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Phytochelatin Synthase Genes from Arabidopsis and the Yeast *Schizosaccharomyces pombe*

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Phytochelatins (PCs), a family of heavy metal–inducible peptides important in the detoxification of heavy metals, have been identified in plants and some microorganisms, including *Schizosaccharomyces pombe*, but not in animals. PCs are synthesized enzymatically from glutathione (GSH) by PC synthase in the presence of heavy metal ions. In Arabidopsis, the *CAD1* gene, identified by using Cd-sensitive, PC-deficient *cad1* mutants, has been proposed to encode PC synthase. Using a positional cloning strategy, we have isolated the *CAD1* gene. Database searches identified a homologous gene in *S. pombe*, and a mutant with a targeted deletion of this gene was also Cd sensitive and PC deficient. Extracts of *Escherichia coli* cells expressing a *CAD1* cDNA or the *S. pombe* gene catalyzing GSH-dependent, heavy metal–activated synthesis of PCs in vitro demonstrated that both genes encode PC synthase activity. Both enzymes were activated by a range of metal ions. In contrast, reverse transcription–polymerase chain reaction experiments showed that expression of the *CAD1* mRNA is not influenced by the presence of Cd. A comparison of the two predicted amino acid sequences revealed a highly conserved N-terminal region, which is presumed to be the catalytic domain, and a variable C-terminal region containing multiple Cys residues, which is proposed to be involved in activation of the enzyme by metal ions. Interestingly, a similar gene was identified in the nematode, *Caenorhabditis elegans*, suggesting that PCs may also be expressed in some animal species.

INTRODUCTION

Plants, like other organisms, have adaptive mechanisms whereby they are able to respond to both nutrient deficiencies and toxicities. One response of plants to heavy metal stress is the induction of phytochelatins (PCs), a family of related peptides that have the structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (where $n > 1$) and are clearly related to the tripeptide glutathione (GSH), for which $n = 1$. These peptides have been referred to variously as cadystins (from *Schizosaccharomyces pombe*), poly-$(\gamma\text{-EC})_n$G peptides, Cd binding peptides, and PCs. They are broadly classified as class III metallothioneins and are enzymatically synthesized, unlike the gene-encoded class I and II metallothioneins (Kägi, 1991). PCs have been identified in a wide variety of plant species, including monocots, dicots, gymnosperms, and algae (Gekeler et al., 1989), in addition to various fungal species (Mehra et al., 1988; Kneer et al., 1992) and marine diatoms (Morelli and Pratesi, 1997), but not in any animal species. PC synthesis is rapidly induced in the presence of a wide range of heavy metals (Grill et al., 1987; Maitani et al., 1996). However, low levels of PCs can be detected in plant tissues or cultures of yeast or plant cells grown in medium containing trace levels of essential heavy metals (Grill et al., 1988; Kneer and Zenk, 1992; Howden et al., 1995a).

Various studies have confirmed that PCs are synthesized from GSH. For example, exposure of whole plants or plant cell cultures to an inhibitor of GSH biosynthesis, namely, buthionine sulfoximine, also inhibited the induction of PCs and/or conferred hypersensitivity to Cd (Steffens et al., 1986; Reese and Wagner, 1987; Scheller et al., 1987; Howden and Cobbett, 1992). In addition, mutants of both *S. pombe* and Arabidopsis having defects in genes encoding GSH biosynthetic enzymes are deficient in PCs and hypersensitive to some metal ions (Mutoh and Hayashi, 1988; Glaeser et al., 1991; Howden et al., 1995b).

The role of GSH as a substrate for PC biosynthesis was confirmed with the identification of an enzyme activity from cultured cells of *Silene cucubalis* that synthesized PCs from GSH in vitro (Grill et al., 1989). The reaction involved the transpeptidation of the γ-Glu-Cys moiety of GSH onto,

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initially, a second GSH molecule to form $PC_{(n = 2)}$ or, in later stages of the incubation, onto a PC molecule to produce an (n + 1) oligomer. This γ-Glu-Cys dipeptidyl transpeptidase (EC 2.3.2.15) has been named PC synthase. The partially purified enzyme was active only in the presence of metal ions. A range of metal ions, all of which were known to induce PC synthesis in plants in vivo, were effective activators of the enzyme in vitro. In these reactions, PC biosynthesis continued until the activating metal ions were chelated either by the PCs formed or by the addition of a metal chelator, such as EDTA (Loeffler et al., 1989). Chelation by PCs provides a mechanism to autoregulate the biosynthesis of PCs in which the product of the reaction chelates the activating metal, thereby terminating the reaction. Similar PC synthase activities have been detected in pea (Klapheck et al., 1995), tomato (Chen et al., 1997), and Arabidopsis (Howden et al., 1995a). PC synthase activity is expressed constitutively in a number of species examined (Grill et al., 1989; Klapheck et al., 1995; Chen et al., 1997).

In addition to GSH and PC biosynthetic activities, a number of other processes important in the function of PCs in vivo have been identified. In both plants and *S. pombe*, PC–Cd complexes are sequestered to the vacuole. In extracts of *S. pombe* and, in some cases, plants (Reese et al., 1992; Speiser et al., 1992a; Howden et al., 1995a) exposed to Cd, high and low molecular weight PC–Cd complexes can be resolved by using gel filtration chromatography. A Cd-sensitive mutant of *S. pombe*, *hmt1*$^-$, which is unable to form high molecular weight complexes, facilitated identification of a gene encoding a member of the family of ATP binding cassette membrane transport proteins. The *HMT1* gene product is located in the vacuolar membrane and is responsible for the transport of PCs and PC–Cd complexes into the vacuole (Ortiz et al., 1992, 1995). High molecular weight complexes also contain acid-labile sulfide, which increases both the amount of Cd in a complex and the stability of the complex (Reese and Winge, 1988; Dameron et al., 1989; Mehra et al., 1994). Other Cd-sensitive mutants of *S. pombe* have allowed identification of genes that are required for the production of high molecular weight PC–Cd complexes. These gene products are proposed to be involved in the biosynthesis of sulfur-containing compounds, which lead to incorporation of sulfide into high molecular weight complexes (Speiser et al., 1992b; Juang et al., 1993).

The isolation and characterization of Cd-sensitive mutants of Arabidopsis have helped to identify two loci involved in PC biosynthesis. The *cad2-1* mutant is partially deficient in γ-Glu-Cys synthetase activity, which is the first of two enzymes in the GSH biosynthetic pathway, and the *cad2-1* mutation lies in the structural gene encoding γ-Glu-Cys synthetase (Cobbett et al., 1998). In contrast, *cad1* mutants retained wild-type levels of GSH and were deficient in PC biosynthesis in vivo and PC synthase activity in vitro (Howden et al., 1995a). Thus, the *CAD1* gene was predicted to encode PC synthase. Here, we describe the isolation of the *CAD1* gene by using a positional cloning strategy and demonstrate that both *CAD1* and a homologous gene in *S. pombe* encode PC synthase activity.

RESULTS

Positional Cloning of the *CAD1* Gene

CAD1 is closely linked to *TRANSPARENT TESTA3* (*TT3*) on chromosome 5 (Howden and Cobbett, 1992). Two restriction fragment length polymorphism (RFLP) markers, m423 and sAt2105 (*CRA1*), flanking *TT3* and the clone pAt5-91.5, which corresponds to *TT3*, were used to continue mapping the position of *CAD1* by using progeny of a cross between the *cad1-1* mutant (Columbia ecotype) and the Landsberg *erecta* ecotype. The relative order of these markers and the calculated genetic distances from *CAD1* are shown in Figure 1. Two recombination events between pAt5-91.5 and *CAD1* were observed in 91 F_3 families scored, whereas no recombinants between *CAD1* and sAt2105 (*CRA1*) were identified in 63 F_3 families. To position *CAD1* more closely with respect to molecular markers in the region, we isolated 26 recombinants between *TT3* and *CAD1*. Additional molecular markers shown in Figure 1 were used to position the 26 recombination events. These included markers corresponding to regions of a genomic clone, MRH10, which had been sequenced as part of the Arabidopsis Genome Initiative.

The rightmost six recombination events occurred between markers corresponding to genes MRH10.7 and MRH10.11, and the recombination points were further mapped using additional markers from this interval (Figure 1). This analysis demonstrated that *CAD1* lay to the right of a marker in the vicinity of base pair position 60,000 of the MRH10 sequence. Two predicted genes, MRH10.11 and MRH10.12, lay on MRH10 to the right of this marker. The latter is predicted to encode a transcription factor, whereas the former is of unknown function. To investigate the possibility that MRH10.11 is the *CAD1* gene, we determined the nucleotide sequence of MRH10.11 in each of four *cad1* mutants. In each case, a different single base pair substitution was identified, indicating that MRH10.11 is the *CAD1* gene. The *cad1-1*, *cad1-4*, and *cad1-5* mutations were base pair transitions generated by ethyl methanesulfonate, whereas the *cad1-3* mutation was a G-C–to–C-G transversion generated by 1,2:3,4-diepoxybutane.

To confirm that MRH10.11 is *CAD1*, we ligated a genomic fragment spanning the gene into the binary vector pBI101.3 and transformed it into the *cad1-3* mutant. The progeny of two kanamycin-resistant transgenic lines were examined in detail. Each line segregated kanamycin-resistant and kanamycin-sensitive and Cd-resistant (wild-type) and Cd-sensitive individuals. Kanamycin-resistant seedlings were uniformly Cd resistant, demonstrating that the transgene complemented the phenotype of the *cad1-3* mutant (data not shown). The progeny of two transgenic lines of the *cad1-3* mutant transformed with pBI101.3 alone were uniformly Cd sensitive.

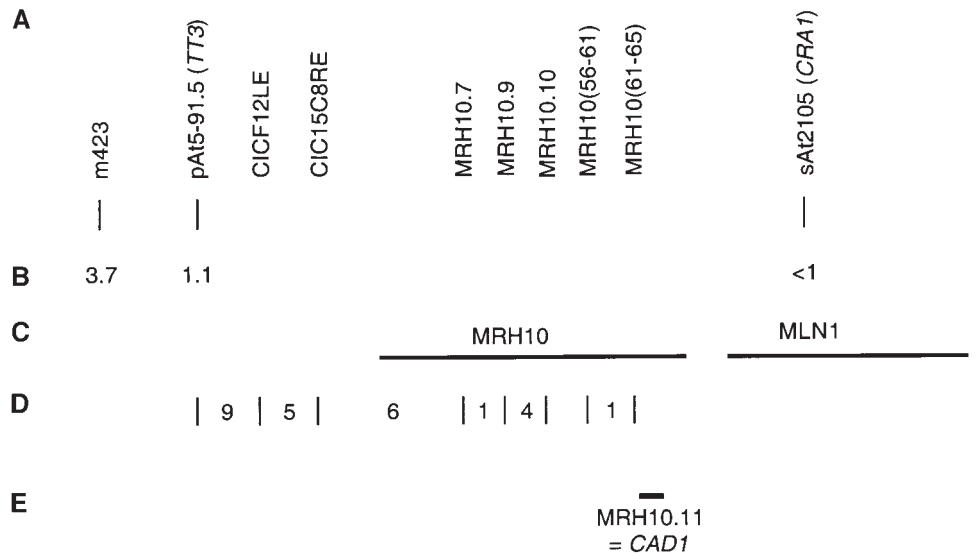

Figure 1. Mapping *CAD1* with Respect to Molecular Markers.

(A) RFLP and PCR-amplified polymorphic markers. Many of these correspond to regions of the genomic clone MRH10 (GenBank accession number AB006703) (not to scale).
(B) Map distances (in centimorgans) between the *CAD1* and RFLP markers indicated.
(C) Genomic clones MRH10 and MLN1 aligned with the markers shown in **(A)**.
(D) Positions of 26 recombination events in the *TT3–CAD1* interval mapped with respect to the markers shown in **(A)**.
(E) Position of the *CAD1* (MRH10.11) gene.

Characteristics of *CAD1* and Homologous Genes from Other Organisms

A genomic polymerase chain reaction (PCR) fragment corresponding to MRH10.11 was used as a probe to identify a cDNA clone containing a 1.6-kb insert. A comparison of the nucleotide sequence of this cDNA with the genomic sequence of MRH10.11 showed the intron/exon structure illustrated in Figure 2A. This structure is different from that predicted in the Arabidopsis Genome Initiative database due to the misidentification of the 5′ splice site of the first intron and the consequent inclusion of an additional 5′ exon to complete an open reading frame. This predicted exon is not present in the cDNA, and reverse transcription–PCR (RT-PCR) experiments using a primer corresponding to part of this exon failed to generate a product (data not shown), suggesting that this region is not present in the mature mRNA. The cDNA contains an open reading frame that would encode a 55-kD protein of 485 amino acids. Database searches identified genes encoding similar proteins in *S. pombe* and the nematode *Caenorhabditis elegans.* An alignment of the deduced amino acid sequences of these gene products is shown in Figure 2B. The N-terminal region of each is well conserved. A pairwise comparison of the region corresponding to the first 220 amino acids of the Arabidopsis sequence showed 40 to 50% identity. Of these, 71 residues are identical across the three gene products. In contrast, the C-terminal region shows no apparent sequence conservation. The only identifiable common feature of the C-terminal region is the occurrence of multiple Cys residues. The plant, yeast, and nematode gene products have 10, 7, and 10 Cys residues, respectively, in the C-terminal region, including two, three, and two adjacent pairs, respectively. However, there is no apparent conservation of the positions of these Cys residues relative to each other.

The effects of the mutations identified in the four *cad1* mutant alleles are also shown in Figure 2B. The *cad1-1* and *cad1-3* mutations change residues conserved across the three sequences. The *cad1-4* mutation alters a residue that is not conserved but is immediately adjacent to a highly conserved block of sequence. The *cad1-5* mutation would result in premature termination of translation. The truncated product would contain the conserved N-terminal domain but would lack the greater part of the C-terminal domain, including nine of the 10 Cys residues.

Characterization of a PC-Deficient Mutant of *S. pombe*

S. pombe has also been used as a model organism for the genetic analysis of PC biosynthesis and function. Because the Arabidopsis *cad1* mutants have a Cd-sensitive phenotype,

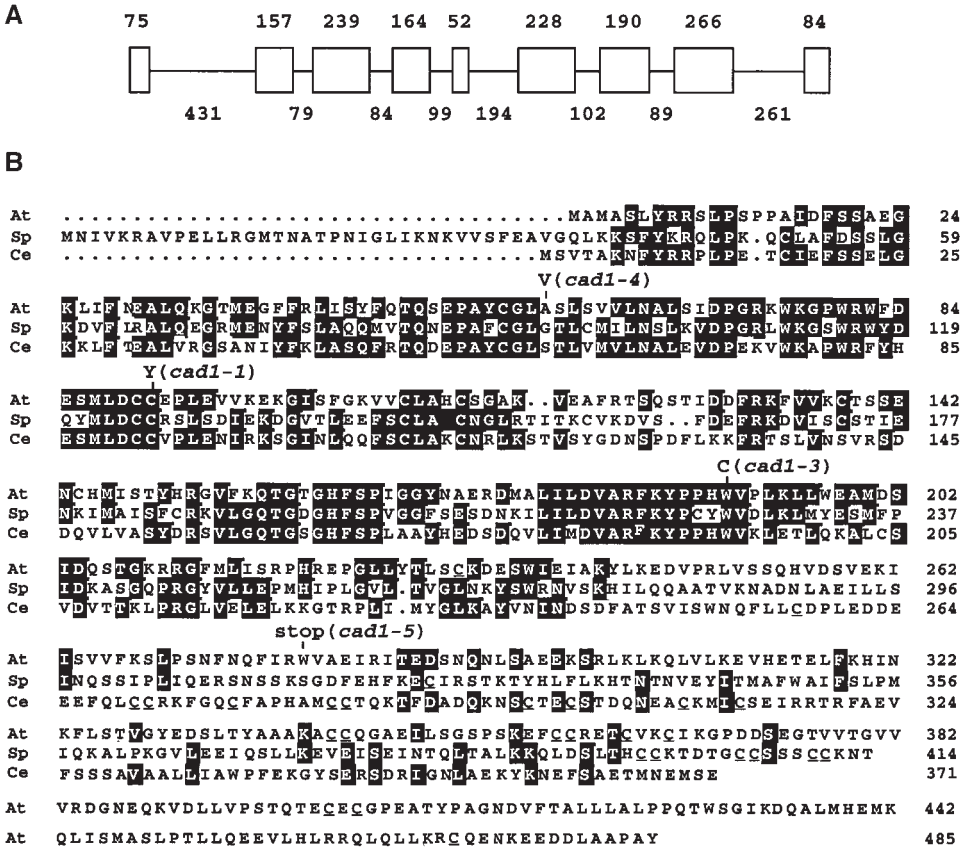

Figure 2. Structure and Sequence of *CAD1.*

(A) The exon/intron structure of *CAD1.* Lengths of exons (above) and introns (below) are in base pairs. The ends of the schematic correspond to the ends of the cDNA sequence.

(B) Amino acid sequences were derived from nucleotide sequences of the Arabidopsis (At) *CAD1* cDNA (GenBank accession number AF135155), the *S. pombe* (Sp) gene *SPAC3H1.10* (GenBank accession number Z68144), and the *C. elegans* (Ce) gene *F54D5.1* (GenBank accession number Z66513). Dots have been inserted to maximize alignment. Residues identical in two or more of the sequences are highlighted. In the C-terminal regions, Cys residues are underlined. The amino acid changes in the *cad1* mutant alleles are indicated above the Arabidopsis sequence.

a similar phenotype would be expected for the analogous *S. pombe* mutant. To test this hypothesis, we constructed a targeted deletion of the *S. pombe* gene and tested the mutant derivative for growth in the presence of Cd. Figure 3A shows that the mutant is Cd sensitive. Whereas the wild-type strain showed inhibition of growth only on medium containing 300 μM $CdSO_4$, the mutant derivative was inhibited by concentrations of $CdSO_4$ as low as 30 μM. The mutant was also more sensitive than the wild type to AsO_4^{2-} (Figure 3A) but not to Cu (Figure 3A), Zn, Hg, SeO_3^{2-}, Ag, or Ni ions (data not shown). Furthermore, because *cad1* mutants of Arabidopsis are PC deficient, the *S. pombe* mutant was tested for PC induction in the presence of Cd. Figure 3B shows the mutant was deficient in PCs (<2 nmol of γ-Glu-Cys equivalents per mg of protein) compared with the wild-type strain (153 ± 4 nmol γ-Glu-Cys equivalents per mg of protein). This demonstrates that the function of these genes in the two organisms is analogous.

To further compare the involvement of PCs in the detoxification of different heavy metals in *S. pombe* and Arabidopsis, we tested the *cad1-3* mutant, which lacks detectable PCs, for sensitivity to a similar range of heavy metal ions. The relative concentrations of metal ion in the medium, which resulted in equivalent inhibition of growth of *cad1-3* and wild-type seedlings, allowed a semiquantitative measurement of the degree of sensitivity of the mutant. Figure 4, for example, shows the data obtained for AsO_4^{2-}, Hg, and Ni. The mutant is ~10- to 20-fold more sensitive than is the wild type to AsO_4^{2-} and approximately twofold more sensitive to Hg; no difference between mutant and wild type was observed in the presence of Ni. Like the *S. pombe* mutant, *cad1-3* was highly sensitive to Cd (20- to 40-fold) and

AsO_4^{2-} and no more sensitive than was the wild type (less than twofold) to Zn, SeO_3^{2-}, and Ni ions. In contrast to the *S. pombe* mutant, *cad1-3* was slightly sensitive to Cu (twofold) and Hg (twofold) and showed intermediate sensitivity to Ag (approximately eightfold) ions.

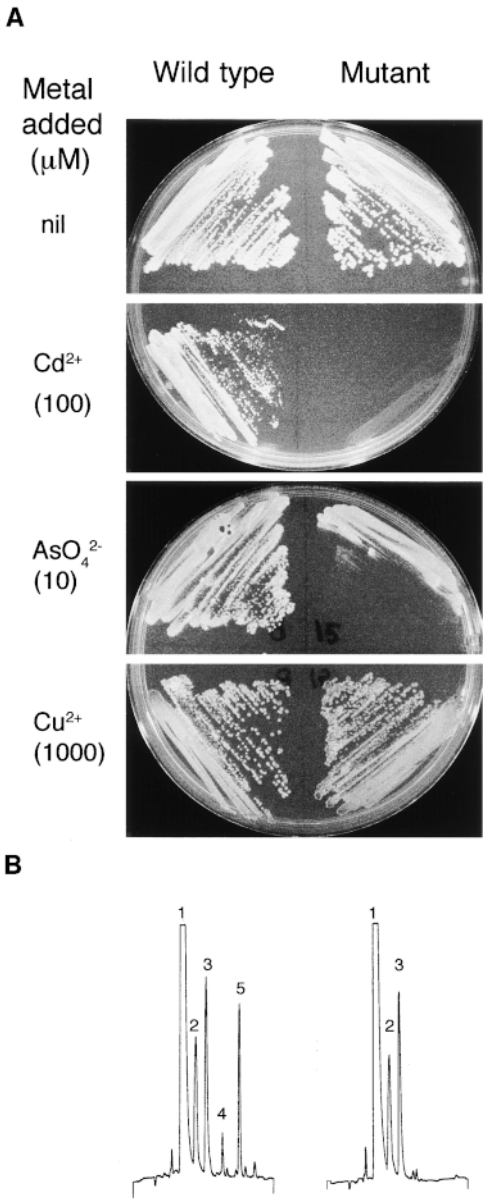

Figure 3. Heavy Metal Sensitivity of an *S. pombe* PC-Deficient Mutant.

(A) Mutant and wild-type strains were grown on medium containing the added heavy metal ions, as indicated. nil; no added metal ions.
(B) Mutant and wild-type strains were assayed by HPLC for PC accumulation. Peaks are identified as follows: peak 1, β-mercaptoethanol; peak 2, GSH; peak 3, standard; peak 4, $PC_{(n = 2)}$; and peak 5, $PC_{(n = 3)}$.

Expression of PC Synthase Activity in *Escherichia coli*

To confirm that both the *S. pombe* and Arabidopsis genes encode PC synthase activity, we expressed the proteins encoded by the predicted open reading frames of these genes in *E. coli*. The open reading frames were ligated into the expression vector pTrc99A, in which translation initiating at the predicted Met residues shown in Figure 2B is driven from a bacterial Shine-Delgarno sequence. Protein extracts prepared from bacteria transformed with these constructs were able to catalyze the synthesis of $PC_{(n = 2 \text{ to } 4)}$ in vitro. No activity (<0.1 nmol γ-Glu-Cys transferred per min per mg of protein) was detected in the absence of GSH or if the extract were boiled or in extracts of cells containing pTrc99A alone. Both the expressed plant and yeast enzymes were activated to variable extents by a range of metal ions known to induce PC synthesis in vivo in plants and *S. pombe* (Table 1). PC synthase activity in extracts from *E. coli* cells expressing *CAD1* was up to 40-fold higher than that observed in extracts from cells expressing the *S. pombe* gene.

Expression of the *CAD1* Gene

A number of previous studies have shown that PC synthase activity is expressed constitutively in plant tissues or cell cultures (Grill et al., 1989; Klapheck et al., 1995; Chen et al., 1997). To examine the expression of the *CAD1* gene in response to exposure to Cd, we isolated total RNA from roots and leaves of plants grown in the presence or absence of Cd. RT-PCR was used to generate cDNA and amplification products using primers specific for *CAD1* and, as a control, primers corresponding to absolutely conserved regions of all the known actin cDNAs in Arabidopsis. Each primer pair flanked an intron, allowing products amplified from cDNAs to be distinguished from those arising from genomic DNA contamination. Preliminary experiments demonstrated that both the number of PCR cycles and the amount of cDNA template used limited the amount of product obtained (data not shown). The results are shown in Figure 5. Only a single fragment corresponding to the predicted cDNA product was observed for each primer pair. Similar amounts of actin-specific product were obtained for all samples. A greater amount of *CAD1*-specific product was obtained with root compared with leaf cDNA. No differences were observed between samples from tissues exposed or not exposed to Cd, indicating that expression of the gene is not induced in the presence of Cd.

Sequences Homologous to *CAD1* in Arabidopsis

The absence of any detectable PC expression in the *cad1-3* mutant in the presence of Cd suggested that the Columbia ecotype has only a single gene expressing this function. However, a search of the Arabidopsis Genome Initiative database

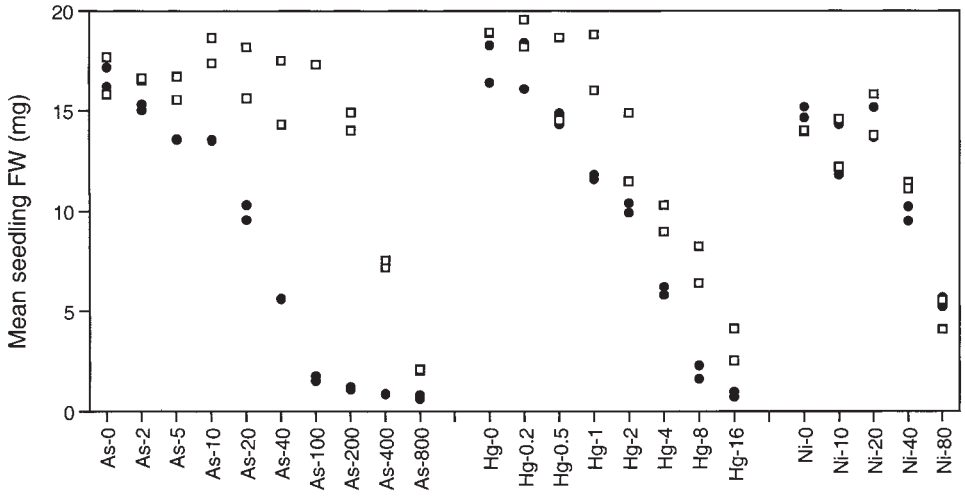

Figure 4. Sensitivity of the *cad1-3* Mutant to Different Heavy Metals.

Fresh weight (FW) of wild-type (open squares) and *cad1-3* (filled circles) plants was measured after growth in the presence of $NaAsO_4$ (As), $HgSO_4$ (Hg), or $NiCl_2$ (Ni) at various concentrations (given in micromoles per liter). Note that the x axis is not to scale. Each data point represents the mean fresh weight of 10 plants. Standard errors are not shown for clarity. The largest standard error calculated for any data set was 1.6 mg.

indicates that there are at least two additional sequences in the Arabidopsis genome that are related to *CAD1*. The first is a 360-bp region of the 1.1-kb intergenic region between *CAD1* (MRH10.11) and MRH10.12. This region is similar to portions of exons 7 and 8 of *CAD1* (data not shown). This sequence does not appear to contain an intact gene and may have arisen from the duplication of part or all of *CAD1*.

The second region of similarity is a predicted gene on clone F21M11 from chromosome 1 (GenBank accession number AC003027), which is similar in both sequence and apparent structure to *CAD1.* The positions of the predicted introns of this second gene are identical to those of *CAD1.* However, the sizes and sequences of the introns vary considerably between the two genes, suggesting that one is not a recent duplication of the other. Predicted exons differ in size by at most a single codon, with the exception of an apparent 90-bp deletion within exon 8 of the *CAD1* homolog, that would result, compared with *CAD1*, in an in-frame deletion of part of the C-terminal variable domain of the predicted gene product. The deduced amino acid sequence for the *CAD1* homolog is 84% identical to *CAD1*.

DISCUSSION

The previous demonstration that *cad1* mutants had normal levels of GSH, were PC deficient, and lacked detectable PC synthase activity in vitro led to the prediction that the *CAD1* gene encodes the PC synthase activity (Howden et al., 1995a). Using a positional cloning strategy, we have demonstrated that the gene MRH10.11, predicted from the Arabidopsis Genome Initiative genomic sequence, is *CAD1*. In support of this conclusion, we demonstrated that four independent *cad1* mutants have base pair substitutions in the MRH10.11 gene and that the wild-type gene can complement the *cad1* Cd-sensitive phenotype. To confirm that *CAD1* encodes PC synthase activity, we expressed the *CAD1* cDNA in *E. coli.* Previous studies on plant PC synthase activities have shown that PC synthesis is dependent on GSH as a substrate and is activated by various heavy metal ions (Grill et al., 1989; Klapheck et al., 1995; Chen et al., 1997). Consistent with these observations, GSH-dependent, heavy metal–activated synthesis of PCs was catalyzed by extracts of *E. coli* cells expressing the *CAD1* gene product. A similar range of metal ions activated the enzyme with variable efficiency.

In addition to Arabidopsis, *S. pombe* has been used as a model organism for the genetic analysis of PC biosynthesis and function. Whereas a number of genes influencing the formation of PC–Cd complexes have been identified by screening for Cd-sensitive mutants (Ortiz et al., 1992; Speiser et al., 1992b), the gene encoding PC synthase has not been isolated using this approach. A homolog of the *CAD1* gene was identified in *S. pombe*; to strengthen the conclusion that this family of genes encodes PC synthase, we have included here studies of the *S. pombe* gene. As for *cad1* mutants of Arabidopsis, the corresponding *S. pombe* mutant was heavy metal sensitive and PC deficent. Hayashi et al. (1991) proposed that there are two pathways for PC biosynthesis in *S. pombe.* In addition to the pathway found in plants (transfer of γ-Glu-Cys from GSH to an acceptor),

they present evidence for a pathway in which γ-Glu-Cys is polymerized and then a terminal Gly is added by GSH synthetase. However, because mutation of the *S. pombe* homolog of *CAD1* effectively abolishes PC expression, it is unlikely that such a pathway is of any physiological significance. Interestingly, whereas PCs have been reported in *Saccharomyces cerevisiae* (Kneer et al., 1992), there is no gene in the *S. cerevisiae* genome with similarity to *CAD1* or the *S. pombe* homolog. This may indicate that PCs can, in some organisms, be synthesized via an alternative pathway.

GSH-dependent, metal-activated PC synthesis was also detected in extracts of *E. coli* expressing the *S. pombe* gene. Activity in extracts from cells expressing the *CAD1* cDNA was up to 40-fold higher than that observed with the *S. pombe* expression construct. This may be due to differences in the level of expression or stability of the enzymes in *E. coli*, possibly resulting from differential mRNA stability, translational efficiency influenced by codon usage, or inappropriate protein folding. Alternatively, it may simply reflect the intrinsic activity of the different gene products under the assay conditions used. In addition, whereas a wide range in the efficiency of activation of the expressed plant enzyme by different heavy metals—for example, a 50-fold difference for Ag^+ compared with Pb^{2+}—was observed, considerably smaller differences in activation of the expressed yeast enzyme by the same metal ions were detected. However, in view of the uncertainty of the structure and stability of the expressed polypeptides, further investigation is required to characterize the apparent differences between these activities.

The degree of sensitivity of the PC-deficient mutants to specific heavy metals gives an indication of the role of PCs in detoxification of these metals in vivo. A comparison of the relative sensitivity of the Arabidopsis and *S. pombe* mutants to different heavy metals revealed a similar but not identical pattern. In both organisms, PCs appeared to play an important role in Cd and arsenate detoxification but had no apparent role in the detoxification of Zn, Ni, and selenite ions. Differences between the two organisms were observed with respect to Cu, Hg, and Ag.

Table 1. Expression of PC Synthase Activity in *E. coli*

	PC Synthase Activity ±SE[a] (nmol γ-Glu-Cys Transferred min^{-1} mg^{-1} Protein)	
Metal Ion (μM)	Arabidopsis	*S. pombe*
None	<0.1 (<0.1)	0.3 ± 0.2 (5)
Cd^{2+} (100)	116 ± 30 (100)	6.5 ± 0.1 (100)
Cu^{2+} (50)	253 ± 2 (218)	4.6 ± 0.1 (71)
Ag^+ (100)	230 ± 17 (198)	3.6 ± 0.5 (55)
Hg^{2+} (50)	97 ± 20 (84)	2.8 ± 0.4 (43)
Zn^{2+} (100)	9.7 ± 2.3 (8)	3.7 ± 0.4 (57)
Pb^{2+} (100)	4.7 ± 0.8 (4)	4.2 ± 0.5 (65)

[a] The Arabidopsis cDNA and *S. pombe* gene encoding PC synthase were expressed in *E. coli*, and the extracts were assayed ($n = 3$) for PC synthase activity in the presence of heavy metal ions. The percentage of activity relative to that in the presence of Cd^{2+} is shown within parentheses.

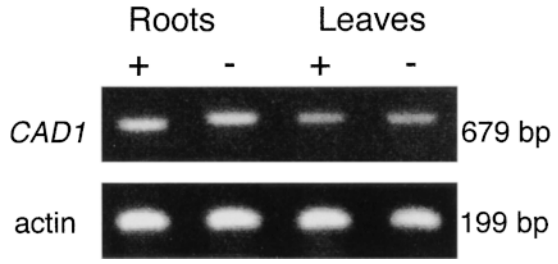

Figure 5. Expression of *CAD1.*

RT-PCRs were conducted using total RNA extracted from wild-type leaf and root tissue of plants exposed (+) or not exposed (−) to 1.5 μM $CdSO_4$ for 3 days. Primers specific to *CAD1* or to actin genes were used.

PC synthesis is activated both in vivo and in vitro by some metal ions to which the corresponding PC synthase mutant is not hypersensitive. For example, both the expressed Arabidopsis enzyme in vitro and PC synthesis in vivo (P.B. Goldsbrough, unpublished data) are efficiently activated by Cu. However, the *cad1-3* mutant is only slightly more sensitive to Cu than is the wild type. Similarly, Zn activates both the expressed *S. pombe* enzyme in vitro and PC synthesis in *S. pombe* in vivo (Grill et al., 1986), but the PC synthase–deficient mutant is not more sensitive to Zn than is the wild type. Although PC synthesis may be activated in vivo by particular metal ions, PCs may play little or no role in their detoxification. This may be due, for example, to inefficient sequestration to the vacuole and an inability to form stable complexes or to the operation of other more effective mechanisms for the detoxification of these metals. Together, these observations indicate that in vitro data on the relative efficiency of activation of PC synthase by various heavy metal ions should not be regarded as reflecting the role of PCs in the detoxification of those metal ions in vivo.

The alignment of the polypeptide sequences derived from the plant, yeast, and nematode genes leads to speculation about the function of the conserved and variable regions of the PC synthase enzymes. The conserved N-terminal domain must possess PC synthase catalytic activity. The protein encoded by the *cad1-5* allele is predicted to terminate at position 280 and lacks almost two-fifths of the wild-type protein. However, this mutant is the least sensitive to Cd. Although no PC synthase activity was detected in extracts of this mutant, it accumulated 33% of the wild-type levels of PCs in vivo, indicating that the mutant gene product retains some activity (Howden et al., 1995a). Because a truncation of part of a catalytic function is likely to completely abolish activity, this observation supports the hypothesis that the C-terminal domain is not absolutely required for catalytic function.

The mechanism by which PC synthase is activated by a wide range of heavy metals and the basis of variations in the specificity and efficiency of activation of enzymes from different organisms are of particular interest. Activation probably arises from metal ions interacting with residues in the conserved, presumably catalytic, domain. Many heavy metal binding motifs contain Cys and His residues. Five Cys residues (two of which are adjacent) and a single His residue are conserved across the three sequences and may play a role in activation of the enzyme. In addition, there is likely to be a role in enzyme activation for the multiple Cys residues in the variable C-terminal region. One hypothesis is that the Cys residues of the C-terminal region provide a local sensor by binding heavy metal ions and bringing them into contact with the activation site in the catalytic domain of the protein. Thus, there may be few constraints on the primary amino acid sequence of this region other than the requirement for multiple Cys residues.

No PCs were detected in the *cad1-3* mutant after prolonged exposure to Cd. This appears to indicate that only a single active PC synthase gene occurs in the Columbia ecotype. However, there is a second predicted gene on chromosome 1 with considerable similarity to *CAD1*. One possible explanation is that this gene encodes a functional PC synthase that is activated by other metal ions but not by Cd. To test this hypothesis, we assayed *cad1-3* seedlings exposed to Zn, Ni, Pb, SeO_3, Ag, and Hg ions for PC accumulation. In no case were PCs detected (data not shown). In view of the lack of specificity of activation of PC synthase by metal ions, it seems unlikely that a functional PC synthase homolog would not be activated by at least some of the metals tested. An alternative hypothesis is that the homologous gene is nonfunctional at some level. However, there are no obvious mutations that would render the gene nonfunctional, such as deletions of highly conserved regions, frameshift mutations, or splice-site mutations. It is not known whether the gene is transcribed. Further analysis of this gene is required to determine whether it is functional.

The identification of a similar gene in *C. elegans* is of considerable interest because PCs have not been detected in any animal species. Currently, we have no evidence that this gene also encodes a protein with PC synthase activity. However, in view of the high level of identity of the nematode gene product with the conserved N-terminal domains of the yeast and plant enzymes as well as the presence of a variable domain containing multiple Cys residues, it seems likely that it too encodes PC synthase. Preliminary HPLC assays of extracts of *C. elegans* have identified a Cd-inducible peak corresponding to $PC_{(n = 2)}$ (A.P. Smith and P.B. Goldsbrough, unpublished data). However, the structure of this compound must be confirmed. In addition, by using PCR, sequences similar to the conserved regions of the three genes have been identified from both the aquatic midge, Chironomus, and earthworm species (W.M. Dietrich and C.S. Cobbett, unpublished data).

Originally, in the absence of evidence for class I or II metallothioneins in plants, PCs were regarded as the functional equivalent of animal metallothioneins (Grill et al., 1987). Subsequently, with the identification of metallothionein-like genes (Robinson et. al., 1993; Zhou and Goldsbrough, 1994) and metallothioneins (Murphy et al., 1997) in plants, it seemed more likely that the two classes of metal binding ligands played different or possibly overlapping roles in metal detoxification or metabolism. Nonetheless, the absence of reports of PCs in animals suggested a phylogenetic divergence between the animal and other kingdoms in the requirement for the two classes of ligand. Together, these observations suggest that this apparent divergence may not be real and that PCs may play a role in heavy metal detoxification in more types of organisms than were previously expected. A more thorough exploration of the spectrum of species containing similar genes will be of interest.

In conclusion, the identification of PC synthase genes in both yeast and plant species, and possibly in animal species as well, will stimulate further studies on the mechanism and regulation of PC biosynthesis. PC synthase is unrelated in sequence, and presumably in structure, to other proteins and thus may identify a new mechanism by which an enzyme may be activated in a relatively nonspecific manner by heavy metal ions. The manipulation of PC synthase expression in transgenic plants may also enhance the capacity of plants to be effective in the phytoremediation of heavy metal–contaminated environments.

METHODS

Plant Strains and Growth Conditions

Arabidopsis thaliana plants were grown as previously described (Howden and Cobbett, 1992). The *cad1* mutants have been described previously (Howden et al., 1995a). To measure the sensitivity of the *cad1-3* mutant compared with the wild type, we transferred 7-day-old seedlings to 25 mL of agar medium containing added heavy metal. Each Petri dish contained 10 mutant and 10 wild-type seedlings. The plates were sealed with gas-permeable tape and incubated for an additional 10 days, when the shoot portions of the plants were weighed.

Mapping *CAD1*

Restriction fragment length polymorphism (RFLP) mapping of *CAD1* was conducted essentially as described by Cobbett et al. (1998) for the *CAD2* locus. Markers were m423 (Chang et al., 1988), pAt5-91.5 (Shirley et al., 1992), and sAt2105 (Pang et al., 1988). Further recombinants were obtained as follows. The *GLABROUS3* (*GL3*), *TRANSPARENT TESTA3* (*TT3*), and *CAD1* loci are linked on chromosome 5 of Arabidopsis. From a cross between strain M24 (*gl3 tz cer3 er*) (Landsberg *erecta* ecotype) and the *cad1-1* mutant (Columbia ecotype), a *gl3-3 cad1-1* double mutant line was generated in which both the *TT3* and *cad1-1* loci were derived from the Columbia parent. Eighty-nine recombinants between the *GL3* and *CAD1* loci were

identified phenotypically from a cross between the *gl3 cad1-1* line and the Landsberg *erecta* ecotype. These were further analyzed for recombination in the *TT3–CAD1* interval by using an EcoRI RFLP between the two ecotypes detected by clone pAt5-91.2. Markers used to further map *CAD1* were CIC5F12LE and CIC15C8RE, both of which detected EcoRV RFLPs on DNA gel blots, and polymerase chain reaction (PCR)–amplified polymorphic markers corresponding to the following regions of MRH10: MRH10.7 (primers 5′-CGGAGAAGAGTAATGTCGTAGA-3′ and 5′-GTCCATAAAATAGGTAGGTAGC-3′; SacI polymorphism), MRH10.9 (primers 5′-ACAAACGCCACAAAGGTAGATT-3′ and 5′-GAAGGATAGACCAAAGAACAAC-3′; HindIII polymorphism), MRH10.10 (primers 5′-TGGGACTAAATAATCGGACTCG-3′ and 5′-AACCTTCACTTTCTTGCGTCTG-3′; PCR product only from Columbia ecotype), MRH10(56-61) (primers 5′-CAGACGCAAGAAAGTGAAGGTT-3′ and 5′-AGAGGAGGAGAAATGAAAGGAC-3′; PCR product size polymorphism), and MRH10(61-65) (primers 5′-GTCCTTTCATTTCTCCTCCTCT-3′ and 5′-GGATGACTAAAGGATTGACGAC-3′; BamHI polymorphism).

Each *cad1* mutant allele was amplified by PCR using primers with the following sequences: 5′-AGCGAAACAAGAAAACACTAAT-3′ and 5′-ATTTGTGGATAGTGGATTCATA-3′. Nucleotide sequences were determined directly from two independently amplified templates.

Complementation of the *cad1-3* Mutant

An 8.4-kb XhoI-SacI fragment spanning the MRH10.11 gene was ligated into pBI101.3 (Clontech, Palo Alto, CA) and transformed into *Agrobacterium tumefaciens* GV3101/pMP90 by electroporation. This construct and the parent plasmid pBI101.3 were transformed into *cad1-3* plants by vacuum infiltration (Bechtold et al., 1993). Transformant seedlings were selected in the presence of 50 μg/mL kanamycin.

Construction and Analysis of the *Schizosaccharomyces pombe* Mutant

A 4.5-kb fragment spanning the *S. pombe* PC synthase gene was ligated into pBluescript SK+ (Stratagene, La Jolla, CA). The SmaI-PstI fragment spanning codons 107 to 277 was deleted and replaced with the *ura4*$^+$ marker as a HincII-PstI fragment. The resulting fragment was transformed into *S. pombe* of genotype *ura4-D18 leu1-32 ade6-704h*, and transformants were selected by uracil prototrophy. Transformants were replica-plated to minimal medium containing 500 μM $CdSO_4$, and ~50% were Cd sensitive. Successful targeting of the phytochelatin (PC) synthase gene in these strains was confirmed by PCR. Methods for propagation and manipulation of *S. pombe* were as described by Moreno et al. (1991). Exponentially growing cells were exposed to 50 mM $CdCl_2$ for 24 hr, centrifuged, resuspended in 50 mM Tris-HCl, pH 7.9, 14% (v/v) glycerol, and 10 mM β-mercaptoethanol, and disrupted by vortexing with glass beads. After removal of cell debris by centrifugation, an aliquot of supernatant was mixed with an equal volume of 10% sulfosalicylic acid, precipitated protein was removed by centrifugation, and the supernatant was analyzed for PCs by HPLC, as described previously (Howden et al., 1995a).

Expression of PC Synthase Genes in *Escherichia coli*

The *CAD1* cDNA was amplified by PCR using primers that introduced BstHI and SacI sites at the putative initiation codon and downstream, respectively, of the open reading frame. Similarly, NcoI and SacI sites flanking the *S. pombe* gene were introduced by amplification from genomic DNA. The resulting fragments were digested and ligated between the NcoI and SacI sites of pTrc99A (Pharmacia) and confirmed by nucleotide sequencing. The expression constructs were transformed into *E. coli* strain DH5α. Cells were grown to an OD_{600} of ~0.6, treated with 100 μM isopropyl-β-D isothiogalactopyranoside, and grown for an additional 3 hr. An extract was prepared by sonicating the collected bacteria in buffer (50 mM Tris-HCl, pH 7.9, 14% [v/v] glycerol, and 10 mM β-mercaptoethanol). The supernatant was assayed for PC synthase activity, as described previously (Chen et al., 1997), except that the reaction time was 15 min.

Reverse Transcription–PCR Analysis of Gene Expression

Ten-day-old Arabidopsis plants were grown in agar medium and transferred to medium containing 1.5 μM $CdSO_4$; root and leaf tissues were harvested separately after another 3 days. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. cDNA was synthesized from 5 μg of total RNA using the SuperScript kit (Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. Independent PCR reactions using equal aliquots of cDNA samples were performed using *CAD1*-specific primers (5′-CTCGTTTCAAGTATCCCCCTCAC-3′ and 5′-GCTTCAGGACCACATTCACACTC-3′) or actin gene–specific primers (5′-GGTAACATTGTGCTCAGTGGTGG-3′ and 5′-CTCGGCCTTGGAGATCCACATC-3′). Reactions contained 1.5 mM Mg^{2+}, 0.2 mM of each deoxynucleotide, 0.2 μM of each primer, and 0.28 units of Taq polymerase. Primers were annealed at 65°C for 30 sec, and DNA was synthesized at 68°C for 3 min for 35 cycles. Preliminary experiments demonstrated that the amount of PCR product increased with increasing numbers of cycles, indicating that the reaction components were not limiting.

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NOTE ADDED IN PROOF

The authors have recently become aware of two other papers (**Vatamaniuk, O.K., Mari, S., Lu, Y.-P., and Rea, P.A.** [1999]. AtPCS1, a phytochelatin synthase from Arabidopsis: Isolation and in vitro reconstitution. Proc. Natl. Acad. Sci. USA. **96,** in press; **Clemens, S., Kim, E.J., Neumann, D., and Schroeder, J.I.** [1999]. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J. **18,** in press.) describing the identification of phytochelatin synthase genes in plants and yeast.