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RESEARCH PAPER

Functional characterisation of two phytochelatin synthases in rice (*Oryza sativa* cv. Milyang 117) that respond to cadmium stress

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ABSTRACT

- Cadmium (Cd) is one of the most toxic heavy metals and a non-essential element to all organisms, including plants; however, the genes involved in Cd resistance in plants remain poorly characterised.
- To identify Cd resistance genes in rice, we screened a rice cDNA expression library treated with $CdCl_2$ using a yeast (*Saccharomyces cerevisiae*) mutant *ycf1* strain (DTY167) and isolated two rice phytochelatin synthases (*OsPCS5* and *OsPCS15*).
- The genes were strongly induced by Cd treatment and conferred increased resistance to Cd when expressed in the *ycf1* mutant strain. In addition, the Cd concentration was twofold higher in yeast expressing *OsPCS5* and *OsPCS15* than in vector-transformed yeast, and OsPCS5 and OsPCS15 localised in the cytoplasm. *Arabidopsis thaliana* plants overexpressing *OsPCS5/-15* paradoxically exhibited increased sensitivity to Cd, suggesting that overexpression of *OsPCS5/-15* resulted in toxicity due to excess phytochelatin production in *A. thaliana*.
- These data indicate that *OsPCS5* and *OsPCS15* are involved in Cd tolerance, which may be related to the relative abundances of phytochelatins synthesised by these phytochelatin synthases.

INTRODUCTION

Contamination by toxic heavy metals is correlated with the degree of industrialisation through mining and combustion of fossil fuels and damages the environment and human health (Nriagu & Pacyna 1988). Among heavy metals, copper (Cu) and zinc (Zn) function as cofactors of enzymes involved in redox reactions and electron transfer reactions in all living organisms (Mengel & Kirkby 2001). Non-essential heavy metals, including arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb), have potentially toxic effects, such as inactivation of enzymes and promotion of oxidative stress (Van Assche & Clijsters 1990; Mengel & Kirkby 2001). Phytoremediation is a technology that uses plants to remove heavy metals from polluted areas and is considered a cost-effective and environmentally friendly technology (Raskin *et al.* 1997; Salt *et al.* 1998; Krämer 2005). Because natural hyperaccumulating plants produce low biomass, grow slowly and only accumulate a specific element (Cunningham *et al.* 1995), genetic and molecular studies are required for the optimisation and improvement of phytoremediation to enhance metal tolerance and accumulation.

Cadmium (Cd) is a hazardous environmental contaminant that is responsible for severe human health problems. It causes intracellular damage *via* inactivation or denaturation of proteins by binding to free sulphydryl residues, by displacing cofactors from a variety of proteins containing transcription factors and by causing oxidative stress, which induces membrane breakage (Liao *et al.* 2002; Kim *et al.* 2008). Animals are exposed to Cd through many different channels, but mainly *via* food intake. Farm products become contaminated with Cd when grown in Cd-contaminated soils (Kim *et al.* 2008). Accumulation of Cd in plants induces chlorosis, wilting, growth reduction and cell death (Herbette *et al.* 2006). Cd detoxification mechanisms in plants include production of cysteine-rich

peptides such as glutathione (GSH), metallothioneins (MT) and phytochelatins (PC) (Rauser 1999; Cobbett 2000a,b).

Phytochelatins are heavy metal-binding peptides with a general structure of (γ-Glu-Cys)n-Gly, where n ranges from 2 to 11, and are synthesised enzymatically following exposure to various heavy metals (Rauser 1990; Cobbett 2000b). These peptides are synthesised from reduced GSH by phytochelatin synthase (PCS), which is induced by heavy metals such as Cd (Cobbett 2000b; Vatamaniuk *et al.* 2000). In addition, PC are required for Cd tolerance in plants, and mutants lacking the ability to synthesise PC are hypersensitive to Cd (Howden *et al.* 1995). In *Arabidopsis thaliana*, *cad1* and *cad2* mutants are deficient in PC synthesis because of mutations in γ-glutamylcysteine synthetase (*cad2*) and PCS (*cad1*), respectively (Howden & Cobbette 1992; Howden *et al.* 1995; Cobbette *et al.* 1998). PC bind heavy metals such as Cd, Cu and As with high affinity, and the complexes formed in the cytosol are subsequently localised to vacuoles for detoxification (Grill *et al.* 1985; Salt 1995; Maitani *et al.* 1996; Zenk 1996; Schmöger *et al.* 2000).

Phytochelatin synthases are essential proteins that play important roles in heavy metal detoxification. *PCS* genes were first isolated from *A. thaliana*, *Schizosaccharomyces pombe* and *Triticum aestivum* (Clemens *et al.* 1999; Ha *et al.* 1999; Vatamaniuk *et al.* 1999), although many *PCS* genes have since been cloned in several plant species (Shen *et al.* 2010). In addition, overexpression of the *AtPCS1* gene in *Escherichia coli*, *Saccaromyces cerevisiae*, *Nicotiana tabacum* and *Brassica juncea* enhances tolerance to and accumulation of Cd (Vatamaniuk *et al.* 1999; Merle *et al.* 2003; Pomponi *et al.* 2006; Gasic & Korban 2007). Expression of *TaPCS1* in *Nicotiana glauca* also enhances tolerance to and accumulation of Cd and Pb (Martinez *et al.* 2006). Overexpression of *AtPCS1* enhances Cd sensitivity with a high level of PC in *A. thaliana* and leads to Cd hypersensitivity without increasing the level of PC in tobacco (Lee *et al.* 2003a,b; Li *et al.* 2004; Wojas *et al.* 2008). Although there are many reports on the functions of PCS in heavy metal detoxification in plants, the effects on plant phenotypes have rarely been explained. Overexpression of *PCS* genes from different species using a model plant such as *A. thaliana* could reveal their properties and functions and address their application in the improvement of phytoremediation.

Yeast cadmium factor (YCF1) is a member of the ATP-binding cassette (ABC) transporter family and confers Cd resistance in *S. cerevisiae*. It functions as a transporter to sequester GSH-conjugated Cd in vacuoles (Li *et al.* 1997). To extend our knowledge of the Cd tolerance mechanism in rice, we constructed a rice cDNA library following treatment with $CdCl_2$, and performed screening using the *S. cerevisiae ycf1* mutant strain DTY167. We isolated two PCS, *OsPCS5* and *OsPCS15*, *via* this screening. *OsPCS15* is a novel PCS in rice. Both genes responded to Cd and the proteins were localised in the cytoplasm in yeast. In addition, *A. thaliana* plants overexpressing *OsPCS5/-15* exhibited hypersensitivity to Cd.

MATERIAL AND METHODS

Yeast, bacterial strains and plant material

The *YCF1* null mutant, DTY167 (*MATα ura3-52 leu2-3,-112 his-200 trp1-901 lys2-801 suc2-9 ycf1::hisG*), and wild type (WT), DTY165 (*MATα ura3-52 his6 leu2-3,-112 his3- Delta 200 trp1-901 lys2-801 suc2- Delta*), *S. cerevisiae* strains were used for rice cDNA library screening and control experiments, respectively (Li *et al.* 1996). The *E. coli* strain XL-1 Blue *MRF'* (Stratagene, La Jolla, CA, USA) was used for cloning. All *A. thaliana* plants used in this study were the Columbia (*Col-0*) ecotype. Plants were germinated on MS (Murashige & Skoog 1962) nutrient medium (pH 5.8) containing 3% sucrose and 0.25% phytagel and maintained in a growth chamber at 22 °C with a 16/8 h light/dark cycle.

Screening of the rice (*Oriza sativa* cv. Milyang 117) cDNA expression library

A rice cDNA expression library was inserted into the *pAD-GAL4-2.1* vector from rice callus treated with $CdCl_2$. *S. cerevisiae* DTY167 cells were transformed with the rice cDNA library using the lithium acetate transformation method (Gietz & Schiestl 1995). The transformants were selected for leucine prototrophy on SD-leu medium, transferred to liquid SD-leu medium, grown overnight at 30 °C and streaked on SD-leu solid medium containing 50 μM $CdCl_2$. Strains exhibiting high tolerance to Cd were transferred to YPD liquid medium and grown overnight at 30 °C. DNA was extracted from the yeast cells and transformed into *E. coli*. The colonies were analysed by restriction digestion and sequencing.

Cloning of *OsPCS* genes

A fragment of *OsPCS5* cDNA was amplified by PCR using the gene-specific primers 5′-CTCGAGATGGCAGCGATGGCATC CCTG-3′ and 5′-TACTAGTCCACCTCCATGGGATTGTGG CACAGGATC-3′, which contained *Xho*I and *Spe*I sites, respectively. In addition, *OsPCS15* cDNA was amplified using the gene-specific primers 5′-CTCGAGATGGCGTCTAAACCAAG CAGCCGAGCGGAA-3′ and 5′-TACTAGTCCACCTCCGCA TTGTTCCCAAGGTTGTGG-3′, which contained *Xho*I and *Spe*I sites, respectively. The genes were then cloned into the *pGEM-T* easy vector (Promega, Madison, WI, USA) and used for various plasmid constructs.

Metal treatments and RNA gel blot analysis

For heavy metal treatments, suspension culture cells induced from rice (*Oryza sativa* L. Milyang 117) embryos were used. The suspension cells were maintained at 25 °C with shaking at 90–100 rpm in 100-ml flasks containing liquid R2 medium supplemented with 2 $mg \cdot l^{-1}$ 2, 4-D and 3% sucrose, and subcultured every week. Rice suspension cells were treated in the dark with 50 μM $CdCl_2$ or 500 μM Na_2HAsO_4 in the original flasks. Each sample was harvested on filter papers by vacuum filtration. Harvested cells were immediately frozen in liquid nitrogen and stored at −70 °C.

Rice suspension cells were ground to a fine powder with a mortar and pestle. RNA gel blot analysis was carried out as previously described (Park *et al.* 2002; Xu *et al.* 2007). A total of 20 μg of total RNA were separated on a denatured 1.5% formaldehyde/agarose gel, transferred onto a nylon membrane (GeneScreenPlus, NEN Life Science Products, Boston, MA, USA), and hybridised with *OsPCS5/-15* gene-specific probes. To verify equal loading, rRNA was visualised by staining with ethidium bromide. Hybridisation and washing were carried

out under high stringency conditions. The blots were air-dried and exposed to X-ray film at −70 °C.

Yeast complementation assay for Cd tolerance

The WT *S. cerevisiae* (DTY165) and *ycf1* mutant (DTY167) cells were transformed with empty vectors (WT/vector and ycf1/vector, respectively). *ycf1* mutant (DTY167) cells were also transformed with *pXY-OsPCS5* and *pXY-OsPCS15*, respectively. The transformed cells were grown overnight to an optical density at 600 nm (OD600) of 1.7. Aliquots of the cell suspensions were then serially diluted and spotted on solid YPD medium containing or lacking 70 μM $CdCl_2$. Colonies were visualised after incubating the plates for 2–4 days at 30 °C. In addition, the strains were grown overnight at 30 °C in liquid minimal selective medium containing 2% glucose. The cultures were diluted in minimal medium to an OD600 of 0.1 in the presence of various concentrations of $CdCl_2$ and incubated for an additional 24 h, after which growth was determined by measuring OD600 (Ghosh *et al.* 1999).

Cadmium content and subcellular localisations of OsPCS5 and OsPCS15

Transformed yeast cells were grown in liquid YPD medium containing 20 μM Cd^{2+}, incubated at 30 °C for 24 h, washed in sterilised deionised water, and harvested by centrifugation. Finally, the yeast samples were acidified with HNO_3 and incubated at 65 °C for 30 min. The samples were centrifuged at 5000 *g* for 10 min to remove debris, and the Cd^{2+} content of the supernatant was determined using inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 4300DV/5300DV; Perkin-Elmer, Waltham, MA, USA).

To observe the subcellular localisations of OsPCS5 and OsPCS15 proteins in yeast, *OsPCS5::GFP* and *OsPCS15::GFP* fusion constructs were prepared, respectively. Each cDNA was fused to the coding region of *GFP* under control of the cauliflower mosaic virus (*CaMV*) *35S* promoter. Transformed yeast cells were grown overnight at 30 °C, subcultured at a 1:1000 dilution in the medium and grown at 30 °C to an OD600 of ~0.7. Cells in log phase were examined at 100× magnification on slides using an Olympus FV1000 confocal microscope (Olympus America, Center Valley, PA, USA) with an excitation wavelength of 488 nm.

Generation of transgenic *A. thaliana* plants and Cd resistance assays

To construct transgenic *A. thaliana* plants, *OsPCS5* and *OsPCS15* cDNA were cloned into the plant GFP expression vector (*pCAMBIA1302*). In this vector, *OsPCS5* and *OsPCS15* cDNA were expressed under the control of the *CaMV 35S* promoter. The recombinant plasmids were transformed into *Agrobacterium tumefaciens* GV3101, and transgenic *A. thaliana* plants were generated using the floral dip method (Clough & Bent 1998). Homozygous T_3 progeny of transgenic plants were selected and used for further experiments following growth under a 16 h/8 h light/dark cycle at 22 °C. The protein levels of OsPCS5/-15 transgenic plants grown for 3 weeks on plates were confirmed by Western blot analysis with an anti-GFP antibody.

To examine the effect of Cd on OsPCS5/-15 transgenic *A. thaliana* plants, WT (ecotype Columbia) and transgenic seeds were germinated on half-strength MS medium solidified with phytagel (2.5 $g \cdot l^{-1}$; Murashige & Skoog 1962) containing 0, 50 or 70 μM $CdCl_2$. After germination, plates were positioned vertically to check root growth. Root lengths of WT and OsPCS5/-15 transgenic *A. thaliana* plants were measured after 10 days.

RESULTS

Isolation of rice PCS and sequence comparison with other plant PCS

To isolate Cd resistance genes in rice, an *O. sativa* cDNA library was introduced into the *ycf1* yeast mutant strain DTY167 (Δ*ycf1*). Approximately 4×10^4 independent transformants were plated on agar medium containing Cd and grown for 4 days. A total of 120 colonies were selected and DNA was extracted and amplified using *E. coli*. The inserts were sequenced and compared with sequences in the GenBank® database. Among them, we isolated two *PCS* that conferred strong Cd tolerance to Δ*ycf1* mutant yeast. These *PCS* encode polypeptides with predicted masses of 55.7 and 57.4 kDa. Based on the nomenclature of *PCS* in rice (Shen *et al.* 2010), one had already been identified as *OsPCS5* and the other was a novel *PCS* gene in rice. Therefore, the latter was named *OsPCS15* because 14 *PCS* or *PCS*-like genes were previously identified in the rice genome using bioinformatics methods (Shen *et al.* 2010).

The deduced amino acid sequences were aligned with those of well-characterised PCS such as OsPCS1 (*O. sativa*), AtPCS1 (*A. thaliana*) and TaPCS1 (*T. aestivum*), and the PC domains were well conserved in the two OsPCS (Fig. 1). The amino acid sequence of OsPCS5 displayed 76.3% identity and 83.5% similarity with the TaPCS1 sequence, and the PC domain was more conserved, with 91.7% identity to the corresponding PC domain of TaPCS1. In addition, OsPCS15 showed 67.5% identity and 73.8% similarity with the TaPCS1 sequence. The PC domain also exhibited 78.8% identity to that of the TaPCS1 protein. Although OsPCS15 showed approximately 10% lower similarity to TaPCS1 than OsPCS5, amino acid sequences in the PC domain of OsPCS15 were well conserved. These results imply that the OsPCS15 protein is a novel PCS family protein and may function as a PCS in rice.

Expression patterns of *OsPCS5* and *OsPCS15* genes following exposure to heavy metals

To investigate whether the *PCS5/-15* genes are expressed in response to heavy metal stress, we performed northern blot analysis with total RNA isolated from rice suspension cells treated with 50 μM Cd^{2+}or 500 μM As^{2+}. As shown in Fig. 2A, expression of the *OsPCS5* transcript was greatly increased at 12 h and remained elevated for 24 h after treatment with Cd or As. *OsPCS15* transcript levels showed similar expression patterns (Fig. 2B). Exposure to these toxic metals increases GSH synthesis and they are detoxified by PC (Rauser 1990; Verbruggen *et al.* 2009). Expression of the *OsPCS5/-15* genes in response to Cd and As supports a role for OsPCS5/-15 proteins in PC synthesis.

Fig. 1. Alignment of the deduced amino acid sequence of plant *PCS* genes. The amino acid sequences of *Oryza sativa* (*OsPCS1, OsPCS5* and *OsPCS15*), *Arabidopsis thaliana* (*AtPCS1, AAD41794*) and *Triticum aestivum* (*TaPCS1, AAD50592*) *PCS* were aligned using the CLUSTAL W alignment program (Thompson *et al.* 1994). Identical amino acid residues are shown in black boxes and similar amino acid residues are shown in grey boxes. The putative PC domain predicted by the domain prediction program (NCBI web server, http://www.ncbi.nih.gov/BLAST/) is underlined.

Complementation of OsPCS5/-15 for Cd tolerance in the yeast ycf1 mutant

Sequestration of Cd and other toxic metals in vacuoles is a well-characterised mechanism of detoxification (Rea *et al.* 1998). YCF1 in *S. cerevisiae* is a crucial factor for the transport of toxic metals into vacuoles and is involved in toxic metal tolerance (Li *et al.* 1997). To characterise the role of OsPCS5/-15 in Cd tolerance, *S. cerevisiae ycf1* mutant (*Δycf1*) cells (*DTY167*) were transformed with constructs containing an empty vector, *OsPCS5* or *OsPCS15*, and WT cells (*DTY165*) were transformed with an empty vector as a control. The yeast cells were serially diluted and spotted on to YPD agar medium lacking or containing 70 μM $CdCl_2$ (Fig. 3A). *Δycf1* cells expressing *OsPCS5* or *OsPCS15* grew much better than those transformed with an empty vector. They also grew better than WT yeast transformed with an empty vector.

To confirm the Cd tolerance phenotype of *OsPCS5*- and *OsPCS15*-expressing yeasts, the cells were grown in liquid SD medium containing various concentrations of $CdCl_2$ and absorbance of cells at 600 nm was measured spectrophotometrically. In medium lacking Cd, growth of *OsPCS5*- and *OsPCS15*-transformed *Δycf1* cells was similar to that of empty vector-transformed WT and *Δycf1* cells. In the presence of 20,

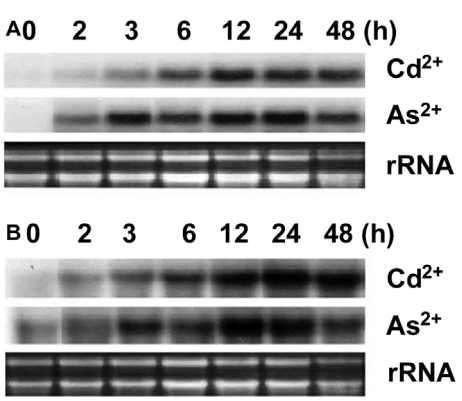

Fig. 2. Expression patterns of *OsPCS5* and *OsPCS15* genes in rice. Northern blot analyses of *OsPCS5* (A) and *OsPCS15* (B) in response to treatment with 50 μM Cd^{2+} and 500 μM As^{5+} in rice cell cultures. Each lane was loaded with 20 μg total RNA prepared from rice suspension-cultured cells at the indicated time points and separated on a 1.5% formaldehyde/agarose gel. The gel was transferred onto a nylon membrane and hybridised with ^{32}P-labelled *OsPCS5* or *OsPCS15* cDNA. To verify equal loading of total RNA, rRNA was visualised by staining with ethidium bromide.

40 or 60 μM $CdCl_2$, the density of empty vector-transformed WT and *Δycf1* cells decreased in a concentration-dependent manner. However, the densities of *OsPCS5*- and *OsPCS15*-transformed *Δycf1* cells were maintained at 1.0 at OD600 in the presence of 300 μM $CdCl_2$ (Fig. 3B). These results indicate that overexpression of *OsPCS5* or *OsPCS15* in *Δycf1* yeast cells confers strong tolerance to $CdCl_2$, suggesting that the OsPCS5 or OsPCS15 protein function in Cd tolerance in yeast.

Cadmium accumulation and subcellular localisation of OsPCS5/-15 proteins

Phytochelatins are synthesised by cytosolic PC synthase and rapidly induced by Cd. Thereafter, PC chelate Cd and form several complexes to inactivate Cd from free Cd ions in plant cells (DalCorso *et al.* 2008). To determine Cd accumulation in *OsPCS5*- and *OsPCS15*-transformed *Δycf1* yeast cells, we analysed the Cd content of empty vector-, *OsPCS5*- and *OsPCS15*-transformed *Δycf1* yeast cells grown in liquid YPD medium containing 20 μM CdCl (Fig. 4). The Cd content of *ycf1-OsPCS5/-15* cells was 2.5-fold higher than that of the *ycf1*-vector control cells. These results imply that *OsPCS5/-15* can contribute to Cd resistance *via* sequestration of Cd in vacuoles through the formation of PC–Cd complexes.

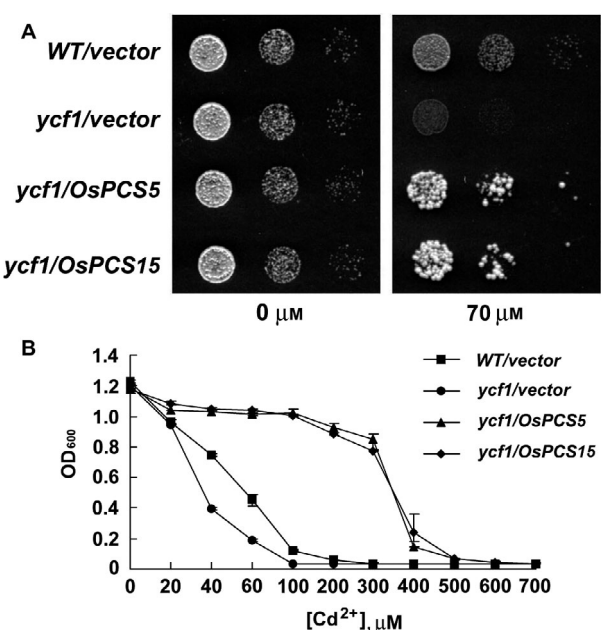

Fig. 3. Expression of *OsPCS5* and *OsPCS15* confers enhanced Cd^{2+} tolerance in a Δ*ycf* yeast mutant. A: Enhanced growth of *OsPCS5*- and *OsPCS15*-transformed yeasts on YPD agar plates supplemented with 70 μM $CdCl_2$. Yeast Δ*ycf* mutant DTY167 and WT DTY165 cells were transformed with a construct containing the empty vector, *OsPCS5* or *OsPCS15*. Ten-fold serial dilutions of Δ*ycf* mutant and WT yeast cells expressing the indicated plasmid were spotted (2 μl) onto plates containing or lacking 70 μM $CdCl_2$, and incubated at 30 °C for 2–4 days. B: Suppression of Cd^{2+} hypersensitivity in the yeast Δ*ycf* mutant DTY167 by plasmid-borne *OsPCS5* and *OsPCS15*. Yeast cells expressing *OsPCS5* or *OsPCS15* were normalised to OD600 and grown in liquid SD media containing different concentrations of Cd^{2+}. After incubation at 30 °C for 24 h, cell growth was determined by measuring OD600. Squares (■), circles (●), triangles (▲) and diamonds (◆) indicate the empty vector (WT), empty vector (*ycf1*), OsPCS5 (*ycf1*) and OsPCS15 (*ycf1*), respectively.

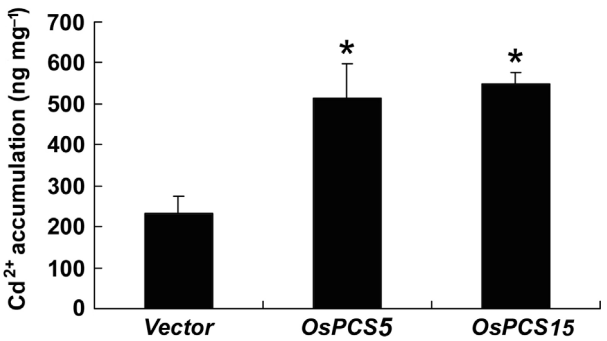

Fig. 4. Yeast cells expressing *OsPCS5* or *OsPCS15* accumulate more Cd^{2+} than control cells of the Δ*ycf* mutant DTY167. Transformed cells were grown in liquid YPD medium containing 20 μM Cd^{2+} at 30 °C for 24 h. The Cd^{2+} content of the samples was measured by ICP-OES. Results are averages (±SE) from three independent experiments performed using three different colonies. Asterisks indicate statistical significance ($P < 0.05$, Student's *t*-test) of differences between yeast cells expressing *OsPCS5/-15* and vector control cells.

Arabidopsis thaliana PCS1 localises in the cytosol and generates metal-binding PC from GSH (Blum *et al.* 2010). The ability of OsPCS5/-15 to complement the *Δycf1* yeast mutant may result from the localisation of heterologous proteins in the cytosol of yeast cells. To investigate the localisations of OsPCS5/-15 proteins, OsPCS5/-15 proteins tagged with GFP were expressed in yeast cells. GFP signals were visualised using confocal microscopy. Free GFP protein was used as a cytosolic marker protein. Fluorescence of the OsPCS5/-15::GFP proteins was observed in the cytosol, as was fluorescence of free GFP protein (Fig. 5). These results strongly indicate that OsPCS5/-15 proteins localise in the cytoplasm in yeast.

Overexpression of OsPCS5 and OsPCS15 and Cd hypersensitivity in *A. thaliana*

To determine the effects of *OsPCS5/-15* overexpression on Cd tolerance in *A. thaliana*, we generated *OsPCS5/-15* transgenic *A. thaliana* plants harbouring plant expression vectors under the control of the *CaMV 35S* promoter. Individual transgenic plants were selected *via* hygromycin resistance, followed by Western blot analysis with an anti-GFP antibody. Three homozygous lines showing the highest expression levels were selected and analysed for Cd tolerance at increasing Cd concentrations (Fig. 6). There were no significant phenotypic differences between *OsPCS5/-15* transgenic plants grown in the absence of Cd. Intriguingly, when the transgenic plant lines were treated with 50 and 70 μM $CdCl_2$, root growth was significantly inhibited. As shown in Fig. 6A, root growth in *OsPCS5* transgenic plants was reduced by approximately 33% following treatment with 50 and 70 μM $CdCl_2$. In the *OsPCS15* transgenic plant lines, root growth was reduced by 26% following exposure to the same Cd treatments (Fig. 6B). Paradoxically, ectopic expression of PCS by *OsPCS5/-15* increased sensitivity to Cd in *A. thaliana*.

DISCUSSION

Heavy metals are a major hazard for ecosystems and human health. Among hazardous toxic metals, Cd is the most toxic to living organisms (He *et al.* 2015; Song *et al.* 2015). In

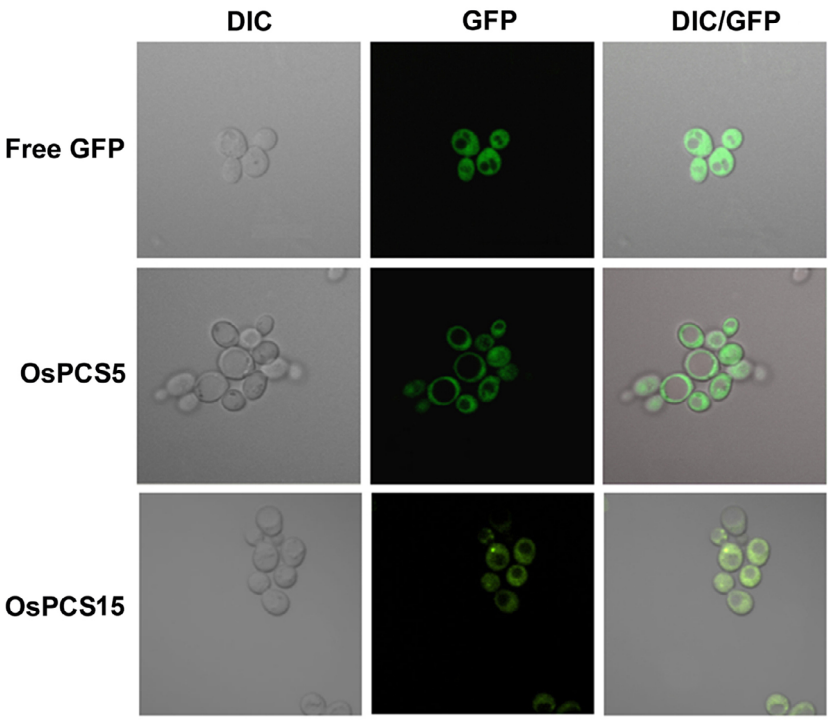

Fig. 5. Subcellular localisations of OsPCS5 and OsPCS15 proteins tagged with GFP in yeast cells. *Saccharomyces cerevisiae* DTY167 cells were transformed with free *GFP*, *OsPCS5::GFP* or *OsPCS15::GFP*, and observed *via* confocal fluorescence microscopy. DIC and GFP merged images were generated using Olympus software. DIC, differential interference contrast; GFP, green fluorescent protein; DIC/GFP, merged images of DIC and GFP.

particular, Cd accumulation causes a range of adverse effects in higher plants. Cd disturbs plant physiological processes, such as respiration, transpiration and photosynthesis (Toppi & Gabbrielli 1999). Understanding the mechanisms responsible for tolerance to and accumulation of Cd is important for developing efficient strategies to tackle Cd stress in plants.

We generated a rice cDNA library with the *ycf1* yeast mutant strain DTY167 (*Δycf1*) and screened approximately 4×10^4 independent transformants. Two PCS (*OsPCS5* and *OsPCS15*) were isolated as candidates that confer strong Cd tolerance in the *Δycf1* mutant yeast (Fig. 1). Examination of the genome of the *O. sativa* subsp. *japonica* variety Zhonghua 11 revealed that *OsPCS15* encodes a novel PCS (Shen *et al.* 2010), suggesting a divergence of genes among varieties within the species. Based on previous reports, Cd is the best activator of PCS (Grill *et al.* 1989; Hayashi *et al.* 1991). In addition, the *A. thaliana* PCS (*AtPCS1*) was identified as a cDNA that suppresses the Cd-sensitive phenotype of Brewer's yeast *ycf1* mutant (Vatamaniuk *et al.* 1999). Therefore, our screen effectively isolated candidates that confer Cd tolerance in the *Δycf1* yeast mutant.

Multiple *PCS* genes have been identified in *A. thaliana* (Ha *et al.* 1999; Vatamaniuk *et al.* 1999), wheat (Clemens *et al.* 1999), soybean (Oven *et al.* 2002) and rice (Shen *et al.* 2010). Transgenic plants overexpressing *PCS* genes exhibit increased synthesis of PC and elevated Cd content (Gisbert *et al.* 2003; Li *et al.* 2006; Pomponi *et al.* 2006), and silencing of a *PCS* gene *via* RNAi reduces Cd accumulation in rice seeds (Li *et al.* 2007). On the other hand, overexpression of *AtPCS1* in *A. thaliana* and tobacco increases hypersensitivity to Cd (Lee *et al.* 2003a; Wojas *et al.* 2008). These results suggest that none of these *PCS* genes would be suitable for transforming plants to

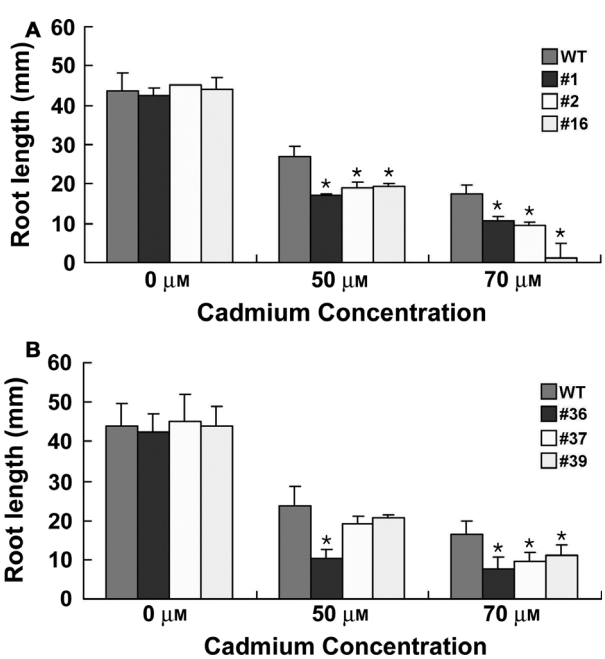

Fig. 6. Effects of *OsPCS5* and *OsPCS15* overexpression on root elongation of plants grown under Cd stress. Seeds were germinated on MS medium containing 0, 50 or 70 μM $CdCl_2$, and Petri dishes were placed in a vertical orientation upon onset of growth. Root lengths of each overexpression line and the WT were measured after 10 days of growth. Results are averages (±SE) of 30 plants. Asterisks indicate statistical significance ($P < 0.05$, Student's *t*-test) of differences between *OsPCS5/-15* overexpressing transgenic plants and WT plants.

tolerate heavy metal accumulation. Further studies on the isolation and function of *PCS* genes in various plant species are required.

Phytochelatin synthases catalyse PC synthesis in plants and PC play an important role in heavy metal homeostasis and detoxification by chelating and sequestering heavy metals (Clemens 2006). Therefore, it is crucial to check PCS expression patterns following treatment with heavy metals. Indeed, *PCS* genes are expressed following exposure to heavy metals such as Cd, Pb and Zn in rice (Shen *et al.* 2010). In this study, *OsPCS5/-15* were expressed after treatment with Cd and As, indicating that *OsPCS5/-15* are specific to Cd and As (Fig. 2). *OsPCS7* expression is only induced by Hg and Pb, while *OsPCS9* expression is induced by Cd and Zn (Shen *et al.* 2010). These results and our data imply that many *PCS* genes exist in the rice genome to respond specifically to different heavy metals.

In addition to the significant expression of *OsPCS5/-15* genes following treatment with Cd and As, *OsPCS5/-15* expression in yeast resulted in the tolerance phenotype upon Cd^{2+} exposure, supporting the suggestion that expression of these genes was responsible for Cd^{2+} resistance (Fig. 3). OsPCS5/-15 expression in the yeast mutant *Δycf1* enabled cells to grow in a ten-fold higher Cd^{2+} concentration than control yeast cells (Fig. 3B). These results concerning the association of metal tolerance with *OsPCS5/-15* expression in yeast provide molecular evidence that PC play a general role in metal homeostasis. PC function as cytosolic chelators and the PC–Cd complexes formed are then sequestered in vacuoles by transporters such as the ABC-type transporter HMT1 (Ortiz *et al.* 1992).

In contrast with overexpression of *OsPCS5/-15* genes in yeast, we generated OsPCS5/-15 transgenic *A. thaliana* plants using the CaMV 35S promoter. Transgenic *A. thaliana* plants expressing *OsPCS5/-15* were hypersensitive to Cd (Fig. 6). Similarly, overexpression of *PCS* genes in *A. thaliana*, tobacco and rice plants decreases Cd tolerance (Lee *et al.* 2003a; Li *et al.* 2004; Wojas *et al.* 2008; Wang *et al.* 2012). These results might indicate the activity of PCS from species-dependent differences generated by the transgenes. The various effects of overexpression may also result from functional differences between PCS from diverse plants. Therefore, not all *PCS* genes will be suitable for transforming plant species for phytoremediation.

In addition, a study has indicated that Cd accumulation in shoots is higher in Cd-tolerant transgenic *Arabidopsis* plants expressing the *AtPCS1* gene than in WT and Cd-intolerant transgenic *Arabidopsis* plants (Lee *et al.* 2003b). This is consistent with previous data that Cd-tolerant transgenic plants overexpressing genes related to synthesis of GSH and PC show increased Cd accumulation in their shoots (Zhu *et al.* 1999a,b; Domínguez-Solís *et al.* 2001). It was also reported that PCS protein is constitutively expressed and activated in the presence of heavy metals (Steffens 1990; Zenk 1996). Therefore, Cd accumulation is correlated with Cd tolerance. Here we speculate that Cd concentration in transgenic *A. thaliana* plants expressing *OsPCS5/-15* will be similar to that of WT plants. Further progress on the biochemical and functional characterisation of *OsPCS5/-15* genes should focus on PC synthesis and Cd accumulation in transgenic *A. thaliana* expressing the *OsPCS5/-15* genes.

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AUTHOR CONTRIBUTIONS

H.C.P. and W.S.C. designed the study and wrote the manuscript. H.C.P., J.E.H., Y.J., Y.J.K. and X.C.N. performed the experiment and analysed the data. C.Y.K. prepared experimental material and analysed the data. All authors have read and approved the manuscript for publication.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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