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In vivo phytochelatins and Hg–phytochelatin complexes in Hg-stressed *Brassica chinensis* L.

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In vivo phytochelatins (PCs) and their corresponding Hg–PC complexes were characterized using RPLC-ESI-MS/MS in the roots of *Brassica chinensis* L. under the stress of a mercury cysteine complex ($HgCys_2$) and/or a mercury humic acid complex (Hg–HA). Results indicated that the presence of Cys and/or HA decreased the Hg uptake in both the roots and shoots of *B. chinensis* but increased the generation of PCs in the roots compared with those where only $HgCl_2$ was in the culture solutions. A series of Hg–PC complexes were synthesized *in vitro* for predicting the possible Hg–PC formed *in vivo* in the $HgCys_2$ and/or Hg–HA stressed roots of *B. chinensis*. The discovery of *in vivo* oxidized PC_2, PC_3 and PC_4 and their corresponding $HgPC_2$, $HgPC_3$, $HgPC_4$ and Hg_2PC_4, which were confirmed by their specific isotope distribution, provided definite evidence for understanding the defense and accumulation mechanism of *B. chinensis* to Hg, in which the induced PCs play an important role not only in Hg detoxification through forming Hg–PC complexes but also for reducing the oxidative stress induced by Hg^{2+}.

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Introduction

Mercury (Hg) pollution is a ubiquitous problem resulting both from natural events and anthropogenic activities. The amount of Hg mobilized and released into the biosphere has gradually increased since the beginning of the industrial age. It has caused deep concern because of its toxicity, mobility, bioaccumulation, methylation process and transport in the biosphere.[1] When absorbed by human bodies, it causes neurological toxicity, kidney damage and even death due to its strong and specific interaction with sulfhydryls in proteins.[2,3] Hg-contaminated soil is believed to contribute to human health risks and major environmental problems. Many studies have shown that plant roots accumulate Hg when exposed to Hg-contaminated soils.[4–6] As a typical soft Lewis acid, Hg^{2+} complexes strongly with reduced sulfur-containing ligands resulting in the predominant Hg chemical form in aquatic and soil environments.[7,8] Humic acids (HA), the predominant fraction of humic substances and well known Hg ligands with cysteine (Cys) in their peptide fragments, tend to increase Hg solubility and mobility, and alter its availability to plants.[9,10] When exposed to heavy metal ions including Hg^{2+}, higher plants and *Schizosaccharomyces pombe* respond by synthesizing Cys sulfhydryl residue-rich peptides and phytochelatins (PCs) in the cytoplasm to defend against their phytotoxicity.[11,12] PCs have the general structure of γ-(Glu–Cys)$_n$–Gly (n = 2–11), and are synthesized from glutathione (GSH) through a constitutively present PC synthase. Subsequently, heavy metal ions such as Hg^{2+} are complexed and sequestered by the induced PCs *via* thiolate coordination due to their high affinity with SH groups.[13] Previous *in vitro* studies demonstrate that Hg^{2+} is facile to transfer from shorter- to longer-chain PCs. The strength of Hg^{2+} binding to GSH and PCs follows the order γ-Glu–Cys–Gly < γ-(Glu–Cys)$_2$–Gly < γ-(Glu–Cys)$_3$–Gly < γ-(Glu–Cys)$_4$–Gly,[12] and GSH and PCs play important roles in resistance in *Hydrilla verticillata* (l.f.) Royle and *Vallisneria spiralis* L. under Hg^{2+} stress.[14] On the other hand, it has been shown that a mutant having a defect in PC synthesis shows significantly enhanced sensitivity to Hg^{2+}.[15] In addition, overexpression of *Escherichia coli* γ-GlyCys synthetase and GSH synthetase in *Arabidopsis thaliana* plants provides significant increases in tolerance and accumulation of Hg^{2+}.[16] However, definite evidence of *in vivo* Hg–PC complexes and their corresponding PC precursors in support of the plant's defense and accumulation mechanisms is still scarce, and this makes it difficult to understand the nature of the Hg–PC complexes present in plant tissues. Among the few studies found in the literature,[12,14,17] only one reports the detection of *in vivo* Hg–PC complexes in *Brassica napus*, but only PC_2 and its Hg complexes are observed in the case of adding chelating agents.[17]

Since HA is the active fraction of soil organic substances and Cys is the most active component towards Hg in HA,[18] in this study we investigated the behavior of Cys and HA on Hg accumulation in *Brassica chinensis* L., and the subsequent synthesis of PCs and the formation of their corresponding Hg complexes for the first time. The hyphenation of RPLC with ESI-MS/MS was used to characterize not only *in vitro* synthesized Hg–PC complexes so as to predict the possible Hg–PC complexes and their binding stoichiometry, but also *in vivo* Hg–PC complexes present in the Hg-treated plant tissues to provide clear and definite evidence of detoxifying and/or deactivating Hg species by PCs in plant tissues.

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Experimental

Chemicals

The HPLC-grade acetonitrile (ACN) and trifluoroacetic acid (TFA) used as the components of the mobile phase in the RPLC experiments were purchased from Merck (Darmstadt, Germany). Ultrapure water (18 Ω) was prepared with a Milli-Q system (Millipore, Bedford, MA, USA), and used throughout this study. Reduced glutathione (GSH) was purchased from Sino-American Technology (Shanghai, China), and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and HA were purchased from Sigma-Aldrich. All other reagents used in this study were at least of analytical-reagent grade. PC standards and a mixture of PCs (100 μM GSH, 30 μM PC_2, 60 μM PC_3 and 15 μM PC_4) used were purified and prepared in our own laboratory from the shoots of *B. chinensis* L. under cadmium stress.[19] The PC mixture was incubated with Hg^{2+} with a 2 : 1 molar ratio of SH and Hg^{2+} to prepare *in vitro* synthesized Hg–PC complexes.

Plant material

B. chinensis seeds (F1 Beauty Crown from Japan) were germinated on filter papers in Petri dishes. Three days after germination, seedlings were carefully transferred to 100 mL pots filled with modified Hoagland nutrient solution.[20] *B. chinensis* seedlings were allowed to grow in hydroponics for one week before treatment with Hg started. Three Hg substrates were added into the nutrient solution to achieve 200 μM $HgCl_2$, (200 μM $HgCl_2$ + 1.5 mg g^{-1} HA), and 200 μM $HgCys_2$ for the studies on the effects of different Hg species on Hg accumulation in *B. chinensis* and on the degree of *in vivo* PC synthesis. The seedlings were grown at a controlled temperature (25 ± 1 °C) with a 16 h per day white light (photon flux, 700 μmol m^{-2} s^{-1}) and humidity of about 60%. After three days of stress, seedling fresh weights were measured and then the roots were immersed in ice-cold 20 mmol L^{-1} EDTA solution for 15 min to displace extracellular Hg. The seedlings were then rinsed with ultrapure water, and blotted to remove excess water before further examination.

Hg determination in *B. chinensis*

Appropriate amounts of cultured roots and shoots of *B. chinensis* were first dried at 40 °C in a conventional electric oven[21] until constant weight was obtained, and then digested in 5 mL HNO_3 in 50 mL closed polypropylene centrifuge tubes in a water bath at 95–100 °C for 2 h. After natural cooling to room temperature (25 °C), the digests were diluted with 2% HNO_3 to 25 mL for Hg determination using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer Elan-DRC II, SCIEX, Canada). Certified reference material BCR 463 (tuna fish) was used for quality control.

Extraction of demetallized PCs and Hg–PC complexes in Hg-stressed *B. chinensis*

Fresh roots and shoots were ground in liquid nitrogen and homogenized in ice-cold 1 M NaOH–0.5% (w/w) $NaBH_4$. Homogenates were centrifuged at 30 000 *g* for 15 min at 4 °C, and then the supernatants obtained were acidified to pH 1.0 with 6 mol L^{-1} HCl. The precipitated materials were centrifuged again and clean extracts were collected for demetallized PC analysis. For the analysis of Hg–PC complexes, the roots of Hg–HA and Hg–Cys treated *B. chinensis* were ground in liquid nitrogen and homogenized in ultrapure water. Homogenates were centrifuged at 30 000 *g* for 15 min at 4 °C, and then the supernatants were collected for *in vivo* Hg–PC analysis.

PC analysis using RPLC

PC analysis was performed with RPLC using a system similar to the method of Grill *et al.*[11] Briefly, PCs were separated on a C18 reverse phase column (2.0 mm I.D. × 150 mm in length; Shimadzu, Japan) at 0.15 mL min^{-1} using a 2 to 20% ACN linear gradient containing 0.02% (v/v) TFA over 25 min. The content of PCs was measured at 410 nm using on-line post-column derivatization with a solution including 1.8 mM DTNB, 15 mM EDTA, 0.3 M K_2HPO_4 (pH = 7.88). Total PCs ($\sum$PCs) were reported as the molar concentration of a sum of γ-GluCys units of PC variants with *n* from 2 to 4. The assignments of the respective peaks were performed with electrospray ionization mass spectrometry (ESI-MS) and ESI-MS/MS (ESQUIRE-LC, Bruker Daltonik, Germany) after RPLC separation without postcolumn DTNB derivatization.

Analysis of *in vitro* synthesized and *in vivo* Hg–PC complexes

In vitro synthesized and *in vivo* Hg–PC complexes were analyzed using RPLC-ESI-MS/MS. The RPLC parameters were used as described above for PC analysis. The eluate was introduced on-line into ESI-MS/MS. The instrument was used as a molecular-specific detector for the detection of Hg–PC complexes *via* their molecular peaks, Hg isotopic distribution assignment and their MS/MS spectra. The parameters used were capillary voltage +3500 V, nebulizer gas (N_2) 11 L min^{-1}, dry gas 21 psi, dry temperature 350 °C, trap drive 70.0, capillary exit offset 70.0 V, skim 1 45.0 V and fragment amplitude 1.0 V.

Results and discussion

Hg accumulation and PC generation in *B. chinensis* under the stress of different Hg species

Concerning the bio-geo-chemical cycle of Hg, most Hg is bound to thio-organics and humic substances in the soil.[22] Growth of *B. chinensis* and uptake of Hg by *B. chinensis* in the culture solutions containing different Hg species of $HgCl_2$, $HgCys_2$ and Hg–HA were investigated first in this study. The biomass and Hg concentrations in roots and shoots of *B. chinensis* were related to the different Hg species in which the total amount of Hg is constant (Table 1). It should be noted that we tried to wash Hg off the epidermis of the roots with EDTA. However, EDTA seemed to be not very effective. It was thus difficult to distinguish between the amount of Hg which was adsorbed onto the epidermis and that taken up into root cells.[17] In this case, the Hg content determined in the roots was thus the sum of that taken up by the cells and that adsorbed onto the epidermis. The results obtained indicated

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Table 1 Plant fresh weight (FW) biomass, Hg concentration and levels of PC peptides induced in the roots and/or shoots of ten day old seedlings of *Brassica chinensis* L. exposed for three days to different Hg substrates: 200 μmol L^{-1} $HgCl_2$; 200 μmol L^{-1} $HgCl_2$ + 1.5 mg g^{-1} humic acids (HA); and 200 μmol L^{-1} $HgCys_2$[a]

Hg species	Biomass[b]/10^{-2} g FW	Hg in root[c]/μg g^{-1} DW	Hg in shoot[c]/μg g^{-1} DW	PCs in the root[c]/nmol g^{-1} FW			∑PC/nmol g^{-1}
				PC_2	PC_3	PC_4	
Control	8.02 ± 1.38	0.33 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
$HgCl_2$	6.10 ± 0.89	26 089 ± 4931	2839.0 ± 184.7	<2.36	<1.36	<0.88	<12.29
$HgCys_2$	7.53 ± 1.64	18 605 ± 3545	2740.4 ± 774.3	24.86 ± 7.17	60.88 ± 6.08	15.00 ± 1.88	292.4 ± 40.1
Hg–HA	8.33 ± 1.36	2279.1 ± 606.7	1946.4 ± 913.5	30.67 ± 4.46	31.30 ± 1.44	7.55 ± 0.42	185.5 ± 14.9

[a] DW, dry weight. n.d., not detected. [b] Mean ± SD of 9 repetitions from triplicate cultivations. [c] Mean ± SD of 6 repetitions from triplicate cultivations. ∑PC, molar concentrations of the sum of γ-Glu–Cys units of the detected PC variants.

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that the presence of Cys and HA depressed the Hg uptake in both the roots and shoots of *B. chinensis* (Table 1). This might be ascribed to the less free Hg^{2+} in the culture solutions because of the high stability contents of $HgCys_2$ ($\log K = 40$)[23] and Hg–HA ($\log K > 30$).[24] But, significantly increased generation of PCs was detected in the $HgCys_2$ and/or Hg–HA stressed roots compared with those of $HgCl_2$-stressed ones, as shown in Table 1. It is reported that PC synthesis produces a detectable depletion in GSH content, and Cys, the GSH precursor thiol molecule, is produced at higher rates in order to support GSH biosynthesis under heavy metal treatment.[25] The low concentration of PCs in the roots of *B. chinensis* under exposure to $HgCl_2$ might have been due to the unavailable Cys, but not to the ineffective activation of PC synthase by Hg^{2+}.

The fact that considerable PCs [(γ-Glu–Cys)$_n$–Gly] with an *n* value from 2 to 4 have been detected in the cases of $HgCys_2$ and Hg–HA suggests that Cys and HA offer the sulfur source to support the GSH biosynthesis as well as the subsequent PCs.[25] It should be noted also that PCs have not been detected in the shoots of *B. chinensis*, indicating that PCs are not the compounds for long-distance root-to-shoot Hg translocation.[26]

RPLC-ESI-MS analyses of *in vitro* synthesized Hg–PC complexes

Immobilization and deactivation of heavy metal by natural compounds in plants, such as PCs, is the major mechanism in counteracting heavy metal toxicity. Structural studies of PC–metal complexes by extended X-ray absorption fine structure spectroscopy[27,28], nuclear magnetic resonance spectroscopy[29] and conventional optical spectroscopy have already documented a ligandation of Cd^{2+}, Ag^{2+}, Pb^{2+} and Hg^{2+} by thiolate coordination, as is known for the corresponding metallothionein–metal complexes.[30] ^{13}C-NMR spectroscopy was also employed to demonstrate the formation and connectivity of *S*,*S*′-bis(glutathionyl)–Hg^{2+} species in solution; Hg^{2+} bonding is exclusive to sulfur, and the amino and carboxyl moieties are remote from Hg^{2+} on account of an extended molecular chain configuration.[31–33] As Hg^{2+} has a linear configuration in coordination compounds, the formation of Hg–PC complexes seems to be through the sequential substitution of the protons of two SHs of PCs by one Hg. Mehra *et al.* also showed that, by UV-VIS detection, the *in vitro* synthesized Hg–PC complexes are in different forms.[12,34] However, just the presence of $HgPC_2$ is ensured by ESI-MS[17] and, for others, more were deduced than definitively detected.

Although our previous results show the presence of *in vitro* synthesized Cd–PC complexes by direct-injection-ESI-MS/MS,[19] those results do offer the possibility for the existence of Cd–PC complexes, but might not indicate the true speciation of Cd–PC complexes in solutions due to the possible generation of mixed complexes of different stoichiometry during the ESI process. In this study, since the Hg–PC complex was highly stable in a wide pH range (even at an acidic condition of pH 2.0),[31] RPLC could be used for the mutual separation of different Hg–PC complexes (Fig. 1). RPLC coupled on-line with ESI-MS/MS was proposed to characterize the stoichiometry of the *in vitro* synthesized Hg–PC complexes, avoiding the possibility of unintelligible mixed Hg–PC complex formation during the ESI process. The *in vitro* synthesized Hg–PC complexes were characterized exactly by their own retention times and by mass spectrometric information. Fig. 1 and 2 show good separation of the Hg–PC complexes under the chromatographic conditions described in the experimental section in their corresponding mass spectra, indicating that $HgGS_2$, $HgPC_2$, $HgPC_3$, $HgPC_4$ and Hg_2PC_4 were formed under *in vitro* conditions. The main signals (as ^{202}Hg) were assigned as follows: $[HgGS_2 + H]^+$ (*m*/*z* 815) and $[HgGS_2 + Na]^+$ (837) in Fig. 2A; $[HgPC_2 + H]^+$ (740) and $[HgPC_2 + Na]^+$ (762) in Fig. 2B; $[HgPC_3 + H]^+$ (972) and $[PC_3 + H]^+$ (772) in Fig. 2C; $[HgPC_4 + H]^+$ (1202), $[HgPC_4 + Na]^+$ (1224) and $[PC_4 + Na]^+$ (1022) in Fig. 2D; and $[Hg_2PC_4 + H]^+$ (1404) and $[Hg_2PC_4 + Na]^+$

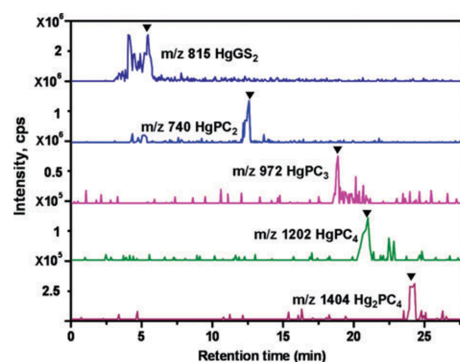

Fig. 1 Analysis of *in vitro* synthesized Hg–PC complexes using RPLC-ESI-MS. Total ion chromatogram (TIC) and extracted ion chromatograms (EIC) of $HgGS_2$, $HgPC_2$, $HgPC_3$, $HgPC_4$ and Hg_2PC_4 analyzed by ESI-MS. ▼, species identified as mentioned in trace.

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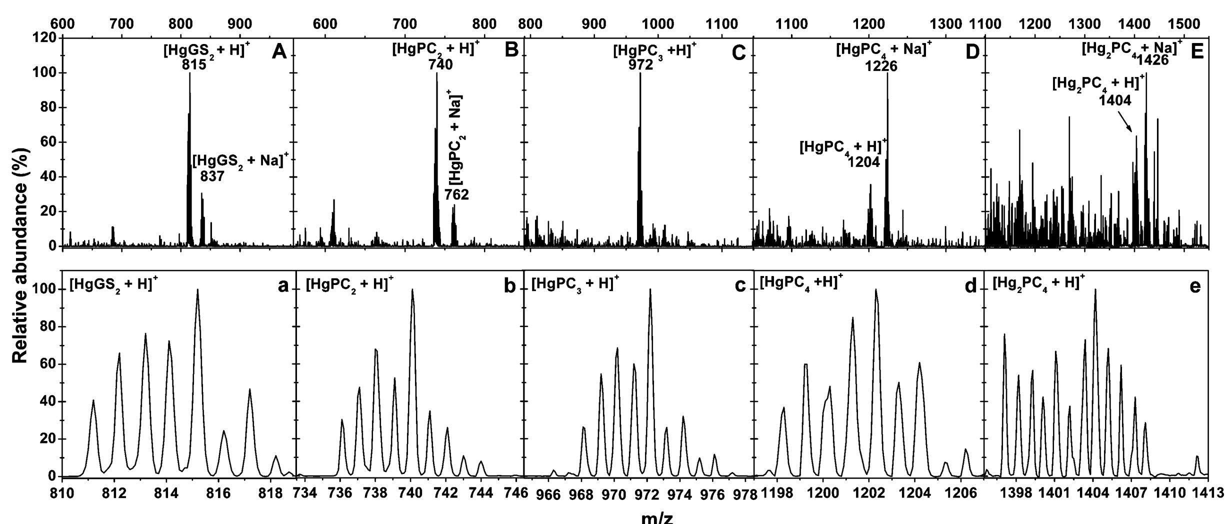

Fig. 2 ESI-MS spectra of the *in vitro* synthesized $HgGS_2$ (A), $HgPC_2$ (B), $HgPC_3$ (C), $HgPC_4$ (D) and Hg_2PC_4 (E) and their isotopic peak distributions of HgGS (a), $HgPC_2$ (b), $HgPC_3$ (c), $HgPC_4$ (d) and Hg_2PC_4 (e).

(1426) in Fig. 2E. The lower part of each figure (Fig. 2a–e) shows the magnification of the corresponding $[HgGS_2 + H]^+$ and $[HgPC_n + H]^+$ (n = 2–5) ions due to the natural isotopic distribution of Hg (^{196}Hg, 0.15%; ^{198}Hg, 10.02%; ^{199}Hg, 16.84%; ^{200}Hg, 23.13%; ^{201}Hg, 13.22%; ^{202}Hg, 29.8%; and ^{204}Hg, 6.85%.). The detected isotopic distribution of $HgPC_2$ (736, 30.4%; 737, 47.3%; 738, 67.7%; 739, 52.4%; 740, 100.0%; 741, 35.1%; 742, 25.7%; 743, 10.6%; and 744, 8.6%), for example, was quite consistent with the theoretical isotopic distribution (734, 0.4%; 736, 27.8%; 737, 53.2%; 738, 78.2%; 739, 58.2%; 740, 100.0%; 741, 26.3%; 742, 31.1%; 743, 7.0%; 744, 3.2%; 745, 0.5%; and 746, 0.2%), which was calculated using an isotope pattern calculator.[35] It is worth noting that the formation of $HgPC_4$ implied that a disulfide bond formed between two Cys residues in the PC_4 monomer. In addition, the capillary voltage was quite important in maintaining the stability of Hg–PC complexes; for example, the intensity of $HgPC_3$ was 2.5×10^5 at 3500 V, while it was 4.8×10^4 at 3600 V. Thus, the high capillary voltage should be fairly tuned, otherwise this would lead to the dissociation or inexplicable formation of Hg–PC complexes.

In vivo Hg–PC complexes induced in the roots of *B. chinensis* under Hg–HA and $HgCys_2$ stress

At the beginning of this study, we found that when the *in vivo* PCs and their corresponding Hg–PC complexes were extracted from the *B. chinensis* roots, they were partly subject to decomposition during the 24 h in the extraction solutions, while they were stable in the intact plant for at least one month at −20 °C. To determine the existence of the *in vivo* PCs and the Hg–PC complexes in the roots of both Hg–HA and $HgCys_2$, stressed *B. chinensis* analysis should be performed within 24 h using RPLC-ESI-MS/MS after extraction. However, only oxidized PC_2 (*m*/*z* 538), PC_3 (*m*/*z* 770) and PC_4 (*m*/*z* 1000) were detected besides the unequivocal identification of *in vivo* $HgPC_2$, $HgPC_3$, $HgPC_4$ and Hg_2PC_4, as shown in Fig. 3A and 3B. The production of active oxygen species (such as H_2O_2) and the oxidative stress generated by

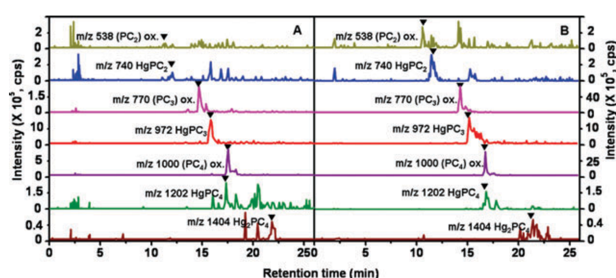

Fig. 3 Analysis of the *in vivo* Hg–PC complexes and PCs in the roots of *B. chinensis* under the stress of Hg–HA (A) and $HgCys_2$ (B) using RPLC-ESI-MS. Total ion chromatogram (TIC) and extracted ion chromatograms (EIC) of oxidized PC_2, oxidized PC_3, oxidized PC_4, $HgPC_2$, $HgPC_3$, $HgPC_4$ and Hg_2PC_4 analyzed by ESI-MS. ▼, species identified as mentioned in trace.

Hg^{2+},[36] which in turn oxidizes the induced PCs, might be responsible for the oxidation of the *in vivo* PCs, since the stronger reaction of PCs with H_2O_2 rather than with GSH or ascorbate has been suggested before.[37] The existence of $[HgPC_2 + H]^+$ (*m*/*z* 740), $[HgPC_3 + H]^+$ (972), $[HgPC_4 + H]^+$ (1202), $[Hg_2PC_4 + H]^+$ (1404) in the mass spectrum (Fig. 4) of the extract of the $HgCys_2$ stressed *B. chinensis* roots was observed and confirmed by their corresponding isotopic distribution pattern. Similar results were obtained in the Hg–HA stressed *B. chinensis* roots. Moreover, Fig. 5 (as an example of an MS/MS spectrum of the Hg–PC complexes) shows there was a match between the fragment ions obtained for the *in vitro* synthetic $HgPC_2$ standard (Fig. 5A) and that for the *in vivo* $HgPC_2$ in the $HgCys_2$ stressed *B. chinensis* roots (Fig. 5B), in addition to the identical retention time of 13.0 min recorded in the corresponding chromatograms shown in Fig. 1 and 3, respectively.

In conclusion, our results demonstrated that Hg accumulation and *in vivo* PC production in *B. chinensis* were related to the kind of Hg species. Although the presence of HA and/or Cys reduced the Hg accumulation in the roots of *B. chinensis*, they improved the generation of *in vivo* PCs in these roots.

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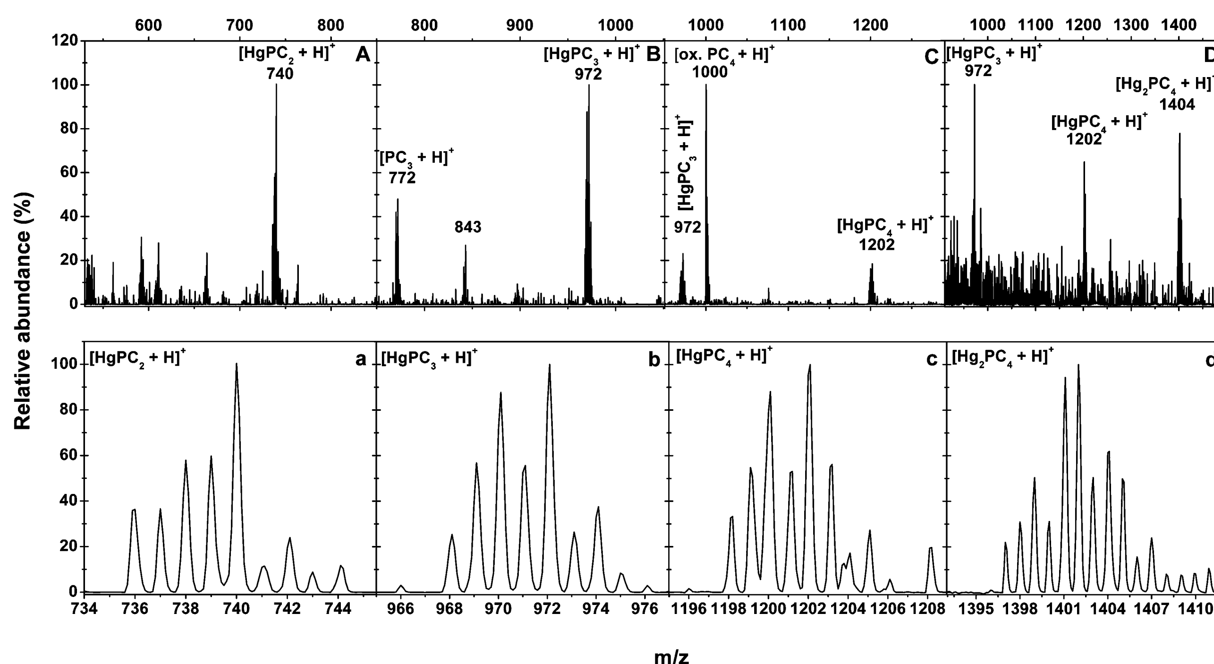

Fig. 4 ESI-MS spectra of the *in vivo* $HgPC_2$ (A), $HgPC_3$ (B), $HgPC_4$ (C) and Hg_2PC_4 (D) and their isotopic peak distributions of $HgPC_2$ (a), $HgPC_3$ (b), $HgPC_4$ (c) and Hg_2PC_4 (d) in the $HgCys_2$ stressed *B. chinensis* roots.

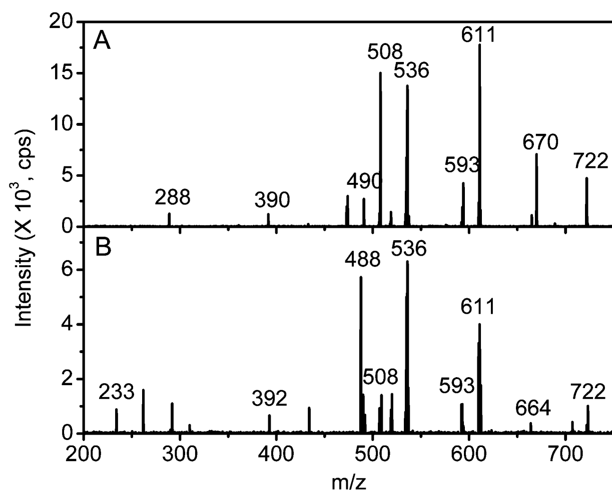

Fig. 5 ESI-MS/MS spectra of the *in vitro* $HgPC_2$ standard (A) and the *in vivo* $HgPC_2$ (B) in the $HgCys_2$ stressed *B. chinensis* roots at *m*/*z* 740.

Positive identification of *in vivo* oxidized PCs and Hg–PC complexes in the $HgCys_2$ and Hg–HA stressed *B. chinensis* roots provided insight into the defense and accumulation mechanisms, elucidating the important roles of PCs in Hg tolerance, not only for their sequestration of free Hg^{2+} but also for reducing the oxidative stress in cells.

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