Phytochelatin synthase (PCS) protein is induced in
Brassica juncea leaves after prolonged Cd exposure

Senta Heiss1,*, Andreas Wachter1,*, Jochen Bogs1, Christopher Cobbett2 and Thomas Rausch1,²

1 Heidelberger Institut für Pflanzenwissenschaften, INF 360, D-69120 Heidelberg, Germany
2 Department of Genetics, University of Melbourne, Victoria 3010, Australia

Received 19 February 2003; Accepted 2 May 2003

Abstract
Higher plants respond to cadmium exposure with the production of phytochelatins (PCn), small heavy metal binding peptides, which are synthesized from glutathione by phytochelatin synthase (PCS). The isolation of a PCS cDNA clone from Brassica juncea L. cv. Vitasso, a candidate species for phytoremediation, is reported here. CLUSTAL analysis revealed a close relationship of BjPCS1 with PCS proteins from Arabidopsis thaliana and Thlaspi caerulescens. BjPCS1 expressed as recombinant protein in E. coli had PCS activity in vitro that was activated by 50 μM Cu and 200 μM Cd to a similar extent. Immunoblot analysis with an antiserum directed against recombinant BjPCS1 showed constitutive PCS expression during plant development. As a percentage of the total protein, the expression was higher in the roots, internodes and petioles in comparison with the leaf tissue. When B. juncea plants were treated with 25 μM cadmium, PCn accumulated increasingly over a 6 d period. Levels in shoots were about 3-fold higher than in roots. Prolonged cadmium exposure caused a significant increase of PCS protein in leaves, whereas in roots PCS protein levels were not affected.

Key words: Brassica juncea, cadmium, heavy metal, phytochelatin synthase.

Introduction
Cadmium ions are toxic to all organisms, however, the mechanisms of Cd toxicity are only partially understood. It has been proposed that Cd can lead to oxidative stress by displacement of essential heavy metal ions in reaction centres of proteins, resulting in the loss of their biological function and the release of free ions (Stadtman and Oliver, 1991; Hall, 2002). In plants, Cd exposure may lead to growth inhibition, root damage, chlorosis, and it may affect transpiration (Das et al., 1998; Haag-Kerwer et al., 1999). Plants have developed defence systems that ensure efficient Cd detoxification by complexation and vacuolar sequestration. In a similar way as some fungal species, plants detoxify Cd by chelation with phytochelatins (PCn) (Grill et al., 1985; Cobbett, 2000). The importance of PCn for Cd detoxification is corroborated by studies on PC-deficient Arabidopsis cad1 mutants which lack PC synthase (PCS) activity and are Cd-hypersensitive (Howden et al., 1995). More recently, it was shown that PCn formation is not limited to plants and fungi. The nematode, Caenorhabditis elegans, in particular, has a functional PCS which contributes to Cd tolerance (Clemens et al., 2001; Vatamaniuk et al., 2001).

PCn are synthesized enzymatically by PCS by the transfer of γ-Glu-Cys moieties to glutathione (GSH) or (γ-Glu-Cys)n-Gly resulting in (γ-Glu-Cys)n+1Gly peptides with n=2–10. In a second detoxification step, the PC-heavy metal complex is transported to the vacuole. An ABC-transporter, Hmt1, accepting low-molecular weight PC-heavy metal complexes as substrate, has been identified in Schizosaccharomyces pombe (Ortiz et al., 1992, 1995), and a MgATP-dependent transport activity for PC3 and PC3–Cd complexes has also been demonstrated in plants (Salt and Raiser, 1995).

PCS genes were first isolated from Arabidopsis thaliana, wheat and the yeast, S. pombe. In vitro studies with purified PCS proteins confirmed earlier biochemical data that the enzyme is activated in vitro by Cd and other heavy metal ions (Grill et al., 1989; Ha et al., 1999; Clemens...
et al., 1999; Vatamaniuk et al., 1999). In wheat, the transcription of TaPCS1 is upregulated in response to Cd exposure while, by contrast, in Arabidopsis, AtPCS1 is constitutively expressed at the level of transcription (Ha et al., 1999; Clemens et al., 1999; Vatamaniuk et al., 2000). In a recent and more detailed study Lee et al. (2002) analysed AtPCS1-promoter GUS-fusions as well as fusions of the AtPCS1-promoter with either AtPCS1-cDNA or -genomic DNA; interestingly, Cd induced an intron-mediated increase of AtPCS1 expression.

Brassica juncea is a heavy metal-accumulator plant with a high biomass, making it a good candidate for application in phytoremediation strategies (Salt et al., 1995, 1998; Pilon-Smits and Pilon, 2002; Clemens et al., 2002). In this species, heavy metal accumulation and concomitant PCn formation have been thoroughly studied (Speiser et al., 1992; Schafer et al., 2002; Clemens et al., 1992; Schaëber et al., 1998). In this study, we present the isolation of a cDNA clone encoding PCS from B. juncea, which is upregulated in response to Cd ions. Furthermore, an antiserum was generated to study the nontarget protein was detected in the presence of Cu and Cd ions. Furthermore, an antiserum was generated to study the nontarget protein was detected in the presence of Cu and Cd ions.

**Materials and methods**

**Plant material and cadmium analysis**

*B. juncea* L. (cv. Vitasso) plants were germinated and grown as previously described (Heiss et al., 1999). Six-week-old plants on hydroponic culture were treated with 25 mM Cd, and the roots and leaves (the third and fourth leaf from the top) were harvested at the time points indicated. After the onset of Cd-stress, the nutrient solution was changed daily. Tissue samples for the expression analysis of PCS protein were taken from 1-week-old seedlings and 5-week-old *B. juncea* plants, combining the first, second and third leaf (young, growing leaves), the fourth and fifth leaf (middle, fully expanded leaves) and the sixth to ninth leaf from the top (old, senescing leaves).

**Screening and sequence analysis**

A cDNA root library (Schafer et al., 1998) was screened using a *B. juncea* PCS synthase cDNA fragment obtained by 5′ RACE-PCR. A biotinylated probe was synthesized with the following primers: sense 5′-TGGCTATGGCATGCTCTACCGG-3′, antisense 5′-ACGAATCAAACGTGCAGGCGG-3′. After sequence confirmation, the expression construct was transformed into the *E. coli* M15 [pREP4] strain. Expression was induced at an OD600 of ~0.6 with 1 mM IPTG, and bacteria were grown for 4 h more. Cells were lysed using an 8 M urea buffer and the recombinant protein was purified on a Ni-Agarose column (Qiagen) under denaturing conditions following the manufacturer’s instructions. For the best recovery, 20 mM 2-mercaptoethanol was added to wash and elution buffers.

**Generation of polyclonal antiserum**

Recombinant BjPCS1 protein was prepared as described above and a polyclonal antiserum was raised by injecting the protein into a rabbit. Pre-immune serum and antiserum were tested at 1:8.000 and 1:15.000 dilutions using western blots of recombinant protein and plant extracts. No non-specific bands were observed and the pre-immune serum showed no cross reactivity with recombinant PCS protein.

**Protein extraction and immunoblot analysis**

For extraction of total soluble protein, 100 mg of frozen plant material (−80 °C) was ground in liquid nitrogen, vortexed with 300 μl extraction buffer (100 mM HEPES pH 7.1, 250 mM sorbitol, 10 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, and 1 mM PMSF), and centrifuged for 20 min at 15 000 g at 4 °C. The protein concentration of the supernatant was measured using the Bradford assay. 10–20 μg total protein were loaded onto a 9% SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane (Immobilon, Millipore) at 3.5 mA cm⁻² and 15 V for 45 min. Protein extraction and immunoblot analysis were performed with the Super Signal® West Dura Detection Kit (Pierce, Rockford, USA), according to the manufacturer’s protocol.

**Phytochelatin synthase enzyme assay**

Expression of BjPCS1 from the pQE30 vector in M15[pREP4] E. coli cells was induced with 1 mM IPTG. After 4 h, cells were resuspended in the lysis buffer (50 mM TRIS-HCl pH 8.1, 1 mM DTT, 14% glycerol) and lysed by freeze/thawing and subsequent sonication for 5 min. The extract was desalted on a Sephadex G25 column, equilibrated with protein extraction buffer (see above). The assay contained 20 μg protein, 200 mM TRIS-HCl pH 8, 2 mM CaCl₂, 1 mM GSH, and 200 μM Cd(NO₃)₂ or 50 μM CuSO₄, respectively, in a total volume of 100 μl. After 5 min pre-incubation at 35 °C, the reaction was started by adding the protein extract. After 0–20 min the reaction was stopped by adding 10 μl 10% trifluoroacetic acid. Phytochelatins were analysed by HPLC.
Fig. 1. Sequence alignment and phylogenetic tree of PCS protein sequences. (A) ClustalW alignment of the conserved N-terminal domains of PCS sequences: BjPCS1 (Brassica juncea, accession number CAC37692), AtPCS1 (Arabidopsis thaliana, accession number AAL78384), AtPCS2 (A. thaliana, accession number AAK94671), TcPCS (Thlaspi caerulescens, accession number BAB93120), TaPCS1 (Triticum aestivum, accession number AAD50592), TlPCS1 (Typha latifolia, accession number AAG22095), AyPCS1 (Athyrium yokoscense, accession number BAB64932), GmPCS (Glycine max, accession number AAL78384), CePCS1 (Caenorhabditis elegans, accession number AAK62992), and SpPCS1 (Schizosaccharomyces pombe, accession number Q10075). (B) Phylogenetic analysis of the PCS protein sequences was performed with the splits decomposition method (Bandelt and Dress, 1992), using distance data calculated by the algorithm of Kimura (Kimura, 1983). Splittable percentage was 0.902.
Results and discussion

Cloning and sequence analysis of BjPCS1
Screening of a B. juncea root cDNA library with a partial B. juncea PCS clone as a probe resulted in the isolation of two related clones, which exhibited 99% homology to the PCS probe. However, only one of them, named BjPCS1, was a full-length clone, encoding a 485 amino acid polypeptide with a predicted molecular mass of 54 kDa. At the amino acid level, BjPCS1 displays an approximately 90% sequence identity with PCS from A. thaliana (AtPCS1) and Thlaspi caerulescens (TcPCS). The plant PCS protein sequences from A. thaliana (AtPCS2), Glycine max (GmPCS), Triticum aestivum (TaPCS1), Typha latifolia (TIPCS1), and Athyrium yokoscense (AyPCS1) shared 77%, 65%, 59%, 55%, and 50% sequence identity, respectively, with BjPCS1. A significantly lower similarity (about 30% sequence identity) was found for CePCS1 from Caenorhabditis elegans and SpPCS1 from Schizosaccharomyces pombe. A partial protein sequence alignment, showing the N-terminal domain, and a phylogenetic tree are presented in Fig. 1. A comparison of the complete protein sequences confirmed the earlier observation that sequence conservation in the putative catalytic N-terminal domain is much higher than in the more variable C-terminal domain (Cobbett, 1999; data not shown); for example, all analysed sequences displayed an N-terminal conserved motif with the consensus sequence [Q-T-G-x-G-H-F-S-P-(11)-L-I-[LM]-D-V-A-R-E-K-Y-P-[PC]-[HY]-W-x-(2)-L]. Cysteine residues are conserved between BjPCS1 and AtPCS1, except for Cys-352, which is not present in the AtPCS1 sequence.

Expression of enzymatically active BjPCS1 in E. coli
To demonstrate the heavy metal-activation of the BjPCS1-encoded enzyme, it was expressed in E. coli. The entire coding sequence was cloned into the pQE30 vector, tagging the protein with six histidine residues at the N-terminus. The Ni-affinity-purified recombinant BjPCS1 protein had an apparent $M_r$ of 59 kDa. PCS enzyme activity was determined in the presence of Cd(NO$_3$)$_2$ or CuSO$_4$, using desalted extracts of E. coli-expressed BjPCS1 protein. Extracts from E. coli cells harbouring the empty pQE30 vector served as a control. No PCS activity could be detected in the latter (not shown) and, similarly, the extract containing recombinant BjPCS1 protein showed no activity in the absence of heavy metals (Table 1). Only after the addition of 200 $\mu$M Cd(NO$_3$)$_2$ or 50 $\mu$M CuSO$_4$ were PCn synthesized. It is noteworthy, that cadmium and copper induced the formation of a different PC product spectrum. While the addition of copper favoured the synthesis of PC$_2$, cadmium predominantly induced PC$_3$ and PC$_4$ synthesis (data not shown). A similar result was reported earlier for PCS activity from Rauwolfia serpentina cell cultures (Grill et al., 1987), indicating that different heavy metal ions affect the affinity of the PCS to different substrates (GSH, PCn), possibly by inducing the slightly different conformational changes.

Tissue-specific expression of PCS protein

Attempts to detect BjPCS1 mRNA in total RNA samples by northern blot analysis were not successful (transcripts could only be detected by RT-PCR; see below). Therefore, expression analysis concentrated on the immunological detection of PCS protein. For immunoblot analysis, the recombinant BjPCS1 protein (see above) was used to generate a polyclonal antiserum. This antiserum was able to detect 10 ng of the recombinant BjPCS1 protein at a dilution of 1:15000. In B. juncea extracts the antiserum detects a single specific band in leaves and roots. Whereas 10 $\mu$g total root protein resulted in a strong band, the detection limit in leaves was much higher, with 20 $\mu$g protein producing only a faint band. The detected protein migrates at a position corresponding to 59 kDa, which is slightly higher than the expected size of 54 kDa. The reason for this discrepancy is not known.

To assess PCS protein expression in different tissues, extracts from total seedlings and different parts of adult plants (roots, internodes, petioles, different leaf stages)
were analysed by immunoblot. Figure 2 shows that PCS protein could be detected in all the tissues of the control plants investigated, confirming the expression of PCS in the absence of Cd. High levels of PCS protein were found in internodes and roots, but the highest protein amounts were detected in petioles. In leaves, PCS amounts were significantly lower and declined further as leaves aged. These results suggest a high expression of PCS protein in vascular tissues.

It has to be noted that the protein expression data (Fig. 2) are based on equal protein amounts and, therefore, reflect PCS abundance in comparison with total protein. As leaf *B. juncea* tissues have a 3–4-fold higher protein content g⁻¹ fresh weight (data not shown), expressing the data as PCS protein g⁻¹ fresh weight would result in a relative increase in leaf samples as compared to root samples.

Although protein amounts do not necessarily correlate with transcript amounts, the data presented here are in agreement with RT-PCR analysis of PCS mRNA in adult *A. thaliana* plants; higher *AtPCS1* transcript amounts were detected in the root than in leaves (Ha et al., 1999). Lee et al. (2002) have characterized the expression of *AtPCS1* during plant development using promoter-GUS fusions. GUS activity was constitutively expressed in leaves, roots, cotyledons, and stems, but not in root tips or root hairs. A particularly strong expression was observed in trichomes. During flowering, GUS expression was absent in petals and stamen, but present in sepals, carpels and during silique development. Recently, a second functional PCS gene from *A. thaliana*, *AtPCS2*, has been characterized (Cazale and Clemens, 2001), which shows low but constitutive expression, however, this gene cannot apparently compensate for the loss of *AtPCS1* function in *cad1* mutant (Howden et al., 1995).

**Phytochelatins and PCS protein expression in Cd-treated B. juncea**

Heavy-metal-treated *B. juncea* plants and seedlings respond by synthesizing high amounts of PCn in leaves and roots (Salt et al., 1995; Schäfer et al., 1998). In a time-course study with 6-week-old Cd-treated *B. juncea* plants, a linear increase in PCn amounts up to 1322 nmol GSH equivalents g⁻¹ fresh weight can be observed in the root after 6 d (Fig. 3). Despite this substantial PC synthesis, PCS protein levels remained unchanged in roots during the entire experiment. In leaves, phytochelatins rose to 3830 nmol GSH equivalents g⁻¹ FW after 120 h Cd-treatment, with the largest increase between 72 h and 96 h. PCS protein levels remained constant for 72 h, but after 96 h...
exposure to Cd, PCS protein levels increased significantly compared with control plants. Protein determinations indicated, that during the entire Cd exposure period, total protein contents in leaves were not affected. During the course of the experiment, plants also showed no visible Cd toxicity symptoms. To quantify the increase in PCS protein expression, a dilution series of leaf samples from control and Cd-treated plants (120 h) was subjected to immunoblot analysis (Fig. 4). The comparison indicates approximately a 4-fold increase of PCS protein in response to Cd treatment.

This is thought to be the first report of a heavy metal-induced increase of endogenous PCS protein in plants. The mechanism of PCS induction in B. juncea is not yet known. Lee et al. (2002) presented evidence for an intron-mediated increase of AtPCS1 mRNA after Cd exposure. AtPCS1-promoter fusions with the genomic AtPCS1 sequence, but not with the AtPCS1-cDNA sequence, showed an increased AtPCS1 mRNA accumulation after Cd exposure.

Unexpectedly, in B. juncea the increase of PCS protein observed in leaves after prolonged Cd-treatment could not be related to an increase in BjPCS1 mRNA, however, quantification of the BjPCS1 transcript level relative to the internal control β-actin2 proved to be difficult because of the altered expression of the reference gene after Cd-treatment extending beyond 96 h (Fig. 5). As the primers used for RT-PCR corresponded to regions highly conserved between BjPCS1, AtPCS1 and AtPCS2, transcripts from other BjPCS genes would probably have been co-amplified. Therefore, it is assumed that the increase in BjPCS protein is due to post-transcriptional regulation.

The kinetics of this Cd exposure experiment suggest that the observed increase in PCS protein between 72 h and 96 h after onset of Cd exposure is responsible for the concomitant strong increase of PCn. It appears that, in leaves, the constitutive PCS enzyme activity may become a limiting factor after prolonged Cd exposure. It was concluded that, despite constitutive expression of PCS protein during most stages of plant development, B. juncea may react to prolonged Cd exposure with an increase of PCS protein in leaves.

Acknowledgements

SH acknowledges scholarships from the Österreichische Akademie der Wissenschaften and from the Graduiertenkolleg Bioteknologie (GK388, DFG). This work was supported by a DFG fund (FOR383) to TR. CC is supported by a grant from the Australian Research Council.

References


Cadmium-sensitive, cad1, mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiology* 107, 1059–1066.


