Functional Characterization of an Unusual Phytochelatin Synthase, LjPCS3, of *Lotus japonicus*[1[W][OA]]

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In plants and many other organisms, phytochelatin synthase (PCS) catalyzes the synthesis of phytochelatins from glutathione in the presence of certain metals and metalloids. We have used budding yeast (*Saccharomyces cerevisiae*) as a heterologous system to characterize two PCS proteins, LjPCS1 and LjPCS3, of the model legume *Lotus japonicus*. Initial experiments revealed that the metal tolerance of yeast cells in vivo depends on the concentrations of divalent cations in the growth medium. Detailed in vivo (intact cells) and in vitro (broken cells) assays of PCS activity were performed with yeast expressing the plant enzymes, and values of phytochelatin production for each metal tested were normalized with respect to those of cadmium to correct for the lower expression level of LjPCS3. Our results showed that lead was the best activator of LjPCS1 in the in vitro assay, whereas, for both assays, arsenic, iron, and aluminum were better activators of LjPCS3 and mercury was similarly active with the two enzymes. Most interestingly, zinc was a powerful activator, especially of LjPCS3, when assayed in vivo, whereas copper and silver were the strongest activators in the in vitro assay. We conclude that the in vivo and in vitro assays are useful and complementary to assess the response of LjPCS1 and LjPCS3 to a wide range of metals and that the differences in the C-terminal domains of the two proteins are responsible for their distinct expression levels or stabilities in heterologous systems and patterns of metal activation.

In plants and other organisms, some metals, such as iron (Fe), copper (Cu), and zinc (Zn), act as cofactors of enzymes involved in electron transfer reactions (Mengel and Kirkby, 2001). The same metals, at supra-optimal concentrations, as well as other nonessential heavy metals and metalloids, such as cadmium (Cd), arsenic (As), mercury (Hg), lead (Pb), silver (Ag), and aluminum (Al), are potentially toxic due to their ability to inactivate enzymes and promote oxidative stress (Van Assche and Clijsters, 1990; Mengel and Kirkby, 2001). General symptoms of phytotoxicity include growth inhibition, leaf chlorosis, loss of photosynthetic activity, membrane disintegration, and induction of stress-related enzymes.

Plants have evolved multiple strategies to maintain physiological concentrations of essential metals and to cope with heavy metal toxicity. One of them involves the chelation of metal ions by polypeptides or proteins, carboxylic acids, and amino acids. Phytochelatins (PCs) are polypeptides of general structure (γGlu-Cys)$_{2\text{-}11}$-Gly that are synthesized from glutathione (GSH; γGlu-Cys-Gly) by PC synthase (PCS). This dipeptidyltransferase catalyzes the net transfer of a γGlu-Cys unit from GSH to another GSH molecule or to an elongating PC polypeptide (Grill et al., 1989; Vatamaniuk et al., 2000). The reaction is strictly dependent on the presence of some metal ions, including Cd^{2+}, Zn^{2+}, and $Cu^{+/2+}$ (Beck et al., 2003). Formation of PC complexes with Cd^{2+}, $Cu^{+/2+}$, As^{3+}, and Ag^{+} in vivo and sequestration of the PC-Cd^{2+} complex to the vacuoles have been observed in yeasts and plants (Schmöger et al., 2000; Cobbett and Goldsbrough, 2002).

The mechanism of the PCS reaction has been studied in detail using Cd and the purified PCS1 enzymes of Arabidopsis (*Arabidopsis thaliana*) and soybean (*Glycine max*). Two major breakthroughs were the identification of Cys-56 as the first acylation (γGlu-Cys donor) site in AtPCS1 and the demonstration that free GSH and a metal-GS thiolate complex are cosubstrates of the enzyme (Vatamaniuk et al., 2004; Romanyuk et al., 2006). An important finding was also that prokaryotic PCS homologs lack both the variable C-terminal domain and the second acylation (γGlu-Cys acceptor) site that is present in the eukaryotic PCS (Harada et al., 2004; Tsuji et al., 2004; Vivares et al., 2005). These and other observations strongly suggest that the second acylation site of the plant enzymes is located at the C-terminal domain (Romanyuk et al., 2006). However, the precise role of the metal ion in the PCS reaction is still contro-

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versial. According to Oven et al. (2002), a direct binding of the metal to the PCS protein would be required for PC formation, whereas Romanyuk et al. (2006) propose that the free metal ion is dispensable for core activity but is needed instead to stimulate enzyme activity by interaction with the C-terminal domain. Additional careful studies with truncated AtPCS1 proteins showed that this domain is important for stabilization of the enzyme and is responsible for its responsiveness to a wide range of metals (Ruotolo et al., 2004).

In a previous work, we identified three functional *LjPCS* genes in the model legume *Lotus japonicus* and found that they were differentially expressed in response to Cd (Ramos et al., 2007). One of the genes, *LjPCS1*, encodes a protein with high homology to GmPCS1 (84% identity in their amino acid sequences), which was characterized in detail previously (Loscos et al., 2006). The two other genes of *L. japonicus*, *LjPCS2* and *LjPCS3*, encode proteins that are closely related to each other (90% identity) but are distant in evolutionary terms (53%–56% identity) from AtPCS1, GmPCS1, and LjPCS1 (Ramos et al., 2007). Phylogenetic analysis also showed that LjPCS2 and LjPCS3 cluster together and separately from the typical PCS enzymes of the Brassicaceae and Leguminosae (Ramos et al., 2007). Furthermore, a close inspection of LjPCS2 or LjPCS3 sequences revealed that they mainly differ from the typical PCS1 enzymes in the C-terminal domain. To determine whether this poor homology determines major differences in their metal specificity and activity, LjPCS3 and LjPCS1 (as a control) were expressed in budding yeast (*Saccharomyces cerevisiae*). We found that LjPCS1 was expressed in soluble form, whereas LjPCS3 was largely insoluble, due probably to the presence of an additional transmembrane domain. Yeast cells were exposed to toxic concentrations of metals and metalloids, and the PCS activity of the recombinant proteins was determined using in vivo and in vitro assays. We conclude that LjPCS1 and LjPCS3 are differentially activated by metals and propose that the differences between the two proteins in solubility and expression levels in heterologous systems as well as in metal responsiveness are due to their distinct C-terminal domains.

RESULTS

Effect of Metal Composition of Yeast Growth Media on PC Production

The complete open reading frame (ORF) of LjPCS3-7N (hereafter, LjPCS3) was initially introduced in the *Escherichia coli* vector TOPO-pET (Invitrogen). However, the recombinant protein was invariably found in inclusion bodies and could be detected on immunoblots only after extraction with urea. This is in contrast to LjPCS1-8R (hereafter, LjPCS1), a typical PCS protein that was largely produced in soluble form, purified, and characterized (Loscos et al., 2006). Because yeast cells are a suitable eukaryotic system to express PCS proteins from plants and determine in vivo tolerance to heavy metals (Clemens et al., 1999; Ramos et al., 2007), the ORF encoding LjPCS3 was introduced in the pYES2.1 TOPO TA vector (Invitrogen) and yeast cells were transformed. Again, immunoblots revealed that LjPCS3 was extracted from yeast cells only with TCA or urea and that the expression level or stability of this protein was much lower than that of LjPCS1 (Fig. 1). Despite repeated attempts to gradually remove the denaturing compounds from the enzyme preparations, the activity of LjPCS3 was lost, and hence the enzyme could not be purified. Instead, in vivo and in vitro PCS assays were developed to determine the effects of metals and metalloids on LjPCS3 activity. For comparison purposes, yeast cells bearing a LjPCS1 construct, produced with identical expression vector, were included in this study. Both recombinant proteins contained an N-terminal poly-His tag, which was expected not to affect their catalytic activities according to our previous data. Thus, purified LjPCS1 proteins having or not poly-His tag were indistinguishable with respect to their metal response (Loscos et al., 2006).

To assay for LjPCS activities, we first needed to select a yeast growth medium with low metal content to avoid interferences with PCS activity determination while allowing high cell growth rates. Two yeast nitrogen base (YNB) media were chosen, and the concentrations of 69 elements were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS). The complete medium ("+metals") and the medium lacking divalent metals contained (per gram): 6 mg sodium, 44 mg potassium (K), 42 mg phosphorus (P), 204 mg sulfur (S), and 10 μg molybdenum. In addition, the "+metals" medium contained (per gram): 4.5 mg calcium (Ca), 13.8 mg magnesium (Mg), 19.1 μg manganese, 8 μg Fe, 2 μg Cu, 17 μg Zn, 4 μg strontium, and 0.6 μg barium, whereas the medium lacking divalent metals had no detectable levels of any of these elements. Yeast cells did not grow in this medium alone due to the lack of essential metal nutrients and had to be supplemented with 5% of a subcul-

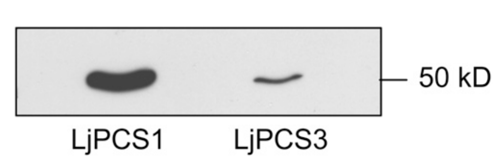


Figure 1. Immunoblot analysis of LjPCS1 and LjPCS3. Recombinant proteins bearing an N-terminal $(His)_6$-tag were extracted with TCA, resolved in 10% SDS gels, and transferred to polyvinylidene fluoride membranes. The primary antibody was a monoclonal anti-poly-His antibody (1:3,000), and the secondary antibody was a goat anti-mouse IgG peroxidase conjugate antibody (1:10,000), both from Sigma-Aldrich. Uniform loading of protein (approximately 25 μg/lane) was verified by Ponceau staining. Proteins were detected on blots using the SuperSignal West Pico chemiluminescent substrate from Pierce. Densitometric analysis of representative blots with ImageJ software (National Institutes of Health) indicated that LjPCS1 was approximately 10-fold more abundant than LjPCS3.

ture of "+metals" medium grown overnight. In the absence of Cd or other toxic heavy metals, yeast cells grew at comparable rates in the "+metals" medium and in the supplemented medium, which is hereafter designated as "−metals" medium.

The effects of PCS expression and metal composition of growth media on PC production were examined in yeast cells carrying the LjPCS1 and LjPCS3 constructs using the in vivo assay (Fig. 2). For this purpose, cells were grown in the "−metals" and "+metals" media under noninducing conditions (−Gal) for 20 h; then, 2% Gal and 50 μM Cd were added and cells were further incubated for 4 h, broken with glass beads in trifluoroacetic acid (TFA), and analyzed for PC content. We conclude that PC synthesis was considerably greater in cells grown in the "+metals" medium for both LjPCS constructs. In particular, PC_2 and PC_3 were found at very high and moderate levels, respectively, in cells expressing LjPCS3 when grown in the "+metals" medium, most probably due to the presence of other metals, especially Zn, that are known to activate PCS (Beck et al., 2003; Loscos et al., 2006).

Because the metal content of the growth media had a major effect on the Cd-induced production of PCs by the recombinant enzymes, we investigated the Cd tolerance of yeast cells grown in "−metals" medium supplemented with Ca and Mg (Fig. 3). These concentrations of divalent cations were added to the "−metals" medium, because they were found to increase cell growth within 24 h to the levels required for a reliable assessment of Cd tolerance of the three yeast constructs. As expected, yeast cells bearing any of the two LjPCS constructs or the empty plasmid (control) grew rapidly in the absence of Cd, whereas, in the presence of 100 μM Cd, only those cells expressing the LjPCS proteins grew at a significant rate (Fig. 3). Qualitatively, the same results were obtained in experiments with cells grown in solid "−metals" medium

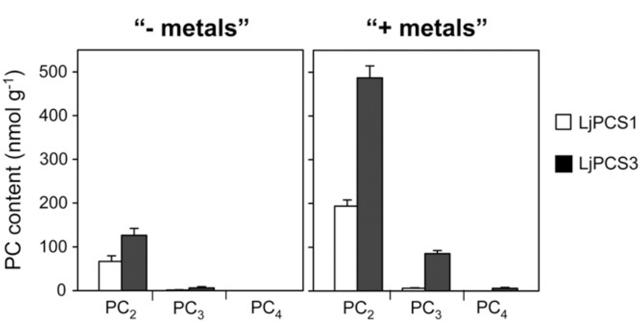


Figure 2. Comparison of PC formation by LjPCS1 (white bars) and LjPCS3 (black bars) expressed in yeast cells grown in the "−metals" and "+metals" media. Cells were grown at 30°C for 20 h, and then media were supplemented with 2% Gal to induce protein expression and with 2 mM GSH to optimize PC formation. Cells were further incubated for 4 h in the absence of added metals. The PC polypeptides were quantified by HPLC with postcolumn derivatization. Values are expressed as nmoles of PCs produced (GSH equivalents) per gram of fresh weight, and represent means ± SE of three to seven replicates, each corresponding to independently grown cell cultures.

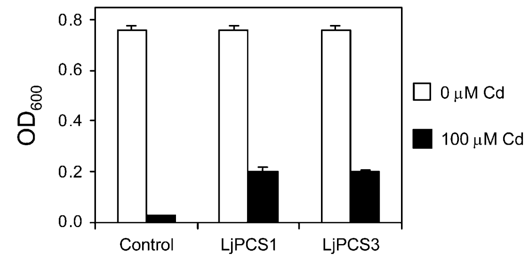


Figure 3. Assay of Cd tolerance in yeast cells bearing the empty plasmid (control) or the LjPCS1 or LjPCS3 constructs. Cells were grown at 30°C for 24 h in "−metals" medium supplemented with 0.75 mM Ca and 3.8 mM Mg and containing 0 μM (white bars) or 100 μM Cd (black bars).

supplemented with Ca and Mg and containing or not 200 μM Cd (data not shown).

Synthesis of PCs by Yeast Cells Not Expressing PCS

Several important control experiments for the in vivo assay of PC production in yeast cells were performed, and the following results were obtained. First, cells bearing the LjPCS1 or LjPCS3 constructs, and which were grown in the "−metals" medium for 24 h under noninducing conditions (−Gal) and without metal activators, were unable to synthesize PCs. The same occurred with cells carrying the empty plasmid and which were grown for 20 h omitting Gal and then for 4 h under inducing conditions (+Gal) in the absence of metal activators. Second, cells lacking the LjPCS constructs, when challenged with 50 μM Cd, produced 25 nmol of PC_2/g of fresh weight. Finally, cells expressing LjPCS1 or LjPCS3, when grown under noninducing conditions in the presence of 50 μM Cd, were able to synthesize 1,103 and 37 nmol of PC_2/g of fresh weight, respectively, indicating that there is some leaky expression of the proteins, particularly of LjPCS1, in the absence of inducer.

Differential Activation of LjPCS1 and LjPCS3 by Metals

Yeast cells expressing LjPCS1 or LjPCS3 were grown in "−metals" medium and used to assess the capacity to activate the enzymes of various metals and metalloids, which are either plant micronutrients (Zn, Cu, and Fe) or environmental pollutants with phytotoxic effects (Cd, Pb, Al, As, Hg, and Ag). Two types of PCS assays were conducted using the same concentrations of GSH and metals for each of them and correcting the activity values for the low levels of PCs produced in the absence of added metals. The in vivo assay with intact cells showed, in the first place, that LjPCS3 is a genuine PCS enzyme that produces copious amounts of PCs (Fig. 4, A and B) despite its poor homology with LjPCS1 (Ramos et al., 2007). Furthermore, in the in vivo assay, LjPCS3 was strongly activated by Zn, As, Fe, and Al (Fig. 4, A and B), especially taking into account that its

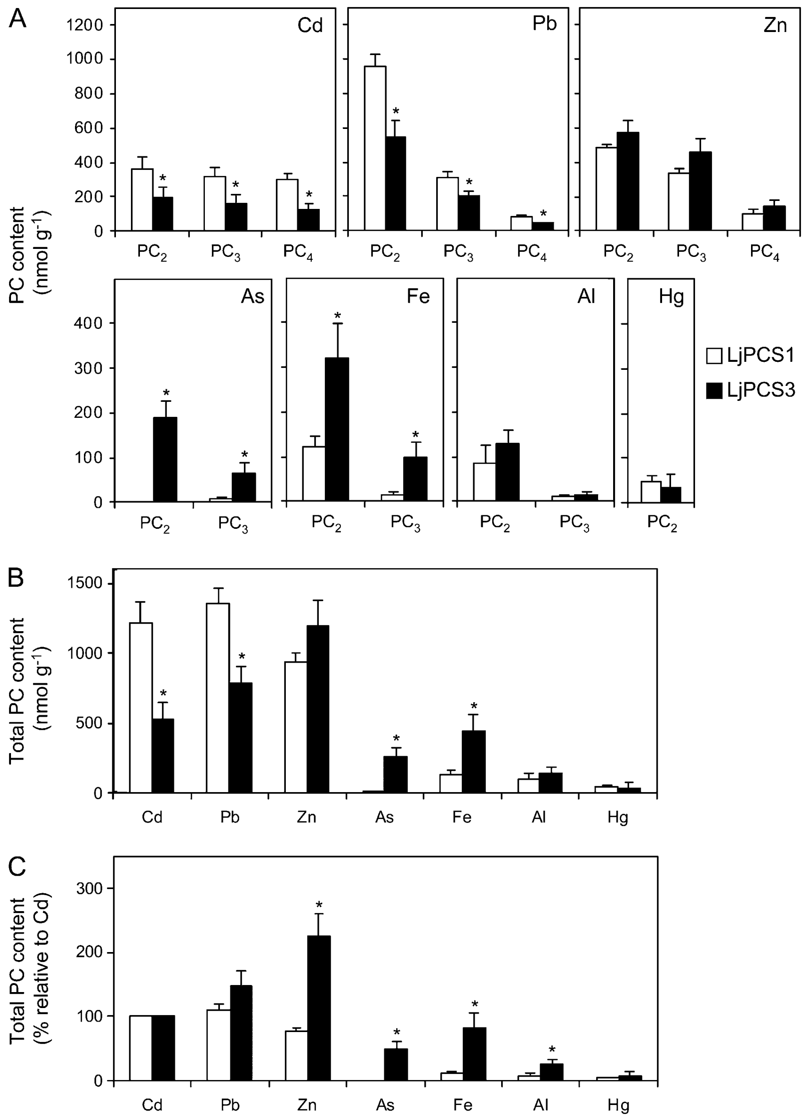


Figure 4. Formation of PC polypeptides by LjPCS1 (white bars) and LjPCS3 (black bars) in yeast intact cells (in vivo assay) exposed to metals and metalloids. Cells were grown in the "−metals" medium as described in the legend to Figure 2, except that they were incubated for 4 h with 50 μM Cd, 200 μM Pb, 200 μM Zn, 50 μM Fe, 200 μM Al, 200 μM As, 50 μM Hg, 100 μM Cu, or 100 μM Ag. Production of PCs was not detected with Cu or Ag for any of the two enzymes. A, Contents of individual PC polypeptides in nmoles of PCs produced (GSH equivalents) per gram of fresh weight. For clarity of the figure, the contents of longer PC polypeptides for Cd, Pb, and Zn were not represented. Values are given in parentheses in the same units as before (for LjPCS1 and LjPCS3, respectively): for Cd, PC_5 (146 and 41), PC_6 (68 and 12), PC_7 (18 and 0), and PC_8 (3 and 0); for Pb, PC_5 (6 and 44); and for Zn, PC_5 (15 and 14). B, Total PC contents, which correspond to the sums of the PC polypeptides indicated in A. C, Normalized PC contents, which correspond to the values of total PCs reported in A but expressed as percentage of those with Cd. For all the panels, values are means ± SE of three to six replicates, each corresponding to independently grown cell cultures, and asterisks denote significant differences between LjPCS1 and LjPCS3 based on ANOVA and Student's *t* test at $P < 0.05$.

expression level was considerably lower than that of LjPCS1 (Fig. 1). The higher activation of LjPCS3 by those metals and metalloids compared to LjPCS1 was confirmed by normalizing the data with those obtained with Cd to account for the differences in the expression levels or stabilities of the two proteins (Fig. 4C). Normalization of PC values also confirmed that Hg activated both enzymes to a similar extent, whereas it suggested that the greater production of PC polypeptides by LjPCS1 in the presence of Pb (Fig. 4B) was due to the higher expression level of this protein, as the normalized values of PC production with Pb were slightly higher for LjPCS3 than for LjPCS1 (Fig. 4C).

Because in the in vivo assay the substrate concentrations that actually reach the PCS enzymes cannot be controlled and it was not possible to purify LjPCS3 in an active form due probably to its association with cell membranes, we developed an in vitro assay of LjPCS1 and LjPCS3 activities. Dialysis of cell extracts was not necessary, as controls without added metals showed negligible PCS activity and the concentrations of endogenous metals in the extracts were below detection limits (<2 μM). The in vitro assay allowed us to compare the activating effects of the various metals on each enzyme (Fig. 5, A and B), as well as the response of the two enzymes to a particular metal when PC values were normalized with respect to Cd (Fig. 5C). The first interesting observation was that, in the in vivo assay, both LjPCS1 and LjPCS3 produced longer PC polypeptides in the presence of Cd, Pb, Fe, and Al than in the vitro assay (compare Figs. 4 and 5). Also, in the presence of As, LjPCS1 produced detectable amounts of PC_2 and PC_3 only in the in vitro assay, whereas LjPCS3 produced larger amounts of the two polypeptides during the in vivo assay (compare Figs. 4A and 5A). In the in vitro assay, normalization of the PC levels produced with the different metals with respect to those found with Cd (Fig. 5C) allowed us to confirm the conclusions drawn from the in vivo assay, namely, that As, Fe, and Al are better activators of LjPCS3 than of

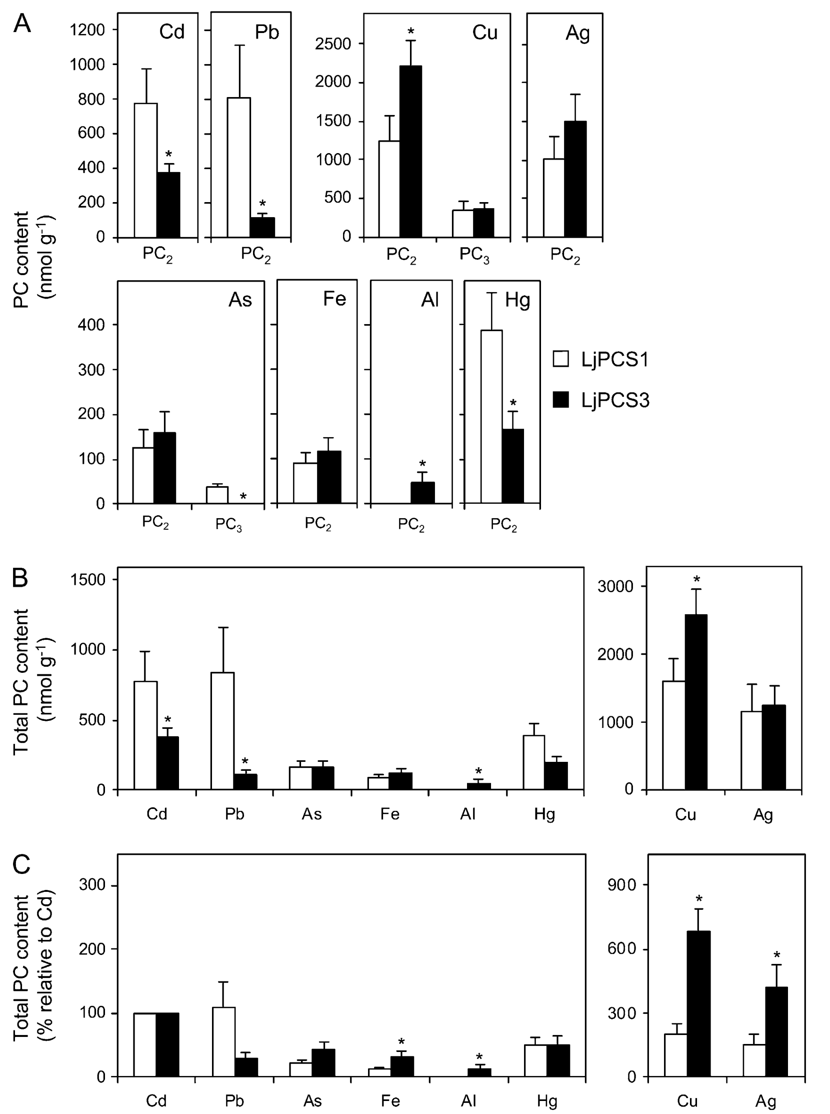


Figure 5. Formation of PC polypeptides by LjPCS1 (white bars) and LjPCS3 (black bars) in yeast cell extracts (in vitro assay) incubated with metals and metalloids. Cells were grown in the "−metals" medium as described in the legend to Figure 2, except that they were broken with glass beads and the cell extracts were incubated for 4 h with 2 mM GSH and the metals and metalloids stated in Figure 4. Production of PCs was not detected with Zn for any of the two enzymes. A, Contents of individual PC polypeptides. B, Total PC contents, which correspond to the sums of the PC polypeptides indicated in A. C, Normalized PC contents, which correspond to the values of total PCs reported in A but expressed as percentage of those with Cd. For all the panels, values are means ± SE of three to six replicates, each corresponding to independently grown cell cultures, and asterisks denote significant differences between LjPCS1 and LjPCS3 based on ANOVA and Student's *t* test at $P < 0.05$.

LjPCS1 and that Hg is similarly active with both enzymes. However, the in vitro assay revealed that Pb was a better activator of LjPCS1, even after normalization of PC values to correct for the lower expression of LjPCS3 (Fig. 5C). The activation of LjPCS proteins by Zn, Cu, Ag, Fe, and Al deserves special attention and is dealt with below.

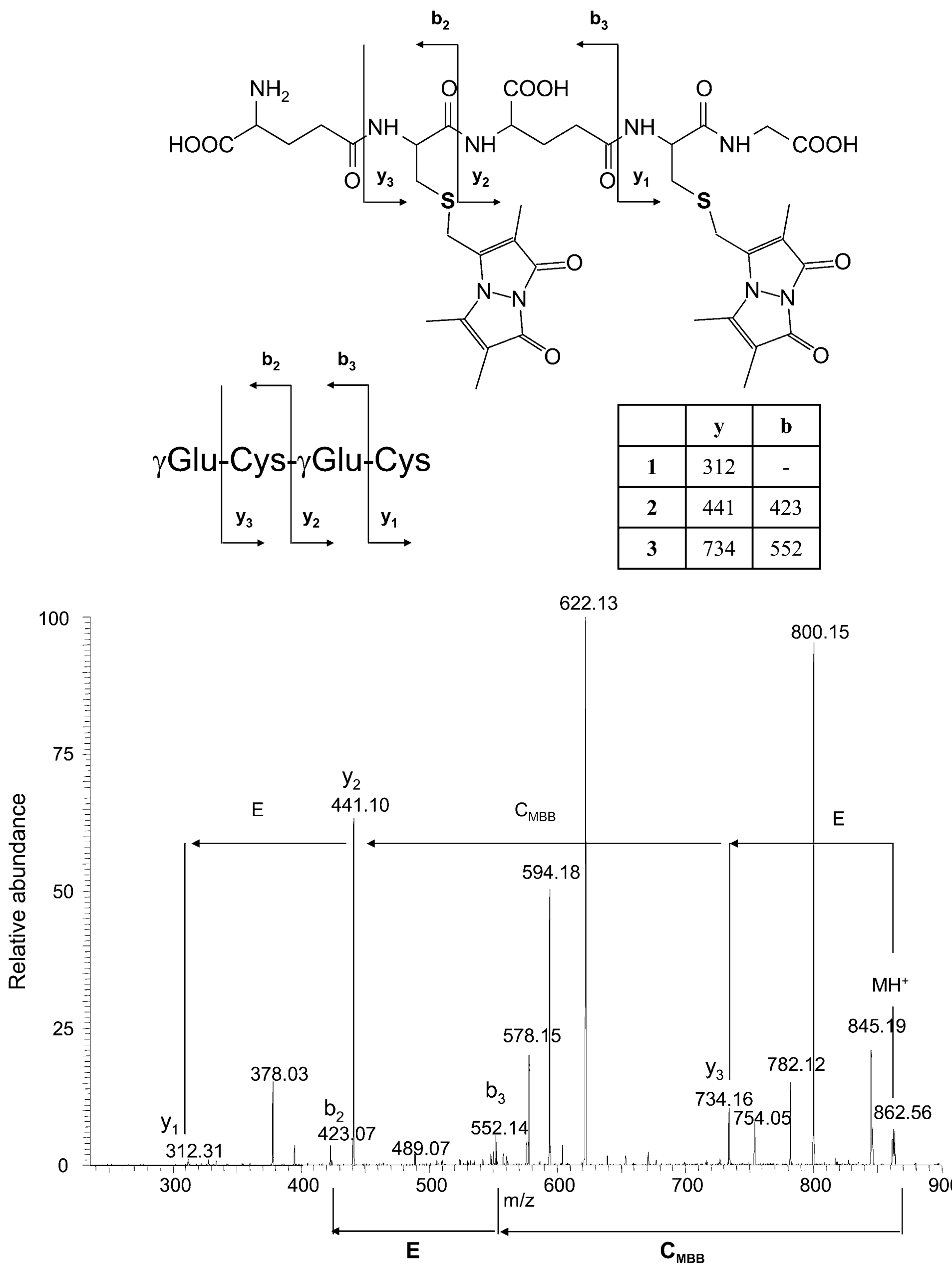
Activation of LjPCS1 and LjPCS3 by Zn, Cu, and Ag

Two major observations were made by comparing the in vivo and in vitro assays of PCS activity in yeast cells. First, we found that Zn was a powerful activator of both enzymes in the in vivo assay (Fig. 4) but not in the in vitro assay (Fig. 5). In fact, PC synthesis was barely detectable in cell extracts upon incubation with Zn. Second, we failed to detect any activation of LjPCS1 or LjPCS3 by Cu or Ag in the in vivo assay (Fig. 4), whereas both metals were by far the most potent activators of the two proteins in the in vitro assay (Fig. 5). Previous work by several groups, including ours, had shown that Zn and Cu activate purified LjPCS1 (Loscos et al., 2006), AtPCS1 (Vatamaniuk et al., 2000; Oven et al., 2002; Beck et al., 2003), and GmPCS1 (Oven et al., 2002). Also, Ag is a strong activator of PCS in yeasts and plants (Grill et al., 1989; Mehra et al., 1996; Ha et al., 1999; Oven et al., 2002). We ruled out that the lack of activating effect of Cu and Ag on the LjPCS enzymes in vivo was due to extreme cellular toxicity, because additional experiments showed that the growth of cells (initial $OD_{600} = 0.4$) exposed to 100 μM Cu or Ag for 4 h only decreased by 25% compared to untreated cells. Likewise, we verified that the absence of effect of Cu and Ag was not due to the inability of intact cells to take up these metals. To this end, cells were incubated with 100 μM Cu or Ag for 4 h, and then metals were quantified in soluble extracts using appropriate controls (cells not treated with metals, and cells treated with metals but then immediately washed). The Cu or Ag content of cells was in the range of 0.05 to

0.10 μg mg^{-1} fresh weight, whereas control cells had no detectable Cu or Ag. Consequently, the two metals did enter yeast cells under the in vivo assay conditions.

Activation of LjPCS1 and LjPCS3 by Fe and Al

The finding that LjPCS1 and LjPCS3 are activated by Fe and Al in the in vivo and in vitro assays, in contrast to previous reports (Grill et al., 1989; Oven et al., 2002), was important and required verification with two additional experiments. First, the putative PC_2 polypeptides produced by both enzymes challenged with Fe or Al were purified by HPLC and subjected to detailed structural analysis by nanoelectrospray ionization ion-trap tandem MS. The fragmentation patterns were found to be identical to those of authentic PC_2 (Supplemental Fig. S1). Second, metal chelators were included in the enzyme assays. Although there are no absolutely specific chelators for Fe and Al, desferrioxamine B (DFO) and ethylenediamine di-(*o*-hydroxyphenyl acetic acid) (EDDHA) have a very high specificity for trivalent cations. In particular, DFO binds Fe^{3+} (Gower et al., 1989) and EDDHA binds Al^{3+} (Rajan et al., 1981) with high stability constants within a broad range of pH. Both in the in vivo and in vitro assays, the addition of DFO to Fe (chelator to metal ratio of 10:1) before initiating the PCS reaction decreased LjPCS1 and LjPCS3 activities by 69% and 46%



	y	b
1	312	-
2	441	423
3	734	552

Figure 6. Identification of des-Gly-PC_2 as the major polypeptide produced by LjPCS1 upon activation by As in the in vivo assay. The polypeptide was purified by HPLC as the bimane derivative and then sequenced on an ion trap mass spectrometer equipped with a nanospray source. The resulting tandem mass spectrum of the parent ion (mass-to-charge ratio of 863) shows singly charged ions for nearly all *b* and *y* series, which were unambiguously assigned. The same mass spectra were obtained for the des-Gly-PC_2 standard and for the corresponding polypeptides produced by LjPCS3 with As or Zn.

with respect to those found with Fe alone. Similarly, preincubation of EDDHA and Al (1:1) decreased the enzyme activities by 71% and 22%, respectively. As expected, preincubation of DFO or EDDHA with Cd instead of Fe or Al had no effect on LjPCS1 activity; however, the chelators inhibited LjPCS3 activity by 47% and 14%, respectively.

Production of Desglycyl-PCs in the LjPCS Assays

Another interesting observation of this work is that yeast cells expressing LjPCS1 and especially LjPCS3 were able to produce polypeptides that are structurally related to typical PCs. These polypeptides were purified and unequivocally identified as desglycyl-PCs (des-Gly-PCs) using tandem MS (Fig. 6), high-resolution MS, and coelution with standards on the HPLC. The amounts of des-Gly-PCs produced relative to those of the typical PCs vary with the LjPCS construct and with the metal added. Thus, in the in vivo assay, particularly As, but also Cd, Pb, Zn, and Fe, elicited a significant production of des-Gly-PCs by LjPCS1 and LjPCS3 (Fig. 7). However, the amounts of des-Gly-PC_2 were considerably lower in the in vitro assay, in general <10 nmol/g of fresh weight (data not shown).

DISCUSSION

Genes encoding PCS or PCS-like proteins are widespread in very distant organisms, including cyanobacteria, algae, ferns, fungi, and nematodes (Cobbett and Goldsbrough, 2002). Interestingly, budding yeast seems to be one of the exceptions and has been used, along with PC-deficient mutants of fission yeast (*Schizosaccharomyces pombe*), to characterize the PCS proteins of plants (Clemens et al., 1999; Ha et al., 1999). We have used budding yeast cells as a heterologous system to express two PCS enzymes of *L. japonicus* and compare their response to an array of metals. For this study, it

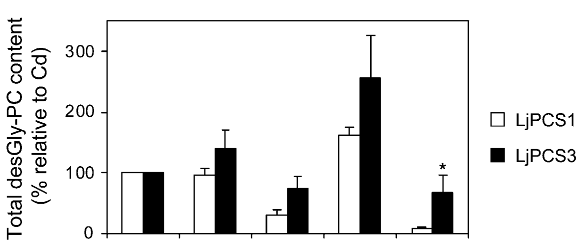


Figure 7. Production of des-Gly-PCs in yeast intact cells (in vivo assay) expressing LjPCS1 (white bars) or LjPCS3 (black bars) upon exposure to metals and metalloids. Values were normalized with respect to Cd, and the 100% values for LjPCS1 and LjPCS3 correspond, respectively, to 198 ± 27 and 106 ± 28 nmol of des-Gly-PCs produced (GSH equivalents) per gram of fresh weight. Des-Gly-PCs were not detected with Cu, Ag, Al, or Hg. Des-Gly-PC_2 accounted for >76% of the total des-Gly-PCs produced by LjPCS1 and LjPCS3. Values are means ± SE of three to six replicates and asterisks denote significant differences between LjPCS1 and LjPCS3 at $P < 0.05$.

was first necessary to verify the metal composition of the yeast growth media and the capacity of wild-type and transformed cells to synthesize PCs. We found that budding yeast cells transformed with the empty plasmid were able to produce PC_2 when challenged with 50 μM Cd but not when the metal was omitted. Because this microorganism is devoid of *PCS* genes (Cobbett and Goldsbrough, 2002), it must possess an alternative metal-dependent mechanism for PC synthesis. Such a mechanism could involve, at least in part, the action of two vacuolar Ser-carboxypeptidases, as recently outlined by Wünschmann et al. (2007). We also conclude from our study that the yeast growth media should be carefully chosen for metal activation assays of PCS enzymes. Otherwise, in a complete ("+metals") medium containing Zn and other divalent metal activators of PCS, background PC levels may hamper detection of PC synthesis when yeast cells are challenged with poorly activating metals.

The expression level and/or stability of LjPCS3 in both *E. coli* and yeast cells was remarkably lower than that of LjPCS1. According to the prediction programs of secondary structure, TMpred (Hofmann and Stoffel, 1993) and TopPred (Claros and von Heijne, 1994), LjPCS3 contains an additional transmembrane region compared to LjPCS1, which is consistent with the different solubility properties of the two proteins. The use of an in vivo assay circumvented the problem of activity loss associated with solubilization of membrane-bound LjPCS3 and allowed for an initial characterization of LjPCS3 as a genuine PCS enzyme. This assay did not permit us, however, to ascertain the concentrations of GSH, metals, and metal-GS thiolates reaching the enzymes, as they may be affected by factors such as the different rates of transport of the metal-GS thiolates through the plasma membrane or the existence in cells of other metal detoxification mechanisms. The study of metal activation was therefore complemented with the measurement of LjPCS activities in cell extracts. Compared to the in vivo assay, the in vitro assay of LjPCS1 and LjPCS3 yielded lower levels of total PCs with Cd, Pb, Fe, or Al and higher levels with Hg. For a specific metal ion, the differences between the in vivo and in vitro assays in the amounts of PCs and in the lengths of the PC polypeptides could be explained by variations in the substrate access to the enzymes, in the protein integrity, and in the pH of the PCS reaction. However, the results of both assays allowed us to conclude that LjPCS3 is more active than LjPCS1 with Fe, Al, and As, and similarly active with Hg. The in vivo assay also showed that Zn is a good activator of both enzymes, especially of LjPCS3. This finding is fully consistent with a strong activation of purified LjPCS1 by Zn (Loscos et al., 2006) and is therefore at odds with the results of the in vitro assay. The lack of any activating effect of Zn in vitro cannot be ascribed to PC degradation by Zn-dependent peptidases, as shown by control experiments in which PC_2, PC_3, and PC_4 standards along with Zn were added to the assay medium. Thus, we cannot offer an explanation for the discrepancy of the effects of Zn between the in vivo and in vitro assays, except perhaps that other mechanisms for Zn scavenging could be activated by breakage of yeast cells and organelles during the in vitro assay. In sharp contrast with Zn, Cu and Ag activated the enzymes only in the in vitro assay. Because these two metals entered the cells, it is likely that additional mechanisms, including chelation of Cu or Ag by metallothioneins, are operating in vivo. Thus, transcription of the metallothionein gene *CUP1* of budding yeast is apparently restricted to Cu and Ag (Fürst et al., 1988), and the Cup1 protein (Cu-thionein) binds Cu^{+} and Ag^{+} ions with high efficiency and is involved in Cu detoxification and homeostasis (Jensen et al., 1996; Calderone et al., 2005). Other explanations for the lack of effect of Cu and Ag may also be invoked. Thus, a rapid transport of the Cu-GS and Ag-GS thiolates to the vacuoles in vivo would prevent PCS activation.

We also conclude in this work that LjPCS1 and, to a greater extent, LjPCS3 are activated by Fe and Al. This was demonstrated by detailed MS analysis of the PC products and by the use of metal chelators. Previous work by Oven et al. (2002) failed to observe any activation of AtPCS1 and GmPCS1 by Fe or Al, a discrepancy that may reflect differences in the proteins themselves or in the activity assays. The particularly high activation of LjPCS3 by Fe and Al could be ascribed to its unusual C-terminal region and may be of physiological interest. In plants, Fe is an essential micronutrient (Mengel and Kirkby, 2001) but can also catalyze, at micromolar concentrations, Fenton reactions (Halliwell and Gutteridge, 2007). On the other hand, Al is a nonessential element that inhibits root cell growth (Ma et al., 2001). Although the major mechanisms for Fe and Al detoxification in plants involve, respectively, ferritin (Briat and Lobréaux, 1997) and exudation and/or chelation with organic acids (Ma et al., 2001), the PCS enzymes could play a complementary protective role under conditions where these metals are largely mobilized, such as in acidic soils of tropical regions.

The aforementioned differences in the activation rates of LjPCS1 and LjPCS3 elicited by most metals and metalloids may rest on the C-terminal (putative metal-sensing) domains of the two proteins, which have only 43% identity in the last 200 amino acid residues. This is consistent with the finding by Ruotolo et al. (2004) that the loss of the C-terminal region of AtPCS1 impairs PC synthesis by some metals (Zn and Hg) but not by others (Cd and Cu), which suggests that the interactions of the proteins with the free metal ions, their thiolates, or both, modulate enzyme activation.

The detection of des-Gly-PCs in the LjPCS assays may also be of interest. These PC structural variants have been reported in certain yeasts (Mehra and Winge, 1988; Hayashi et al., 1991; Barbas et al., 1992) and higher plants (Klapheck et al., 1994; Meuwly et al., 1995; Maitani et al., 1996) exposed to metals, but their origin is unclear. We have not pursued the study of des-Gly-PCs, as it is beyond the scope of this article. However, the finding of lower amounts of these polypeptides in vitro than in vivo would suggest that they can be formed, at least in part, by removal of the C-terminal Gly from PCs by endogenous proteases during the in vivo assay. Alternatively, it has been reported that des-Gly-PCs can be synthesized from GSH and γGlu-Cys by PCS in the fission yeast (Hayashi et al., 1991) and may be also functional in heavy metal detoxification (Mehra and Winge, 1988; Barbas et al., 1992).

In summary, we have found that budding yeast cells expressing LjPCS1 or LjPCS3 show increased in vivo tolerance to Cd. The two proteins are expressed at different levels in cells and show distinct activation responses to a range of metals, including Cu, Ag, Zn, Fe, and Al, probably as a result of the major differences in their C-terminal domains.

MATERIALS AND METHODS

Yeast Growth and Metal Analysis of Growth Media

Yeast (*Saccharomyces cerevisiae* INVSc1) cells (Clemens et al., 1999) were grown in YNB complete "+metals" medium (Pronadisa) or in YNB medium without divalent cations (Q-BIOgene) but containing 5% (v/v) of "+metals" medium. The latter was designated as "−metals" medium and was used throughout the study. Both media were used at a concentration of 6.7 g L^{-1} and were supplemented with 0.77 g L^{-1} of CSM-minus uracil (Q-BIOgene) and 2% D-(+)-raffinose (Fluka).

Semiquantitative analyses of metals in yeast growth media were performed by ICP-AES (Optima 3200RL; Perkin-Elmer) and ICP-MS (ELAN6000; Perkin-Elmer) using conventional protocols. Sodium, Mg, P, S, K, Ca, and Fe were determined by ICP-AES and the other elements by ICP-MS.

Expression of Recombinant Proteins in Yeast Cells and Cd Tolerance Assay

The ORFs encoding of LjPCS1 and LjPCS3 were introduced in the Champion pET directional TOPO bacterial expression vector (Invitrogen), and the DNAs encoding the fusion proteins with N-terminal poly-His tags were PCR amplified, introduced in the pYES2.1 TOPO TA vector, and used to transform yeast cells according to the supplier's protocol (Invitrogen).

For the Cd tolerance assay, cells bearing the empty plasmid or the LjPCS constructs were grown in "−metals" medium containing 0.75 mM Ca, 3.8 mM Mg, and plus or minus 100 μM Cd. The initial OD_{600} was 0.020 and cells were grown at 30°C for 24 h.

MS Analysis of PC and Des-Gly-PC Polypeptides

Nanoelectrospray ionization ion-trap tandem MS experiments were performed using a Finnigan LCQ ion trap mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray source (Protana). The spray voltage applied was 0.85 kV and the capillary temperature was 110°C. The isolation window was 3 mass units wide and the relative collision energy was 25% to 50%.

Assay of LjPCS Activities

For the in vivo assay of PCS activity, yeast cells (initial OD_{600} of 0.1) were incubated overnight at 30°C in "−metals" medium for 20 h, and protein expression was induced for 4 h with 2% Gal. At the time of induction, cultures were supplemented with 2 mM GSH and with one of the following metals or metalloids: 50 μM $CdCl_2$, 200 μM $PbCl_2$, 200 μM $ZnSO_4 \cdot 7H_2O$, 50 μM $FeCl_3 \cdot 6H_2O$, 200 μM $AlCl_3$, 200 μM KH_2AsO_4, 50 μM $HgCl_2$, 100 μM $CuCl_2 \cdot 2H_2O$, or 100 μM $AgNO_3$. After 4 h, cells were collected by centrifugation (5,000g × 2 min, 4°C), washed twice with distilled water, and resuspended in 0.1% (v/v) TFA and 0.5 mM diethylenetriaminepentaacetic acid. Cells were lysed at 4°C by vigorous vortexing with glass beads (425–600 μm, Sigma-Aldrich) in 3 × 4-min pulses. The lysates were cleared by centrifugation (13,000g × 15 min, 4°C), the supernatants were stored overnight at −80°C, and PCs were analyzed by HPLC.

For the in vitro assay of PCS activity, the same protocol as for the in vivo assay was followed for growing yeast cells, with some modifications. After the 4-h induction with Gal but without GSH, cells were collected by centrifugation, washed with distilled water, and resuspended in 300 mM Tris-HCl (pH 8.0) containing 2 mM β-mercaptoethanol. To assay for PCS activity, yeast cells were then broken with glass beads, and to the extracts, without removing the glass beads, an additional 1 mM β-mercaptoethanol, 5 mM GSH, and the metals or metalloids (preincubated for 1 min with GSH), at the concentrations indicated above, were added. After incubation of samples at 35°C for 2 h, the reactions were stopped by the addition of 1.3% TFA (final concentration). Samples were cleared by centrifugation, and the supernatants were stored overnight at −80°C and analyzed for PC content the next day.

Quantification of PC and Des-Gly-PC Polypeptides

The contents of individual PC and des-Gly-PC polypeptides in yeast cell extracts were determined by HPLC using postcolumn derivatization with 5,5′-dithiobis(2-nitrobenzoic acid), as described (Loscos et al., 2006; Ramos et al., 2007). Standards of PC_2, PC_3, des-Gly-PC_2, and des-Gly-PC_3 were synthesized chemically (Biosyntan). For MS structural analyses, the polypeptides were purified either by HPLC with precolumn derivatization with monobromobimane using fluorescence detection with excitation at 380 nm and emission at 480 nm (Else et al., 2000) or by HPLC without derivatization using detection at 220 nm. In the latter case, the isolated polypeptides were treated, at room temperature and in the dark, with 10 mM dithiothreitol for 1 h and then with 55 mM iodoacetamide for 1 h to ensure complete reduction and alkylation of the thiol groups, respectively.

Chelator Studies

To investigate the effect of metal chelators on PCS activity, yeast cells were grown for 20 h as indicated above, and then 2% Gal, 2 mM GSH, and a mixture of the metals and chelators were added. The metal salts (50 μM $FeCl_3 \cdot 6H_2O$, 50 μM $CdCl_2$, 200 μM $AlCl_3$) and chelators (500 μM DFO, 200 μM EDDHA) were preincubated for 30 min before addition to the media to allow chelate formation. Cell cultures were subsequently incubated for 4 h and processed in the same way as above for PC analysis.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY633847 (LjPCS1-8R) and DQ013041 (LjPCS3-7N).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Identification of PC_2 as the major polypeptide formed by LjPCS1 and LjPCS3 upon activation by Al in the in vivo assay.

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