SEC-ICP-MS and ESI-MS/MS for analyzing *in vitro* and *in vivo* Cd-phytochelatin complexes in a Cd-hyperaccumulator *Brassica chinensis*

Liqin Chen,[a] Yifei Guo,[a] Limin Yang[a] and Qiuquan Wang*[ab]

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In this study, *in vitro* synthesized Cd-phytochelatin (PC) complexes and *in vivo* Cd–PC complexes in Cd-stressed *Brassica chinensis*, which has been identified as a Cd hyperaccumulator, were characterized using SEC-ICP-MS and ESI-MS/MS. The PCs (n = 1–5) obtained from Cd-stressed *B. chinensis* together with $CdCl_2$ were used to synthesize the *in vitro* Cd–PC complexes, and the formation of $CdGS_{1-2}$, $(CdGS)_2$, $Cd_{1-2}PC_2$, $Cd_{1-3}PC_3$, $Cd_{1-3}PC_4$ and $Cd_{1-3}PC_5$ was observed. In addition, for the first time, *in vivo* $CdPC_3$ and $CdPC_4$ complexes, as well as Cd-free PCs (n = 2–5) and desGlu-PC_3 were detected in the extracts of Cd-stressed *B. chinensis* and confirmed by means of their corresponding isotopic peak distribution and MS/MS spectra. Nitrogen saturated ammonium bicarbonate buffer (pH 7.8), instead of Tris-HCl and phosphate buffer, was used as a suitable mobile phase in order to stabilize the Cd–PC complexes and effectively avoid possible oxidation of PC analogues during SEC fractionation. Results obtained in this study give definite evidence elucidating the important roles which PCs play in plant defensive mechanisms to Cd stress. SEC-ICP-MS and ESI-MS/MS were demonstrated as powerful and promising techniques for screening and identifying the *in vivo* metallopeptides, with accurate isotopic distribution assignment, in metal toxicological studies.

Introduction

Cadmium (Cd) is a non-essential heavy metal ubiquitously dispersed in the environment by natural and anthropogenic activities.[1] It can accumulate in the human body with a half-life exceeding 10 years, causing renal dysfunction and pulmonary emphysema, and it is also a suspected carcinogen.[2–4] It has been estimated that at least 70% of the Cd uptake by humans originates from plant foods.[1] Most of the higher plants investigated are able to synthesize sulfhydryl-rich peptides called phytochelatins (PCs) induced by Cd stress, having a general structure $(\gamma\text{-Glu-Cys})_n$-Gly, where n is from 2 to 11.[5,6] PCs are synthesized from reduced glutathione (GSH) by the constitutive enzyme PC synthase, activated by a variety of metal ions, among which Cd is the most effective.[7,8] As a thiophilic metal ion, there is a strong tendency for the complexation of Cd with PCs,[9] and this significantly reduces the toxicity of free Cd in plant tissues.[7] It is estimated that Cd–PC complexes localize preferentially in the vacuoles of intact plant cells and that at least 97% of the Cd in plant cell lines which are capable of tolerating high levels of Cd is accumulated as Cd–PC complexes.[10,11] However, confirmation of such a hypothesis needs more definite evidence *in vivo*.

In order to determine PCs and Cd–PC complexes in plant tissues, various analytical approaches have been developed, among which size-exclusion chromatography (SEC) coupled with on-line and/or off-line element-selective detectors such as atomic absorption spectrometry (AAS),[12] atomic fluorescence spectrometry (AFS),[13] and inductively coupled plasma-mass spectrometry (ICP-MS)[14–19] are frequently used for the screening of Cd-containing fractions. Electrospray ionization-mass spectrometry (ESI-MS)[12,14–19] and extended X-ray absorption fine structure (EXAFS) spectroscopy[20] were also used for the structural identification of the Cd-containing species. Those investigations, however, only showed that Cd induced the synthesis of PCs in plant cells. In addition, the *in vitro* synthesized Cd–PC complexes have been studied with EXAFS,[21] potentiometry and NMR spectroscopy,[22] and this indicated the existence of a predominantly tetrahedral coordination complex of Cd with sulfur of PC_3,[21] and the dependence of binding chemistries on pH and Cd : PC molar ratios.[22] Obviously, these results can only predict the existence of Cd–PC complexes and their possible roles in Cd detoxification in plant systems. Compared with other molecule-specific detectors, ESI-MS is a more useful tool which is able to provide structural information regarding the complexes.[23] Table 1 gives a comprehensive list of PCs and their Cd complexes identified to date by SEC-ESI-MS.[12,14–19] However, few studies have reported possible Cd–PC complexes *in vivo*,[12,18] but only the free demetallized PCs in plant tissues or cell extracts.

In this study, we used ESI-MS and ESI-MS/MS for the analysis of PCs and Cd–PC complexes *in vitro* to predict the possible structures of Cd–PC complexes through which Cd might be sequestered in plants. Moreover, a novel SEC separation approach coupled with element-selective ICP-MS

[a] *Department of Chemistry & the MOE Key Laboratory of Analytical Sciences, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen, 361005, China. E-mail: qqwang@xmu.edu.cn; Fax: +86 5922181796; Tel: +86 5922181796*
[b] *State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, 361005, China*

Table 1 A comprehensive list of PCs and their Cd complexes identified by SEC-ESI-MS^n

Separation	Detection	Samples	Observation	Ref.
SEC (Bio Gel P-6, 100 × 2.5 cm id), 50 mM Tris-HCl (pH 7.8)—capillary RPC_{18}, up to 30% ACN, 0.1% TFA	AAS; Nano ESI-MS/MS	PC_5 Standard *Datura innoxia* cell culture	*In vitro* synthesized $Cd_{1-3}PC_5$; PC_{3-6}, $Cd_{1-2}PC_3$, $CdPC_{4-5}$	12
SEC, 10 mM ammonium acetate (pH 7)	ICP-MS (^{114}Cd) ESI-MS	*Silene vulgaris* cell suspension	PC_2, des-PC_2	14
SEC (Superdex Peptide HR 10/30, 300 × 10 mm id), 30 mM Tris-HCl (pH = 7.5)—RPC_{18}, up to 50% ACN, 0.1% TFA	ICP-MS (^{114}Cd, ^{65}Cu) ES-MS/MS	*Silene cucubalus* cell culture	PC_{2-4}	15
SEC (Superdex 75HR 10/30, 300 × 10 mm), 30 mM Tris-HCl/10 mM NaCl (pH 7.4); RPC_{18} (Zorbax 300SB), up to 100% ACN, 0.1% TFA	ICP-MS (^{112}Cd, ^{114}Cd, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn) ESI-MS	*Arabidopsis thaliana*	GSH, iso-PC_{2-5} (γ-Glu), PC_{2-5}, des-PC_{2-6}	16
SEC (Hi-Load Superdex 30 preparation-grade size-exclusion), 25 mM phosphate buffer (pH 8.0)—ion-pair chromatography, 0.04% TBAH	ICP-MS (^{116}Cd) ESI-MS	*Phaeodactylum tricornutum*	PC_{2-7}	17
SEC (Superdex Peptide HR 10/30), 25 mM ammonium acetate (pH 7.8)	ICP-MS (^{111}Cd, ^{114}Cd) ESI-Q-TOF-MS/MS	*Brassica juncea*	PC_2, a possible $CdPC_2$	18
SEC (Superdex Peptide 10/300 GL), 50 mM ammonium acetate (pH 7.5)—RPC_{18} with 0.1% TFA	ICP-MS (^{55}Mn, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{111}Cd, ^{114}Cd) ESI-TOF-MS	*Hordeum vulgare* L.	desγ-Glu-PC_2, PC_{2-3}, *in-vitro* synthesized $CdPC_{2-3}$	19

and structure-specific ESI-MS/MS was developed for the identification of *in vivo* Cd–PC complexes present in a Cd-hyperaccumulating crop, *Brassica chinensis*. The structure of the *in vivo* complexes was elucidated with accurate Cd-isotopic distribution assignment in order to provide clear evidence of the detoxifying function of these Cd–PC complexes in plant systems.

Experimental

Instrumentation

Chromatographic separations were performed on an HPLC system (Agilent 1100 Series, Agilent Technologies, UK) equipped with a UV detector and a 100 μL loop. Two columns, a Diol-300 size exclusion column (7.9 × 500 mm) (Shimadzu, Japan) and a C_{18} reverse phase column (2.0 × 250 mm) (Shimadzu, Japan), were used. An ELAN-DRC II ICP-MS (PerkinElmer, SCIEX, Canada) was used as an element-selective detector in SEC. The column eluate was introduced on-line into the ICP-MS instrument through a concentric pneumatic nebulizer. An ESI-MS^n (ESQUIRE-LC, Bruker Daltonik, Germany) was used for mass detection and structural analysis. Detailed instrumental operating conditions are summarized in Table 2. A Hermle Z36HK refrigerated centrifuge (Wehingen, Germany) was used to obtain the extracts.

Reagents and materials

HPLC-grade acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany); 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Sigma (USA); acetic acid was HPLC grade from TEDIA (Fairfield, OH, USA); ultrapure water (18 MΩ) was prepared with a Milli-Q system (Millipore, Bedford, MA, USA); and reduced glutathione (GSH) was purchased from Sino-American Technology (Shanghai, China). All other reagents used in this study were at least of analytical-reagent grade.

Plant cultivation under Cd stress

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 one-quarter-strength Hoagland culture solution.[24] *B. chinensis* seedlings were allowed to grow in hydroponics for two weeks before Cd stress commenced. Appropriate amounts of $3CdSO_4 \cdot 7H_2O$ were added into the nutrient solution to achieve a 50 μM Cd concentration in the culture solution. The seedlings were grown at a controlled temperature (25 ± 1 °C) with 16 h d^{-1} white light (photon flux, 700 μmol m^{-2} s^{-1}) and humidity of about 60%. The culture solution was renewed every two days. After six days of Cd stress, the roots of seedlings were immersed in ice-cold 20 mM

Table 2 Operating conditions for HPLC, ICP-MS, and ESI-MS

HPLC parameters	
Reverse phase column	Shim-pack VP-ODS 250 × 2.0 mm ID, 5 μm particle size
Mobile phase	A. H_2O–0.02% TFA B. ACN–0.02% TFA
Injection volume	100 μL
Gradient	2%–20% B in 20 min
Flow rate	0.15 mL min^{-1}
Postcolumn derivatization	1.8 mM DTNB, 15 mM EDTA, 0.3 M K_2HPO_4 (pH 7.88)
Detection	UV (410 nm)
Size exclusion column	Shim-pack Diol-300 size exclusion column 500 × 7.9 mm ID, 5 μm particle size
Mobile phase	50 mM ammonium bicarbonate (pH 7.8)
Flow rate	0.75 mL min^{-1}
Injection volume	100 μL
Detection	UV (214 nm and 254 nm) and ICP-MS
ICP-MS parameters	
ICP RF Power	1150 W
Plasma gas flow	15 L min^{-1}
Auxiliary gas flow	1.2 L min^{-1}
Isotope monitored	^{111}Cd, ^{34}S, ^{63}Cu, ^{66}Zn, ^{60}Ni
ESI-MS parameters	
Solvent	5 mM ammonium bicarbonate (pH 7.8)
Scan type	Positive MS and MS/MS
Ion-spray voltage	4.0 kV

EDTA solution for 15 min to remove extracellular Cd. The seedlings were then rinsed with ultrapure water, and blotted to remove excess water before further use.

Preparation of metal-free PCs and synthesis of the Cd–PC complexes

Since no PC standards are commercially available, we prepared individual PC samples from the Cd stressed *B. chinensis*. The fresh Cd stressed *B. chinensis* plants were ground in liquid nitrogen (N_2) and homogenized in ice-cold and N_2 saturated 0.1 N HCl. Homogenates were centrifuged at 20 000*g* for 15 min at 4 °C, and then the supernatants obtained were used for thiol peptide analysis using HPLC in a system similar to that described by Grill *et al.*[5] Thiol peptides were separated with C_{18} reversed-phase chromatography (RPC) using a 2 to 20% ACN gradient in 0.02% (v/v) TFA (pH 2.0) over 20 min. The content of thiol peptides was measured using on-line postcolumn derivatization with 1.8 mM DTNB, and detected at 410 nm. The assignments of the respective peaks were performed with ESI-MS and ESI-MS/MS without postcolumn derivatization. Then the fractions containing the individual PCs (PC_2, PC_3, PC_4 and PC_5) were heart-cut and collected under an N_2 atmosphere and subjected to lyophilization. The lyophilized heart-cut fractions were redissolved in N_2 saturated water. The sum of the sulfhydryl (SH) concentrations of each PC was analyzed using the method described by Meuwly and Rauser.[25] For the synthesis of Cd-GSH/PC complexes, a standard solution of 50 mM Cd^{2+} was prepared by dissolving the corresponding $CdCl_2$ in N_2 saturated ultrapure water. Reduced GSH was also prepared in the N_2 saturated water. Then the GSH and PC solutions obtained were incubated with Cd ions with a 1 : 1 molar ratio of SH and Cd in an N_2 saturated 5 mM ammonium bicarbonate buffer (pH 7.8) on ice in order to avoid any possible oxidation.

Analysis of *in vivo* Cd–PC complexes

Fresh Cd stressed *B. chinensis* (20 g) was ground in liquid N_2 and homogenized in ice-cold and N_2 saturated 50 mM ammonium bicarbonate buffer. Homogenates were centrifuged at 20 000*g* at 4 °C for 15 min, and then the supernatant obtained was centrifuged again using the same conditions. The final supernatant obtained was subjected to lyophilization and then re-suspended in the 2-mL ice-cold and N_2 saturated 50 mM ammonium bicarbonate buffer for SEC-ICP-MS and ESI-MS/MS analysis.

Results and discussion

Screening of Cd-containing fraction in Cd-stressed *B. chinensis* by SEC-ICP-MS

In this study, *B. chinensis* is chosen as a model because it hyperaccumulates Cd up to 1348.3 ± 461.8 mg kg^{-1} dry weight in shoots at 200 μM of Cd exposure (our unpublished data). The coupling of SEC to ICP-MS was selected as a screening method to study the possible Cd–PC complexes in *B. chinensis* tissues after Cd stress. Doreák *et al.* indicated that the distribution of free Cd ions in the mixture of Cd and PC_2 at pH 5.0 and 7.3 are 98.7 and 0.28%, respectively.[22] N_2 saturated 50 mM ammonium bicarbonate (pH 7.8) was thus used as a mobile phase for the stabilization of Cd-thiolate during SEC. Fig. 1 shows the simultaneous measurements of ^{111}Cd, ^{63}Cu, ^{66}Zn, ^{60}Ni and ^{34}S isotopes in *B. chinensis* extracts with and without 50 μM Cd stress. The intensities of ^{66}Zn and parts of ^{63}Cu (t_r = 20.5 min) were increased while the intensities of ^{60}Ni and the rest of ^{63}Cu (t_r = 24.3 min) were decreased in the Cd-stressed *B. chinensis* when compared with the control. As shown in Fig. 1A, the levels of the essential metals were not seriously changed in *B. chinensis* under Cd stress, suggesting that Cd may not affect the absorption of these essential metals in *B. chinensis* compared with observations by Persson *et al.*[19] This may be due to the different plant species used in the experiments. An additional peak of ^{34}S and a ^{111}Cd peak were coeluted at the same retention time (t_r = 23.5 min), as shown in Fig. 1A of the Cd-stressed *B. chinensis* when compared with that of the control shown in Fig. 1B, suggesting a strong induction of phytochelatin-like compounds produced under Cd stress. This Cd/S containing fraction was collected for further study.

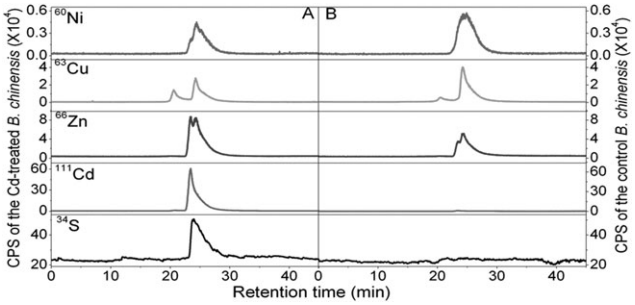

Fig. 1 ^{60}Ni, ^{66}Zn, ^{63}Cu, ^{34}S and ^{111}Cd specific monitoring in the extracts of the Cd-stressed *B. chinensis* (A) and the control *B. chinensis* (B) using SEC-ICP-MS.

Characterization of *in vitro* synthesized Cd–PC complexes

To understand the nature of the possible Cd–PC complexes in Cd-stressed *B. chinensis*, the *in vitro* synthesized Cd–PC_n complexes (n = 1–5) were infused into the ESI source at 2 μL min^{-1} for structural analysis. The main signals in Fig. 2 were assigned as follows: $[GSH + H]^+$ (m/z 308), $[(GSH)_2 + H]^+$ (m/z 615), $[CdGS + H]^+$ (m/z 420), $[Cd(GS)_2 + H]^+$ (m/z 727) and $[(CdGS)_2 + H]^+$ (m/z 839) in Fig. 2A; $[PC_2 + H]^+$ (m/z 540), $[CdPC_2 + H]^+$ (m/z 652) and $[Cd_2PC_2 + H]^+$ (m/z 764) in Fig. 2B; $[PC_3 + H]^+$ (m/z 772), $[CdPC_3 + H]^+$ (m/z 884), $[Cd_2PC_3 + H]^+$ (m/z 996) and $[Cd_3PC_3 + H]^+$ (m/z 1106) in Fig. 2C; $[PC_4 + H]^+$ (m/z 1004), $[CdPC_4 + H]^+$ (m/z 1116), $[Cd_2PC_4 + H]^+$ (m/z 1228) and $[Cd_3PC_4 + H]^+$ (m/z 1338) in Fig. 2D; and $[PC_5 + H]^+$ (m/z 1236), $[CdPC_5 + H]^+$ (m/z 1348), $[Cd_2PC_5 + H]^+$ (m/z 1459) and $[Cd_3PC_5 + H]^+$ (m/z 1570) in Fig. 2E. The lower part of each figure (Fig. 2a–e) shows the magnification of the isotopic peak distribution of the corresponding $[CdPC_n + H]^+$ (n = 1–5) ion due to the isotopic distribution of Cd (^{106}Cd, 1.25%; ^{108}Cd, 0.89%; ^{110}Cd, 12.49%; ^{111}Cd, 12.80%; ^{112}Cd, 24.13%; ^{113}Cd, 12.22%; ^{114}Cd, 28.73%; ^{116}Cd, 7.49%.).

It is notable that an interesting complex, $(CdGS)_2$, was found, as shown in Fig. 2A, implying that Cd–PC complexes

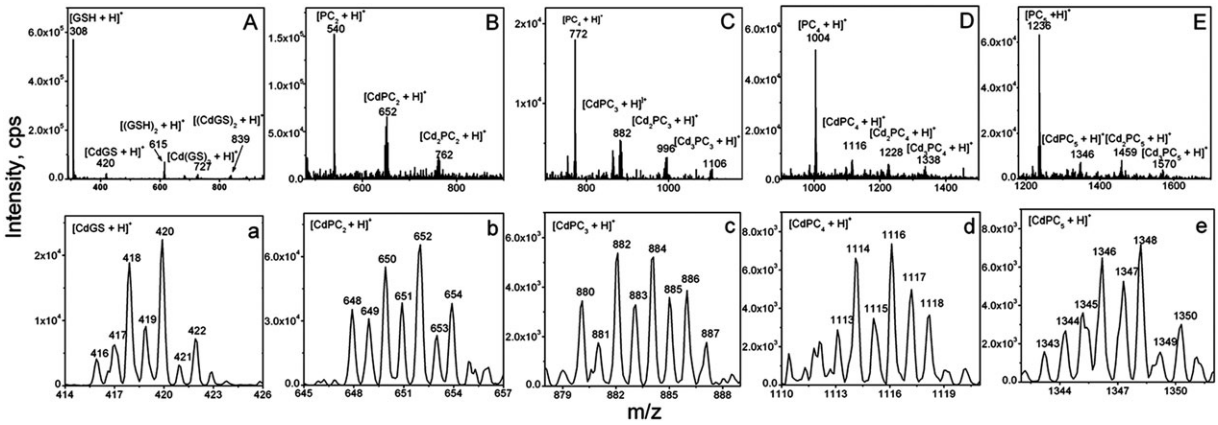

Fig. 2 ESI-MS spectra of the *in vitro* synthesized Cd-GSH (A), Cd–PC_2 (B), Cd–PC_3 (C), Cd–PC_4 (D) and Cd–PC_5 (E) and their isotopic peak distribution of CdGS (a), $CdPC_2$ (b), $CdPC_3$ (c), $CdPC_4$ (d) and $CdPC_5$ (e).

might exist in the form of a dimer.[21] Furthermore, we performed MS/MS analysis on this precursor ion at m/z 839 to confirm its existence and characterize its possible structure (Fig. 3). Besides the substitution of the proton on the SH of Cys in GSH by Cd, the electrostatic interactions between Cd and carboxyl and the hydrogen bonding between the hydrogen of the carboxyl of one GSH and the nitrogen of the amino of the other, as in the $(GSH)_2$, may be involved in the formation of $(CdGS)_2$. Fig. 2B indicates that 1 : 1 and 2 : 1 Cd–PC_2 complexes are formed, as reported by other groups showing the stoichiometry of 0.5–5.0 Cd atom binding per PC_2 molecule.[22,26–28] The formation of $CdPC_2$ seems to be through the sequential substitution of the protons of two SHs of the PC_2 by Cd, while that of Cd_2PC_2 is through the sequential substitution of the protons of one SH and one Glu carboxyl of the PC_2 for each Cd.[26–28] Similar results have also been obtained by EXAFS studies showing that it was possible for a Cd atom to combine 1–4 PC units.[20] Results from Fig. 2C–E indicate that $Cd_{1–3}PC_{3–5}$ complexes can be formed; however, the formation of complexes containing more than 3 Cd atoms has not been observed, which might be ascribed to the poor ESI ionization efficiency for larger polynuclear Cd–PC complexes.[29] It should be noted that these results from ESI-MS might not indicate the true speciation of Cd–PCs in solutions due to the possible generation of complexes of different stoichiometry during the ESI process. A clue, however, is caught for one to elucidate what have been identified *in vivo*.

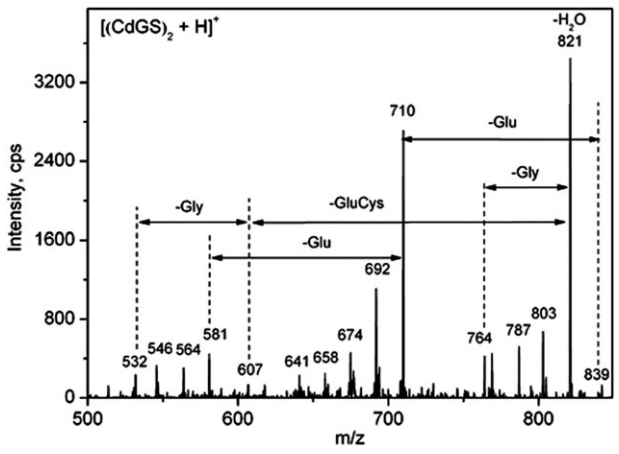

Fig. 3 ESI-MS/MS spectrum of $[(CdGS)_2 + H]^+$ at m/z 839.

Analysis of *in vivo* Cd–PC complexes in *B. chinensis* by ESI-MS/MS

At the beginning of this study, we attempted to use a complementary separation mechanism-RPC, to separate the *in vitro* synthesized Cd–PC complexes prior to ESI-MS analysis. However, the Cd–PC complexes dissociated in RPC with a mobile phase containing 0.02% TFA (pH = 2); alternatively, the Cd–$PC_{3–5}$ standards could not be eluted out of the column using a mobile phase with or without 0.1% acetic acid. This may be ascribed to the possible dissociation of the uncovered silicon hydroxide in the stationary phase when the pH is higher than 3 when it might interact with Cd–PC complexes.[30] It seems that SEC is the only choice for fractionation of *in vivo* Cd–PC complexes in Cd-stressed *B. chinensis*. It should be noted that ammonium bicarbonate buffer instead of Tris-HCl and/or phosphate buffer was used as the mobile phase to perform the chromatographic separation in this study. The ammonium salt in the effluent can not only be freeze-dried off by lyophilization but also is compatible with ESI-MS analysis, while the non-volatile Tris and phosphate salts would seriously suppress the ESI-MS signal. Consequently, the fraction containing Cd–PC-like complexes collected from the SEC column was completely lyophilized, resuspended in 100 μL 5 mM ammonium bicarbonate under an N_2 atmosphere, and finally analyzed by ESI-MS and ESI-MS/MS. The results shown in Fig. 4A not only indicate that $[PC_2 + H]^+$ (m/z 540), $[PC_3 + H]^+$ (772), $[PC_4 + H]^+$ (1004), $[PC_5 + H]^+$ (1236) and $[desGlu\text{-}PC_3 + H]^+$ (643) have been induced in *B. chinensis* under Cd stress, but also contain rich information of *in vivo* Cd–PC complexes. The existence of $CdPC_3$ (Fig. 4B) and $CdPC_4$ (Fig. 4C) complexes were observed at 884 m/z ($[CdPC_3 + H]^+$) and 1116 m/z ($[CdPC_4 + H]^+$), and confirmed by their corresponding isotopic distribution pattern. Their MS/MS spectra were also consistent with those of the corresponding *in vitro* synthesized Cd–PC standards (data not shown). The signal intensities of

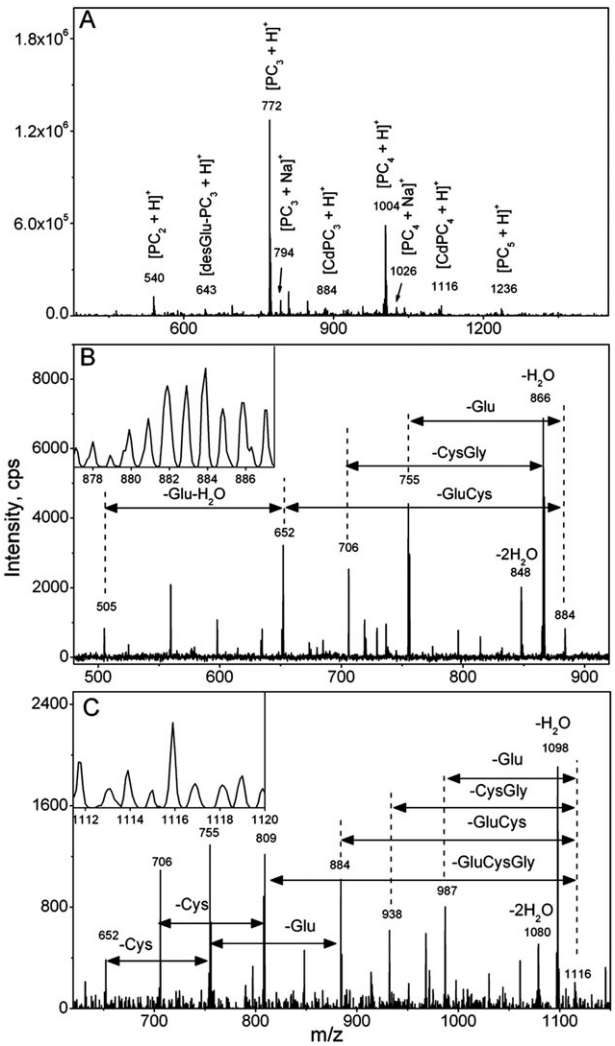

Fig. 4 ESI-MS and ESI-MS/MS of the SEC fraction of the Cd-stressed *B. chinensis*. The full-scan mass spectrum (A); the MS/MS spectra for $CdPC_3$ at *m*/*z* 884 (B) and $CdPC_4$ at *m*/*z* 1116 (C); the inset of each mass spectrum is the isotopic peak distribution of the corresponding complex.

the *in vivo* Cd–PC complexes are much weaker than those of the PCs without Cd due to the poor volatility of the Cd–PC complexes,[29] resulting in low ionization efficiency; on the other hand, the enzymatic PC biosynthesis is known to occur in the cytosol, and the Cd–PC complexes are subsequently sequestrated into the vacuole (typically at pH 5.0–5.5), where the Cd–PC complexes can dissociate somewhat.[7,31] Moreover, unlike the *in vitro* synthesized Cd–PC_{3-4} complexes, the $Cd_{2-3}PC_{3-4}$ complexes were not detected in the Cd-stressed *B. chinensis* extracts. Lack of the complexes containing more Cd may, in turn, reflect the acidic pH conditions in the vacuole.

Conclusions

SEC-ICP-MS and ESI-MS/MS have been demonstrated to be powerful and promising techniques for screening and characterizing *in vitro* synthesized Cd–PC complexes and *in vivo* Cd–PC complexes in the Cd-stressed *B. chinensis*, giving definite evidence of the complexation of PCs with Cd in this plant species. The structure-specific identifying ability, with accurate isotopic distribution assignment obtained using ESI-MS and ESI-MS/MS, offers insights not only into an understanding of the coordination chemistry of Cd and PCs, but also regarding plant defensive mechanisms under Cd stress. We expect that, after suitable modifications, SEC-ICP-MS and ESI-MS/MS will find wide application in metal toxicology studies.

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