of the mutant. Furthermore, all of the mutants were deficient in PC synthase activity. Thus, it is likely that *cad1* encodes the PC synthase activity itself. Although a regulatory role for *CAD1* may be possible, no other locus has been identified that confers a phenotype similar to that of *cad1* mutants and that also affects PC synthase activity. Thus, *CAD1* is most likely to be the structural gene for PC synthase. Recently, a possible alternative pathway for the biosynthesis of PCs was demonstrated in vitro using crude extracts of *S. pombe* (Hayashi et al., 1991). From the studies described here it appears that *Arabidopsis* has only a single pathway for PC biosynthesis. If there are two pathways, as proposed for *S. pombe*, then these must share a common step that is affected by the mutations at the *CAD1* locus.

These mutants confirm the essential role played by PCs in Cd detoxification in *Arabidopsis*. Previous observations have shown that *cad1* mutants are also sensitive to Hg but only slightly sensitive to Cu and Zn (Howden and Cobbett, 1992). Although these studies were done using the *cad1–1* mutant, the same is true for the *cad1–3* mutant (R. Howden, P.B. Goldsbrough, C.R. Andersen, and C.S. Cobbett, unpublished observations). PC biosynthesis is induced by both Cu and Zn (Grill et al., 1987), although the observation of the latter has been contradicted by other studies (Reese and Wagner, 1987b). Furthermore, PC synthase activity is also activated by Cu and Zn in vitro (Grill et al., 1989). However, the observation that *cad1* mutants are only slightly sensitive to Cu and Zn indicates that PCs play a comparatively minor role in the detoxification of these heavy metals and suggests the presence of some other mechanism(s) for their detoxification. Recently, MT genes were identified in *Arabidopsis* (Zhou and Goldsbrough, 1994) and these may well fulfill such a role. Notwithstanding these observations, PCs may play a role in essential heavy-metal homeostasis. It has also been proposed that PCs are involved in aspects of sulfur reduction and transport (Robinson, 1989). The *cad1–3* mutant, in which no PCs are detectable, is of particular interest since it will allow such hypotheses about the functions of PCs to be tested directly. This mutant has no mutant phenotype in the presence of only trace concentrations of essential micronutrients such as Cu, Zn, and Co. This suggests that under "normal" laboratory conditions PCs do not play an essential role in heavy-metal homeostasis. However, it will be of further interest to examine the growth of the *cad1–3* mutant under conditions of heavy-metal deficiency.

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