특강6 Molecular genetic analysis of phytochelatin synthase genes in *Arabidopsis*

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Abstract

This study has investigated the biosynthesis and function of the heavy metal binding peptides, the phytochelatins, in plants. PCs are synthesised enzymatically from glutathione by the enzyme PC synthase in the presence of heavy metal ions. Using *Arabidopsis thaliana* as a model organism cadmium-sensitive, phytochelatin-deficient mutants have been isolated and characterised in previous studies. The *cad1* mutants have wildtype levels of glutathione, are PC deficient and lack PC synthase activity. Thus, the *CAD1* gene has been proposed to encode PC synthase. The *CAD1* gene was isolated by a positional cloning strategy. The gene was mapped and a candidate identified. Each of four *cad1* mutants had a single base pair change in the candidate gene and the cadmium-sensitive, *cad1* phenotype was complemented by the candidate gene. This ,demonstrated the *CAD1* gene had been cloned. A homologous gene in the fission yeast, *Schizosaccharomyces pombe* was identified through database searches. A targeted-deletion mutation of this gene was constructed and the mutant, like *cad1* mutants of Arabidopsis, was cadmium-sensitive and PC-deficient. A comparison of the predicted amino acid sequences reveals a highly conserved N-terminal region presumed to be the catalytic domain and a variable C-terminal region containing multiple Cys residues proposed to be involved in activation of the enzyme by metal ions. Similar genes were also identified in animal species. The Arabidopsis *CAD1/AtPCS1* and *S. pombe SpbPCS* genes were expressed in *E. coli* and were shown to be sufficient for glutathione-dependent, heavy metal activate PC synthesis *in vitro*, thus demonstrating these genes encode PC synthase enzymes. Using RT-PCR, *AtPCS1* expression appeared to be independent of Cd exposure. However, at higher levels of Cd exposure a *AtPCS1-GUS* reporter gene construct appeared to be more highly expressed. Using the reporter gene construct, *AtPCS1* was expressed most tissues. Expression appeared to be greater in younger tissues and some higher levels of expression was observed in some regions, including carpels and the base of siliques. *AtPCS2* was a functional gene encoding an active PC synthase. However, its pattern of expression and the phenotype of a mutant (or antisense line) have not been determined. Assuming the gene is functional then it has clearly been maintained through evolution and must provide some selective advantage. This implies that, at least in some cells or tissue, it is likely to be the dominant PC synthase expressed. This remains to be determined

Introduction

Plants take up heavy metals from their environment. Some essential heavy metals, such as Cu and Zn, are vital to plant metabolism particularly as components of metalloproteins. However, an excess of essential heavy metals, and non-essential metals, such as Cd and Hg, can have toxic effects on cellular processes. Metal ions can interact with sulphydryl groups and other motifs of important metabolic enzymes and transcription factors, and inhibit their function. Heavy metals also cause oxidative damage, generating harmful oxygen radicals which disrupt cell membranes and organelles. The capacity of heavy metal ions to react with and damage biological molecules requires that essential metals must be transported into the plant cell for biological function but must be regulated to avoid toxicity and non-essential metals must be removed from cellular activities through detoxification mechanisms. Plants have evolved mechanisms to respond to heavy metal toxicity. Possible mechanisms for the detoxification of heavy metals include extracellular binding or chelation, regulation of influx or efflux systems, and intracellular sequestration or chelation of the heavy metal (Sanita di Toppi and Gabbrielli, 1999).

One common general mechanism for heavy metal detoxification in numerous organisms, including plants, is the chelation of the metals by a ligand and, in some cases, the subsequent compartmentalisation of the ligand-metal complexes. A variety of metal chelators in plants have been identified, for example, organic acids, amino acids, peptides, polypeptides and are well reviewed (Rauser, 1999). Heavy metal binding peptides, phytochelatins (PCs) are the focus of the work described in this report. These and the polypeptides, metallothioneins (MTs), are considered to be important molecules in metal detoxification. In the following sections PCs and, to a lesser extent, MTs, are described in greater detail.

Phytochelatins were first identified in the yeast, *Schizosaccharomyces pombe* by Murasugi *et al.*,1981. Numerous physiological, biochemical, and genetic studies have confirmed that GSH (or, in some cases, related compounds) is the substrate for PC biosynthesis (Rauser, 1995, 1999; Zenk, 1996). The pathway of GSH biosynthesis (Figure 1) consists of two steps catalysed by the ATP-dependent enzymes, g-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS). Studies have used a variety of plant species, sometimes as intact plants but often in the form of *in vitro* cell cultures. Early studies with cell cultures demonstrated that induction of PCs in the presence of Cd coincided with a transient decrease in levels of GSH. By the use of L-buthionine sulfoximine (BSO), an inhibitor of GCS activity, the PC biosynthetic pathway was shown to be necessary for heavy metal detoxification. BSO inhibits both glutathione and PC synthesis and increases the sensitivity of the cell to added heavy metals. The addition of glutathione reverses this effect (Huang *et al.*, 1987; Steffens *et al.*, 1986). Glutathione-deficient mutants in *S. pombe* (Mutoh and Hayashi, 1988; Glaeser *et al.*, 1991) and Arabidopsis (Cobbett *et al.*, 1998) are also PC deficient and Cd hypersensitive (Howden *et al.*, 1995). In particular, the *cad2-1* mutant of Arabidopsis is partially deficient in GSH and in GCS activity. The *cad2-1* mutation is a 6 bp deletion

within an exon of the GCS gene affecting residues in the vicinity of the presumed active site of the enzyme (Cobbett *et al.*, 1998).

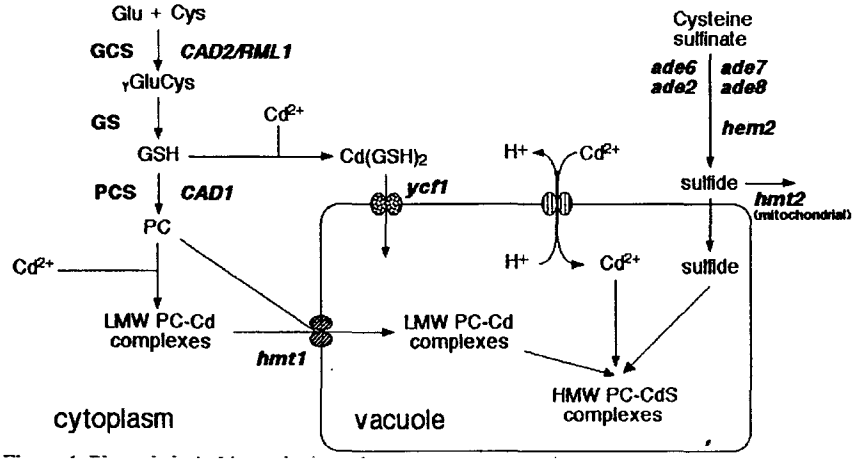

Figure 1. Phytochelatin biosynthetic pathway, and genes and functions involved in Cd detoxification in yeast and plants. This figure is drawn on the basis of findings from different organisms. Identified components are described in the text. Enzyme abbreviations: GS, GSH synthetase; PCS, phytochelatin syntyhase; GCS, ã -glutamylcysteinyl synthetase.

HMW PC-Cd complexes are sequestered to the vacuole (Figure 1). Studies of the subcellular localisation of PC-Cd complexes in tobacco have shown that most are sequestered to the cell vacuole and transport studies using oat root vacuoles have demonstrated a mechanism for transporting Cd into the vacuole. In mesophyll protoplasts derived from tobacco plants exposed to Cd, almost all of both the Cd and PCs accumulated is confined to the vacuole (Vogeli-Lange and Wagner, 1990). An ATP-dependent, proton gradient-independent activity, similar to that of HMT1, capable of transporting both PCs and PC-Cd complexes into tonoplast vesicles derived from oat roots has been identified (Salt and Rauser, 1995). In yeast, YCF1 is also a member of the ABC family of transporters (Figure 1.3) and transports both GSH conjugates and $(GSH)_2Cd$ complexes to the vacuole (Li *et al.*, 1997). In plants, members of this family of transporters have also been identified (Vatamaniuk *et al.*, 1999) and may be involved in the transport of heavy metal complexes to the vacuole.

PCs clearly have important roles in heavy-metal detoxification processes because the PC-deficient mutants of Arabidopsis and *S. pombe* are sensitive to heavy metals. Other roles of PCs have been proposed, such as metal homeostasis, protective effects on metal-sensitive enzymes, iron metabolism and sulfur metabolism. *In vivo* and *in vitro*

experiments suggest a role for PCs in the homeostasis of essential metal ion metabolism (Zenk, 1996; Thumann *et al.*, 1991) and the protection of metal-sensitive enzymes by PCs has been reported (Kneer and Zenk, 1992). In addition, the involvement of PCs in iron and sulfur metabolism has been proposed (Zenk, 1996; Sanita di Toppi and Gabbrielli, 1999). However, no conclusive evidence that PCs have functions other than in metal detoxification, has been found. The isolation and characterisation of cadmium-sensitive mutants of Arabidopsis has advanced our understanding of metal detoxification mechanisms in plants (Howden and Cobbett, 1992).

Results and discussion

An allelic series of *cad1*, Cd-sensitive mutants has previously been isolated and characterised both phenotypically and biochemically. Mutants at the *cad1* locus in Arabidopsis, are Cd-sensitive and deficient in the formation of Cd-binding complexes and in PC biosynthesis. The amount of PCs accumulated by each mutant correlated with its degree of sensitivity to Cd. Particularly in the *cad1-3* mutant, no PCs were detected after exposure to Cd. The *cad1* mutants have wildtype levels of GSH, suggesting a defect in PC synthase. The enzyme PC synthase was first identified by Grill *et al.*, (1989) and had been characterised in subsequent studies (Klapheck *et al.*, 1995, Chen *et al.*, 1997). However, a PC synthase gene had not been isolated in the subsequent period. Previous characterisation of *cad1 mutants led to the prediction that the CAD1* gene encoded a PC synthase. The mutants had *wildtype* levels of GSH, were PC-deficient, and lacked detectable PC synthase activity *in vitro* (Howden *et al.*, 1995). The aim of this study was to isolate the *CAD1* gene using a positional cloning strategy. A panel of recombinants between the *TT3* and *CAD1* loci were used to map *CAD1*. Development of PCR markers and subsequent application of these markers defined a left-hand boundary and two candidate genes positioned on the right hand end of P1 clone, MRH10 and close to this boundary, were examined. One of these, MRH10.11, was identified as the *CAD1* gene. This conclusion was supported by demonstrating all the *cad1* mutants sequenced had mutations in the MRH10.11 gene and by complementing the *cad1* Cd-sensitive phenotype with the wildtype MRH10.11 gene.

S. pombe has been used as a model organism to study the genetic analysis of PC biosynthesis and function. A homologue of the *CAD1* gene was identified by database searches and a targeted-deletion mutant was generated by replacing part of the homologous gene with *URA4*' marker. This mutant, like the *cad1* mutants of Arabidopsis, was Cd-sensitive and PC-deficient (Figure 2). Mutoh and Hayashi (1988), have described an alternative PC biosynthetic pathway in *S. pombe* in which ã-Glu-Cys is polymerised and a terminal Gly is added by GSH synthetase. However, because the PC synthase mutant described here did not have any detectable PCs, this alternative pathway does not appear to be of physiological importance. Interestingly, PCs have been also been detected in *S. cerevisiae* (Kneer *et al.*, 1992) although its genome contains no sequences homologous to the plant or *S. pombe* PC synthases. Thus an alternative biosynthetic pathway might be important in some organisms.

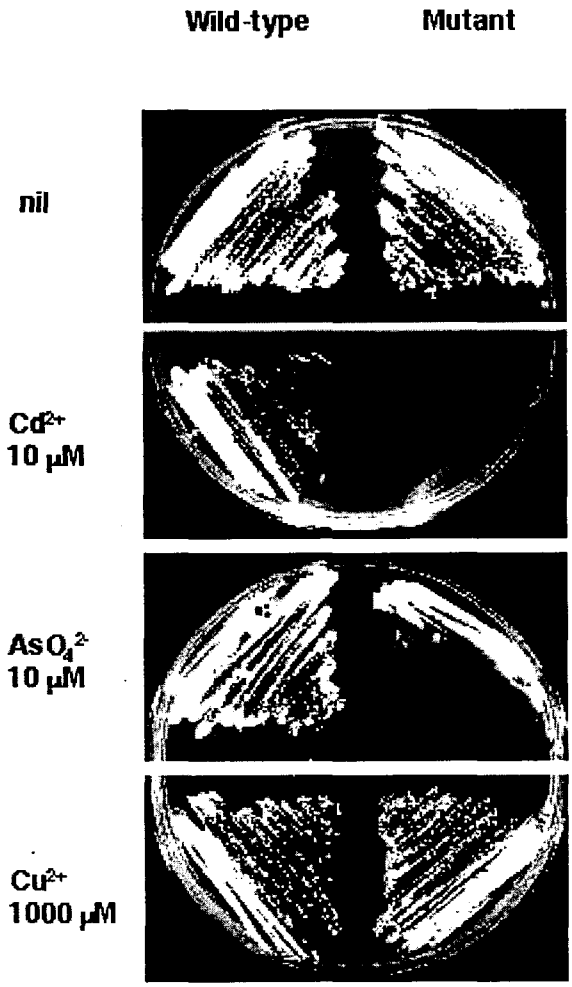

Figure 2. Heavy metal sensitivity of the *S. pombe* PC-deficient mutant. Mutant and wildtype strains were grown for 3 days at 300C on medium containing the added heavy metal ions, as indicated on the left side.

Early models for the activation of PC synthase assumed a direct interaction between metal ions and the enzyme but raised the question of how the enzyme might be activated by such a wide range of metals. A significant recent study has provided evidence for an alternative model that provides a solution to this dilemma (Vatamaniuk *et al.*, 2000). Using purified recombinant AtPCS1 these authors demonstrated that, in contrast to earlier models of activation, metal binding to the enzyme *per se* is not responsible for catalytic activation. The kinetics of PC synthesis is consistent with a mechanism in which heavy metal glutathione thiolate (eg. $Cd.GS_2$) and free GSH act as

g-Glu-Cys acceptor and donor. The observation that S-alkylglutathiones can participate in PC biosynthesis in the absence of heavy metals is consistent with a model in which blocked glutathione molecules (metal thiolates or alkyl substituted) are the substrates for PC biosynthesis. Thus the role of metal ions in enzyme activation is as an integral part of the substrate rather than interacting directly with the enzyme itself. In this way any metal ions which form thiolate bonds with GSH have the capacity to activate PC biosynthesis. The proposed PC synthase enzyme action is shown in Figure 3. This model incorporates data arising from the characterisation of the *cad1-5* mutant in Arabidopsis. This mutant would lack the entire C-terminal domain but of all the *cad1* mutants it was the least sensitive and expressed up to 30% of wildtype PC levels (Howden *et al.*, 1995). This suggests the conserved N-terminal domains possess the catalytic activity. However, the C-terminal domain must have some role in activity. Cobbett (2000) suggested that this domain may act as local sensor by binding heavy metal ions or, more probably, GSH-metal ion complexes via interaction with the multiple Cysteine residues thus bringing them into contact with the catalytic domain.

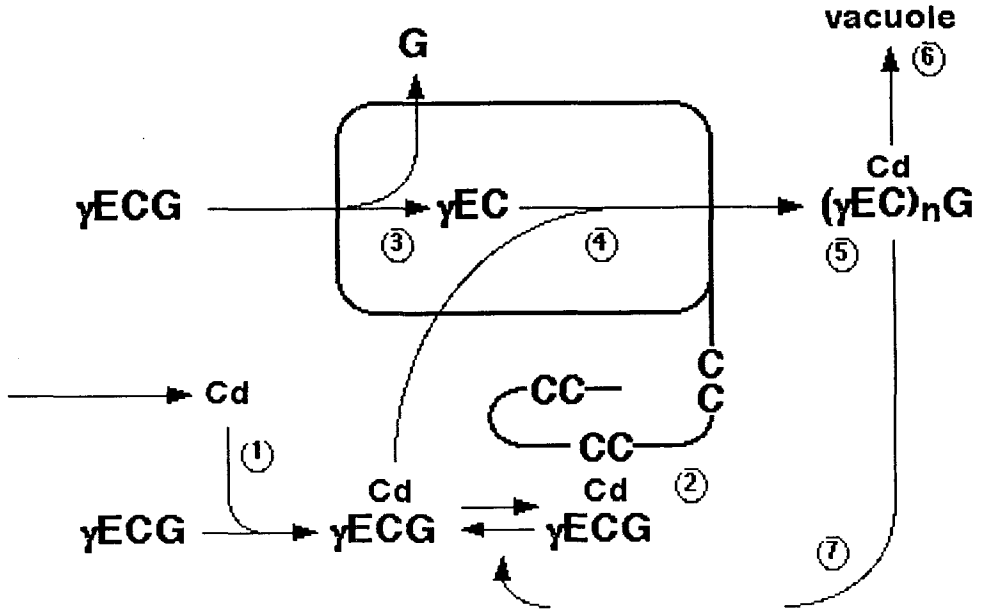

Figure 3. Proposed model of PC synthase action. A schematic model for phytochelatin synthase function (adapted from Vatamaniuk *et al.*, 2000). The box represents the conserved N-terminal region of the enzyme with a tail depicting the variable C-terminal containing multiple Cys (C) residues.

The PC-deficient mutants of Arabidopsis and *S. pombe* showed similar, but not identical, patterns of sensitivity to different heavy metals. PCs were essential for cadmium and arsenate detoxification but not zinc, nickel and selenite detoxification.

However, differences between the two organisms were observed with respect to copper, mercury and silver. This may indicate that some alternative mechanisms involved in the detoxification of these metals may be more or less effective in these two organisms. 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 enzymes *in vitro* and PC synthesis *in vivo* are efficiently activated by Cu. However, the *cad1-3* mutant is only slightly more sensitive to Cu than the wild type. This suggests that, 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. In the case of Cu this could include MTs.

There have been few previous studies of the tissue specificity of PC synthase expression in plants. In tomato, PC synthase activity was detected in roots and stems, but not in leaves or fruits (Chen *et al.*, 1997). A database search for expressed sequence tags (ESTs) corresponding to *AtPCS1* gives some indication of the spatial and temporal expression of *AtPCS1*. ESTs have been identified in libraries from a wide range of tissues suggesting broad expression of *AtPCS1* during development. *AtPCS1* expression was studied using RT-PCR and promoter-reporter gene expression constructs in transgenic plants. In this study, *AtPCS1* was expressed in both root and leaf tissue at approximately equal levels as measured by RT-PCR. This was supported by the promoter-*GUS* experiments. However, with the promoter-*GUS* lines, GUS activity in leaves appeared to be more intense than in roots. This may be an artifact of the staining protocol or may indicate that sequences controlling root expression were not included in the construct. In younger plants (7 to 21 days old) most tissues were stained for GUS activity. In older senescing leaves, however, activity appeared low and may, again, have reflected differences in promoter activity or GUS turnover. GUS activity was also detected in stems and floral tissues, particularly the carpels. This latter observation is in contrast to the apparent absence of PC synthase activity in tomato fruits. In addition, the RT-PCR experiment indicated *AtPCS1* expression was not responsive to Cd exposure. This too has been supported by other workers (Vatamaniuk *et al.*, 1999, 2000) and is consistent with reports of constitutive levels of PC synthase enzyme activity in plant tissues (Grill *et al.*, 1989, Klapheck *et al.*, 1995, Chen *et al.*, 1997). However, in contrast to the RT-PCR experiment, the promoter-*GUS* construct appeared to be induced after exposure to extreme levels of Cd which caused considerable growth inhibition. This is similar to the observation that *TaPCS1* expression was increased five- to tenfold in plant roots after exposure to 100 M Cd for 6 hours (Clemens *et al.*, 1999). It is possible that the apparent increase in *GUS* expression reflects differential turnover of the enzyme under highly stressed conditions and may not reflect expression of the endogenous gene. Overall, the promoter-*GUS* experiments must be considered with some caution. However, they do point to some further experiments measuring expression by RT-PCR or by tissue-in situ hybridisation to examine the questions of Cd induction of gene expression and the tissue specificity of

expression.

Database searches identified two *AtPCS* genes in Arabidopsis. The existence of *AtPCS2* was unexpected because PCs were not detected in the *cad1-3* mutant even after prolonged exposure to Cd. This might indicate that *AtPCS2* is a pseudogene which is no longer expressed, or is expressed in a highly tissue-specific manner or is only expressed in particular growth conditions. From the genomic and cDNA nucleotide sequences there was no indication that *AtPCS2* might be a pseudogene. The positions of introns of *AtPCS2* were identical to those of *AtPCS1*. However, the sizes and sequences of the introns varied between two genes. Predicted exons differed in size by at most a single codon, with the exception of an apparent 90 bp deletion within exon 8 of *AtPCS2*. This 90 bp deletion resulted in an in-frame deletion of part of the variable C-terminal domain of *AtPCS2*. This deletion is unlikely to abolish function, particularly since a comparison of the wheat and *AtPCS1* sequences shows a similar deletion (at a different position) which has no apparent effect on function. The deduced amino acid sequence for AtPCS2 was 84 % identical to AtPCS1. There were few substitutions of amino acids which appeared to be highly conserved across PC synthase genes from different species. None of these differences would suggest that the *AtPCS2* gene is non-functional. In particular, there were no apparent frame-shift or nonsense mutations which might be expected were the gene an evolutionary relic that was no longer functional.

AtPCS2 was transcribed at a low level compared to *AtPCS1* in all the tissues tested. Low amounts of RT-PCR products, amplified with *AtPCS2*-specific primers were detected in leaf, root, stem and inflorescence tissue. Nonetheless, the gene may be expressed at a greater level than *AtPCS1* in a highly tissue-specific manner. This may not be evident in the relatively undiscriminating tissue samples made, for example whole leaf and whole inflorescence. The low level of *AtPCS2* expression was not affected by Cd treatment (data not shown). However, it is possible the gene is only expressed under other particular environmental conditions.

AtPCS2 can express an active PC synthase enzyme. When *AtPCS2* was expressed in *S. cerevisiae* it conferred a level of Cd-resistance comparable to that obtained with *AtPCS1*. Measurements of PCs showed a greater level of PCs expressed from *AtPCS2* than from *AtPCS1*. Expression in the PC synthase-deficient mutant of *S. pombe* also complemented the Cd-sensitive phenotype as effectively as expression of *AtPCS1*. Furthermore, expression of *AtPCS2* in *cad1-3* mutants partially complemented Cd-sensitive phenotype. These transgenic plants should be analysed further to determine whether or not the partial complementation is due to low levels of transgene expression or reflects intrinsic differences in the activities of the two gene products. Nonetheless, together this data supports the conclusion that *AtPCS2* encodes an enzymatically active PC synthase.

*AtPCS2*promoter-*GUS* transgenic plants were generated to observe the *AtPCS2*

expression pattern. Two of 83 lines examined showed GUS activity at root tips. It is likely that these two lines do not reflect the endogenous pattern of *AtPCS2* expression but are artifacts arising from the insertion of the transgenes in those particular lines. The absence of GUS staining in any of the 10 lines examined more extensively is consistent with the extremely low level of endogenous expression observed using RT-PCR. However, this may also result from important regulatory sequences having been omitted from the construct; particularly since only about 650 bp of upstream sequence was used. New promoter constructs using more extensive sequences might be worthwhile.

The cloning and characterisation of PC synthase genes in this study is an important advance in this field and will contribute to a greater understanding of heavy metal detoxification mechanism in plants, and other organisms, in the future. This understanding may contribute to the development of phytoremediation processes.

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