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A Cadmium-Sensitive, Glutathione-Deficient Mutant of *Arabidopsis thaliana*[1]

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The roots of the cadmium-sensitive mutant of *Arabidopsis thaliana, cad1–1*, become brown in the presence of cadmium. A new cadmium-sensitive mutant affected at a second locus, *cad2*, has been identified using this phenotype. Genetic analysis has shown that the sensitive phenotype is recessive to the wild type and segregates as a single Mendelian locus. Assays of cadmium accumulation by intact plants indicated that the mutant is deficient in its ability to sequester cadmium. Undifferentiated callus tissue was also cadmium sensitive, suggesting that the mutant phenotype is expressed at the cellular level. The level of cadmium-binding complexes formed in vivo was decreased compared with the wild type and accumulation of phytochelatins was about 10% of that in the wild type. The level of glutathione, the substrate for phytochelatin biosynthesis, in tissues of the mutant was decreased to about 15 to 30% of that in the wild type. Thus, the deficiency in phytochelatin biosynthesis can be explained by a deficiency in glutathione.

In plants and in some yeast species a class of inducible peptides, sometimes termed PCs, with the molecular structures $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ is known to play an important role in heavy-metal detoxification processes. The biosynthesis, structure, and function of PCs have been extensively reviewed (Rauser, 1990; Steffens, 1990). Their biosynthesis in vivo is rapidly induced in the presence of heavy metals (Grill et al., 1987) and appears to result from the activation by heavy metals of an enzyme, PC synthase, that synthesizes PCs from the substrate GSH (Grill et al., 1989). Use of BSO, an inhibitor of the enzyme γ-Glu-Cys synthetase and thus of GSH 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 GSH and PCs and increases the sensitivity of cells to added heavy metals. Furthermore, this effect can be reversed by the addition of GSH (Scheller et al., 1987; Mendum et al., 1990).

Additional aspects of the function of the PCs include the stabilization of the PC-Cd complex by sulfide ions and the sequestration of the complex into the vacuole. In both the yeast *Schizosaccharomyces pombe*, which is also known to synthesize PCs (Mehra et al., 1988; Mutoh and Hayashi, 1988), and in some plant species (Reese et al., 1992; Speiser et al., 1992) two PC-Cd complexes of different mol wts can be resolved by gel-filtration chromatography. The HMW complex generally has a higher sulfide:Cd ratio than the LMW complex and is more stable in vitro (Murasugi et al., 1983; Reese and Winge, 1988). Studies of the subcellular localization of PC-Cd complexes in tobacco have shown that most are sequestered to the cell vacuole (Vogeli-Lange and Wagner, 1990) and transport studies using oat root vacuoles have demonstrated a mechanism for transporting Cd into the vacuole (Salt and Wagner, 1993).

Genetic studies of *S. pombe* have proven valuable in identifying a number of genes involved in the biosynthesis or function of PCs. GSH-deficient mutants with reduced activity of γ-Glu-Cys synthetase or GSH synthetase were found to be Cd sensitive (Mutoh and Hayashi, 1988; Glaeser et al., 1991). One interesting Cd-sensitive mutant, LK100, lacks the HMW PC-Cd complex, and the *hmt1* gene was found to encode a transport protein located in the vacuolar membrane (Ortiz et al., 1992). Some purine auxotrophs have also been found to be Cd sensitive (Speiser et al., 1992) and the biochemical pathway affected in these mutants is believed to be involved in the biosynthesis of intermediates or carriers in the transfer of sulfide to the PC-Cd complex (Juang et al., 1993).

We have initiated similar genetic studies using the higher plant *Arabidopsis thaliana* to identify genes and processes important in the detoxification of Cd and other heavy metals and have previously described a Cd-sensitive mutant, *cad1–1* (Howden and Cobbett, 1992). In the accompanying paper (Howden et al., 1995) we show that *cad1* mutants are deficient in PCs, but not in GSH biosynthesis, because of a defect in PC synthase activity. Here we describe the identification of a new Cd-sensitive mutant, *cad2–1*, with a decreased level of PCs that appears to result from a decrease in the level of endogenous GSH.

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Abbreviations: BSO, L-buthionine sulfoximine; HMW, high molecular weight; LMW, low molecular weight; PC, phytochelatin $[(\gamma\text{-Glu-Cys})_n\text{-Gly}]$; PC_2, $(\gamma\text{-Glu-Cys})_2\text{-Gly}$.

MATERIALS AND METHODS

Plant Materials

The *Arabidopsis thaliana* strains used in this study were wild type and the *cad1–1* (Howden and Cobbett, 1992), *cad1–3* (Howden et al., 1995), and *cad2–1* mutants (all var Columbia). The *cad2–1* mutant was identified in a population of M_2 seed derived from wild-type (var Columbia) seed that had been exposed to 60 krad of x-irradiation and were the generous gift of A. Chaudary (see "Results"). This mutant has been backcrossed once to the wild type.

Methods for the growth and genetic analysis of plants and the induction and maintenance of callus tissue were as described previously (Somerville and Ogren, 1982; Howden and Cobbett, 1992). Growth measurements of seedlings and callus tissue were as described previously (Howden and Cobbett, 1992) except that seedlings were transferred to the test medium 8 d after imbibition and seedling fresh weights were measured after an additional 8 d. Cd accumulation assays were as described previously (Howden and Cobbett, 1992) except that 8-d-old plants with intact root systems were transferred to medium containing 0.02 μCi of $^{109}CdCl_2$/mL.

Biochemical Techniques

Gel-filtration chromatography of Cd-binding complexes, HPLC quantitation of GSH and PCs, and the enzymatic assay of GSH were as described in the accompanying paper (Howden et al., 1995).

RESULTS

Isolation of the *cad2* Mutant

When the *cad1–1* mutant was grown on agar medium in the presence of inhibitory levels of Cd the roots grew but developed a distinct brown pigment. This phenotype has been used to identify a number of new *cad1* alleles generated by chemical mutagenesis (Howden et al., 1995). In an attempt to isolate x-ray-induced alleles of the *cad1* locus, 20,000 M_2 seedlings were screened for the brown-root phenotype using the technique described by Howden et al. (1995). A single mutant was identified, rescreened in the subsequent generation, and proved to have both the brown-root and Cd-sensitive phenotypes. Genetic analysis (see below) demonstrated that this mutation affected a second locus, *cad2*. Initial phenotypic characterization was carried out using an M_4 population, and later biochemical analysis was done using the backcrossed line, which is indistinguishable from the original mutant.

Sensitivity of the *cad2–1* Mutant to Heavy Metals

Measurements of growth of the *cad2–1* mutant in comparison with the wild type and *cad1–1* mutant in the presence of various concentrations of $CdSO_4$ are shown in Figure 1A. In the absence of Cd no significant difference in the growth or appearance of the mutant compared with the wild type was observed. At Cd concentrations up to 6 μM the wild type showed no inhibition of growth, whereas the

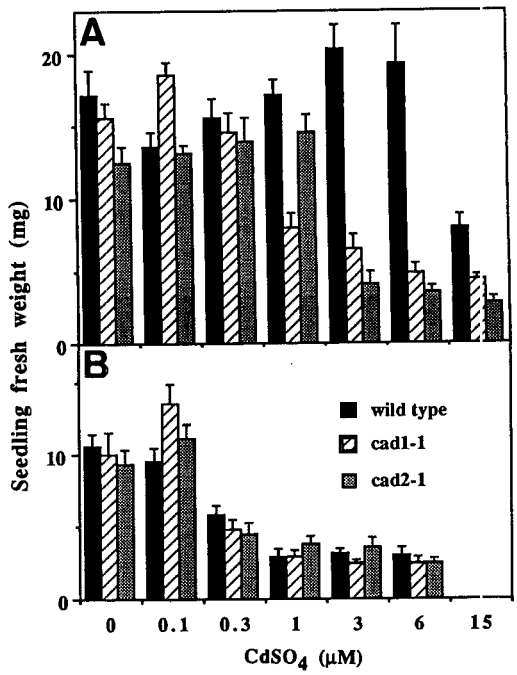

Figure 1. Sensitivity of the *cad2–1* and *cad1–1* mutants to Cd. Eight-day-old seedlings were transferred to medium containing added $CdSO_4$ in the absence (A) or presence (B) of 0.5 mM BSO. Total leaf fresh weight (mean ± SE, n = 5) was determined after an additional 8 d of growth.

cad1–1 mutant showed reduced growth on 1 μM Cd. The *cad2–1* mutant had an intermediate phenotype. Growth was inhibited on 3 μM Cd but, unlike *cad1–1*, the *cad2–1* mutant showed no effects on medium containing 1 μM Cd. Under conditions in which the *cad2–1* mutant exhibited the sensitive phenotype the brown pigment was clearly visible in the roots; however, it was a less intense color compared with the roots of the *cad1–1* mutant grown under the same conditions (not shown).

BSO is an inhibitor of the enzyme γ-Glu-Cys synthetase and has been shown to inhibit the biosynthesis of PCs by plant cells, resulting in increased sensitivity to heavy metals (Mendum et al., 1990). If the *cad2–1* mutation also results in a deficiency in PC biosynthesis, then in the presence of BSO it should not be more sensitive than wild type. Alternatively, if the defect in the *cad2–1* mutant affects a different mechanism for Cd detoxification, then in the presence of BSO the mutant might be expected to be more sensitive than wild type if the two mechanisms function in an additive manner. Figure 1B shows the effect of 0.5 mM BSO in the medium on the growth of wild-type and mutant seedlings in the presence of various concentrations of added $CdSO_4$. BSO reduced plant growth by about 30% in the absence of Cd. In the presence of Cd the sensitivity of the wild type was significantly increased and the sensitivity of both the *cad1–1* and *cad2–1* mutants was further enhanced such that sensitivity to 0.3 μM Cd was observed. Under these conditions the mutants were indistinguishable from the wild type. This is consistent with the hypothesis that the *cad2–1* mutation affects the biosynthesis of PCs.

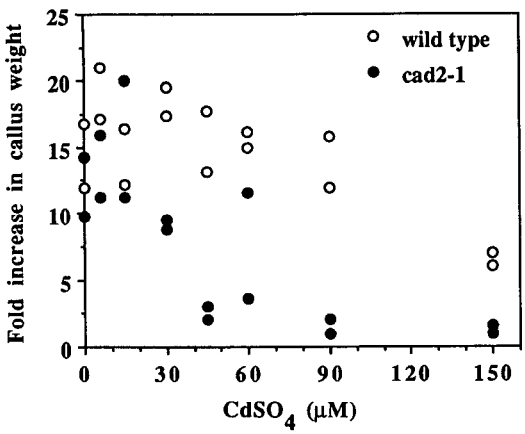

Figure 2. Sensitivity of callus tissue to Cd. Weighed callus derived from wild-type and *cad2–1* seedlings was incubated on callus maintenance medium containing added $CdSO_4$ for 14 d. The fold increase in callus weight is shown for each of duplicate samples.

The effect of Cd on the growth of undifferentiated callus derived from both the *cad1–1* and *cad2–1* mutants and wild type was also measured. The results in Figure 2 show that, as previously reported for *cad1–1* (Howden and Cobbett, 1992), *cad2–1* mutant callus tissue also has increased sensitivity to Cd. This demonstrates that the sensitive phenotype is a cellular defect not dependent on the differentiation of complex plant structures such as roots. Previously it was also found that, in addition to Cd, the *cad1–1* mutant is significantly more sensitive than wild type to Hg and slightly more sensitive to Cu and Zn than wild type (Howden and Cobbett, 1992). Similar results were obtained for the *cad2–1* mutant (data not shown), suggesting that in the *cad2–1* and *cad1–1* mutants the same mechanism for heavy-metal detoxification may be affected.

Genetic Characterization of the *cad2* Mutant

In crosses of the *cad2–1* mutant to the wild type, the F_1 progeny exhibited a wild-type phenotype, indicating that the Cd-sensitive phenotype is recessive to the wild type. F_1 plants were allowed to self-fertilize, and the F_2 progeny gave a 3:1 ratio of resistant to sensitive individuals expected for a single recessive Mendelian locus (Table I). F_1 progeny from crosses between the *cad1–1* and *cad2–1* mutants exhibited the wild-type phenotype, demonstrating that they complemented each other and that the new mutation identified a second locus. These F_1 individuals were allowed to self-fertilize and the F_2 seedlings were scored for sensitivity on medium containing 6 μM Cd. The expected ratio of 9:7 wild-type:sensitive individuals for two independently assorting loci was obtained (Table I). Among the sensitive individuals the darker brown *cad1–1* root phenotype could be distinguished from the lighter brown *cad2–1* phenotype; however, it was difficult to assign some individuals unequivocally to one class or the other.

Table I. *Genetic segregation of the Cd-sensitive phenotype*

Strain	Total	Observed[a]		χ^2
		R	S	
Wild type	270	270	0	
cad2–1	189	0	189	
cad1–1	234	0	234	
cad2–1 × wild type (F_1)	27	27	0	
F_2 (F_1 selfed)	1030	778	252	0.2[b]
cad2–1 × *cad1–1* (F_1)	30	30	0	
F_2 (F_1 selfed)	883	471	412	1.7[c]

[a] Seven-day-old seedlings were transferred to medium containing 6 μM CdSO4 and scored R (resistant) or S (sensitive) after an additional 7 to 10 d. [b] χ^2 based on an expected ratio of 3:1 R:S individuals; $P > 0.5$. [c] χ^2 based on an expected ratio of 9:7 R:S individuals; $P > 0.1$.

To isolate a *cad1–1,cad2–1* double mutant, 10 plants with the lighter brown-root phenotype were grown and allowed to self-fertilize, and these F_3 families were scored for sensitivity to Cd. All individuals tested from each family on 3 μM Cd were sensitive, demonstrating that these populations were homozygous for one of the mutant alleles. Approximately one-quarter of the individuals in 5 of the 10 families exhibited the more sensitive *cad1–1* phenotype on 1 μM Cd. Thus, the 10 F_2 individuals chosen were assumed to be *cad2–1/cad2–1* and 5 of the 10 were heterozygous at the *CAD1* locus. The more sensitive F_3 individuals were, therefore, assumed to be *cad1–1/cad1–1,cad2–1/cad2–1* double mutants. These individuals were grown and their genotype was confirmed by their inability to complement either of the two mutant parents (data not shown).

The sensitivity of two independently derived double-mutant lines was tested in comparison with the *cad1–1* and *cad2–1* parent lines. The two double-mutant lines were indistinguishable from each other (not shown) and were sensitive to 0.3 μM Cd in contrast to both of the parent lines, which were not visibly affected at this concentration (Fig. 3). In the presence of 3 μM Cd the roots of the double mutant also had a brown color that individually appeared to be intermediate between the intensity of the root colors

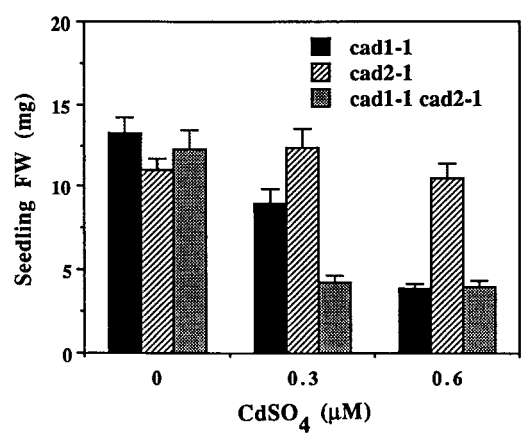

Figure 3. Sensitivity of the *cad1–1,cad2–1* double mutant to Cd. Eight-day-old *cad1–1,cad2–1* and *cad1–1,cad2–1* seedlings were transferred to medium containing added $CdSO_4$. Total leaf fresh weight (FW; mean ± SE, $n = 5$) was determined after an additional 8 d of growth.

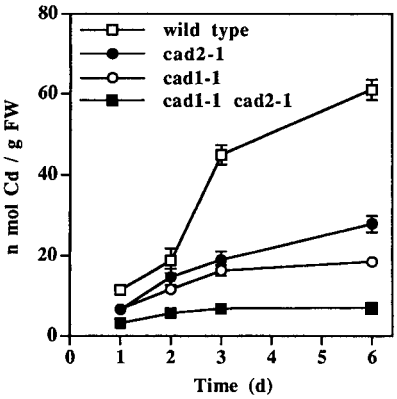

Figure 4. Accumulation of Cd by wild-type and mutant plants. Eight-day-old wild-type, *cad1–1,cad2–1*, and *cad1–1,cad2–1* seedlings were transferred to medium containing added 6 μM $CdSO_4$ and labeled $^{109}CdCl_2$. Cd accumulation into leaves was determined (mean ± SE, n = 5) at the time intervals shown. FW, Fresh weight.

of the parents. This was confirmed when 10 root systems from each of the parents and the double mutant were massed together and viewed adjacent to each other on a white background (not shown).

Cd Accumulation by Wild-Type and Mutant Plants

The accumulation of Cd by plants grown in the presence of 6 μM $CdSO_4$ and $^{109}CdCl_2$ was measured as described in "Materials and Methods." As observed previously, Cd was accumulated progressively by the wild type but accumulation in the *cad1–1* mutant was decreased and appeared to plateau at an approximately constant level (Fig. 4) (Howden and Cobbett, 1992). Accumulation in the *cad2–1* mutant was intermediate between that observed for the wild type and *cad1–1*. In the *cad1–1,cad2–1* double mutant accumulation of Cd was less than in either of the singly mutant parent strains. In each case the level of Cd accumulation reflected the minimum concentration of Cd to which each mutant was sensitive. For example, the *cad2–1* mutant, which shows growth inhibition only at 3 μM Cd, exhibited the highest level of Cd accumulation of all mutants tested. However, it is not possible to distinguish whether the inability to accumulate Cd is in fact the cause of the Cd-sensitive phenotype or, conversely, whether the sensitivity of each strain prevents Cd accumulation.

Biochemical Characterization of the *cad2–1* Mutant

To compare the profiles of Cd-peptide complexes in the wild type and *cad2–1* mutant, seedlings were grown in liquid culture and exposed to Cd in the presence of the ^{109}Cd isotope. Extracts were chromatographed on a gel-filtration Sephadex G-50 column as described in "Materials and Methods." Figure 5 shows the column profiles obtained for seedlings exposed to 6 and 30 μM $CdSO_4$. In the wild type two peaks, referred to as HMW and LMW, of Cd-binding material were observed. In seedlings exposed to 6 μM CdSO4 (Fig. 5A) the LMW peak was predominant, whereas at 30 μM (Fig. 5B), the LMW peak was visible only as a shoulder on the larger HMW peak. For the *cad2–1* mutant at both Cd concentrations only a peak broadly corresponding to the wild-type LMW peak was observed. These observations indicated the *cad2–1* mutant was partially deficient in its ability to form Cd-binding complexes. The *cad1–1* mutant showed a similar but more extreme deficiency (Fig. 5C; Howden et al., 1995) and in the *cad1–1,cad2–1* double mutant the Cd-binding complexes were almost completely absent (Fig. 5C).

Extracts obtained from seedlings unexposed or exposed to Cd were assayed for glutathione and total PC content by reversed-phase HPLC as described in "Materials and Methods." Representative HPLC chromatograms for the wild

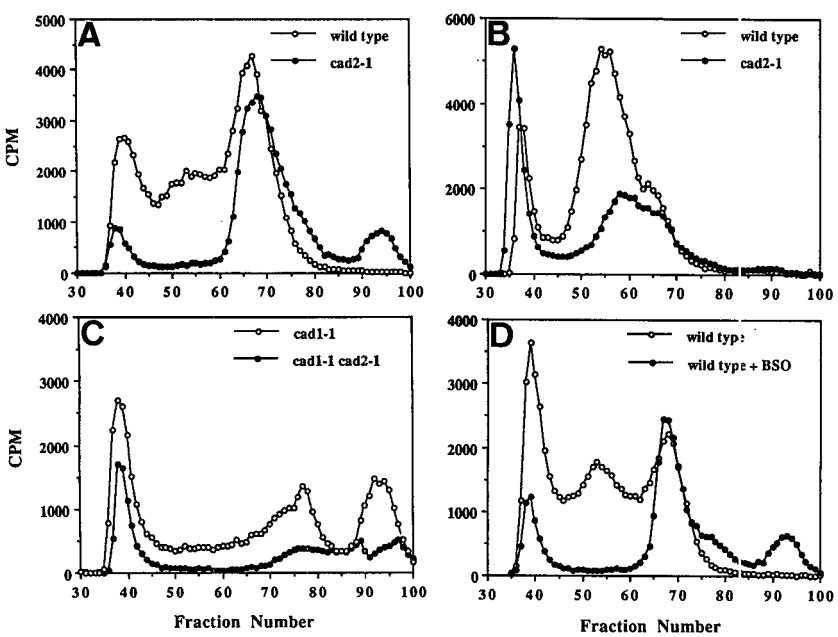

Figure 5. Cd-binding complexes of wild-type and mutant seedlings detected by gel-filtration chromatography. Seedlings were exposed to Cd in liquid culture in the presence of ^{109}Cd. Extracts were chromatographed on a Sephadex G-50 column. ^{109}Cd in the indicated fractions was measured by using a γ-irradiation 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. Extracts of wild type and *cad2–1* were exposed to 6 μM Cd (A) and 30 μM Cd (B); extracts of *cad1–1* and *cad1–1,cad2–1* were exposed to 6 μM Cd (C); and extracts of wild type were exposed to 6 μM Cd in the presence or absence of 0.5 mM BSO (D).

Table II. *PC accumulation in wild type and cad2–1 seedlings*

Total PCs (measured as GSH equivalents) were assayed in extracts of seedlings exposed to Cd for 24 h as described in "Materials and Methods." Results are mean values ± SE for assays performed on at least five independent samples of each genotype.

Cd	Total PCs		
	Wild type	*cad2–1*	*cad1–1*
μM	*μmol GSH equivalents/kg fresh weight*		
0	5 ± 1	1 ± 0	5 ± 0
30	150 ± 13	16 ± 1	37 ± 5

type are shown in Howden et al. (1995), and the quantitative data are presented in Table II. Low levels of PC_2 were detected in both wild type and *cad1–1* in the absence of Cd. In *cad2–1* seedlings PC_2 was barely detectable. After exposure to 30 μM Cd for 24 h there was a significant increase in the level of PCs in the wild-type seedlings. However, accumulation of PCs was greatly reduced in *cad2–1*, reaching only about 10% of the level in the wild type. In the accompanying paper (Howden et al., 1995) we have shown that the activity of PC synthase in *cad2–1* approaches that of the wild type, whereas *cad1* mutants are deficient in this activity. This suggests that the low level of PCs accumulated in *cad2–1* is the result of a deficiency not in PC synthase but in another part of the PC biosynthetic pathway. Therefore, we examined the level of GSH in *cad2–1* (Table III). The GSH concentration in *cad2–1* seedlings was measured by HPLC analysis as less than 20% of that in either wild type or *cad1–1*. The observation that the GSH level of the *cad2–1* mutant is decreased in the absence of Cd and the fact that the *cad1* mutants had increased levels of GSH in the presence of Cd (Howden et al., 1995) indicated that the low levels of GSH in the *cad2–1* mutant are not simply a consequence of exposure of a sensitive mutant to Cd. Unlike the *cad1* mutants the level of GSH in the *cad2–1* mutant compared with the wild type was unchanged after exposure to Cd (Table III).

To confirm these observations extracts of roots and leaves of wild-type and *cad2–1* plants grown in liquid medium and on solid agar medium and not exposed to Cd were assayed enzymatically for total glutathione levels. These results are shown in Table III. The GSH levels obtained for the seedlings grown in liquid culture were 2- to 3-fold higher than for the intact plants or for the HPLC assay. This may simply reflect the different conditions under which the plants were grown. In addition, the HPLC assay measures only GSH and not GSSG. The results confirmed that the *cad2–1* mutant contains 15 to 30% of the wild-type levels of glutathione depending upon the growth medium. Thus, it appeared that the mutant was unable to accumulate PCs to wild-type levels because of a deficiency in GSH.

To demonstrate that the deficiency in glutathione cosegregated with the Cd-sensitive phenotype, a segregating F_2 population was germinated in the presence of Cd. Seedlings were scored for the Cd-sensitive phenotype after 7 d and then transferred to medium in the absence of Cd to grow for an additional 10 d. Sensitive plants were indistinguishable from resistant plants at this stage and were individually assayed for their glutathione content. Of 8 phenotypically wild-type plants assayed, all contained wild-type glutathione levels (range, 208–341 μmol/kg fresh weight), whereas of 12 Cd-sensitive plants assayed, all contained decreased levels of glutathione (range, 22–62 nmol/g fresh weight). Wild-type and *cad2–1* control plants grown under the same conditions contained 229 ± 6 (mean ± SE, $n = 5$) and 56 ± 4 (mean ± SE, $n = 6$) μmol/kg fresh weight, respectively. This indicates that the two phenotypes, Cd sensitivity and GSH deficiency, are genetically linked and are probably the result of the same mutation.

Since the decrease in glutathione levels in the *cad2–1* mutant apparently results in the absence of the HMW Cd-binding complex, we attempted to mimic this observation using the inhibitor BSO. Wild-type seedlings were grown in the presence of 0.5 mM BSO and exposed to 6 μM Cd as described in "Materials and Methods." This concentration of BSO decreased the GSH concentration to about 5 to 10% of the level in the absence of BSO (data not shown). The Sephadex G-50 profile of Cd-binding complexes in wild-type seedlings in the presence or absence of BSO is shown in Figure 5D. In the culture exposed to BSO the HMW peak is absent and the profile is very similar to that from *cad2–1* seedlings grown in the absence of BSO.

Table III. *GSH content of wild-type and mutant plants*

GSH was assayed by HPLC or by the enzymatic recycling assay as described in "Materials and Methods." The HPLC assay was on extracts of seedlings grown in liquid culture for 10 or 11 d and exposed (+Cd) or not exposed (−Cd) to 30 μM $CdSO_4$ for 1 d. Results are mean values ± SE for assays performed on 11 independent samples of each genotype. The enzymatic assay was on extracts of leaves and roots of 15-d-old plants grown in agar medium and of seedlings grown in liquid culture for 14 d without exposure to Cd. Results are mean values ± SE for duplicate assays performed on two tissue samples in each case. NT, Not tested.

Strain	Glutathione				
	HPLC assay		Enzymatic assay		
	−Cd	+Cd	Leaves	Roots	Liquid culture
	μmol/kg fresh weight (% wild type)				
Wild type	231 ± 17 (100)	154 ± 5 (100)	325 ± 8 (100)	271 ± 13 (100)	555 ± 7 (100)
cad2–1	41 ± 6 (18)	30 ± 2 (20)	71 ± 4 (22)	44 ± 28 (16)	188 ± 21 (34)
cad1–1	225 ± 12 (97)	567 ± 32 (368)	NT	NT	606 ± 101 (109)
cad2–1,cad1–1	NT	NT	NT	NT	248 ± 40 (44)

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. The screening procedure based on this observation has been used to identify new alleles of *cad1* (Howden et al., 1995), as expected, but has also identified a mutant at a second locus, *cad2*, described here. The basis of this phenotype is not yet understood. Preliminary data (R. Howden, C.R. Andersen, and C.S. Cobbett, unpublished data) suggest that the brown pigment is probably a deposit of various metal-sulfide complexes. Since both *cad1* and *cad2* mutants are unable to form sufficient amounts of PCs the production of high levels of sulfide may be an adaptive response to the presence of free Cd in plants. This screening procedure may, therefore, be useful in isolating any mutants that are Cd sensitive because of a decrease in PC biosynthesis. However, some Cd-sensitive mutants may not form brown roots in the presence of Cd and would thus be overlooked by this procedure.

The *cad2–1* accumulated less PCs than *cad1–1* in the presence of 30 μM Cd. However, in growth assays the *cad1–1* mutant is more sensitive than the *cad2–1* mutant at lower levels of Cd. This inconsistency probably reflects the different amounts of GSH, and thus PCs, formed by the *cad2–1* mutant grown under the different conditions, including the different Cd concentrations, required for these two assay procedures. Similarly, the callus tissue derived from *cad2–1* appeared to be equally as sensitive to Cd as that from the *cad1–1* mutant. This too may reflect differences in the biosynthesis of GSH under these different conditions, particularly since the callus tissue was not photosynthetically active.

The double mutant is more sensitive to Cd than either of its parents. This might suggest that the two mutations affect unrelated pathways, each separately contributing to the wild-type level of Cd tolerance, and that these two defects have an additive effect in the double mutant. However, both mutations affect the biosynthesis of PCs. The *cad1–1* mutant has wild-type levels of glutathione but decreased levels of PCs, whereas in *cad2–1* both are decreased. If, in *cad1–1*, GSH plays a role, albeit minor, in the detoxification of Cd, then in the double mutant the decrease in GSH superimposed on the deficiency in PC synthesis may enhance the sensitive phenotype. This is not likely to be the case, since experiments using BSO suggest that GSH itself does not contribute to Cd-detoxification in the *cad1–3* mutant, which is lacking detectable PCs (Howden et al., 1995). Alternatively, since *cad1–1* has a residual capacity to synthesize PCs, the decrease in GSH levels in the double mutant may further decrease the ability of the *cad1–1* mutant to make PCs. These observations with the double mutant mirror the effect of BSO, which also decreases the level of GSH, and enhances the sensitivity of the *cad1–1* mutant. It would be informative to construct a double mutant using the *cad1–3* mutant, in which no PCs can be detected.

In *Schizosaccharomyces pombe*, the *hmt1* mutant and a number of mutants affected in purine biosynthesis are unable to synthesize HMW Cd-binding complexes. These mutants have defects in a vacuolar transport protein (Ortiz et al., 1992) and in aspects of sulfide metabolism (Speiser et al., 1992), respectively. It is interesting that when GSH levels are decreased both in the *cad2–1* mutant and in the wild type treated with BSO the HMW Cd-binding complex is also absent. This may directly reflect the reduced capacity to synthesize PCs under both of these conditions and may indicate that formation of HMW complexes is dependent on accumulation of a minimum level of PCs. Alternatively, it is possible that some metabolic or regulatory process is affected by the decrease in GSH, thereby influencing, for example, a function homologous to HMT1 or some aspect of sulfide metabolism.

The function of the *CAD2* gene product has not yet been determined. Indeed, since the *cad2–1* mutation is x-ray induced, it is possible that the mutation affects more than one gene. The most obvious possibility is in the GSH biosynthetic pathway itself or in its regulation. GSH biosynthesis proceeds from its constituent amino acids, Glu, Cys, and Gly, in two successive steps catalyzed by the enzymes γ-Glu-Cys synthetase and GSH synthetase (Alscher, 1989). If the defect is indeed in either of these two enzymes, the fact that there remains some GSH in the *cad2–1* mutant may suggest that the mutation is leaky, but it is more likely to reflect that this pathway probably occurs in both the cytosol and the chloroplast (Alscher, 1989; Hell and Bergmann, 1990). A mutant blocked in GSH synthetase might be expected to accumulate γ-Glu-Cys as is seen in the GSH synthetase-deficient mutant of *Saccharomyces cerevisiae* (Ohtake et al., 1990). However, this was not observed in the HPLC profiles. This may indicate that the defect is more likely to affect the first step in the pathway.

There is evidence in pea and maize that the activities of GSH biosynthetic enzymes increase during exposure to Cd, suggesting that this pathway is subject to regulation (Ruegsegger et al., 1990; Ruegsegger and Brunold, 1992). Thus, it is possible that the *CAD2* gene is involved in regulating this pathway. Other formal possibilities include defects in the reduction of GSH or its breakdown; however, it is difficult to see how loss-of-function mutations affecting these processes would decrease the total levels of GSH.

GSH is proposed to play a significant role in a number of processes in plants. Among these is a role in responses to oxidative stress. GSH is the major reducing agent within plant cells and is presumed to play an important role in the reduction of activated oxygen species, especially in the reduction of hydrogen peroxide via the Asada-Halliwell pathway involving dehydroascorbate reductase and GSH reductase (Alscher, 1989). GSH is also the substrate for glutathione-*S*-transferases, which are involved in the inactivation of xenobiotics in many organisms but particularly herbicides in plants (Timmerman, 1989). In addition, GSH has been proposed to play a role in sulfur storage and transport in plants (Rennenberg, 1982). It is of considerable interest that in the absence of Cd under laboratory conditions the *cad2–1* mutant has no apparent phenotype. This mutant may provide valuable insights into the proposed roles of GSH in plants.

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