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# Cadmium-Sensitive Mutants of *Arabidopsis thaliana*[1]

**Ross Howden and Christopher S. Cobbett***

*Department of Genetics, The University of Melbourne, Parkville, Australia, 3052*

## ABSTRACT

**A screening procedure for identifying Cd-sensitive mutants of *Arabidopsis thaliana* is described. With this procedure, two Cd-sensitive mutants were isolated. These represent independent mutations in the same locus, referred to as *CAD1*. Genetic analysis has shown that the sensitive phenotype is recessive to the wild type and segregates as a single Mendelian locus. Crosses of the mutant to marker strains showed that the mutation is closely linked to the *tt3* locus on chromosome 5. In addition to Cd, the mutants are also significantly more sensitive to mercuric ions and only slightly more sensitive to Cu and Zn, while being no more sensitive than the wild type to Mn, thus indicating a degree of specificity in the mechanism affected by the mutation. Undifferentiated callus tissue is also Cd sensitive, suggesting that the mutant phenotype is expressed at the cellular level. Both wild-type and mutant plants showed increased sensitivity to Cd in the presence of buthionine sulfoximine, an inhibitor of the biosynthesis of the cadmium-binding ($\gamma$-glutamyl-cysteine)$_n$-glycine peptides, suggesting that the mutant is still able to synthesize these peptides. However, the effects of a *cad1* mutation and buthionine sulfoximine together on cadmium sensitivity are essentially nonadditive, indicating that they may affect different aspects of the same detoxification mechanism. Assays of Cd uptake by intact plants indicate that the mutant is deficient in its ability to sequester Cd.**

The ability to respond to potentially toxic levels of heavy metal ions appears to be ubiquitous in biological systems. Possible mechanisms for such a response include extracellular binding or chelation, regulation of influx or efflux systems, and intracellular sequestration or chelation of the heavy metal (23). In plants, considerable attention has been paid to a class of inducible peptides, sometimes termed phytochelatins, with the molecular structure ($\gamma$EC)$_n$G,[2] which are able to bind heavy metals. The biosynthesis, structure, and function of these peptides have been extensively reviewed (15, 20). In addition to comprehensive studies of the ability of these peptides to complex heavy metals in vitro, there is evidence that they play a role in heavy metal detoxification in vivo. Biosynthesis of these peptides in vivo is rapidly induced in the presence of heavy metals and appears to result from the activation by heavy metals of an enzyme, phytochelatin synthetase, which synthesizes the ($\gamma$EC)$_n$G peptides from glutathione (6, 7). Use of BSO, an inhibitor of the enzyme $\gamma$-glutamyl-cysteine synthase and, thus, of glutathione biosynthesis, has provided strong evidence that this biosynthetic pathway is necessary for heavy metal detoxification. The addition of BSO to cell cultures inhibits the biosynthesis of both glutathione and ($\gamma$EC)$_n$G peptides and increases the sensitivity of the cells to added heavy metal. Furthermore, this effect can be reversed by the addition of glutathione (9, 21). Nevertheless, it is not clear whether it is the reduction of the levels of ($\gamma$EC)$_n$G peptides or of glutathione itself that is important in this effect. In addition to a role in heavy metal detoxification, ($\gamma$EC)$_n$G peptides may also play a role in the homeostatic provision of essential heavy metals to plant tissues and cells. Furthermore, it may be that the primary role of ($\gamma$EC)$_n$G peptides in plants is something other than heavy metal detoxification or homeostasis, and a role in sulfate reduction and transport has also been proposed (18).

Studies of metal-tolerant plant populations or of laboratory-selected, metal-tolerant cell lines indicate that although increases in the amounts of ($\gamma$EC)$_n$G peptides or the rates at which they form complexes with metals may account for some resistance mechanisms, other factors may be involved (see refs. 15, 20 for reviews). Such factors may include metallothionein-like proteins, which have recently been described in plants (3, 13). Although these proteins may be able to bind heavy metals, there is no evidence to indicate a role in heavy metal detoxification or homeostasis in vivo. In addition, a role for organic acids, such as malate and citrate, in heavy metal detoxification has been proposed (5, 10).

Thus, although this field of research is rapidly expanding, there is currently uncertainty about the relative importance of the various potential mechanisms for heavy metal detoxification in plants and their roles with respect to different metals. To explore this area further, we have adopted a genetic approach to the study of heavy metal detoxification mechanisms by the isolation of heavy metal-sensitive mutants of the model genetic organism *Arabidopsis thaliana*. The rationale behind this approach assumes that *Arabidopsis* has some intrinsic mechanism(s) for both heavy metal detoxification and homeostasis and that a mutation that interferes with any single component of those mechanisms may result in a sensitive phenotype. We believe this approach has the potential to identify a greater number of such components than the approach of selecting for heavy metal resistance, which may result only in a limited number of, perhaps rare, mutants in which the capacity of "rate-limiting" steps of a mechanism may be increased. A similar genetic approach has been described for the yeast *Schizosaccharomyces pombe*, which is known to synthesize ($\gamma$EC)$_n$G peptides (14). In this study, we chose to use Cd because, first, it is a nonessential toxic metal and, thus, any mechanism for Cd detoxification

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[2] Abbreviations: ($\gamma$EC)$_n$G, ($\gamma$-glutamyl-cysteinyl)$_n$-glycine; BSO, L-buthionine sulfoximine; EMS, ethyl methanesufonate; CIM, callus induction medium; CMM, callus maintenance medium.

is likely to be less complex than a detoxification mechanism for an essential metal, which would require, in addition, homeostatic regulation. Second, the majority of previous studies of the responses of plants and plant cells to heavy metals have used Cd. Here we describe the isolation and characterization of Cd-sensitive mutants that appear to be deficient in their ability to sequester Cd.

## MATERIALS AND METHODS

### Plant Materials

The *Arabidopsis thaliana* (L.) Heynh. strains used in this study were wild type (var Columbia), the Cd-sensitive mutants CC5 and CC10 (var Columbia), W100 (var Landsberg), which is homozygous for the following markers: *an, ap1, er, py, hy2, gl1, bp, cer2, ms1,* and *tt3* (11), and NW84 (*er, tt3*). CC5 has been back-crossed three times, and CC10 once, to the wild type. Since subsequent genetic analysis has demonstrated that these two mutations are allelic, the former has been more extensively studied. The in vivo and in vitro growth responses and Cd accumulation data were obtained with the back-crossed lines.

$M_2$ seed derived from wild type (var Columbia) was generated with EMS as described by Haughn and Somerville (8). The $M_2$ seed gave rise to pigment mutants at a frequency of approximately 1%. Plants were grown at 23 to 26°C under continuous fluorescent lighting (150–250 $\mu$E $m^{-2}s^{-1}$) unless otherwise indicated. In pots, plants were grown in a sand/compost mix (Debco, Australia) watered with nutrient medium (8). The harvesting of seed and cross-pollination were as described in Somerville and Ogren (19). In Petri dishes, plants were grown on nutrient medium containing 2% sucrose solidified with agar (0.8%, Difco). For callus induction and maintenance, Murashige and Skoog medium (Gibco) containing 2% sucrose and supplemented with *myo*-inositol (10 mg/L), nicotinic acid (100 $\mu$g/L), thiamine-HCl (1 mg/L), pyridoxine-HCl (100 $\mu$g/L) and glycine. Glycine (400 $\mu$g/L) was used with the addition of 0.23 $\mu$M kinetin and 4.5 $\mu$M 2,4-dichlorophenoxyacetic acid for CIM and 0.46 $\mu$M kinetin and 2.25 $\mu$M 2,4-dichlorophenoxyacetic acid for CMM.

### Screening Procedure for Cd-Sensitive Mutants

To screen for Cd-sensitive mutants, it was necessary that the sensitive phenotype be detected using a relatively short exposure to toxic levels of Cd and that, even if growth of a sensitive mutant were completely inhibited during this period, there was a reasonable expectation that the mutant could be recovered. Preliminary measurements of wild-type root growth on medium containing various levels of $CdSO_4$ led to the choice of 90 $\mu$M $CdSO_4$ as the optimal concentration on which wild-type root growth was partly inhibited, but uniform. Furthermore, after 24 h of exposure to a concentration of $CdSO_4$ (210 $\mu$M) that completely inhibited root growth, wild-type seedlings uniformly recovered and resumed growth after transfer to Cd-free medium. Thus, it was hoped that sensitive mutants completely inhibited by the lower level of Cd might also be recovered.

To screen for $M_2$ seedlings that were sensitive to 90 $\mu$M $CdSO_4$, seeds were first sown in rows on Cd-free medium in Petri dishes that were incubated standing on edge to allow the roots of the germinated seedlings to grow vertically down the surface of the agar. Three to 4 d after germination, when the roots were 1.5 to 2 cm in length, the seedlings were transferred, again in rows, to medium containing 90 $\mu$M $CdSO_4$. These Petri dishes were rotated through almost 180° and incubated for approximately 24 h standing on edge with the seedlings upside down. Seedlings were then scored for root growth with a dissecting microscope. Growth of the seedling root during the 24-h period in the presence of Cd resulted in a "hook" of about 3 to 5 mm at the end of the root. The absence of this "hook" was indicative of an absence of growth during this period, suggesting a Cd-sensitive phenotype. Putative mutants were transferred to Cd-free medium to recover, and 5 to 7 d later, were transplanted to soil in pots, grown to maturity, and retested in the $M_3$ generation.

### In Vivo Growth Measurements

Seeds were germinated on nutrient medium containing sucrose and transferred at 5 d postimbibition onto medium containing various concentrations of the compound being tested. In most cases, plants were weighed after 4 d, and in others, after 7 d of further growth. Total seedling fresh weight was determined after gently drawing the root system out of the agar medium. $F_1$ seedlings were plated only on media containing 0 or 90 $\mu$M $CdSO_4$ and weighed after 4 d of growth. Ten seedlings were weighed for each data point. When measuring the effect of BSO on Cd sensitivity, 8-d-old seedlings grown in a 16-h/8-h light/dark cycle were transferred to medium containing $CdSO_4$ with or without 1 mM BSO. After a further 7 d, the leaf portions of plants were excised at the agar surface and weighed. Five plants were weighed for each data point.

### In Vitro Growth Measurements

Callus was generated from both the wild type and mutant by sowing seeds directly on CIM. After 2 weeks, the callus was transferred to CMM and transferred to fresh CMM at 2-week intervals thereafter. To measure the effect of Cd on callus growth, samples (50–70 mg) were transferred onto CMM containing various concentrations of $CdSO_4$, incubated for 14 d, and weighed again. The results are standardized to the fold increase in callus weight.

### Genetic Crosses

Crosses for the purpose of mapping the Cd-sensitive locus were done with the W100 and NW84 strains as the maternal parents. $F_1$ progeny were grown under nonselective conditions, allowed to self-pollinate, and harvested individually. $F_2$ progeny were examined as described in "Results." For the linkage analysis, recombination percentages were calculated by the linkage analysis program of Suiter et al. (22).

### Cd Uptake Assays

Eight-day-old seedlings of the mutant and wild type were transferred to nutrient medium containing added $CdSO_4$ and 0.5 $\mu$Ci of $^{109}CdCl_2$. Small perspex combs were used to

prevent the leaves from contacting the labeled medium. These plates were incubated under a 16-h light/8-h dark cycle. At appropriate time intervals, seedlings were harvested and separated into roots and leaves. The leaves were weighed and directly assayed for radioactivity. The roots were washed extensively with a jet of water to remove any adhering agar particles, blotted dry, weighed, and assayed for radioactivity. Samples of the medium were also assayed for radioactivity, and the specific activity of the labeled Cd in the medium was used to calculate the total amount of Cd accumulated in the plant tissues. For autoradiography, seedlings were placed on paper, covered with a plastic sheet, and exposed to film at −70°C for 4 d.

## RESULTS

### Isolation of Cd-Sensitive Mutants

To identify Cd-sensitive mutants among an $M_2$ population, we adopted a procedure for scoring root growth during a 24-h period of exposure to a concentration of $CdSO_4$ that was partially inhibitory to the wild type (see "Materials and Methods"). A total of about 15,000 $M_2$ seedlings from two independent batches of seed were screened for Cd-sensitive mutants. Of these, about 120 putative mutants were chosen, grown to maturity, and re-examined for the sensitive phenotype in the $M_3$ generation. Due to the death or infertility of some plants, only 83 were rescreened. Of these, two were Cd-sensitive mutants, one from each of the independent seed batches. Of the remainder, six were found to be agravitropic mutants that had presumably grown during exposure to Cd, but not to give the characteristic "hook" of the root seen with the wild type. The remainder were neither Cd sensitive nor agravitropic. The most likely explanation for them having been chosen in the initial screening procedure was that their roots had been damaged during the transfer to cadmium-containing medium and had not grown during the subsequent 24-h period.

The growth of mutant (CC5) and wild-type seedlings in the presence of Cd is shown in Figure 1. For this, 5-d-old seedlings were transferred to medium containing various levels of Cd as described in the "Materials and Methods." four days later, individual seedlings were removed and weighed (Fig. 2). The growth of the wild type was partially inhibited at about 90 μM $CdSO_4$, and growth was still observed at 150 μM; for the mutant, partial inhibition of growth was seen at 15 or 30 μM, and growth was completely inhibited at 90 μM $CdSO_4$. (The baseline of about 2 mg for the mutant seedlings seen in Fig. 2 represents the weight of the 5-d-old seedlings at the time of transfer.) The differences in root growth of the mutant and wild-type seedlings can be seen in Figure 1. On concentrations of $CdSO_4$ as low as 6 μM, inhibition of leaf growth was observed over a 10-d period with leaves becoming progressively chlorotic. Nevertheless, no obvious inhibition of root growth was observed at these low concentrations. In the absence of Cd, either in soil (data not shown) or in agar medium (Fig. 1), no difference in growth between the wild type and mutants was observed. The Cd-sensitive phenotype of CC10 was indistinguishable from CC5 (data not shown).

### Genetic Analysis of the Cd-Sensitive Mutants

$F_1$ progeny were generated from reciprocal crosses of both CC5 and CC10 to the wild-type parent. Neither qualitative scoring nor quantitative measurements (data not shown) of the growth of $F_1$ progeny in the presence of 90 μM $CdSO_4$ indicated any differences from the wild-type phenotype (Table I), indicating that the mutant phenotype is recessive to the wild type. No maternal effects were observed. $F_1$ plants were grown in soil and allowed to self fertilize to produce $F_2$ seed. These gave the 3:1 ratio of resistant:sensitive progeny expected for a single recessive Mendelian locus (Table I). Similar results were obtained for all $F_2$ populations examined for the reciprocal crosses of both mutants (data not shown). $F_1$ and $F_2$ progeny of a cross between the two mutants were sensitive to Cd, indicating that these were mutant alleles of the same locus (Table I). We have designated this locus *CAD1* and its mutant alleles *cad1-1* (CC5) and *cad1-2* (CC10), respectively.

To map the *CAD1* locus, CC5 was crossed with the multiply marked mapping strain, W100, which is homozygous for two recessive markers on each linkage group. $F_1$ plants were allowed to self-fertilize, and the resulting $F_2$ seed was germinated on medium containing sucrose and thiamine. Thiamine was included in the medium to avoid any possible interference of the thiamine auxotrophic phenotype derived from W100 with the Cd-sensitive phenotype from CC5. The markers *tt3*, *hy2*, and *gl1* were scored before transfer to

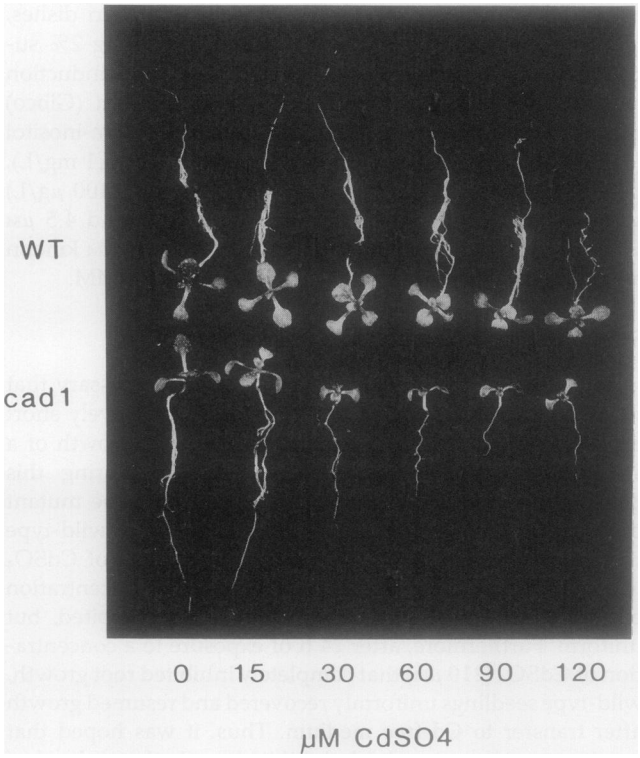


**Figure 1.** Cd-sensitive phenotype. Five-d-old wild-type (top row) and Cd-sensitive (CC5) (bottom row) seedlings were transferred to medium containing $CdSO_4$ and are pictured after an additional 4 d.

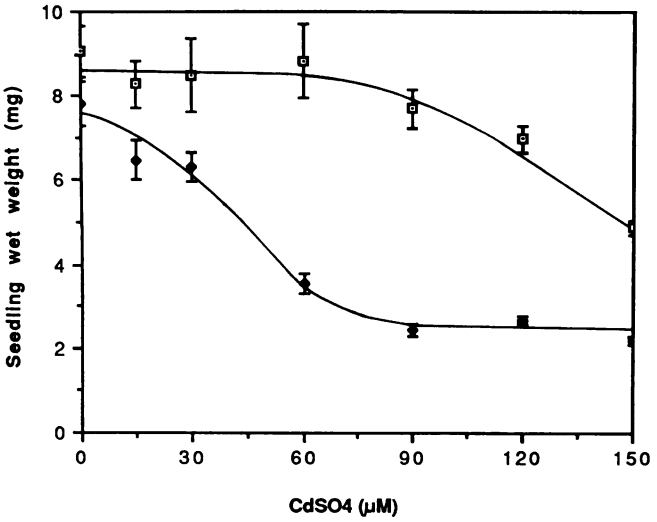


**Figure 2.** Sensitivity of wild-type and mutant seedlings to Cd. Five-d-old wild-type (□) and mutant (CC5) (◆) seedlings were transferred to medium containing added $CdSO_4$. Total seedling fresh weights (mean ± SE, $n$ = 10) were determined after an additional 4 d of growth.

**Table II.** *Genetic Linkage of the CAD1* Locus

| $F_2$ Progeny | Phenotype[a] | | | | $\chi^{2b}$ |
|---|---|---|---|---|---|
| | Resistant | | Sensitive | | |
| | $M^+$ | $M^-$ | $M^+$ | $M^-$ | |
| W100 × CC5 ($F_2$) | | | | | |
| M = tt3 | 121 | 65 | 55 | 0 | 27.8[c] |
| M = hy2 | 144 | 42 | 40 | 15 | 1.23[d] |
| M = gl1 | 138 | 48 | 41 | 14 | 0.66[d] |
| M = an | 142 | 44 | 55[e] | | 0.70[d] |
| M = cer2 | 148 | 38 | 55[e] | | 2.57[d] |
| M = bp | 148 | 38 | 55[e] | | 2.57[d] |
| M = ap1 | 147 | 39 | 55[e] | | 2.11[d] |
| M = er | 135 | 51 | 55[e] | | 1.23[d] |
| M = ms1 | 138 | 48 | 55[e] | | 0.65[d] |
| NW84 × CC5 (F2) | | | | | |
| M = tt3 | 2085 | 849 | 957 | 0 | >20[c] |

[a] $M^+$ and $M^-$ represent $F_2$ progeny phenotypically wild type and mutant, respectively, for the markers of W100 or NW84 identified in column 1 by M =. [b] $\chi^2$ values are based on an expected ratio of 9:3:3:1 or 9:3:4 of the classes listed across the top of the table. [c] $P < 0.01$. [d] $P > 0.2$. [e] Sensitive plants could not be scored for these markers of W100.

medium containing Cd. To unequivocally score the Cd phenotype, 3 d of exposure were required, which resulted in the death of the sensitive individuals. Therefore, only the resistant individuals were scored for the remaining markers. These results are shown in Table II. Linkage between *tt3* on chromosome 5 and *CAD1* was clearly observed. For the remaining markers, including *ms1*, which is also on chromosome 5, the 9:3:3:1 (or 9:3:4) ratio of the four (or three) phenotypic classes expected for independently assorting loci was observed.

To determine the distance between *cad1* and *tt3*, $F_2$ progeny from a cross between CC5 and NW84 were scored for these markers (Table II). Each marker segregated with the expected 3:1 ratio of mutant to wild-type individuals. Because no individuals in the double mutant class of progeny were identified, only a maximum recombination frequency between these two loci of about 3% could be calculated. Preliminary restriction fragment-length polymorphism mapping indicates the *cad1* locus is on the centromere-distal side of *tt3* (unpublished data).

**Table I.** *Genetic Segregation of the Cd-Sensitive Phenotypes*

| Strains | | Total | Observed[a] | | $\chi^{2b}$ |
|---|---|---|---|---|---|
| | | | R | S | |
| WT Columbia | | 335 | 335 | 0 | |
| CC5 | | 312 | 0 | 312 | |
| CC10 | | 253 | 0 | 253 | |
| WT × CC5[c] | ($F_1$) | 119 | 119 | 0 | |
| | ($F_2$) | 158 | 120 | 38 | 0.13[d] |
| CC5 × WT | ($F_1$) | 113 | 113 | 0 | |
| CC10 × WT | ($F_1$) | 32 | 32 | 0 | |
| | ($F_2$) | 301 | 229 | 72 | 0.17[d] |
| CC5 × CC10 | ($F_1$) | 35 | 0 | 35 | |
| | ($F_2$) | 205 | 0 | 205 | |
| W100 × CC5 | ($F_2$) | 241 | 186 | 55 | 0.56[d] |

[a] R, resistant, or S, sensitive, on medium containing 90 μM $CdSO_4$. [b] $\chi^2$ values are based on an expected ratio of 3:1 for R:S individuals. [c] In crosses, the maternal parent is represented first. [d] $P > 0.3$.

## Growth Responses to Other Heavy Metals

To determine the specificity of the Cd-sensitive phenotype, the effect of heavy metals other than Cd on the growth of mutant seedlings compared with the wild type was also assayed (Fig. 3). The mutant (CC5) was significantly more sensitive to $HgSO_4$, and, on concentrations of $HgSO_4$ greater than 4 μM, the anthocyanin pigments of the mutant were strongly induced (not shown). This was not observed on medium containing Cd. This may be indicative of different types of biological damage caused by Hg and Cd. Also, the mutant was consistently, although only slightly, more sensitive to $CuSO_4$ and $ZnSO_4$ than the wild type. On medium containing 60 μM $CuSO_4$, a slight browning of the root tips of the mutant was observed, whereas no visible difference between the mutant and wild type was detected on medium containing Zn. On medium containing $MnSO_4$, there was no difference between the sensitivity of the mutant and that of the wild type. Essentially identical results were obtained for CC10. These observations suggest that the defect in these mutants is relatively specific and is not manifested simply as an increased sensitivity to environmental stresses in general.

## Effect of Cd on Callus Tissue

The effect of Cd on the growth of undifferentiated callus from both the mutant and wild type was also measured. The results in Figure 4 show that, as for intact seedlings, mutant callus tissue also has increased sensitivity to Cd. The greatest

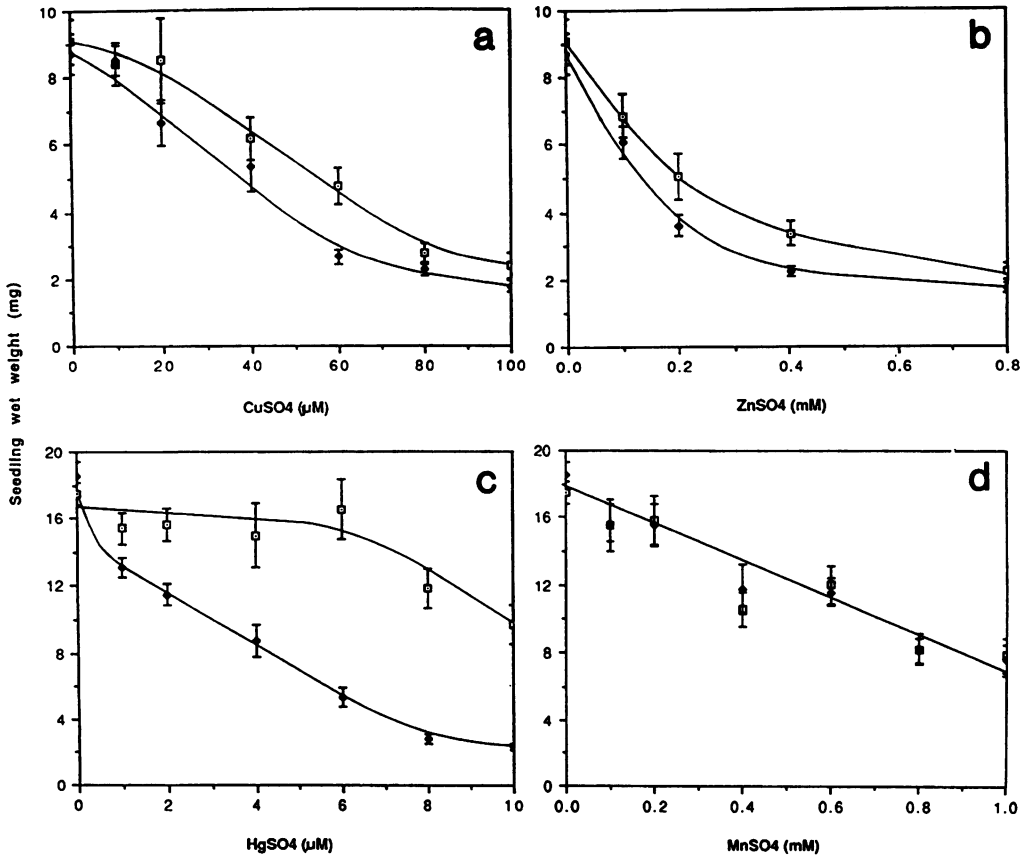


**Figure 3.** Sensitivity of wild-type and mutant seedlings to other metal ions. Five-d-old wild-type (⊡) and mutant (CC5) (◆) seedlings were transferred to medium containing the added metal ion as indicated. Total seedling fresh weights (mean ± SE, $n$ = 10) were determined after an additional 4 (a, b) or 7 d (c, d) of growth.

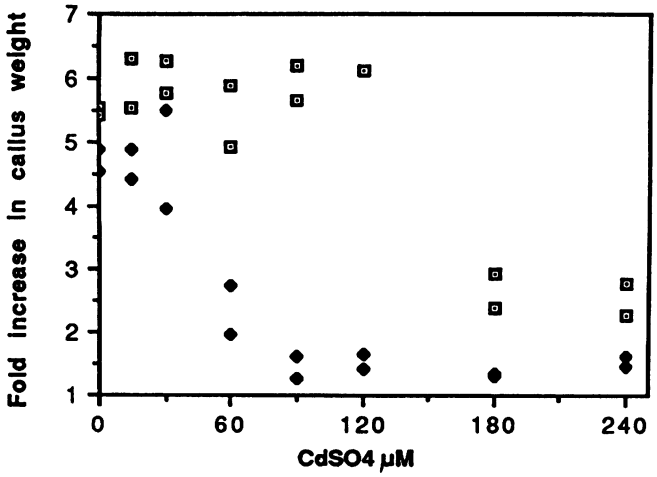


**Figure 4.** Sensitivity of wild-type and Cd-sensitive callus to Cd. Weighed callus derived from wild-type (⊡) and mutant (CC5) (◆) seedlings was incubated on CMM containing added $CdSO_4$ for 14 d. The fold increase in callus weight is shown for duplicate samples.

difference in growth between the mutant and wild-type callus was observed at 90 and 120 μM $CdSO_4$. At 90 μM, the sensitive callus tissue produced patches of brown pigment. This was a nonuniform response in the mutant and was not observed in the wild type, even at concentrations of Cd that had an equivalent effect on its growth. Thus, it seems unlikely that the brown patches simply resulted from cell death. The nature of this pigment has not yet been investigated.

## Effect of BSO on Seedling Growth

BSO is an inhibitor of the enzyme γ-glutamyl cysteine synthetase and has been shown to inhibit the biosynthesis of $(\gamma EC)_nG$ peptides by plant cells, resulting in increased sensitivity to heavy metals (16). If the sensitive phenotype of the *cad1* mutant is due to an inability to synthesize $(\gamma EC)_nG$ peptides, one would expect BSO to have no further effect on the sensitivity of the mutant. Figure 5 shows the effect of BSO on the growth of wild-type and mutant seedlings in the presence of various concentrations of added $CdSO_4$ with or without 1 mM BSO. BSO had no significant effect on plant growth in the absence of Cd. Over a concentration range of 0.15 to 3 μM $CdSO_4$, no effect on growth of the wild type was observed in the absence of BSO, whereas at all these

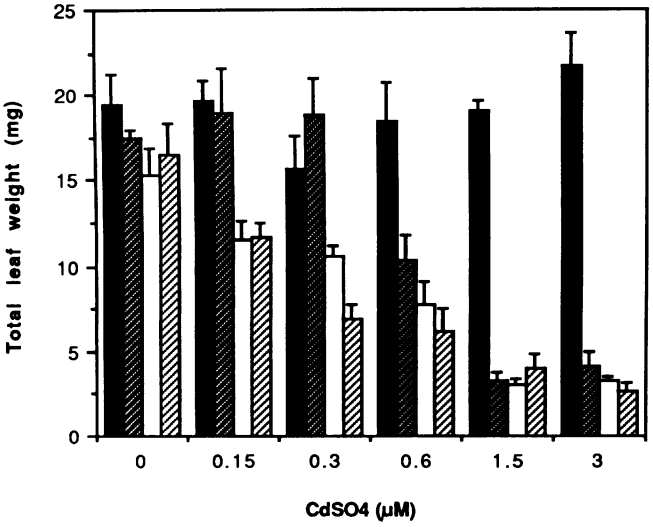


**Figure 5.** Effect of BSO on Cd sensitivity. Eight-d-old wild-type and mutant (CC5) seedlings were transferred to medium containing added $CdSO_4$ with or without 1 mM BSO (wild-type without BSO [■]; wild type with BSO [▩]; CC5 without BSO [□]; CC5 with BSO [▨]. Total leaf weights (mean ± SE, $n = 4$) were determined after 7 d of additional growth.

concentrations in the presence of 1 mM BSO, growth of the wild-type leaves was significantly retarded, and visible effects of Cd toxicity, particularly chlorosis, were observed to increasing extents at increasing concentrations of Cd. BSO also further increased the sensitivity of the *cad1* mutant seedlings to Cd at concentrations of 0.15 to 0.6 μM $CdSO_4$, whereas at higher concentrations, no further increase in sensitivity due to BSO was observed. In general, the growth of the mutant and wild type in the presence of BSO was very similar. However, it was clear that at 0.3 μM $CdSO_4$, the mutant was visibly more sensitive than the wild type. This enhanced sensitivity is reflected in the difference in mean weights seen in Figure 5.

### Cd Uptake by Growing Seedlings

To investigate the nature of the defect in the Cd-sensitive mutants, we measured the accumulation of Cd from the medium by growing plants. Plants were grown in agar medium in the presence of 6 μM $CdSO_4$ and carrier-free $^{109}CdCl_2$, and the amount of Cd accumulated per fresh weight of tissue in roots and leaves was measured over a period of 3 d (Fig. 6). This concentration of Cd progressively inhibited the growth of the leaves of the mutant two- to threefold, but did not inhibit root growth over this period (data not shown, but see Fig. 7). Both the wild-type and mutant leaves accumulated Cd at approximately the same rate over the first 36 h, after which the Cd concentration continued to increase at an approximately linear rate in the wild type, but appeared to plateau at a constant level in the mutant. Similar results were obtained for roots (data not shown, but see Fig. 7). In this experiment, the equilibrium concentrations of Cd in leaves and roots were about 30 and 150 nmol/g tissue, respectively. A second experiment (data not shown) confirmed that in the mutant, these levels remained unchanged during a period of 8 d of growth in the presence of Cd, whereas in the wild type, accumulation of Cd continued to increase over this period. The difference in the amounts of Cd taken up by the mutant and wild type is illustrated in Figure 7, which shows an autoradiograph of roots and leaves of plants grown for 8 d in the presence of 6 μM $CdSO_4$ containing $^{109}Cd$. Assays of Cd uptake in the presence of 6 μM $CdSO_4$ were repeated

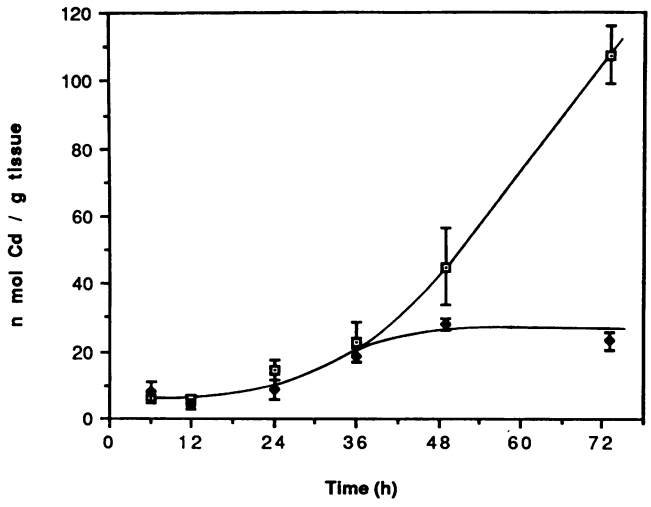


**Figure 6.** Uptake of Cd by mutant and wild-type plants. Eight-d-old wild-type (□) and mutant (CC5) (◆) seedlings were transferred to medium containing added 6 μM $CdSO_4$ and labeled $^{109}CdCl_2$. Cd uptake into leaves was determined (mean ± SE, $n = 5$) at the time intervals shown.

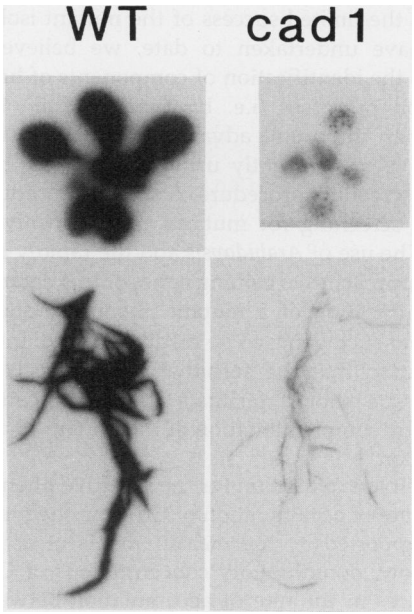


**Figure 7.** Autoradiographs of wild-type and mutant (CC5) plants after 8 d of exposure to 6 μM $CdSO_4$ and labeled $^{109}CdCl_2$.

twice, and in each case, the same relative effect was observed. The equilibrium Cd concentration in leaves was, in each case, about 30 to 40 nmol/g tissue, whereas in roots, the Cd concentrations varied from 50 to 150 nmol/g tissue. At lower concentrations of Cd (3 and 1.5 $\mu$M), a similar difference between mutant and wild type was observed, but accumulation in the mutant did not plateau at a constant level until about d 5 and d 7, respectively; in both cases, the final concentration of Cd in the leaves was approximately equal to that found in plants grown on 6 $\mu$M $CdSO_4$. At 0.03 $\mu$M $CdSO_4$, accumulation in the mutant was indistinguishable from the wild type over an 8-d period (data not shown).

## DISCUSSION

The screening procedure described here has led to the isolation of Cd-sensitive mutants of *Arabidopsis*. Of the 15,000 $M_2$ seedlings screened, two independent alleles of only a single locus were identified. Compared with other mutant screening programs, this frequency appears to be somewhat low (2). This may be indicative of some shortcomings in the screening procedure itself. Of the putative mutants chosen for rescreening in the next generation, about 20% died. It may be that these included other, more sensitive mutants that did not recover from their relatively brief exposure to Cd. This may be overcome by using a lower concentration of Cd in the screening process. Alternatively, other mutants may exhibit a less extreme phenotype and may have gone undetected. The limited number of loci identified may reflect the lethality of other mutations, which could affect gene products necessary for both the detoxification and the normal homeostasis of essential heavy metals such as Cu and Zn. In this respect, it is of interest that the *cad1* mutants showed only a slightly increased sensitivity to Cu and Zn. Another explanation is the possibility of genetic redundancy for some components of the detoxification process. Despite the limited success of the mutant isolation program we have undertaken to date, we believe that this approach to the identification of components of heavy metal detoxification processes (i.e. by the isolation of sensitive mutants) is an important advance in the analysis of these processes. We are currently using the existing mutants to modify our screening procedure. A similar procedure may be suitable for screening for mutants more sensitive to other inhibitors. The use of *Arabidopsis* and the rapidly developing molecular approaches to cloning genes of this organism based on the identification of a mutant phenotype should allow this and other such genes to be readily isolated. In particular, the observation that the sensitive phenotype is expressed in callus tissue should facilitate the identification of the *CAD1* gene by complementation of the mutant phenotype by transformation.

One possible explanation for the sensitive phenotype is an enhanced rate of accumulation of Cd from the medium. This was not supported by the measurements of accumulation, which, at low, noninhibitory concentrations of Cd, showed no differences in the rate of accumulation between mutant and wild type and, at inhibitory levels of Cd, showed that initially, the rate of Cd accumulation by the mutant was indistinguishable from that of the wild type (Fig. 7). Subsequently, the Cd concentration in the wild type continued to increase, whereas in the mutant, an apparent equilibrium was reached at which the Cd concentration remained constant. This suggests that the mutant is deficient in its ability to sequester Cd. It is likely that this inability to sequester Cd is the cause of the sensitive phenotype and that the *CAD1* gene is directly involved in a Cd detoxification mechanism. However, it is possible that the mutant phenotype is directly due to some other effect, and the resulting toxicity of the Cd may prevent normal sequestration. Circumstantial evidence suggests that the observed difference in Cd accumulation is not simply related to a decrease in plant growth rate caused by the nonspecific toxic effects of Cd on plant tissues. First, the diminished Cd accumulation in the mutant was observed in both roots and leaves, whereas no inhibition of root growth was detected at the Cd concentrations used. Second, on 6 $\mu$M $CdSO_4$, for example, leaves continued to grow, although their growth was retarded, whereas there was no further increase in Cd accumulation after day 2.

There was a two- to threefold variation across experiments in the equilibrium concentrations of Cd reached in the mutant. Nevertheless, the minimum equilibrium concentrations of Cd reached in the mutant were about 30 nmol Cd/g tissue and 50 nmol Cd/g tissue in leaves and roots, respectively, of plants grown in the presence of 6 $\mu$M $CdSO_4$. By equating 1 g of tissue with a maximum volume of 1 mL, and by assuming that all the Cd added to the medium was available, it can be seen that the Cd was concentrated at least five- to tenfold in the mutant compared with the concentration in the medium. This may imply some residual ability to sequester Cd, but also suggests that such a mechanism is more easily saturated.

Although the biochemical basis of the Cd-sensitive phenotype has not yet been determined, the observation that undifferentiated callus tissue also exhibits the sensitive phenotype suggests that the defect in these mutants occurs at the cellular level. This is supported by the observation that both leaves and roots of the mutant showed reduced accumulation of Cd. Obvious possible defects may be in the synthesis or intracellular distribution of $(\gamma EC)_nG$ peptides or their precursors or in the ability of these peptides to function adequately. If the biosynthesis of the $(\gamma EC)_nG$ peptides is completely blocked in the mutant, the addition of BSO to the medium would have no further effect on Cd sensitivity. Thus, because BSO increased the sensitivity of both the mutant and the wild type to Cd, it would seem that this biosynthetic pathway is functional, or at least partially functional, in the mutant. However, it may be that both the $(\gamma EC)_nG$ peptides and glutathione itself play a role in Cd detoxification. There is some evidence that glutathione plays a direct role in heavy metal detoxification in mammalian cells (4). Thus, the *cad1* mutation may abolish peptide biosynthesis, whereas BSO would further increase Cd sensitivity by reducing glutathione biosynthesis. If, on the other hand, *cad1* and BSO were affecting entirely unrelated mechanisms of Cd detoxification, their effects on Cd sensitivity would be additive. However, these effects are largely nonadditive, as seen by the overall similarity in the growth of the wild type and mutant in the presence of Cd and BSO. Together these observations suggest that the *cad1* mutation and BSO are affecting different aspects of the same "pathway" of Cd detoxification.

One possibility is that peptides are synthesized normally, but their function is in some way restricted. Previous studies have shown that $(\gamma EC)_nG$ complexes are better able to bind Cd in the presence of sulfite or sulfide (17), and there is evidence that $(\gamma EC)_nG$-Cd complexes can be sequestered in the cell vacuole (24). Defects in these functional aspects of the $(\gamma EC)_nG$ peptides may prevent the effective sequestration of Cd in the mutant. Thus, the existing peptides may provide some limited, saturable mechanism for binding Cd, which could be further reduced in the presence of BSO. A thorough biochemical investigation of the mutant should resolve some of these possibilities.

The mutants described here are significantly more sensitive to both Cd and Hg, only slightly more sensitive to Cu and Zn, and not more sensitive to Mn (Figs. 2 and 3). These observations suggest that they are specifically affected in the processes of detoxification of heavy metals and, more particularly, nonessential heavy metals. In view of the slight effect observed for Cu and Zn, and because both the recessive nature of the mutant phenotype and the identical phenotypes of the two independent mutants suggest these are loss of function mutations, these results indicate that for Cu and Zn, there may be an additional detoxification mechanism. Such a mechanism may be coordinated with the homeostatic provision of these essential trace metals to plant cells. Other studies have also suggested that the mechanisms for Cd detoxification may be different from mechanisms of Cu or Zn detoxification. For instance, in tobacco cells, BSO has been shown to increase sensitivity to Cd, but not to Cu or Zn (16). Also, both naturally isolated Cd-resistant plant species (1) and laboratory-selected Cd-resistant cell lines (9) may not exhibit cross-resistance to other heavy metals. Although there is no clear evidence for such a dichotomy in detoxification mechanisms in higher plants, the yeast *Torulopsis glabrata* responds to Cd by the induction of $(\gamma EC)_nG$ peptide biosynthesis, but to Cu by the induction of two metallothionein-like proteins (12). Although in higher plants, $(\gamma EC)_nG$ peptides are inducible by both Cd and Cu (7), it may be that different mechanisms have different but overlapping specificities for different heavy metals. The *cad1* mutants may allow further components of detoxification mechanisms to be identified by the isolation of second-site mutations that further increase the sensitivity of the mutant to Cd or to other heavy metals such as Cu and Zn.

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