Title	Chelation of cadmium ions by phytochelatin synthas : role of the cystein-rich C-terminal
Author(s)	VESTERGAARD, Mûn'delanji; MATSUMOTO, Sachiko; NISHIKORI, Shingo; SHIRAKI, Kentaro; HIRATA, Kazumasa; TAKAGI, Masahiro
Citation	Analytical Sciences, 24(2): 277-281
Issue Date	2008-02
Type	Journal Article
Text version	publisher
URL	http://hdl.handle.net/10119/8550
Rights	Copyright (C) 2008 . Mûn'delanji VESTERGAARD, Sachiko MATSUMOTO, Shingo NISHIKORI, Kentaro SHIRAKI, Kazumasa HIRATA, Masahiro TAKAGI, Analytical Sciences, 24(2), 2008, 277-281. http://dx.doi.org/10.2116/analsci.24.277
Description	

Notes

Chelation of Cadmium Ions by Phytochelatin Synthase: Role of the Cystein-rich C-Terminal

Mun'delanji Vestergaard,[*1] **Sachiko Matsumoto,**[*1] **Shingo Nishikori,**[*2] **Kentaro Shiraki,**[*3] **Kazumasa Hirata,**[*4] **and Masahiro Takagi**[*1†]

[*1] *School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923–1292, Japan*
[*2] *Division of Molecular Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Kumamoto 862–0976, Japan*
[*3] *Institute of Applied Physics, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305–8573, Japan*
[*4] *Department of Environmental Bioengineering Laboratory, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565–0871, Japan*

The interactions between Cd^{2+} and the C-terminal region of phytochelatin (PC) synthase using recombinant wild-type and mutant PC synthase were studied. We show that site-directed mutagenesis of Cys residues at $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif decreases the number of Cd^{2+} and other heavy metal ions interacting with the enzyme, and that the motif binds the metals discriminatingly. The optimum binding ratio of PC synthase to Cd^{2+} was also determined. The findings indicate that Cys exists as a free SH residue and that it is involved in the regulation of PC enzyme activity by transferring the metals into closer proximity with the catalytic domain. These results are important in understanding heavy metal detoxification mechanisms in higher plants, a step towards phytoremediated-applications.

Cadmium (Cd) is a heavy metal naturally present in soil. Since it is available for uptake by plants and subsequent human uptake, it poses a serious health risk to humans. It is a carcinogen that accumulates in the kidney cortex and is a cause of end-stage renal disease.[1] Unlike essential heavy metals such as copper and zinc which are required for a range of plant physiological processes, Cd is not required, but instead interferes with those that are. Plants can provide the much needed remediation by stabilizing the Cd in the soil, and/or by extracting the Cd from the soil. The biomass containing Cd can then be harvested afterwards.[2,3] Heavy metal cations impede cation movement in vascular plants, particularly in the negatively charged cells of the xylem. A general solution to this problem is chelation, which is generally understood as the process of a cation binding to a compound resulting in a neutrally-charged complex that can move more freely through a variety of substrates. Several chelators, both natural and synthetic, are known to perform this very function in soil and in plants. Natural chelators include phytochelatins (PC), metallothionein (MT) and organic acids.[4,5]

Binding sites for metal ions are found in a myriad of proteins. Characterization of the interaction between metal cations and proteins provides interesting and valuable information about structure-function relationships of metal proteins. Preferred ligands for soft and borderline ions such as Cu^{2+}, Zn^{2+} and Cd^{2+} are thiolates and amines. Consequently, known metal-binding sites in most cases contain Cys or His residues. Many of these sites consist of contiguous short stretches of amino acids.

Sequence overlaps between neighboring peptides with binding activity are used to tentatively determine major binding motifs. The majority of proteins and peptides that function in the uptake, distribution, storage or detoxification of essential and non-essential metal ions possess one or several metal-binding sites. The -Cys-X-X-Cys- and -Cys-Cys- motifs of various metallothioneins are well known.[6,7] Putative metal-binding sites have also been identified in several metal transporters. Members of the ZIP (ZRT-, IRT-like proteins) and the cation diffusion facilitator (CDF) families show His-rich cytoplasmic loops between transmembrane domains.[8,9] Most metal-pumping CPx-type ATPases such as RAN1[10] display varying numbers of N-terminal metal-binding domains consisting of -Cys-X-X-Cys-motifs.[11] These are also found at the surface of metallochaperones such as Atx1 and its plant homolog CCH that deliver copper ions to copper-transporting ATPases.[12,13]

Phytochelatins (PC), small polypeptides (1.5 – 4 kDa), were first isolated from cell suspension cultures in 1985.[14] They are produced inductively in plants by PC synthase upon exposure to Cd and other heavy metals and oxyanions (Fig. 1).[15] In higher plants, although PC synthase is a constitutive enzyme in general, its expression can be regulated in some organs, upon exposure to toxic heavy metal ions. PC synthase genes in higher plants and in fission yeast *Schizosaccharomyces pombe* were identified and characterized, for the first time, in 1999.[14] Phytochelatin chelate Cd ions and the formed chelate (Cd:PC) is translocated to the vacuole, where high molecular weight complexes are formed with the incorporation of sulfide (Fig. 2).[16] A highly homologous N-terminal region of PC synthase has been reported among eukaryotes[17] and that the conserved Cys^{56}, Cys^{90}, Cys^{109}, Cys^{113}, residues are reported to play an important

† To whom correspondence should be addressed.
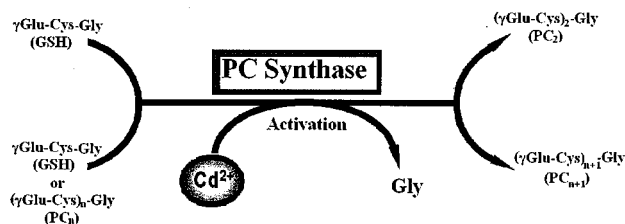E-mail: takagi@jaist.ac.jp

Fig. 1   Phytochelatin synthesis catalyzed by phytochelatin synthase upon activation by cadmium ions.
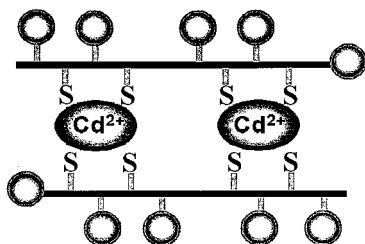

Fig. 2   PC-Cd complexes.   Spheres denote the bulky anionic carboxylate groups on the higher order Cd-PC complexes that accumulate.

roles in heavy metal-induced PC catalysis.[18]   In slight contrast, Vatamaniuk *et al.* and Tsuji *et al.* reported that of the four Cys residues, only Cys[56] is involved in PC synthesis.   Also, the exact point at which dependency of Cd in PC synthesis is manifested differed between the work by Vatamaniuk and colleagues and that of the Tsuji group.[19,20]

It is generally accepted that the active site region of PC synthases is located in the N-terminal region, which is well-conserved among PC synthases.   However, the Cys-rich C-terminals of *Schizosaccharomyces pombe* and *Arabidoptis spp* suggest that PCs may play a wider role in heavy metal detoxification than previously expected, through interactions involving the C-terminal.   Cobbett proposed a model for the function of the C-terminal domain of PC synthase.[17]   The model presents the C-terminal domain as a local sensor of heavy metal ions, such as Cd.   The Cys residues bind Cd ions, bringing them into closer proximity and transferring them to the activation site in the N-terminal, catalytic domain.   Previously, we reported that the C-terminal region of PC synthase exhibited homology to functional domains of metallothionein and metallochaperone, and we hypothesized that the C-terminal region might play an important role in the regulation of PC synthesis.[21]   Derivatives of PC synthase in which Cys was replaced with Ala at the C-terminal region were prepared.   Interactions of the native and mutant enzymes with metal ions were analyzed by HPLC; and the results suggested that the C-terminal region might have metal specificity properties.   In this paper, we discuss the interactions between Cd and the C-terminal region of the PC synthase.   Further, we identify the potential role of Cd in enzymatic activation of PC synthase, through the C-terminal.

Experimental

Materials

Unless otherwise stated, all other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).   All solutions were prepared and diluted using ultra-pure water (18.3 MΩ cm^{−1}) from a Millipore Milli-Q system.

Apparatus

All cadmium and zinc ion analyses were carried out using an SPS 7700 plasma spectrometer (Seiko Instruments Inc., Japan) for inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Procedure

Expression and purification of wild-type and mutant phytochelatin synthase proteins.   A recombinant wild-type ($C^{358}C^{359}XXXC^{363}XXC^{366}$) PC synthase protein and a mutant ($A^{358}A^{359}XXXA^{363}XXA^{366}$) PC synthase protein were prepared as described by Matsumoto *et al.*[21]   Protein concentration was measured using a Pierce BCA™ protein assay reagent kit according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Assay for phytochelatin synthase activity.   PC synthase activity was measured according to the method of Oven *et al.*[22]   The reaction mixture (125 μL) contained 200 mM Tris–HCl buffer (pH 8.0), 10 mM 2-mercaptoethanol (2-ME), 10 mM glutathione (GSH), 0.5 mM metal chloride ($CdCl_2$, $CuCl_2$, $ZnCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $CaCl_2$ or NaCl), and 0.2 μg of the wild-type or mutant PC synthase enzyme.   The reaction mixture was incubated at 35°C and the reaction was terminated by addition of 125 μL of 3.6 N HCl.   The supernatant obtained after centrifugation (10000*g* for 10 min at room temperature) was analyzed using HPLC.

Cadmium and zinc contents of wild-type and mutant phytochelatin synthase proteins.   Determinations of the cadmium and zinc ion concentrations in wild-type and mutant PC synthase proteins were carried out according to the manufacturer's instructions, at wavelengths of 225 and 213.9 nm, respectively ($\lambda_{226.5nm}$ and $\lambda_{213.9nm}$), using ICP-AES.   The reaction mixture (1.5 mL) contained 200 mM Tris–HCl buffer (pH 8.0), 10 mM 2-ME, 10 ppm Cd or Zn, and 0.5 mM the wild-type or mutant PC synthase proteins.   Each reaction mixture was incubated for 3 h at 35°C.   After incubation, the mixture was dialyzed for 12 h against 10 mM Tris–HCl buffer (pH 8.0) containing 10 mM 2-ME, 1 mM dithiothreitol (DTT), 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM phenyl methyl sulfonyl fluoride (PMSF).   Each reaction was terminated by addition of 1.5 mL of 3.6 N HCl.

Results and Discussion

In PC biosynthetic reactions, PC synthase is active only in the presence of metal ions.   Although the exact mechanism of enzyme activation by free metal ions has not been determined,[18-23] reactions continue until the activating metal ions have been chelated.[24,25]   Affinity-purified PC synthase preparations require divalent heavy metal ions such as Cd II, Zn II or Cu II.[26]   The C-terminal regions of AtPCS1, a PC synthase from *Arabidopsis spp* have 10 Cys residues of which 4 are adjacent pairs.   It seems that the less-conserved Cys residues, often presented in pairs, have a role as a binding sensor for heavy metals.   However, no clear role of these Cys residues has been characterized.   To investigate the role of the Cys residues in the C-terminal region of the AtPCS1, we carried out site-directed mutagenesis; Cys[358], Cys[359], Cys[363] and Cys[366] were changed to Ala residues (Fig. 3).

Wild-type

-Cys-Cys-Arg-Glu-Thr-Cys-Val-Lys-Cys
358 359   363   366

NH₂ ▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬ COOH
     1   358 366   485

Mutant

-Ala-Ala-Arg-Glu-Thr-Ala-Val-Lys-Ala
358 359   363   366

NH₂ ▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬ COOH
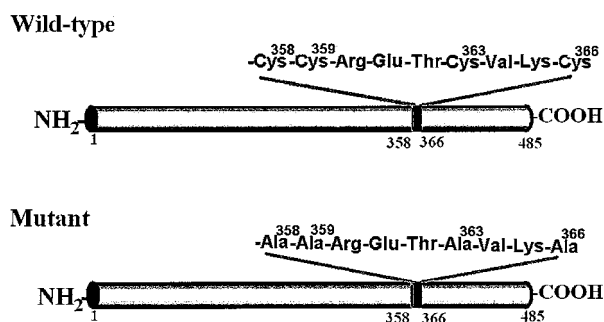   1   358 366   485

Fig. 3   Illustration of wild-type and mutant PC synthases. The PC synthase protein has a Cys-rich region in the C-terminal domain. The mutant replaced Cys^{358}, Cys^{359}, Cys^{363} and Cys^{366} with Ala.

Fig. 4   HPLC profiles of the interaction between wild-type and mutant PC synthases and metal ions. 0.5 mM $CdCl_2$, $CuCl_2$, $ZnCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $CaCl_2$ or NaCl was used for the analysis.

Table 1   Melting temperatures of the wild-type and mutant enzymes with and without Cd^{2+}

Protein	T_m/°C
Wild-type	54.8 ± 0.2
Mutant	55.5 ± 0.2
Wild-type with Cd	57.8 ± 0.2
Mutant with Cd	56.6 ± 0.2

Melting temperatures (T_m) were calculated from the temperature-induced curves obtained by monitoring the absorbance of Cd^{2+} at 222 nm.

Table 2   Cadmium to protein and zinc to protein ratios

Sample type	[Heavy metal]/[PC synthase]
Wild-type with Cd	7.54 ± 0.03
Mutant with Cd	5.73 ± 0.07
Wild-type with Zn	7.78 ± 0.31
Mutant with Zn	4.83 ± 0.11

The metal content determined by ICP-AES was 226.5 nm Cd and 213.9 nm Zn. The protein at concentration of 0.5 µM was dialyzed against a buffer containing 200 mM Tris–HCl (pH 8.0), 10 mM 2-ME and 10 ppm Cd or Zn.

Effect of Cd^{2+} binding on protein stability

First, we examined the effect that the elimination of the Cys residues at positions Cys^{358}, Cys^{359}, Cys^{363} and Cys^{366} had on Cd^{2+} binding and the stability of PC synthase, as described by Kanaya et al.[27] The melting temperatures (T_m) of wild-type and mutant enzymes were 54.8 and 55.5°C, respectively (Table 1). This was determined by following the denaturation profile of the enzymes using circular dichroism (CD), and T_m represents the mid-point denaturation temperature of the enzymes. There was no significant difference in enzyme stability between the wild-type and mutant enzymes in the absence of Cd^{2+}, indicating that the introduction of point mutations into the cysteine residues does not affect the global protein structure of the wild-type PC synthase. In the presence of 5 µM Cd^{2+}, the T_m values of the wild-type and mutant proteins were 57.8 and 56.6°C, respectively.   The T_m of the wild-type protein increased by 3.0°C in the presence of Cd^{2+}, while the T_m of the mutant protein increased by 1.1°C. These results show that introduction of mutations did not affect stability of the enzymes and that subsequent work could be carried out without concern. The results also show that the overall capacity for Cd^{2+} to the protein was reduced by the elimination of the cysteine residues at Cys^{358}, Cys^{359}, Cys^{363} and Cys^{366}, indicating a strong correlation between the C-terminal Cys-rich region and its capacity for Cd^{2+}.

Metal specificity of $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif

Interactions of the native $C^{358}C^{359}XXXC^{363}XXC^{366}$ and mutant motifs with metal ions (Cd^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} Mg^{2+}, Mn^{2+}, Ca^{2+} and Na^+) were analyzed using HPLC.   Figure 4 shows the amount of thiol groups detected in the complexation mixture. The number of thiol groups present is directly proportional to the PC synthesis activity and the catalytic activity of PC synthase. Our results clearly show that the PC-synthesizing reaction was markedly influenced by the type of metal ion present. In the
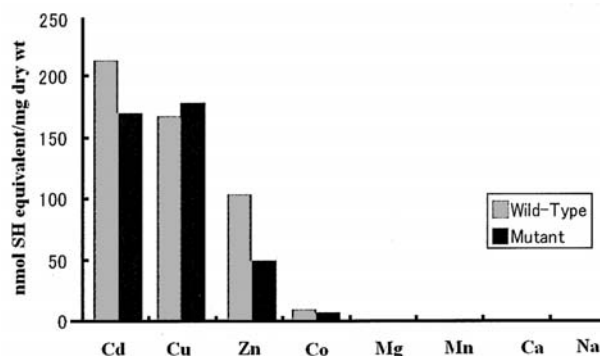
presence of Mg^{2+}, Mn^{2+}, Ca^{2+} or Na^+, PCs were not synthesized by either wild-type or mutant PC synthase. In the presence of Cd^{2+} and Zn^{2+}, more PCs were synthesized by the wild-type PC synthase than by the mutant enzyme. In contrast, the presence of Cu^{2+}, triggered production of more PCs by mutant enzymes than by the wild-type enzymes. The results demonstrate that the specificity of PC synthase was changed by the elimination of the Cys residues at the positions Cys^{358}, Cys^{359}, Cys^{363} and Cys^{366}. We therefore affirm that the $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif of the PC synthase serves as a sensor for heavy metal ions. These results are in agreement with a model proposed by Cobbett.[17] Our work further demonstrates not only that the C-terminal has a role, but also confirms unequivocally through site-directed mutagenesis, that the Cys residues play a role in binding cadmium and other heavy metal ions. Further, the differential metal-binding capacity is presented.

Determination of the ratios of heavy metals to wild-type and mutant PC enzymes

We examined the effect that elimination of the Cys residues, at the $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif of PC synthase, had on Cd^{2+} or Zn^{2+} binding to the enzyme.   Using ICP-AES, we analyzed the interaction between Cd^{2+} and Zn^{2+} ions and the wild-type and mutant enzymes according to Washabaugh and Collins.[28] We determined the ratio of wild-type PC synthase to Cd^{2+} at 1:7, and the ratio of mutant enzyme to Cd^{2+} at 1:5. The results suggest that two Cd^{2+} ions bind at the C-teminal $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif of the enzyme. In comparison, the ratio of wild-type enzyme to Zn^{2+} was 1:7, and the ratio of mutant enzyme to Zn^{2+} was 1:4 (Table 2). The stoichiometry of Cd^{2+} to PC synthase protein was larger than that of Zn^{2+}. Interestingly, the number of Zn^{2+} ions interacting with the mutant enzyme was less than the number for Cd^{2+}. This might
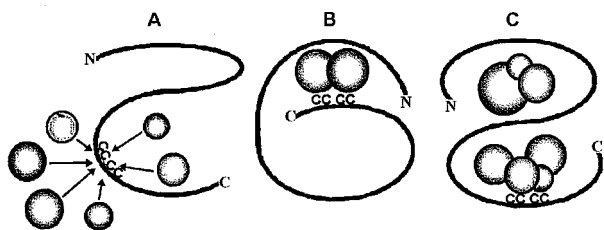
Fig. 5   Model of heavy metal binding mechanism of C-terminal region. (A) Metal sensor function, (B) metallochaperone function, (C) metallothionein function. N-terminal region consists of residues 1 – 221 and the C-terminal region consists of residues 222 – 485. Filled circles show heavy metal.   The four key Cys residues are indicated as CC CC.

be because the enzyme has a higher specificity to Cd^{2+} and Cys residues in the C-terminal region, which might help in transferring the cadmium ions to the N-terminal for interaction. The results indicate that the overall capacity of the heavy metal to the protein is also reduced by the elimination of the Cys residues at the $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif. The C-terminal region binds Cd ions at high capacity, but has a much lower capacity for other metal ions, such as Zn^{2+}, which are equally effective activators for PC synthesis.

   From the above, we propose an advanced model of PC synthesis. As shown in Fig. 5, a putative metal sensor located in the C-terminal region may function to determine the type of heavy metal ion (A).   After sensing the presence of a heavy metal ion, the C-terminal region transfers the heavy metal ions, essential for enzymatic activity, to the N-terminal catalytic domain. The C-terminal region may function as the metallochaperone to transfer metal ions (B).   Although transition elements can be quite toxic, these metal receptors are not detoxification proteins; they clearly function in a metallochaperone-like manner, guiding and protecting the metal ion while facilitating appropriate partnerships. Models have been proposed in which the C-terminal region, perhaps in concert with the N-terminal region, directly inserts Cd^{2+} into the active site of the PC synthase.   Metallothioneins are ubiquitous, low-molecular-weight Cys-rich proteins, which bind metal ions in *meta*-thiolate clusters (C).   Metallothioneins are known to confer Cd^{2+} tolerance. It has been hypothesized that the differences among Cys motifs (*e.g.*, -Cys-Cys-, -Cys-X-Cys- and -Cys-X-X-Cys-) could account for differences in metal specificities.[39]

   Various amino acid sequence motifs which may function as interaction sites with heavy metals have been reported. Some representative motifs could also be found in the amino acid sequence of the PC synthase. For example, ZntA, a P_{1B}-ATPase transporter from *E. coli*, mediates resistance specifically to Pb^{2+}, Zn^{2+} and Cd^{2+} by active efflux.[30] ZntA has a hydrophilic N-terminal domain that contains the -Gly-X-X-Cys-X-X-Cys- motif that has been shown to be the binding site for metal ions, and an additional cysteine-rich motif, -Cys-Cys-Cys-Asp-Gly-Ala-Cys-. When the first 46 amino acids containing four extra cysteine residues are deleted, the isolated fragment, which still has the signature P1-type ATPase metal-binding motif, -Gly-X-X-Cys-X-X-Cys-, can bind Cd^{2+} and Zn^{2+} but not Pb^{2+}. The -Cys-X-X-Cys- motif is not suitable for binding the cupric form of Cu^{2+} or Co^{2+}.[31]   Dipeptidyl peptidase III (DPP III, EC 3.4.14.4) preferentially cleaves dipeptide residues (Arg-Arg-, Ala-Arg-, Asp-Arg-, or Tyr-Gly-) from the amino termini of oligopeptides or proteins at pH 6 – 8.[32] DPP III has a novel zinc-binding motif (-His^{450}-Glu^{451}-X-X-X-His^{455}-) and one of the seven Cys

residues in its molecule is involved in activity regulation. Cys^{176} exists as a free SH residue that is involved in the regulation of DPP III enzyme activity.[33] PC synthase has a similar motif, -His^{313}-Glu^{314}-X-X-X-X-X-His^{320}-, and suitable Cys-rich residues.   These studies support the possibility of several functions in the C-terminal Cys-rich region, including the role proposed in our present study.

We have studied the interaction between cadmium ions and the C-terminal region of phytochelatin (PC) synthase using recombinant wild-type and mutant PC synthase, for the first time. Previous studies have reported interaction of cadmium ions with PC and the homologous domain of the N-terminal region of PC synthase.   Our results clearly show that elimination of the Cys residues at the $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif, through site-directed mutagenesis, decreases the number of heavy metal ions interacting with the enzyme, and that the $C^{358}C^{359}XXXC^{363}XXC^{366}$ motif binds heavy metals discriminatingly. In addition, we have determined the optimum binding ratio PC synthase to $Cd.^{2+}$ Our findings also indicate that Cys exists as a free SH residue and that it is involved in the regulation of PC enzyme activity. Although the C-terminal domain is not absolutely required for catalysis, it clearly has an important role in regulating the enzyme activity. This domain acts to enhance activity by promoting metal binding, and transferring the metals into closer proximity with the catalytic domain. These findings support our previous model proposing that the cadmium-binding activation sites are in the C-terminal region of PC synthase proteins. These results are important in understanding heavy metal detoxification mechanisms in higher plants, a step towards phytoremediated-applications.

Acknowledgements

References

1. D. Il'yasova and G. G. Schwartz, *Toxicol. Appl. Pharmacol.*, **2005**, *207*, 179.
2. R. L. Chaney, "*Plant Uptake in Inorganic Waste. In: Land Treatment of Hazadous Waste*", ed. J. E. Parr, P. B. Marsh, and J. M. Kla, **1983**, Noyes Data Corporation, Park Ridge, NJ, 50.
3. A. Kabata-Pendius and H. Pendius, "*Trace Elements in Soils and Plants*", **1992**, CRC Press Inc., Boca Raton.
4. S. Eapen and S. F. D'Souza, *Biotechnol. Adv.*, **2005**, *23*, 97.
5. W. E. Rauser, *New Phytologist*, **2003**, *158*, 269.
6. Y. Kojima, P. A. Binz, and J. H. Kägi, in "*Klaassen C (ed) Metallothionein IV*", **1999**, Birkhäuser, Basel, 7.
7. C. Cobbett and P. Goldsbrough, *Annu. Rev. Plant Physiol. Plant Mol.*, **2002**, *53*, 159.
8. I. T. Paulsen and M. H. Saier Jr., *J. Memb. Biol.*, **1997**, *156*, 99.
9. M. L. Guerinot and D. Eide, *Curr. Opin. Plant Biol.*, **1999**, *2*, 244.
10. T. Hirayama, J. J. Kieber, N. Hirayama, M. Kogan, P.

Guzman, S. Nourizadeh, J. M. Alonso, W. P. Dailey, A. Dancis, and J. R. Ecker, *Cell*, **1999**, *97*, 383.
11. M. Solioz and C. Vulpe, *Trends Biochem. Sci.*, **1996**, *21*, 237.
12. E. Himelblau, H. Mira, S. J. Lin, V. C. Culotta, L. Penarrubia, and R. M. Amasino, *Plant Physiol.*, **1998**, *117*, 1227.
13. D. L. Huffman and T. V. O'Halloran, *J. Biol. Chem.*, **2002**, *275*, 18611.
14. K. Hirata, N. Tsuji, and K. Miyamoto, *J. Biosci. Bioeng.*, **2005**, *100*, 593.
15. F. B. Salisbury and C. W. Ross, "*Plant Physiology*", 4th ed., **1992**, Wadsworth Publishing Company, Belmont, CA, 126.
16. D. Hussain, M. J. Haydon, Y. Wang, E. Wong, S. M. Sherson, J. Young, J. Camakaris, J. F. Harper, and C. S. Cobbett, *Plant Cell*, **2004**, *16*, 1327.
17. C. S. Cobbett, *Plant Physiol.*, **2000**, *123*, 825.
18. T. Maier, C. Yu, G. Kullertz, and S. Clemens, *Planta*, **2003**, *218*, 300.
19. O. K. Vatamaniuk, S. Mari, A. Lang, S. Chalasani, L. O. Demkiv, and P. A. Rea, *J. Biol. Chem.*, **2004**, *279*, 22449.
20. N. Tsuji, S. Nishikori, O. Iwabe, S. Matsumoto, K. Shiraki, H. Miyasaka, M. Takagi, K. Miyamoto, and K. Hirata, *Planta*, **2005**, *222*, 181.
21. S. Matsumoto, K. Shiraki, N. Tsuji, K. Hirata, K. Miyamoto, and M. Takagi, *Sci. Technol. Adv. Mat.*, **2004**, *5*, 377.
22. M. Oven, J. E. Page, M. H. Zenk, and T. M. Kutchan, *J. Biol. Chem.*, **2002**, *277*, 4747.
23. O. K. Vatamaniuk, S. Mari, Y. P. Lu, and P. A. Rea, *J. Biol. Chem.*, **2002**, *275*, 31451.
24. E. Grill, S. Löffler, E. L. Winnacker, and M. H. Zenk, *Proc. Natl. Acad. Sci. U. S. A.*, **1989**, *86*, 6838.
25. S. Loeffler, A. Hochberger, E. Grill, E. L. Winnacker, and M. H. Zenk, *FEBS Lett.*, **1989**, *258*, 42.
26. A. Beck, K. Lendzian, M. Oven, A. Christmann, and E. Grill, *Phytochemistry*, **2003**, *62*, 423.
27. S. Kanaya, M. Oobatake, and Y. Liu, *J. Biol. Chem.*, **1996**, *271*, 32729.
28. M. W. Washabaugh and K. D. Collins, *J. Biol. Chem.*, **1986**, *261*, 5920.
29. N. J. Robinson, A. M. Tommey, C. Kuske, and P. J. Jackson, *Biochem. J.*, **1993**, *295*, 1.
30. L. Banci, I. Bertini, S. Ciofi-Baffoni, L. A. Finney, C. E. Outten, and T. V. O'Halloran, *J. Mol. Biol.*, **2002**, *323*, 883.
31. J. Liu, A. J. Stemmler, J. Fatima, and B. Mitra, *Biochemistry*, **2005**, *44*, 5159.
32. J. K. McDonald and A. J. Barrett, "*Mammalian Proteases: a Glossary and Bibliography*", **1986**, Vol. 2, Academic Press, London, 111.
33. Y. H. Li, T. Maeda, T. Yamane, and I. Ohkubo, *Biochem. Biophys. Res. Commun.*, **2000**, *276*, 553.