Multi-elemental speciation analysis of barley genotypes differing in tolerance to cadmium toxicity using SEC-ICP-MS and ESI-TOF-MS

Daniel P. Persson,[a] Thomas H. Hansen,[ab] Peter E. Holm,[b] Jan K. Schjoerring,[a] Hans Christian B. Hansen,[b] John Nielsen,[b] Ismail Cakmak[c] and Søren Husted*[a]

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Plants respond to Cd exposure by synthesizing heavy-metal-binding oligopeptides, called phytochelatins (PCs). These peptides reduce the activity of Cd^{2+} ions in the plant tissues by forming Cd chelates. The main objective of the present work was to develop an analytical technique, which allowed identification of the most prominent Cd species in plant tissue by SEC-ICP-MS and ESI-TOF-MS. An integrated part of the method development was to test the hypothesis that differential Cd tolerance between two barley genotypes was linked to differences in Cd speciation. Only one fraction of Cd species, ranging from 700–1800 Da, was detected in the shoots of both genotypes. In the roots, two additional fractions ranging from 2900–4600 and 6700–15 000 Da were found. The Cd-rich SEC fractions were heart-cut, de-salted and de-metallized using reversed-phase chromatography (RPC), followed by ESI-MS-TOF to identify the ligands. Three different families of PCs, *viz*. (γGlu-Cys)$_n$-Gly (PC$_n$), (γGlu-Cys)$_n$-Ser (iso-PC$_n$) and Cys-(γGlu-Cys)$_n$-Gly (des-γGlu-PC$_n$), the last lacking the *N*-terminal amino acid, were identified. The PCs induced by Cd toxicity also bound several essential trace elements in plants, including Zn, Cu, and Ni, whereas no Mn species were detected. Zn, Cu and Ni-species were distributed between the 700–1800 Da and 6700–15 000 Da fractions, whereas only Cd species were found in the 2900–4600 Da fraction dominated by PC_3 ligands. Although the total tissue concentration of Cd was similar for the two species, the tolerant barley genotype synthesized significantly more $CdPC_3$ species with a high Cd specificity than the intolerant genotype, clearly indicating a correlation between Cd tolerance and the Cd–PC speciation.

Introduction

Cadmium (Cd) is a heavy metal, which is present in almost all terrestrial environments. It is highly toxic to almost all organisms, including humans, even at very low concentrations.[1] It is estimated that the dietary intake accounts for up to 50% of the tolerable weekly intake of Cd in Europe, of which plant products are the main Cd source.[1]

Higher plants, algae, bacteria, yeast and some fungi, are able to induce the synthesis of sulfur-rich compounds in response to exposure to toxic elements like Cd, As, and Pb, but also in response to excess of essential trace elements, such as Zn, Cu and Se. The synthesis of the detoxifying oligopeptides, known as phytochelatins (PCs), is considered one of the most abundant mechanisms for Cd detoxification among higher plants.[2–4] A Cd^{2+} ion bound to the thiol group of a PC molecule becomes approximately 1000 times less toxic to most plant enzymes than "free" Cd^{2+} aqua ions.[4]

PCs are a group of short, non-protein, metal-binding peptides, with the general formula (γ-Glu-Cys)$_n$-X, where n = 2–11 and X can be glycine,[2] β-alanine,[2] serine,[5] glutamate,[6] glutamine[7] or missing.[8] The *N*-terminal linked γ-Glu can also be missing (des-γ-Glu-PCs).[9] Thus, seven families of PCs exist to date, which vary among plant species.[10,11] All of these families function analogically by chelating metals, forming oligopeptide based coordination complexes.[12]

The synthesis of PCs is controlled by the enzyme PC-synthase (γ-glutamyl-cysteine-dipeptidyl-transpeptidase), which is constitutively present in the cytoplasm of plants and activated by elevated metal ion concentrations.[2] The precursor is reduced glutathione (GSH), which undergoes a transpeptidal reaction catalyzed by PC synthase, resulting in PC-chains of various lengths. Cd is regarded as the most potent inducer of PC synthesis in higher plants,[4] and biosynthesis of PCs typically occurs within minutes after Cd-exposure, as it is independent of *de novo* protein synthesis.[13] PC synthase is deactivated when all Cd ions are bound to PCs.[3]

The low molecular weight Cd–PC species are transported across the tonoplast into the vacuole. This transport is mediated by an ATP-binding cassette-type transport protein, but free Cd^{2+} ions are also pumped into the vacuole by a Cd^{2+}/H^+ antiporter protein. Together with additional γ-Glu-Cys-peptides and free sulfide, Cd–PC complexes with a higher molecular weight are formed.[3] The molecular mass of Cd–PC complexes

[a] *Plant and Soil Science Laboratory, Department of Agricultural Sciences, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark. E-mail: shu@kvl.dk; Fax: +45 35283460; Tel: +45 35283498*
[b] *Department of Natural Sciences, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark*
[c] *Faculty of Engineering and Natural Sciences, Sabanci University, Tuzla-Istanbul, Turkey*

is reported to range from 2500 to 10 000 Da, depending on plant species[14] and the physiological age of the plants.[15]

Much effort has been put into the analysis of the free de-metallized PCs induced by metal toxicity. Reversed-phase chromatography, *e.g.*, RP-HPLC of fluorochrome-tagged PCs,[16] has frequently been used. Obviously, the major drawback of this approach is that no structural information is obtained on the coordination complex. The classical procedure for analysing coordination complexes in biological matrices has been fractionation with size exclusion chromatography (SEC), followed by off-line metal detection of the collected fractions by atomic absorption spectrometry (AAS) or atomic fluorescence spectrometry (AFS).[17] In recent years, SEC hyphenated to ICP-MS, ICP-OES or ESI-MS has been used to characterize coordination complexes in plants.[14,17–21] Among the new generation of stationary phases in SEC, several attractive materials have become available, which in comparison with the ones used in ion exchange chromatography (IEC) and reversed phase chromatography (RPC), have proved to be far more gentle in maintaining the structural integrity of the coordination complexes.[22,23] However, SEC still suffers from a relatively low resolution in comparison with IEC and RPC, and the analytical conditions during SEC need to be carefully optimized in order to maintain the structural integrity of the coordination complex and to produce reproducible chromatograms. Thus, in extracts of biological matrices a diverse range of metal ions are retained on the column, which might lead to ligand exchange and formation of artefact coordination complexes. Consequently, it is a major analytical challenge to develop robust and reliable SEC-ICP-MS based techniques to study Cd induced coordination complexes in biological matrices.

The objective of the present work was to develop a robust analytical technique, which allows the identification of the dominating Cd coordination complexes formed in plants exposed to Cd, using SEC-ICP-MS and ESI-TOF-MS. The multi-elemental capacity of SEC-ICP-MS was used to analyse the Cd-toxicity-induced speciation of several essential trace elements in plants and, subsequently, ESI-TOF-MS was used to determine the identity of the ligands. The biological importance of Cd-peptide speciation for tolerance towards excessive Cd is demonstrated using two genotypes of barley (*Hordeum vulgare* L.).[26]

Experimental

Instrumentation

SEC-ICP-MS experiments were performed on a HPLC (Agilent 1100 Series, Agilent Technologies, UK) coupled to a diode array detector (DAD) and an ICP-MS (Agilent 7500c, Agilent Technologies, UK) equipped with a PFA micro-flow nebulizer. The ICP-MS was tuned in standard mode (no reaction/collision gas used) to achieve a sensitivity on the masses ^{7}Li, ^{89}Y and ^{205}Tl higher than 18 000, 36 000 and 18 000 cps ppb^{-1}, respectively, and at the same time ensuring that the oxide level at m/z 156/140 was below 0.5%. The plasma power was operated at 1450 ± 50 W and the carrier and make-up gases were typically set at 0.83 and 0.17 L min^{-1}. Sample uptake was maintained at approximately 0.1 ml min^{-1} by the self aspirating PFA nebulizer. The detection limits (DL) for all ions (Mn, Ni, Cu and Cd) were below 0.2 μg L^{-1}, whereas the DL for Zn was about 1 μg L^{-1} due to background contamination, mainly originating from the SEC mobile phases.

All connections were constructed of PEEK tubing (0.17 mm id). All SEC-ICP-MS chromatographic data were processed using Plasma Chromatographic Software v. B-02-04 (Agilent Technologies, UK).

Fractionated peaks were automatically collected and pooled using the Agilent 1100 Series Fraction Collector (Agilent Technologies, UK).

The exact mass of ligands in the collected fractions was determined using ESI-TOF-MS connected to a Waters 2795 HPLC equipped with a Waters 996 PDA detector. The ESI-TOF-MS was a Micromass LCT apparatus (Waters, USA) equipped with an ESI probe and run in positive mode. The total ESI-TOF-MS spectra were acquired in the range from 100–1500 Da. The following basic ESI settings were used: capillary voltage, 2900 V; cone voltage, 33 V; ion energy, 35 V. The instrument was calibrated daily using a mixture of polyethylene glycol (PEG) standards (Sigma–Aldrich).

Materials

Analytical grade reagents were purchased from Sigma–Aldrich and demineralised water was prepared by a Milli-Q element filtration system (Millipore, USA).

For validation of the ICP-MS multi-elemental analysis of acid digested barley tissue, certified reference material was used (NIST 1515 Apple Leaves, US Department of Commerce, National Institute of Standards and Technology, USA).

Extracted samples for speciation analysis were centrifuged and subsequently ultra-filtered, using a Microcon centrifugal filter device YM-50 (Millipore, USA), with a 50 kDa cut-off.

Several analytical columns were tested for their ability to separate/fractionate the Cd-species including high resolution SEC columns (>20 000 theoretical plates), such as the TSK-GEL-BioAssist G3SW, which is a PEEK column with 5 μm beads (Tosoh Bioscience, Germany). Anion exchange was also tested with various buffers and at different pH-values using a Mono Q 5/50 GL (Amersham Biosciences, USA). However, none of these columns were suitable as Cd-species were unstable and data was obtained with poor reproducibility. However, stable analytical conditions were obtained by SEC using a Superdex Peptide 10/300 GL (Glass, 10 × 300 mm, 13 μm cross-linked agarose/dextran, Amersham Biosciences, USA), with an optimum separation range between 100 and 7000 Da. For de-salting and de-metallization of collected fractions prior to the ESI-TOF-MS analysis, RPC on an XTerra Phenyl C18 Column (2.1 × 100 mm, 3.5 μm, Waters, USA) was used.

Freeze-drying of plant tissue and rotational vacuum concentration of liquid samples were done on a RVC-Alpha (Christ, Germany) and the following digestion of samples was done in a microwave oven (Multiwave 3000, Anton Paar GmbH, Austria).

Analytical procedures

Cultivation of plants. Two genotypes of barley, *i.e.*, the Cd-tolerant Tokak and the intolerant Hamidiye, were grown in a nutrient solution for 26 days; thereafter half of the plants were exposed to 15 μM Cd for 4 days. Before cultivation, all the seeds were surface-sterilized by 3% NaOCl for 30 min. Seedlings were pre-cultivated in vermiculite for 6 days in a greenhouse (18 °C/15 °C) and then transferred to non-transparent cultivation units of a HD-PE polymer. Five plants were cultivated in each unit. The nutrient solution contained 0.2 mM KH_2PO_4, 0.1 mM NaCl, 0.6 mM $Mg(NO_3)_2$, 1.1 mM $Ca(NO_3)_2$, 0.6 mM KNO_3, 1 μM H_3BO_3, 0.2 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.02 μM Na_2MoO_4, 100 μM Fe-EDTA and 2 mM Na_2SO_4. The solution was renewed once a week, and the position of the cultivation units was randomized every third day. The pH was regulated to 6.5 ± 0.2 and controlled every second day. The nutrient solution was constantly aerated through sparging tubes. On day 26, Cd was added as $CdCl_2$ in a 15 μM concentration. Before the onset of the Cd treatment one plant from each unit was harvested in order to monitor the net uptake of Cd during the four days of Cd treatment.

Harvest and freeze-drying. After 4 days of Cd treatment the plants were harvested and the roots were washed, first in 5 mM $CaCl_2$, and then twice in water. Wash solutions were renewed between +Cd and −Cd plants, and between genotypes. The wash procedure was applied in order to reduce the amount of extra-cellular Cd bound to the root surface and to cell walls in the root apoplast, since this would interfere with the uptake measurements. Shoots and roots were then separated and rapidly weighed, placed in plastic bags and immersed in liquid N_2 in order to reduce the risk of oxidization. The plastic bags were filled with Ar and subsequently stored at −80 °C. Before extraction plants were homogenized in liquid N_2 and freeze-dried for 48 hours.

Digestion of tissue samples. 10.00 mg of freeze-dried plant material from each sample was transferred to clean, capped Savillex PTFE 6 ml vials, and was digested with 750 μL of HNO_3 (69–70% Baker Instra). The vials were left standing overnight for 16 hours in a fume-hood, loosely capped. Then, the samples were transferred to micro-oven vessels containing 8 ml of water as ballast. Digestion was performed using the following temperature program: 30 min (100 °C), 30 min (50 °C), 30 min (25 °C) and 30 min (50 °C), which ends up with a total sample preparation time of 150 min, including three 10 min-ramps between the temperature regimes. After digestion, the samples were left to cool, and thereafter they were diluted to a final volume of 10.5 mL, resulting in a 5% HNO_3-concentration. Five samples with certified reference material were included and 10 samples without any plant material were also prepared as blanks. If the blanks exceeded the LOD, analytical data were background corrected.

Extraction for speciation analysis. 40 mg of freeze dried plant material per sample was mixed and extracted in an ice-cold mortar with 2 mL of ice-cold and de-gassed 50 mM ammonium acetate (pH = 7.5) and 500 mg acid washed quartz sand. The homogenate was centrifuged immediately after extraction at 16 000*g* (2 °C) in 15 min and the supernatant was subjected to ultra-filtration for 20 min at 13 000*g* (2 °C).

SEC-ICP-MS analysis. The SEC column was mass calibrated using cytochrome c (12 500 Da), aprotinin (6512 Da), vitamin B12 (1355 Da), glutathione (307 Da) and cysteine (121 Da) by DAD at 214 nm.

The ICP-MS was tuned and run in standard mode and the following masses and elements were monitored: ^{55}Mn, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{111}Cd and ^{114}Cd. Several different buffers (phosphate, TRIS), concentrations (1–100 mM) and pH values (4–8) were tested to obtain the best SEC resolution and, based on this, ammonium acetate (50 mM, pH 7.5) was selected as the mobile phase with a flow rate of 0.5 ml min^{-1}. The sample was kept cool on a 2 °C thermostatic autosampler and 20 μL of sample was injected on the SEC-column, using a 55 min runtime. SEC fractions with high ion intensity were collected and immediately stored at −80 °C until used for further analysis. After each run the column was rinsed by an automatic injection program set at 5 repetitive 20 μL injections of a 5 mM EDTA–50 mM ammonium acetate solution (pH = 7.5), with a 3 min delay between each injection.

Peak fractionation and identification. The collected SEC fractions were lyophilized and re-suspended in 200 μL 0.4% TFA solution (pH 1.8) in order to dissociate the coordination complexes. In order to reduce signal suppression in the ESI-TOF-MS analysis, ligands were de-metallized and de-salted by off-line RPC. 20 μL of sample was injected on the XTerra column with a flow rate of 0.3 ml min^{-1} using buffer A (0.1% TFA) for 10 min, causing salts and free metal ions to be washed out. During the following 20 min the gradient was linearly changed to 20% buffer B (0.1% TFA–acetonitrile), and finally washed with 100% buffer B. Fractions were collected every 3 min in vials containing 5 mM dithiothreitol (DTT) as a reducing agent and lyophilized. Samples were then re-suspended in 200 μL (50% acetonitrile–0.1% formic acid) and 50 μL was injected into the ESI-TOF-MS.

Extraction efficiencies. In order to estimate the amount of Cd species included in the SEC-ICP-MS analysis relative to the total Cd amount found in the plant tissue, a mass balance was established. The total concentrations for each element obtained after microwave digestion and ICP-MS determination were set at index 100. Plant tissue used for speciation analysis was transferred quantitatively to Falcon vials, washing the mortar twice with buffer. The samples were freeze dried and re-suspended in 2 mL of water. After centrifugation, the pellet and the supernatant were split, and 600 μL of the supernatant was ultra-filtered with a 50 kDa cut-off, generating a low molecular weight fraction, LMW, and a high molecular weight fraction, HMW. Samples representing all fractions were digested and the concentrations of Cd, Cu, Mn and Zn (Table 1) were determined as described above.

Table 1 The concentrations and extraction efficiencies of four elements (Mn, Cu, Zn and Cd) in the root and shoot tissues of two barley genotypes differing in Cd tolerance. The plants were exposed to 15 μM $CdCl_2$ for 4 days before analysis. The concentrations were measured in total tissue and in the low molecular weight fraction (LMW < 50 kDa) of the tissue extract. Concentrations are given as mean values of 3 replicates and extraction efficiencies are listed as percentage of total tissue concentrations ±SE

		Elemental concentrations/μg g^{-1} tissue DM		
Genotypes and tissue	Element	Total tissue	LMW elemental species	Extraction efficiency (%)
Hamidiye root	Mn	120	48	40 ± 3
	Cu	24	17	71 ± 16
	Zn	82	47	58 ± 18
	Cd	1138	251	22 ± 3
	Cd in control	1.3		
Hamidiye shoot	Mn	35	28	80 ± 4
	Cu	6	4	78 ± 3
	Zn	55	46	84 ± 3
	Cd	33	21	65 ± 5
	Cd in control	0.05		
Tokak root	Mn	116	43	37 ± 2
	Cu	24	14	58 ± 3
	Zn	39	20	51 ± 8
	Cd	952	189	20 ± 1
	Cd in control	0.9		
Tokak Shoot	Mn	49	30	61 ± 7
	Cu	7	5	71 ± 17
	Zn	50	16	32 ± 3
	Cd	40	16	39 ± 5
	Cd in control	0.07		

Results and discussion

Cd toxicity symptoms

There were clear visual differences between the tolerant genotype, Tokak, and the intolerant one, Hamidiye, in their response to Cd (Fig. 1). Tokak showed almost no visual symptoms of Cd toxicity throughout the 4 days of Cd exposure, whereas Hamidiye showed visual toxicity symptoms already after 24 hours. These symptoms were initially whitish areas in the middle of the older leaves, which evolved to necrotic spots and spread along the ribs to the leaf tip (Fig. 1).

Cd in plant tissue

No significant difference was found in the shoot tissue concentrations of Cd between the genotypes (Table 1) despite the obvious difference in visual appearance (Fig. 1). In both genotypes, only approximately 3–4% of the absorbed Cd was translocated to the shoots. This shows that most of the Cd was prevented from reaching the shoot, where biochemical processes such as photosynthesis are highly sensitive to Cd. The observed difference in Cd tolerance between the two genotypes could thus not be explained by differences in uptake or translocation.

Fig. 1 Cd toxicity symptoms of the two investigated genotypes; the tolerant Tokak (upper leaf) and the intolerant Hamidiye (lower leaf). The plants were exposed to 15 μM of $CdCl_2$ for four days.

Extraction efficiencies of elements

Relative to the other trace elements, plants were exposed to high Cd concentrations in order to specifically induce Cd-toxicity. Removal of exchangeable Cd^{2+} in the root cell walls was found to be important in order to reduce the risk of ligand exchange during extraction and analysis. Thus, roots were carefully washed several times with 5 mM $CaCl_2$, but still it was evident that large amounts of Cd were trapped, relative to the other trace elements, apparently in the non-exchangeable ion pool of the root tissue (Table 1).

In the roots approximately 20% of total tissue Cd was extractable to the LMW-fraction of both genotypes (Table 1), and in the shoots the corresponding values were 65% and 39% for Hamidiye and Tokak, respectively. Interestingly, only a negligible amount of Cd occurred in the HMW-fraction after ultra-filtration (<1% of Cd in the LMW), indicating that most Cd species in the supernatant had a molecular size below 50 kDa.

Precision, recovery and polyatomic interferences

The Cd speciation was determined in the LMW fraction extracted from shoots and roots. However, it was initially impossible to reproduce the chromatography due to an excessive amount of free metal ions being retained on the SEC column, leading to ligand exchange and destabilization of the

injected Cd coordination complexes. For removal of metals on the column, the procedure by Vaccina *et al.* (1999)[14] was tested. This procedure is based on a column cleaning procedure in which the mobile phase of 30 mM TRIS is switched to a reconditioning mobile phase of 2 mM β-mercaptoethanol–30 mM TRIS after each sample injection. Reconditioning and re-equilibration of the column was allowed for 2 times 30 min, which tripled the analytical run time between samples from 30 to 90 min. However, this procedure was not sufficient in our study, as repeatedly Cd could be eluted from the column after injecting β-mercaptoethanol. Moreover, we wanted to avoid the switch of mobile phase in order to reduce the overall run time. Thus, several wash-procedures were tested; the fastest and most efficient one was repetitive injections of 5 mM EDTA dissolved in a mobile phase of 50 mM ammonium acetate at a pH of 7.5. In between the analytical runs, the EDTA-solution was automatically injected and the size of the eluting Cd–EDTA-peaks were recorded by ICP-MS. Optimization of the procedure showed that it was possible to bring the Cd levels down to the background ion intensity of 500 counts s^{-1} by injecting a 20 μL EDTA-solution repeatedly five times with a delay of 3 min between injections, which resulted in Cd–EDTA peaks with decreasing area (Fig. 2). Each injection reduced the levels of Cd and Cu on the column by approximately 85%. This rinse procedure provided chromatographic results with a high reproducibility. The standard deviation of the integrated peak areas of fraction 1, 2 and 3 was 3%, 2% and 4%, respectively, for the four repetitions (Fig. 3). The stability of Cd complexes during SEC-fractionation was checked by collecting and re-injecting some of the fractions. The recovery of these re-injected fractions ranged from 86% for the smallest Cd species, Cd–GSH, to nearly 100% for the larger Cd species, based on peak area.

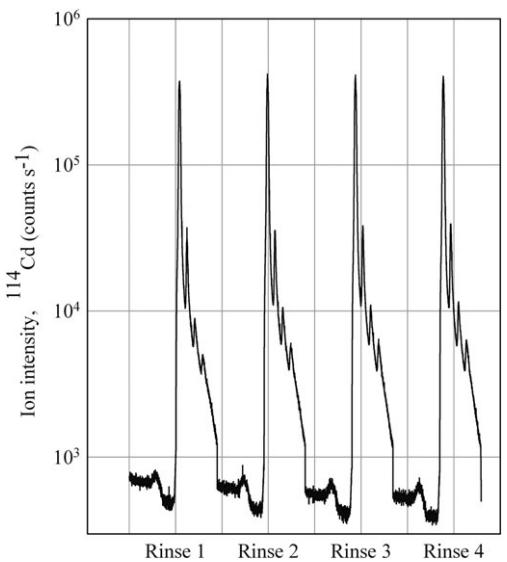

Fig. 2 A composite SEC-ICP-MS chromatogram showing the result of the EDTA injections used to regenerate the SEC-column between samples. There were five repetitive injections in four different analytical runs, each one run immediately after a root sample analysis. The peaks represent the amount of column-bound, EDTA-extractable Cd originating from the previous root sample. See Fig. 3 for comparison.

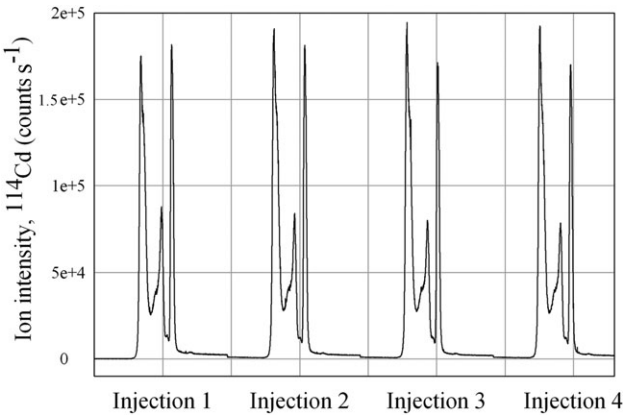

Fig. 3 A composite chromatogram showing the reproducibility of the SEC-ICP-MS plant sample analyses. The peaks represent the different Cd fractions in four repetitive analyses of a root sample. An EDTA-injection programme was run in between samples in order to regenerate the column. See Fig. 2 for comparison.

The measurement of ^{111}Cd and ^{114}Cd isotopes during SEC fractionation of the PCs showed a close agreement between the ^{111}Cd/^{114}Cd isotope ratios found in peaks 1, 2 and 3 (0.439, 0.441, 0.443) and the theoretical value of 0.446, which documents the absence of any significant analytical bias on the Cd ion intensity from polyatomic ion interferences in the tissue matrix (Fig. 8).

Fractionation of Cd species by SEC-ICP-MS

The chromatograms of the LMW fractions of root samples displayed three distinct peaks with highly consistent retention times and peak areas in both Hamidiye and Tokak (Fig. 4). The approximate molecular weights of these three Cd-binding coordination complexes were 8700–15 000 Da, 2900–4600 Da and 700–1800 Da, according to the SEC calibration curve (Fig. 4, right axis). In the following, they will be referred to as fraction 1, 2 and 3, respectively. In contrast, only one fraction appeared in the shoot samples, with the same retention time as fraction 3 in the root samples (Fig. 4, lower chromatogram). The size of this fraction was only 4–5% the size of the corresponding peak in root fraction.

The distribution of Cd between the different fractions is shown in Table 2. As Cd was quantitatively eluted from the column (Fig. 3), these values represent the distribution and recovery of all Cd species found in the LMW-fraction. In roots, the Cd tolerant genotype had more Cd bound in species eluting in the first two fractions relative to the intolerant genotype. The genotypes had similar amounts of Cd bound to the third fraction.

In order to verify the presence of Cd binding ligands in the observed fractions, we used a mixture of PCs extracted from a cell culture of the plant Bladder Campion (*Silene vulgaris*), previously described by Zenk *et al.* (1996)[4] as standard. The identity of the isolated and de-metallized ligands was analysed by ESI-TOF-MS and found to contain a mixture of glutathione (GSH): m/z calculated 308.0916, found 308.0921, PC_2; m/z calculated 540.1434, found 540.1414, PC_2 lacking

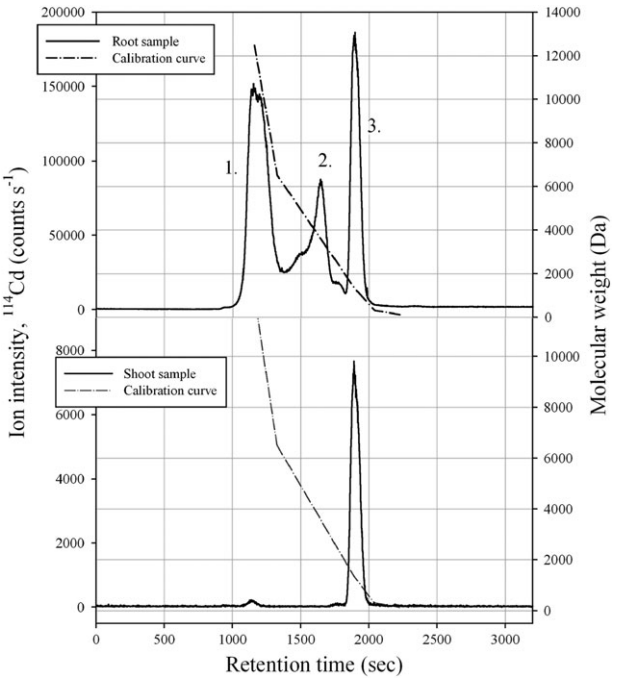

Fig. 4 SEC-ICP-MS chromatograms showing the different Cd fractions in barley root (upper chromatogram) and shoot tissues (lower chromatogram). Fractions 1, 2 and 3 are indicated in the figure and the right axis is showing the approximate molecular size of the Cd species (Da).

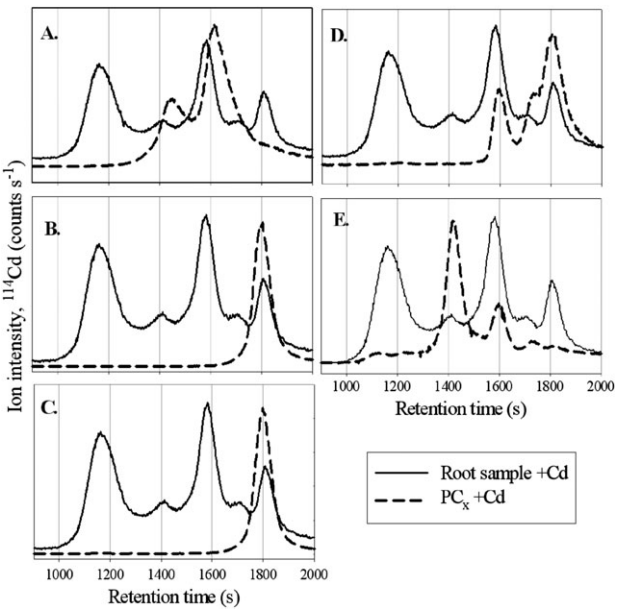

Fig. 5 SEC-ICP-MS chromatograms showing purified phytochelatins reconstituted with Cd in equimolar concentrations. (A) A sample containing all isolated phytochelatins, (B) GSH and des-γGlu-PC_2, (C) PC_2, (D) PC_3 and (E) a mixture of PC_4, PC_5 and PC_6. For comparison of retention times, each chromatogram is displayed together with a barley root sample (solid line).

the *N*-terminal linked γ-Glu (des-γGlu-PC_2); *m*/*z* calculated 411.1008, found 411.1037, PC_3; *m*/*z* calculated 772.1952, found 772.1926, PC_4; *m*/*z* calculated 1004.2470, found 1004.2433, PC_5; *m*/*z* calculated 1236.2987, found 1236.2927, PC_6; *m*/*z* calculated 1468.3505, found 1468.2777, all with glycine as the terminal amino acid.

PC_2, PC_3 and PC_4 were isolated by semi-quantitative RPC but, due to insufficient chromatographic resolution, GSH and des-γGlu–PC_2 were collected in the same fraction and PC_5 and PC_6 were collected together in a later eluting fraction. The collected fractions were incubated for 16 hours under Ar-gas with a $Cd(NO_3)_2$ solution in an approximate 1:1 molar relationship. The samples were subsequently analyzed with the same SEC-ICP-MS set-up as was used for the root and shoot samples. All of the Cd species formed under these conditions either co-eluted or overlapped with the Cd containing fractions of the barley root samples, indicating the likely presence of similar PCs in the tissue samples (Fig. 5). The shortest PC-chains (GSH, des-γGlu-PC_2 and PC_2) all had the same retention time as fraction 3 in the root sample (Fig. 5 B and C), supporting a theory of fraction 3 being a mixture of Cd species with short peptide chains. Incubating Cd with PC_3 produced Cd species which co-eluted with both fractions 2 and 3 in our sample, but also in-between these fractions, indicating that several additional combinations of ligands and Cd existed, compared with the PC_2s (Fig. 5D). The long-chained PCs (PC_4, PC_5 and PC_6) (Fig. 5E) overlapped mostly with the small signal between fraction 1 and 2 found in the root sample (Fig. 4), but traces were found in all fractions of the root tissue sample. Thus, the general tendency was that the peptide chain length of the Cd-species decreased gradually from fraction 1 to fraction 3. We can conclude from these observations that Cd coordination complexes in fraction 3 are mainly composed of PCs with a peptide chain length in the range of PC_2 or less, fraction 2 contains mainly PCs in the range from PC_3 to PC_6 and, finally, that fraction 1 contains PCs with the chain length

Table 2 The retention times, mass ranges and fractional distribution in root and shoot samples, analyzed by SEC-ICP-MS. The distribution of fractionated Cd-species is presented as mean percentages (±SE) of the whole LMW-fraction (< 50 kDa), based on three replications

			Distribution of fractionated Cd-species (% of LMW)			
			Root		Shoot	
			Genotypes			
SEC-fraction	Retention time/s	Mass range/Da	Tolerant	Intolerant	Tolerant	Intolerant
Fraction 1	1220	6700–15000	19 ± 1	12 ± 1		
Fraction 2	1640	2900–4600	27 ± 4	18 ± 2		
Fraction 3	1880	700–1800	4 ± 1	4 ± 1	67 ± 1	72 ± 9
Total speciation			50 ± 4	34 ± 3	67 ± 1	72 ± 9

PC_5 to PC_6, or possibly even longer. We can also conclude that the amount of different Cd species increases with the PC chain length, illustrated by the fact that a chain length of PC_3 or more generates three or more species of different sizes, whereas chain lengths of PC_2 or shorter only lead to the formation of one single fraction.

When comparing these observations, there seems to be a correlation between Cd tolerance and the distribution of Cd-species found in the three LMW root fractions. The tolerant genotype clearly had a higher amount of accumulated Cd bound to PCs with a chain length of PC_3 or longer (fraction 1 and 2), whereas both the intolerant and the tolerant genotype had similar amounts of accumulated Cd bound to PCs with a short chain length (PC_2 or shorter) (Table 2).

The molecular weights of the Cd species found in fraction 1–3, which were estimated by calibration of the SEC-column, strongly indicate that the Cd species found generally contain more than one PC ligand, and possibly also more than one Cd atom per species. For example, PC_2 bound to Cd in a 1:1 ratio would have the molecular weight 652 Da, but elutes as a 700–1800 Da complex, rather indicating a 2:1 or a 2:2 ligand:-metal ratio. For Cd bound to PC_3, the latest eluting Cd-species in fraction 3 might be a 1:1 complex (MW_{Cd-PC_3}: 884 Da), whereas the PC_3 containing Cd-species found in fraction 2 must contain more than one PC_3 ligand in order to elute as a 2900–4600 Da Cd species.

Identification of Cd species by ESI-TOF-MS

ESI-TOF-MS analysis of the three heart-cut, de-salted and de-metallized Cd containing SEC fractions showed that three different families of PCs were present: $(\gamma Glu\text{-}Cys)_n$-Gly ($PC_n$), $(\gamma Glu\text{-}Cys)_n$-Ser (iso-$PC_n$) and Cys-$(\gamma Glu\text{-}Cys)_n$-Gly (des-$\gamma Glu\text{-}PC_n$), the latter lacking the *N*-terminal amino acid. The quantitatively most important ligands were identified as des-$\gamma Glu\text{-}PC_2$ (m/z calculated 411.1008, found 411.1009) and PC_3 (m/z calculated 772.1952, found 772.1929). These ligands dominated the peptide fractions ranging from 700–1800 Da (fraction 3) and 2900–4600 Da (fraction 2), and also occurred as traces in fraction 3 ranging from 6700 to 15 000 Da (Fig. 6). This indicates that the ratio between Cd and ligands must deviate significantly from a 1:1 stoichiometry and that the same ligands are likely to be involved in several different Cd species differing considerably in molecular weights. It was not possible to estimate the Cd/S ratio in the SEC fractions because the ESI-TOF-MS measurements do not provide quantitative data. In addition, the ion intensity of ^{34}S obtained by SEC-ICP-MS was too weak due to the poor ionization potential of S and the low S concentration in the samples. Whereas des-$\gamma Glu\text{-}PC_2$ was only present in the reduced form, PC_3 occurred in both the reduced and oxidized forms (m/z calculated 770.1795, found 770.1758) together with a significant amount of iso-Ser-PC_3, where the C terminal Gly had been substituted by Ser (m/z calculated 802.2057, found 802.1913).

To obtain further information about the identity of the Cd species formed in fractions 2 and 3, the individual ligands, isolated and purified by RPC as listed above, were reconstituted with Cd in equimolar concentrations and analysed by direct injection ESI-TOF-MS (Fig. 7A). For des-$\gamma Glu\text{-}PC_2$ both the free ligand and stochiometric ligand:Cd ratios of 1:1 and 1:2 were formed, as evidenced by the accurate mass determiner for the species and by the distinct Cd isotope profile found in the mass spectrum (Fig. 7 (A) and (C)), which closely matched the analytical Cd isotope profile (Fig. 7(B)). For PC_3, only the free ligand and the 1:1 ratio between Cd and ligand were found (Fig. 7(A) and (D)). There was no indication of the presence of Cd species with PC_{4-6}, probably due to the low ligand concentrations found in the Cd reconstituted samples.

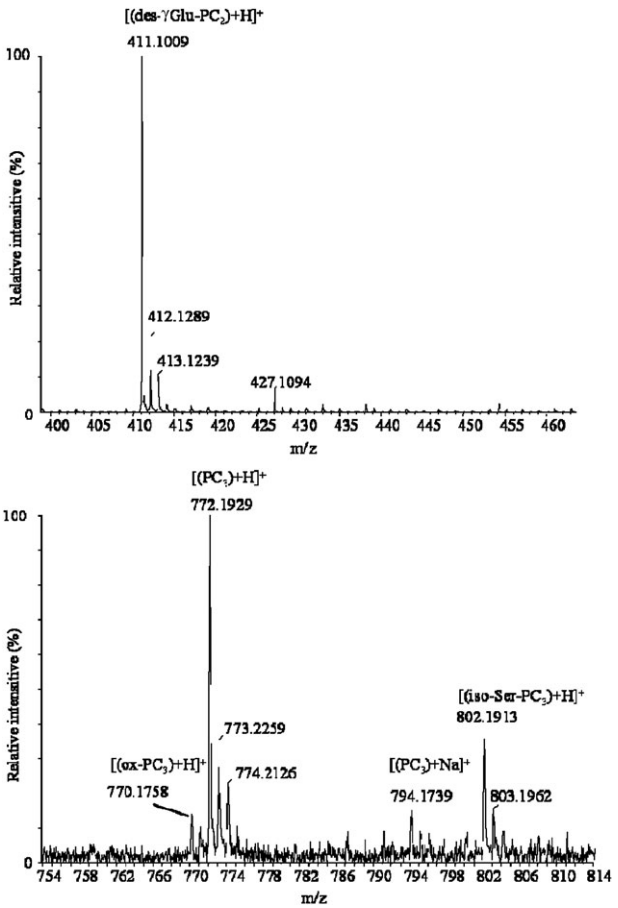

Fig. 6 ESI-TOF-MS spectra of the phytochelatins found in the root extracts of the barley genotypes Hamidiye and Tokak. The upper spectrum is the fraction eluting between 12 and 15 min from the RPC-column, dominated by des-$\gamma Glu\text{-}PC_2$, and the lower spectrum is the fraction eluting from 15 to 18 min, dominated by PC_3 and iso-Ser-PC_3.

The identities of a few Cd–PC species which are likely to be present in fractions 2 and 3 can theoretically be deduced using the information on PCs and Cd–PC species obtained by ESI-TOF-MS and SEC-ICP-MS and assuming that Cd preferably coordinates with two thiol groups (Fig. 7). A Cd-species consisting of four PC_3 molecules bound to 6 Cd atoms would have the molecular weight 3749 Da, which is in good agreement with the observed molecular weight of 3600 Da found by SEC-ICP-MS. Cd-containing peptide fractions of this size have previously been found in plants by other authors.[2,14] Following the same approach for fraction 3, the most likely

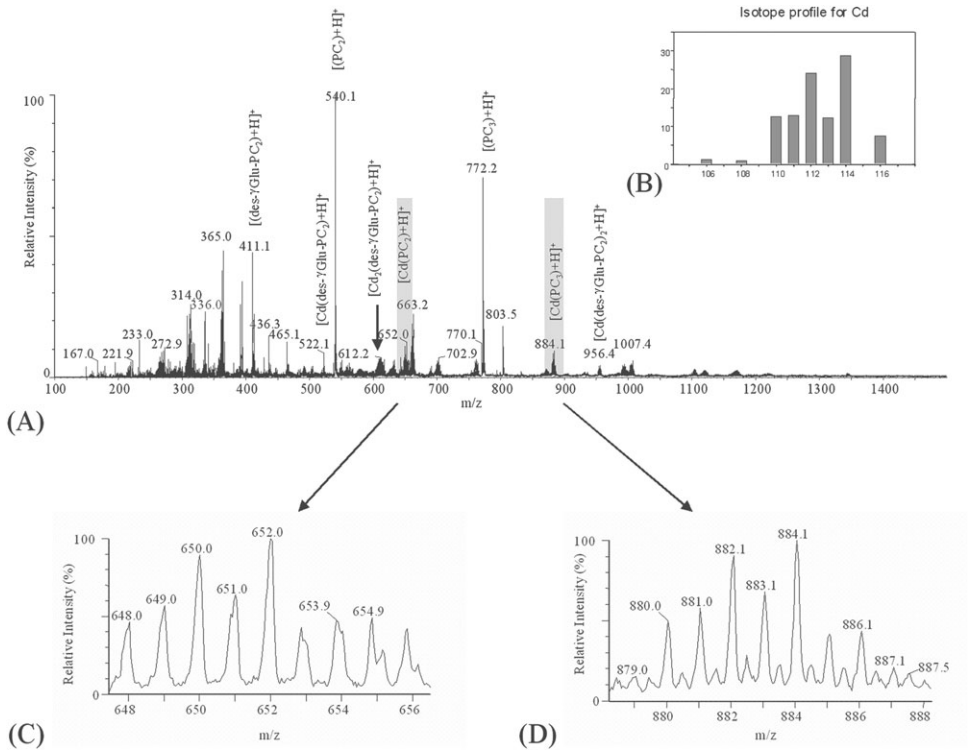

Fig. 7 ESI-TOF-MS spectra of synthesized Cd–phytochelatin complexes. (A) is the whole spectrum for a mixture of various phytochelatins reconstituted with an equimolar Cd concentration, and (B) is the theoretical isotopic distribution for Cd. (C) and (D) are close-ups of the most prominent Cd species $[Cd(PC_2) + H]^+$ and $[Cd(PC_3) + H]^+$.

Cd species would be either $[Cd_2(des\text{-}\gamma Glu\text{-}PC_2)_2]$, $[Cd_3(des\text{-}\gamma Glu\text{-}PC_2)_3]$ or a 1:1 Cd:PC_3 complex, all combinations lying within the 700–1800 Da range.

In fraction 2 both PC_3 and des-γGlu-PC_2 were observed, which may indicate the presence of a Cd species consisting of both PC_3 and des-γGlu-PC_2, rather than two separate, co-eluting Cd-species, since des-γGlu-PC_2 reconstituted with Cd eluted only as fraction 3. Assuming that each Cd binds to two thiol groups, and that unchelated thiol groups are highly unlikely in the Cd rich environment of the root cytosol, only three coordination complexes in the range 2900–4600 Da, containing both PC_3 and des-γGlu-PC_2, are theoretically possible. These are $[Cd_6(PC_3)_2(des\text{-}\gamma Glu\text{-}PC_2)_3]$, (MW: 3437 Da), $[Cd_7(PC_3)_2(des\text{-}\gamma Glu\text{-}PC_2)_4]$, (MW: 3958 Da) or $[Cd_8(PC_3)_2(des\text{-}\gamma Glu\text{-}PC_2)_5]$, (MW: 4479). However, the actual existence of such complexes has yet to be confirmed. Thus, fraction 2 in this work was possibly dominated by a 4:6 PC_3:Cd complex, but with traces of the $[Cd_x(PC_3)_x(des\text{-}\gamma Glu\text{-}PC_2)_x]$ species listed above.

In fraction 3, the ESI-TOF-MS mass-spectrum showed the presence of both des-γGlu-PC_2 and PC_3, suggesting a possible co-elution of $[Cd_x(des\text{-}\gamma Glu\text{-}PC_2)_x]$ species and the simplest form of $[Cd(PC_3)]$.

Earlier work by several groups has used ESI-MS to elucidate how PCs form coordination complexes with Cd. Yen *et al.* (1999)[18] showed that PC_5 coordinates with up to three Cd atoms and that the PC_{3-4} can coordinate 1–2 Cd atoms. Burford *et al.* (2005)[24] showed that also GSH can coordinate with Cd, and in a later study by Navaza *et al.* (2006)[20] it was shown that GSH actually can coordinate with up to four Cd atoms. This group also presented data suggesting the prescence of a Cd–PC_2 species in *Brassica juncea*, even though no Cd isotope profile could be shown. The perfectly matching Cd isotope profile obtained in the present work (Fig. 7 (B) and (C)) confirms the existence of a Cd–PC_2 complex. In addition, the presence of several new 2:1 and 1:2 Cd-species is documented and it is shown that PC chains larger than PC_2 may form several Cd species, differing in size and Cd:PC ratio. Comparison of the Cd speciation in barley genotypes with differential Cd tolerance provide strong evidence that Cd speciation is linked to Cd tolerance *in planta* with the biologically relevant Cd species generally being the largest possible having PC chains with $n > 2$.

Several minor peaks in the ESI-TOF-MS spectra were observed which could not be assigned to any PCs (data not shown). A similar observation was made by Vacchina *et al.* (2000),[19] who also analyzed Cd species in plant samples fractionated by SEC. This is presumably related to the relatively low resolution of SEC causing some co-elution of oligopeptides other than PCs. Thus, it cannot be excluded that ligands other than PCs might be of minor importance in controlling the binding of Cd in plant tissue.

Multi-elemental speciation analysis by SEC-ICP-MS

The simultaneous measurements of the ^{66}Zn, ^{60}Ni, ^{63}Cu, ^{55}Mn, ^{111}Cd and ^{114}Cd isotopes generated new information on the speciation of elements other than Cd in root tissue

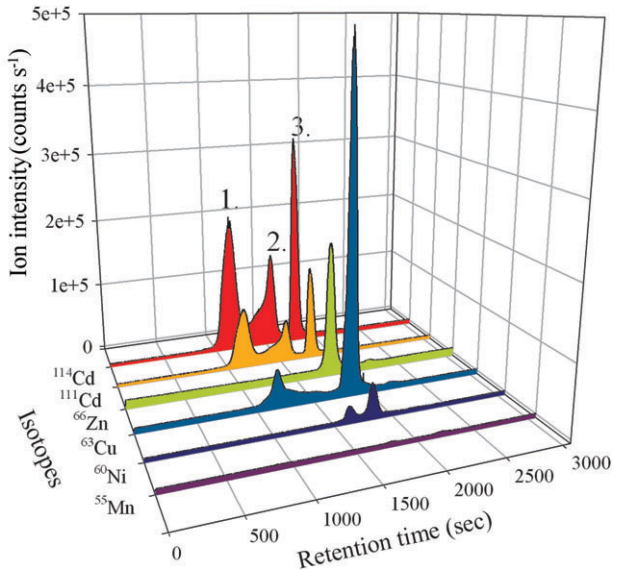

Fig. 8 SEC-ICP-MS chromatogram showing the binding of two Cd isotopes, Zn, Cu, Ni and Mn to ligands in a Cd treated root sample. For comparison with a non-Cd treated sample, see Fig. 9.

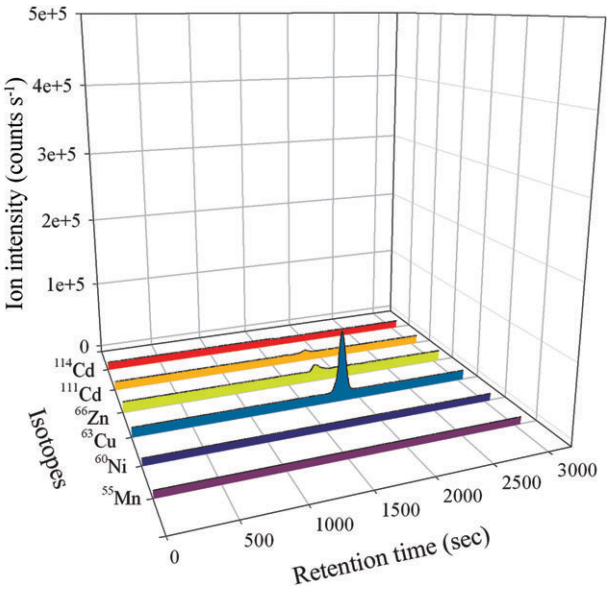

Fig. 9 SEC-ICP-MS chromatogram showing the negligible binding of two Cd isotopes, Zn, Ni and Mn to ligands in a non Cd-treated root sample. The only element which clearly speciated with ligands was Cu.

exposed to Cd toxicity. Cd induced a 1000-fold increase in tissue Cd concentration (Table 1) and was followed by a marked increase in the synthesis of metal ion scavenging peptides (Fig. 8). Apart from Cu only trace amounts of the other elements were found in coordination complexes in the control plants (Fig. 9). However, Cd exposure induced a remarkable change in the speciation of the other trace elements due to the increased intercellular concentration of PCs (Fig. 8). Approximately five times more Cu was bound to PCs in the Cd treated samples compared with the control. The chelation of Ni and Zn by PCs was also markedly increased due to Cd exposure. No Mn speciation was observed in any of the treatments, confirming the poor ability of Mn to form thiol-based species. Thus, Cd-induced PC synthesis may affect the bioavailability of essential trace elements in plants, which subsequently may lead to secondary deficiencies of essential elements adding to the cellular stress level imposed by Cd toxicity.

The highest ion intensities of Zn, Cu and Ni were found in fraction 3, where they co-eluted with Cd, suggesting that these elements primarily formed coordination complexes with des-γGlu-PC_2, or a single PC_3 ligand. In particular, the Cu/Cd ratio was high despite the much lower bulk tissue concentration of Cu compared with Cd (Table 1). The Cd species in fractions 1 and 2, containing many of the longer peptide chains, had a higher affinity for Cd than for the other metals under the conditions given. This indicates that PC_3, being the dominating ligand in fraction 2, had a high affinity for Cd. Fraction 1, consisting of PCs with $n > 6$ and possibly also other non-phytochelatin ligands, was dominated by Cd and Cu-species, whereas the ion intensity of Ni and Zn was not significantly different from the background. This shows that important elements such as Zn, Cu and Ni with essential metabolic functions in plants form complexes with different PCs and with highly variable affinities and that major differences even occur in the speciation of closely related elements such as Cd and Zn. Chiefly, the PCs in fraction 2 with peptide chain lengths >3, in this particular case dominated by PC_3, predominantly chelated Cd, whereas only trace amounts of Cu were measured. The tolerant genotype synthesized significantly more of these Cd-specific complexes (Table 2), clearly indicating a correlation between Cd tolerance, PC chain length and the amount of PCs formed. It is noteworthy that none of these ligands occurred in shoot tissue where only Cd species, ranging from 700–1800 Da, were identified (Fig. 4). The higher affinity for Cd than for Cu of PCs with $n > 3$ invalidates the general assumption that Cu is always preferentially speciated.[25] This seems only to be the case for PCs with $n < 3$.

Conclusions

This study confirms that plants respond to Cd toxicity by synthesizing metal ion scavenging oligopeptide phytochelatins, which reduce the Cd ion activity in tissue by forming Cd based coordination complexes. Ligand exchange on the column used for size exclusion chromatography (SEC) was a major challenge as several artefact Cd species were formed which could not be efficiently removed by the hitherto reported methods. An effective automatic injection program of repetitive EDTA injections between samples was developed, which ensured a rapid and efficient removal of non-chelated metal ions and produced data with an excellent precision. The amount of extractable Cd bound in coordination complexes was 70% in the shoot and 34–50% in the root tissue, depending on the genotype considered. No certified Cd–phytochelatin species are commercially available, but re-injection of isolated Cd species with known compositions showed recoveries of 86% for Cd–GSH and nearly 100% for Cd species with ligands larger than PC_2. In the shoot tissue only one fraction of Cd species was found, ranging from 700–1800 Da, whereas at least two additional fractions ranging from 2900–4600 and 6700–

15 000 Da were found in the root tissue. Three different families of phytochelatins, *viz*. (γGlu-Cys)$_n$-Gly (PC$_n$), (γGlu-Cys)$_n$-Ser (iso-PC$_n$) and compounds lacking the *N*-terminal amino acid Cys-(γGlu-Cys)$_n$-Gly (des-γGlu-PC$_n$) were identified. The quantitatively most important ligands were des-γGlu-PC$_2$ (m/z calculated 411.1008, found 411.1009), and PC$_3$ (m/z calculated 772.1952, found 772.1929), dominating the peptide fractions ranging from 700 to 1800 Da and 2900–4600 Da. This indicates that the ratio between Cd and ligands deviated significantly from a 1:1 stoichiometry and that the same ligands were involved in several Cd-species differing considerably in molecular weights. The phytochelatins induced by Cd toxicity also bound several essential trace elements in plants, including Zn, Cu, Ni, whereas no Mn species were found. Zn, Cu and Ni-species were found in the 700–1800 Da and 6700–15 000 Da fractions, whereas only Cd species were found in the 2900–4600 Da fraction dominated by PC$_3$ ligands. In contrast, the des-γGlu-PC$_2$ ligands dominating the 700–1800 Da peptide fraction chelated much more Cu than Cd, despite the fact that the Cu tissue concentration was approximately 50 times lower than those observed for Cd. Finally, we conclude that the tolerant genotype synthesized significantly more Cd–PC$_3$ and that the PC$_3$ ligand had a high Cd-specificity, clearly indicating a correlation between Cd tolerance and the Cd speciation.

Acknowledgements

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