Arabidopsis thaliana expresses a second functional phytochelatin synthase

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Abstract Phytochelatins represent a major detoxifying pathway for heavy metals in plants and many other organisms. The *Arabidopsis thaliana CAD1* (= *AtPCS1*) gene encodes a phytochelatin synthase and *cad1* mutants are phytochelatin deficient and cadmium hypersensitive. The *Arabidopsis* genome contains a highly homologous gene, *AtPCS2*, of which expression and function were studied in order to understand the apparent non-redundancy of the two genes. Low constitutive *AtPCS2* expression is detected in all plant organs analyzed. The *AtPCS2* gene encodes a functional phytochelatin synthase as shown by expression in *Saccharomyces cerevisiae* and the complementation of a *Schizosaccharomyces pombe* phytochelatin synthase knockout strain. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Heavy metal tolerance; Metal chelator; Metal homeostasis

1. Introduction

The potential toxicity of heavy metal ions requires tight control of their cytosolic concentrations. Chelation and subsequent sequestration of metal–ligand complexes represent one of the general mechanisms of metal homeostasis and tolerance. The formation of phytochelatins (PCs), small metal binding peptides of the general structure $(\gamma\text{-Glu-Cys})_n$-Gly ($n = 2$–11) [1], is a well-established response to toxic metal exposure of plants, fungi, marine diatoms and, more recently, *Caenorhabditis elegans* [2,3]. PCs are synthesized non-translationally from glutathione in a transpeptidation reaction by the enzyme phytochelatin synthase (PCS) [4] that is activated by the binding of glutathione–cadmium (Cd) chelate [5]. Cd–PC complexes are translocated to the vacuole, where high molecular weight complexes are formed under incorporation of sulfide [6]. In *Schizosaccharomyces pombe*, the respective transport is mediated by Hmt1, an ABC-type transporter localized in the vacuolar membrane [7,8]. A corresponding transporter in plants has not yet been identified. The contribution of PC synthesis to plant metal tolerance became evident with the isolation of the Cd hypersensitive *Arabidopsis* mutant *cad1* [9], the phenotype of which was attributable to the deficiency in PC synthesis [10]. The *CAD1* (= *AtPCS1*) gene and other plant and fungal genes encoding *PCS* have recently been cloned by positional cloning [11] and screening for plant cDNAs mediating Cd tolerance in *Saccharomyces cerevisiae* [12,13]. *CAD1* restores PC synthesis in *cad1* plants and confers PCS activity when expressed in *Escherichia coli*. Expression of *AtPCS1* from *Arabidopsis*, *SpPCS* from *S. pombe* or *TaPCS1* from wheat in *S. cerevisiae* cells results in metal inducible PC formation. Furthermore, purified recombinant AtPCS1 and SpPCS catalyze the formation of PCs from glutathione. The analysis of a *S. pombe PCS* knockout strain, and of *S. cerevisiae* cells expressing *PCS* genes from different sources, showed that PC formation confers Cd tolerance and, to a more limited degree, copper, arsenate and mercury tolerance. *AtPCS1* and *TaPCS1* transcripts can be detected in roots and shoots of *Arabidopsis* and wheat plants, respectively.

Considering the fact that *cad1* plants are Cd hypersensitive and PC deficient, it was surprising to find a second *PCS* homolog (*AtPCS2*) in the *Arabidopsis* genome (chromosome 1, GenBank accession number AC003027, gene F21M11.9 [11,13]). In order to understand this apparent non-redundancy of function, we initiated a study on this gene. Here we report on the expression analysis and functional characterization of *AtPCS2*.

2. Material and methods

2.1. Yeast cultures, transformation and growth assays

The *S. cerevisiae* strain INVSc1 (*MATα his3Δ1 leu2 trp1-289 ura3-62*), the *S. pombe* strain FY254 (*h⁻ ade6-M210 leu1-32 ura4-Δ18 can1-1*) and the corresponding *SpPCS* knockout strain [12] were used in this study. *S. cerevisiae* cells were grown at 30°C in yeast nitrogen base (YNB) supplemented with the appropriate amino acids. The pGAL1 promoter was induced with 1% galactose and 1% sucrose or repressed using 2% glucose. *S. pombe* cells were grown at 30°C in Edinburgh's minimal medium (EMM) supplemented appropriately. Transformation was performed as described [14,15]. To assay for metal sensitivity, cells grown to log phase were diluted to an OD_{590} of 0.1 (*S. pombe*) or 0.05 (*S. cerevisiae*) and incubated in the presence of various metal concentrations. Cell density was measured after 18–24 h.

2.2. Plant material and treatments

Arabidopsis thaliana ecotype Columbia wild-type and *cad1-3* mutants [10] were grown in hydroponic conditions in 1/10 Hoagland medium (Sigma H2395) with 8 h light and 16 h dark. Roots and shoots were collected from 6 week old *Arabidopsis* plants. The plants were harvested 3 or 24 h after addition of 10 μM $CdCl_2$ in the hydroponic medium. Stems, leaves and flowers were collected from flowering plants grown on soil in the greenhouse.

2.3. DNA manipulations

E. coli strain DH5α was used for all DNA manipulations. Genes were expressed in *S. cerevisiae* using the inducible expression vector pYES2 (Invitrogen, Carlsbad, CA, USA). DNA sequencing was performed on a LI-COR 4200 sequencer. PCR analysis followed established procedures [16]. Homologous sequences were identified within

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Abbreviations: PC, phytochelatin; PCS, phytochelatin synthase

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the DDBJ/EMBL/GenBank database using BLAST [17]. Sequence analyses were performed using the DNASTAR package (DNASTAR, Madison, WI, USA).

2.4. RT-PCR analysis and full-length cloning of AtPCS2

Total RNA was extracted using the Trizol reagent protocol (Life Technologies, Gaithersburg, MD, USA). The cDNA was synthesized from 1.5 μg of total RNA that was treated by DNase I before reverse transcription with the Superscript II reverse transcriptase (Life Technologies). For RT-PCR analysis, PCR reactions using equal amounts of cDNA samples were performed using *AtPCS1* specific primers (5′-GTCTGTTTGGCTCATTGTTCAGG-3′ and 5′-GATCCAGCTTTCATCCTTGCAG-3′, 35 cycles), *AtPCS2* specific primers (5′-AAGGATGAGAGCTGGATCAGC-3′ and 5′-CTACCACTGTCACTTTCTCCTC-3′, 40 cycles) or β-tubulin specific primers (5′-GTCCAGTGTCTGTGATATTGCACC-3′ and 5′-GCTTACGAATCCGAGGGTGCC-3′, 22 cycles). The samples were analyzed after separation on agarose gel and transfer to nylon membranes. Membranes were hybridized with ^{32}P radiolabeled *AtPCS2* or β-tubulin probes (Megaprime kit, Amersham Pharmacia Biotech, Freiburg, Germany) and quantified with a phosphorimager system (Molecular Dynamics, Sunnyvale, CA, USA). For semi-quantitative RT-PCR reactions were performed incorporating [^{33}P]dATP. Specificity of the *AtPCS2* primers was tested using plasmid DNA as template. No amplification was seen using *AtPCS1* as a template for the *AtPCS2* primers. Also, the fragment was cloned, sequenced and found to match the expected gene sequence.

To isolate a full-length cDNA clone of *AtPCS2*, two different PCR reactions were performed with *AtPCS2* specific primers and the *Pfu* polymerase (Promega, Madison, WI, USA). The 5′ cDNA end was amplified using the primers 5′-ATGTCTATGGCGAGTTTGTATCGGCGG-3′ and 5′-CTACCACTGTCACTTTCTCCTC-3′ and the 3′ cDNA end with 5′-TCCCGGATGGGTAAGCTACCAC-3′ and 5′-CTCGAGGGTTGGTTGTGTTTGATTAGG-3′. Both fragments were cloned in pGEM-T (Promega) and sequenced. A full-length cDNA was constructed by ligation of the two partial cDNAs after digestion with *Bpu*10I and *Pae*I. For functional expression in yeast, the full-length cDNA was cloned under the pGAL1 promoter in the pYES2 vector in the *Sac*I-*Pae*I restriction sites.

2.5. Measurement of PCs

S. pombe cultures were diluted to an OD_{590} of 0.1, grown for 3 h and treated with 10 μM $CdCl_2$, 200 μM KH_2AsO_4, 10 μM $CuCl_2$, 100 μM $NiCl_2$, 4 mM $MnCl_2$ or 2 mM $ZnCl_2$. The cells were harvested after 24 h of growth and lyophilized. Hydroponically grown *Arabidopsis* Col-O wild-type and *cad1-3* plants were treated with 10 μM $CdCl_2$ for 24 h. The PCs in trifluoroacetic acid extracts were assayed fluorimetrically by HPLC after monobromobimane derivatization [12,18]. Standards were used for the identification of glutathione and PCs $(\gamma\text{-EC})_2$G (=PC2), $(\gamma\text{-EC})_3$G (=PC3). The detection limit of the HPLC assay was 5 pmol SH. ESI-Q-TOF-MS analysis was performed as described [3].

2.6. Expression of HA-tagged AtPCS2

AtPCS2 was subcloned into pSGP72 (kindly provided by Dr. Susan Forsburg, Salk Institute, La Jolla, CA, USA) to express AtPCS2 protein with a C-terminal triple HA tag in the *S. pombe SpPCS* knockout strain. Protein fractions were resolved by SDS–PAGE and transferred to nitrocellulose membranes using standard procedures [16]. HA-tagged AtPCS2 was detected with an HA monoclonal antibody (BAbCo, Berkeley, CA, USA).

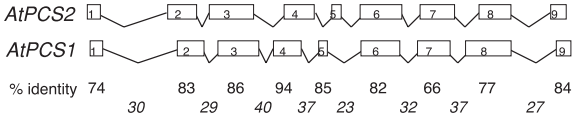

Fig. 1. Comparison of *AtPCS2* and *AtPCS1* gene structures. *AtPCS2* and *AtPCS1* cDNA lengths are 1.36 and 1.46 kb, respectively, and are drawn to scale. Exons are indicated as boxed numbers and introns as broken lines. The percentage (%) of identity of exons (plain) and introns (italic) was determined with the DNASTAR package.

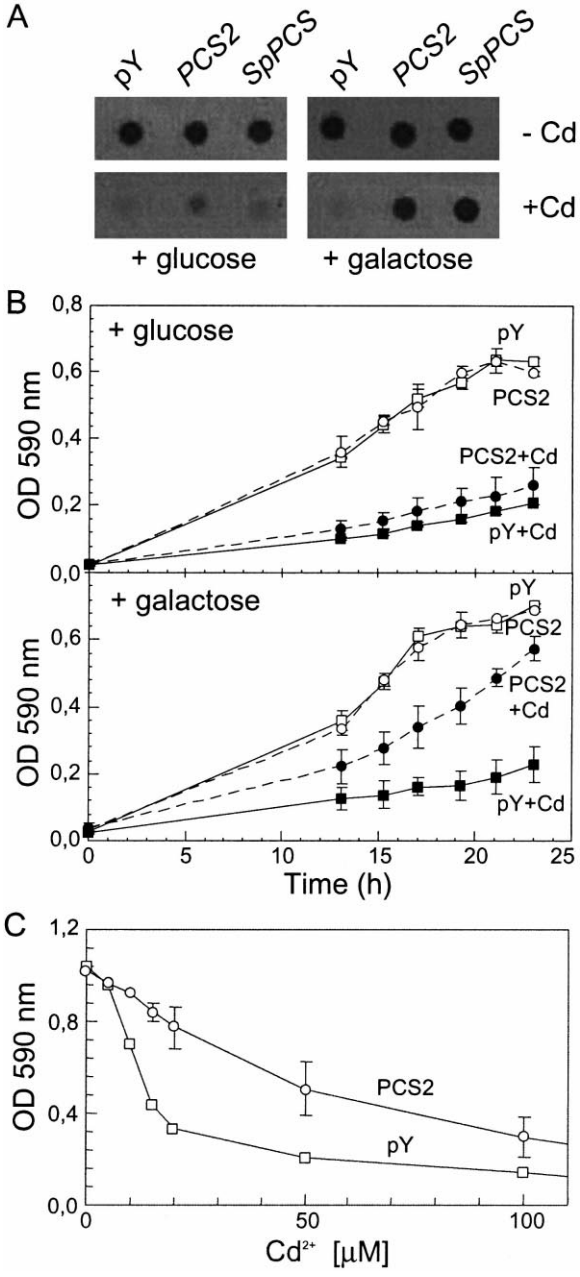

Fig. 2. Cd^{2+} tolerance of *S. cerevisiae* cells expressing *AtPCS2*. A: Yeast cells carrying the empty pYES2 vector (pY) and yeast cells expressing either *AtPCS2* (PCS2) or *SpPCS* (SpPCS) were grown on YNB−ura plates without (−Cd) or with 100 μM $CdCl_2$ (+Cd) under inducing (1% galactose/1% sucrose) and repressing (2% glucose) conditions. B: Growth of pY and PCS2 cells in liquid YNB−ura without or with 20 μM $CdCl_2$ (+Cd) in the presence of glucose or galactose. C: Growth of pY and PCS2 cells in liquid YNB−ura with galactose and varying concentrations of $CdCl_2$. Error bars represent S.D. between three different strains.

3. Results

3.1. Search for trace amounts of PCs in cad1-3 mutant plants

No PCs could be detected in the *cad1-3* mutant plants by HPLC following post-column derivatization of extracts with Ellman's reagent [10]. It was recently shown that fluorescence labeling of thiols by monobromobimane allows for a more sensitive detection of PCs [18]. In order to confirm the PC

deficiency of the *cad1-3* mutant, hydroponically grown plants were challenged with Cd^{2+} and the extracts subjected to HPLC analysis after monobromobimane derivatization as well as ESI-Q-TOF-MS analysis. In both cases, no trace of PC accumulation was detectable whereas wild-type control plants showed PC accumulation of about 4.6 μmol/g d.w. (data not shown). These data confirmed the apparent non-redundancy of the two *PCS* genes. Thus, *AtPCS2* expression and function were investigated.

3.2. AtPCS2 is expressed

The *AtPCS2* gene exhibits an exon–intron structure very similar to *AtPCS1* and the predicted coding sequence is 84% identical to *AtPCS1* (Fig. 1). The intron sequences are diverged and *AtPCS2* does not appear to be the result of a recent duplication event in the *Arabidopsis* genome [19], indicating that the two genes have co-existed for a long time. However, no expressed sequence tag corresponding to *AtPCS2* and hence no evidence for *AtPCS2* expression could be found in databases. Specific primers were designed based on the predicted coding sequence of *AtPCS2* to analyze possible expression by RT-PCR. A PCR fragment was obtained which matched the expected *AtPCS2* cDNA fragment, demonstrating expression of the gene. A full-length cDNA was cloned, sequenced and found to correspond to the predicted coding sequence (GenBank accession number AY044049).

3.3. Functional characterization of AtPCS2 in yeast

The *AtPCS2* full-length cDNA was expressed in *S. cerevisiae* under control of the inducible pGAL1 promoter to test for its PCS activity. Growth assays showed that *AtPCS2* expression, like *SpPCS* expression, supported growth of *S. cerevisiae* cells in the presence of Cd^{2+} concentrations that almost completely inhibited growth of cells transformed with the empty pYES2 plasmid (Fig. 2A,B). This effect was dependent on strong *AtPCS2* expression in galactose medium. Under repressing conditions (glucose medium), no difference was detectable between the strains carrying either the empty vector, *AtPCS2* or *SpPCS*. In liquid culture, *AtPCS2* expression resulted in a 3.6-fold increase of IC_{50} values for Cd^{2+} tolerance (14–50 μM; Fig. 2C). *AtPCS2* was also expressed in a *S. pombe SpPCS* knockout strain. When *SpPCS* is disrupted, the tolerance of *S. pombe* to Cd^{2+} decreases dramatically (Fig. 3, [11,12]). The expression of *AtPCS2* restored the Cd^{2+} tolerance of the *SpPCS* knockout (Fig. 3).

The ability of PCS proteins to bind Cd^{2+} and Cd–glutathione complexes with high affinity [5,13] could potentially lead to an increase in Cd^{2+} tolerance in cells highly overexpressing *AtPCS2* even in the absence of any catalytic activity. Therefore, extracts from control and *AtPCS2* expressing *SpPCS* knockout cells treated with Cd^{2+} for 24 h were analyzed for thiols by HPLC. PCs (PC2 and PC3) could be detected in *AtPCS2* expressing *pcs*$^-$ cells but not in cells carrying the empty plasmid or in untreated cells (Fig. 4). Similarly, *S. cerevisiae* cells expressing *AtPCS2* synthesized PC2 and PC3 upon Cd^{2+} exposure (data not shown). Thus, the *AtPCS2* gene encodes a functional PCS and this enzyme is able to synthesize PCs in the presence of Cd^{2+}.

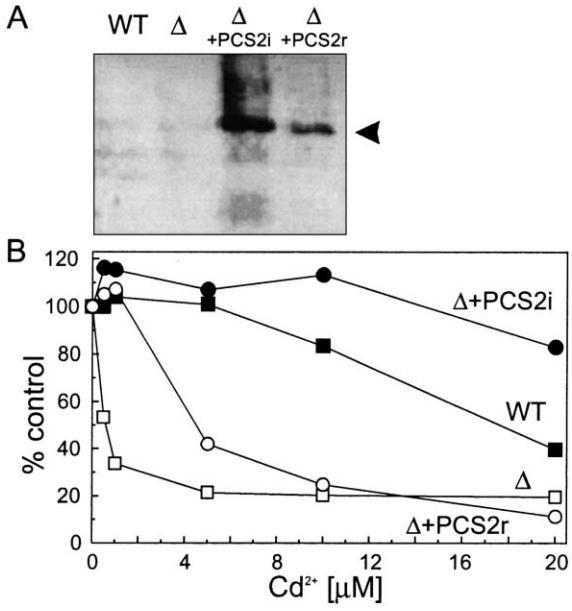

Fig. 3. Expression of *AtPCS2* restores Cd tolerance to the *S. pombe PCS* knockout strain. A: Western blot analysis of HA-tagged *AtPCS2* expression. B: Yeast wild-type strains (WT), *SpPCS* knockout strains (Δ), and *AtPCS2* expressing *SpPCS* knockout strains with (Δ+PCS2r) or without thiamine (Δ+PCS2i), which represses the *nmt1* promoter, were grown in liquid EMM−ura−leu with varying concentrations of $CdCl_2$.

Table 1
Activation of PC synthesis by different metal ions

Metal ion	*S. pombe* strain[a]		
	Wild-type	*AtPCS1*	*AtPCS2*
Cd^{2+}	100[b,c]	118.3	83.3
Cu^{2+}	15.1	9.8	n.d.
AsO_4^{3-}	43.0	39.8	6.2
Zn^{2+}	n.d.[d]	n.d.	n.d.
Ni^{2+}	n.d.	n.d.	n.d.
Mn^{2+}	n.d.	n.d.	n.d.

[a]*S. pombe* wild-type and *pcs*$^-$ cells expressing either *AtPCS1* or *AtPCS2* were treated with different metal ions.
[b]The amount of PCs was measured after 24 h.
[c]Amount of PCs in wild-type cells following Cd^{2+} exposure = 100% (2.8 μmol/g d.w.).
[d]Not detectable.

3.4. Specificity of AtPCS2 activation by metals

One of the characteristic biochemical properties of PCSs is the activation by a variety of metals and metalloids. In order to compare AtPCS1 and AtPCS2 in this respect, we challenged *S. cerevisiae* and *S. pombe PCS* knockout cells expressing either *AtPCS1* or *AtPCS2* with a range of different metal salts and subsequently analyzed PC formation. Both genes conferred the capacity to synthesize PCs in the presence of Cd^{2+}, the most stimulating ion. In *S. pombe* cells, the degree of both AtPCS1 and AtPCS2 dependent PC formation upon Cd^{2+} challenge was comparable to that in wild-type cells (Table 1). Similarly, AsO_4^{3-} treatment elicited PC formation in all three strains whereas exposure to Zn^{2+}, Mn^{2+} and Ni^{2+} did not result in any detectable accumulation of PCs. A notable difference was observed for Cu^{2+} ions, which activated *S. pombe* PCS and AtPCS1 but not AtPCS2. Qualitatively, the same results were obtained for *S. cerevisiae* cells with respect to Cd^{2+} and Cu^{2+} (data not shown). Again, only *AtPCS1* expressing cells synthesized PCs following Cu^{2+} exposure. In addition, Zn^{2+}, Ni^{2+} and Sb^{3+} activation of PC synthesis was only observable in *S. cerevisiae* cells expressing *AtPCS1*.

3.5. *AtPCS2* expression in Arabidopsis

Given the demonstrated functionality of the AtPCS2 protein, an explanation for the *cad1-3* phenotype might be a very localized or tightly controlled expression of *AtPCS2*. Therefore, tissue specificity and regulation of *AtPCS2* were investigated. The expression of *AtPCS2* could be detected by RT-PCR analysis in different parts of the plant, with higher levels in roots than in shoots (Fig. 5A). *AtPCS1* transcripts were also present in these organs (Fig. 5A). *AtPCS2* expression in leaves and total seedlings was far lower compared to *AtPCS1*. Routinely, up to five more cycles of PCR were used to detect *AtPCS2* message. A semiquantitative RT-PCR analysis yielded AtPCS1/AtPCS2 message abundance ratios of about 0.5 for roots and >20 for shoots. No significant (more than twofold) transcriptional regulation of *AtPCS2* could be seen upon Cd^{2+} treatment (Fig. 5B). Other conditions of heavy metal stress (Zn or Cu), oxidative stress, salt stress, jasmonic acid or salicylic acid treatment also failed to significantly induce *AtPCS1* or *AtPCS2* expression (data not shown).

4. Discussion

Cd contamination of the environment is largely due to industrial activities over the last 150 years. Thus, one may ask whether Cd detoxification represents an actual evolutionary function for PCSs, just as for other factors such as metallothioneins [20]. It has long been suggested that PCs may play a more general role in metal homeostasis [6,21]. In this context, and given the fact that AtPCS1 dysfunction results in Cd^{2+} hypersensitivity in *Arabidopsis* [10], it was interesting to find a second putative *PCS* gene in the *Arabidopsis* genome [11,13]. We tested several possible explanations for the PC deficiency of plants lacking an intact *AtPCS1* gene. RT-PCR data showed that *AtPCS2* is expressed. Its expression does not appear to be tightly controlled as transcripts could be detected in all organs analyzed and at different developmental stages. Expression is constitutive and no evidence for up-regulation upon stress treatment could be found, as was previously reported for *AtPCS1* [5,11]. Presence of AtPCS2 protein has not been tested yet.

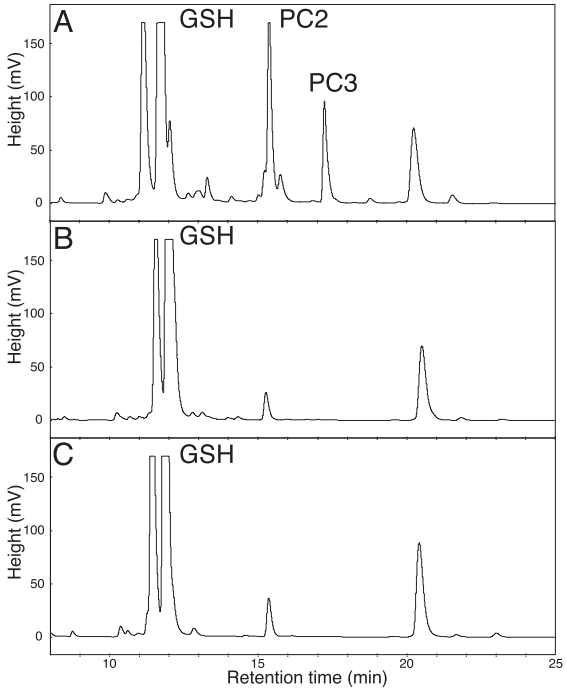

Fig. 4. PC synthesis in *S. pombe* cells expressing *AtPCS2*. After 24 h of growth in the presence (A) or absence of 10 μM $CdCl_2$ (B,C), extracts of *pcs*$^-$ cells carrying the empty pSGP72 vector (B) or expressing *AtPCS2* (A,C) were labeled with monobromobimane and analyzed by HPLC using fluorescence detection. PCs $(\gamma\text{-EC})_2$G (PC2), $(\gamma\text{-EC})_3$G (PC3) and glutathione (GSH) are indicated.

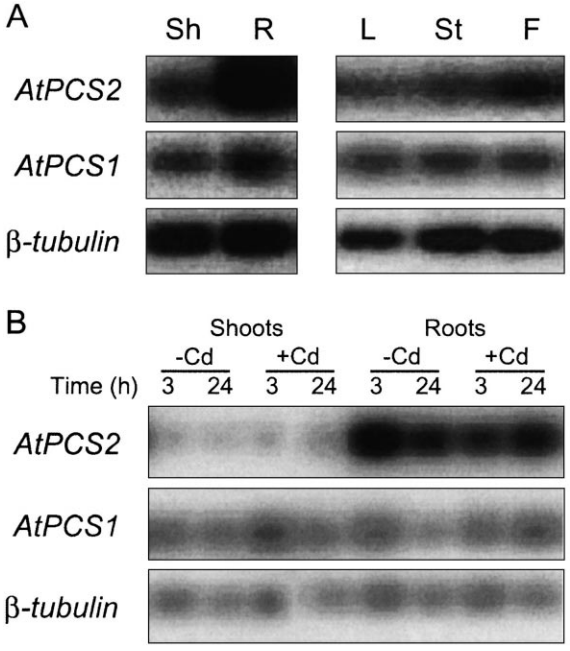

Fig. 5. Comparative RT-PCR analysis of *AtPCS2* and *AtPCS1* expression in *Arabidopsis* tissues. A: Localization of transcripts in shoots (Sh) and roots (R) from hydroponically grown plants and in leaves (L), stems (St) and flowers (F) from plants grown in the greenhouse. B: Hydroponically grown plants were treated for 3 or 24 h with 10 μM $CdCl_2$ before shoots and roots were harvested and analyzed for *AtPCS1* and *AtPCS2* expression. RT-PCR samples were separated on agarose gel and transferred to membranes that were hybridized with an *AtPCS2* probe for *AtPCS2* and *AtPCS1* analysis and a β-tubulin probe.

Heterologous expression in two different systems demonstrated that *AtPCS2* encodes a functional PCS. Also, as already known for other characterized PCS proteins, Cd^{2+} ions are the most effective inducers of PC formation. The significance of the observed differences in activation by some of the other metal ions has to be analyzed in more detail. They cannot, however, explain the PC deficiency of *cad1-3* plants.

AtPCS2 transcript abundance could simply be too low to result in any measurable PC accumulation. Given the similar levels of *AtPCS1* and *AtPCS2* message abundance in roots, apparent from our semiquantitative RT-PCR, however, this appears to be unlikely. Alternatively, expression might be highly localized and confined to a few cells within the different tissues where transcript was detected. Another possibility is that, unlike in a heterologous system, interaction with other factors is required for AtPCS2 function in the plant and a mutated AtPCS1 protein interferes with this interaction.

Catalyzing the formation of Cd–PC complexes might not be the physiological function of AtPCS2. Localization in a cellular compartment with significantly less available Cd^{2+} than the cytosol could explain why no PCs are formed upon Cd exposure in *cad1-3* plants. Algorithms such as TargetP [22] or

PSORT [23], however, did not provide any conclusive indication concerning the subcellular localization of AtPCS2. Analysis of a knockout line will be required to gain a better understanding of the role of the *AtPCS2* gene. This will potentially lead to new insights into the function of PCSs. *Arabidopsis* appears to be the first organism where two functional *PCS* genes are described.

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