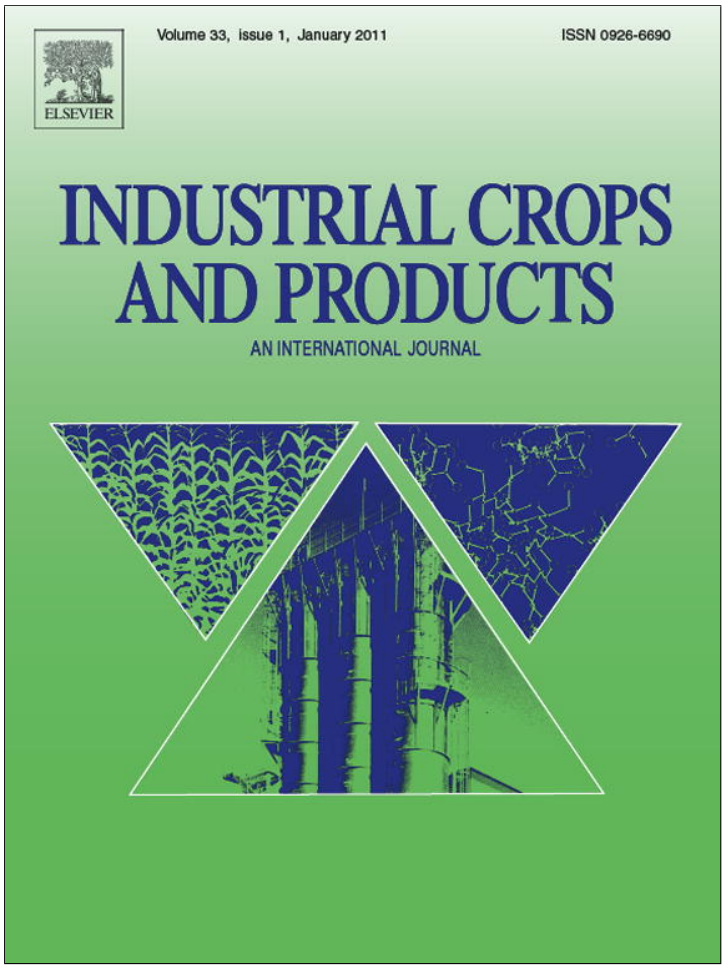

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Cadmium-induced production of phytochelatins and speciation of intracellular cadmium in organs of *Linum usitatissimum* seedlings

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ABSTRACT

The capacity of most flax (*Linum usitatissimum* L.) varieties and cultivars to accumulate cadmium (Cd), underlined by their Cd-tolerant phenotype, brought an interest in their use in phytoextraction of soil Cd to aboveground biomass. The aim of this study was to gain an insight into the mechanism of intracellular detoxification of Cd, by exploring the response of flax seedlings, cultivar AGT 952, to 12 days growth at 50–500 μM Cd. The majority of accumulated Cd (67–74%) was retained in roots, which showed reduced elongation (by 50% at 100 μM Cd) and 3.5 (at 50 μM Cd) to 9.6 (at 500 μM Cd) times higher concentration of Cd than in shoots. Labeling of roots with Leadmium fluorescent marker visualized intracellular localization of Cd. High-performance liquid chromatography of extracts from Cd-treated seedlings revealed induced synthesis of phytochelatins, small intracellular peptides binding Cd through cysteine of their repeating $(\gamma\text{-Glu-Cys})_n$ motives (PCn). Regardless the treatment, PC3 was dominant in all organs and PC2 was restricted to cotyledons. Larger PC4 and PC5 accumulated only in roots grown at 500 μM Cd. Size exclusion chromatography of the organ extracts from seedlings exposed to 100 μM revealed the presence of 2-kDa complexes equated to low-molecular weight (LMW) Cd–PC complexes. In roots, the majority of Cd was stored in ≥9-kDa complex corresponding in apparent molecular mass to stable high-molecular weight (HMW) CdS–PC complexes of yeasts and plants. Data demonstrate that, like in most other plants, the response of flax to Cd involved phytochelatins and suggested that the deposition of Cd in HMW complex should be considered the intrinsic feature reducing translocation of Cd to shoots.

1. Introduction

Flax (*Linum usitatissimum* L.) is a traditional annual culture crop with long-established use in the textile, chemical and food industries (Singh et al., 2011). Apart from this, flax fibers are emerging as a renewable alternative to synthetic and glass fibers in fiber-reinforced polymer composite materials especially in building and automotive industry (Aamr-Daya et al., 2008; Satyanarayana et al., 2009; Lazko et al., 2011). As Cd-accumulating and Cd-tolerant plant, flax is also gaining increasing attention for potential use in phytoremediation of soils polluted with Cd, highly toxic and abundant environmental contaminant (Broadley et al., 2001; Kos et al., 2003; Angelova et al., 2004; Shi and Cai, 2009; Bjelková et al., 2011). Of particular interest is phytoextraction of Cd that relies upon accumulation of Cd in aboveground harvestable parts of the plant. The cost effectiveness of flax-based phytoextraction would be further reinforced by utility of the fiber in many industrial applications, rendering the metal immobilized in relatively safe form. Certain limitation of flax in phytoextraction is due to retention of substantial amount of Cd in roots (up to 60%) of plants grown on heavily polluted soils, which restricts techno-economic suitability of flax to soil Cd concentrations ranging from 1 to 10 mg kg^{-1} (Angelova et al., 2004; Jiao et al., 2004; Bjelková et al., 2011).

Interest in phytoextraction potential of flax brought efforts directed towards screenings of flax varieties and cultivars for Cd-accumulating and Cd-tolerant phenotypes (Smykalova et al., 2010; Soudek et al., 2010; Bjelková et al., 2011). However information about the mechanism directly involved in detoxification of Cd, underlying its accumulation, and about speciation of Cd in organs is quite limited. Douchiche et al. (2010) have recently shown that growth of seedlings of tolerant flax cultivar Hermes on media with 500 μM Cd induce in all organs cell wall thickening, pectin cross-linking and increase of cell-wall associated pectin methylesterase and peroxidase. These changes may improve the tissue cohesion and provide cell walls with higher Cd biosorption capacity, allowing storage of the metal in apoplasm and reduce its uptake into the

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cytoplasm. In a wide variety of plants, certain fungi and nematodes, phytochelatins (PCs) play a pivotal role in detoxification of intracellular Cd (Clemens and Simm, 2003; Clemens, 2006). These are peptides of general structure $(\gamma\text{-Glu-Cys})_n$X with n being mostly 2–5 (PCn; X represents Gly, Ser, β-Ala, Glu, Gln or no residue), sequestering Cd through the metal thiolate coordination. Their synthesis occurs by chain extension in a transpeptidation reaction from glutathione (GSH) or its homologues by the constitutive PC synthase in a metal-dependent manner. The Cd–PC complexes thus contain PCs of various lengths. Two Cd–PC complex species that differ in apparent molecular mass are formed in yeasts *Schizosaccharomyces pombe* and *Candida glabrata* as well as in plants. These are referred to as low molecular weight (LMW) complexes of apparent molecular mass ranging from 2 to 4 kDa and high molecular weight (HMW) complexes of molecular mass typically ranging from 6 to 9 kDa (Grill et al., 1989; Clemens and Simm, 2003). Formation of HMW complex, essential for maximum Cd detoxification in *S. pombe*, involves production of LMW complex in cytosolic compartment, which is further transported to vacuoles where immobile HMW complexes of CdS crystallites covered with PC are formed under the incorporation of S^{2-} (Ortiz et al., 1995). Several lines of evidence suggest that, like in *S. pombe*, Cd sequestration in plants involves vacuolar HMW complexes of apparent molecular mass up to 13 kDa (Clemens and Simm, 2003; Rauser, 2003).

Hradilová et al. (2010) have recently scored the proteomic response of flax cells in suspension culture exposed to Cd and identified 14 differentially produced proteins. Among them, glutamine synthetase, *S*-adenosyl-L-methionine synthetase and methionine synthetase, accumulated in the cell suspension cultures exposed to Cd. These enzymes that directly or indirectly enhance biosynthesis of GSH, and hence of PCs, are implicated in Cd detoxification in *Arabidopsis thaliana* (Sarry et al., 2006). It prompted our efforts to assess the role PCs may play in intracellular binding of Cd in flax seedlings and in high capacity of roots to sequester the metal. Here we report the characterization of the physiological response of flax cultivar AGT 952 to high Cd doses, organ-specific accumulation of Cd accompanied by induction of PC synthesis and complex formation and visualization of Cd deposition in root vacuoles by fluorescent tracer.

2. Materials and methods

2.1. Plant material, growth conditions and treatments

Seeds of *L. usitatissimum* (L.), cv AGT 952, were obtained from Agritec Ltd. (Šumperk, Czech Rep.). Seeds were axenically sawn (30 seeds dm^{-2}) on Murashige and Skoog (MS) agar medium (Sigma–Aldrich, Steinheim, Germany) containing 50, 100, 250, 500 or 1000 μM $CdCl_2$ and on the same medium without metal supplement. After 12 days at 25 °C and a 16-h photoperiod (irradiance 50 μmol m^{-2} s^{-1} by cool white fluorescent tubes), the produced seedlings were harvested (40–50 seedlings per Cd concentration per replica), thoroughly washed with deionized water, and the length of the longest root of individual seedlings was measured. Roots, hypocotyls and cotyledons were separated, briefly blotted dry and weighted. When necessary, separated tissues were frozen in liquid nitrogen and stored at −80 °C for maximum 2 weeks. Dry tissue masses were determined after freeze-drying using a model FreeZone 2.5 Liter Benchtop Freeze Dry Systems (Labconco, Kansas City, MO, USA).

2.2. Cadmium analysis in plant tissues

Dried tissues were homogenized by grinding with mortar and pestle and 150–200 mg of the material was decomposed by dry ashing procedure at 400 °C and in the presence of oxygen, ozone, and nitrous oxides using a Dry Mode Mineralizer APION (Tessek Ltd., Prague, Czech Rep.). Ash was dissolved in 20 ml of 0.35 M HNO_3 and Cd concentrations were measured by atomic absorption spectrophotometry ([AAS] model SpectrAA-400, Varian, Inc., Palo Alto, CA, USA) with flame ionization. Blanks and Cd standard (Sigma–Aldrich) were run with all samples for quality control.

2.3. Fluorescence imaging of Cd in roots

Freshly harvested roots were incubated for 3 min in excess of 5 mM EDTA, thoroughly washed with deionized water and hand-sectioned. For Cd staining, the sections were incubated for 90 min in dark and at room temperature with 50 mM HEPES (pH 6.0) containing 100 μg ml^{-1} of Leadmium (Molecular Probes, Inc., Heyden, The Netherlands), the Cd/Pb-specific fluorescent marker. Sections were then washed with excess of 50 mM HEPES (pH 6.0) for 5 min and sections mounted in the same buffer were analyzed by fluorescence microscopy (excitation filter: 495/15 nm, emission filter: 530/30 nm) by using a BioSystems Imaging station CellR̂ with MT20 illumination and DSU semiconfocal unit on IX-81 microscope (Olympus BioSystems, Planegg, Germany) equipped with model C9100 EM-CCD camera (Hamamatsu Photonix, Hamamatsu, Japan).

2.4. Phytochelatin extraction and analysis

Freshly harvested and separated tissue (0.5 g) was ground in liquid nitrogen using a mortar and pestle; the disintegrated tissue was extracted with 1 ml of 0.2 M phosphate buffer (pH 7.2) by applying 10 pulses of 12 W for 5 s with a Misonix MicroTip of a model 3000 sonicator (Misonix, Inc., Farmingdale, NY, USA). The cell debris was removed by centrifugation (20,000 × *g*, 30 min, 4 °C) and 20 μl aliquots of supernatant were subjected to reverse-phase high-performance liquid chromatography with electrochemical detection (RP-HPLC-ED), essentially conducted as described previously (Diopan et al., 2010). The RP-HPLC-ED analyses were performed with a HPLC equipment from ESA Inc. (Chelmsford, MA, USA), consisting of a Model 542 autosampler, two Model 582 solvent delivery pumps, a Model 5600A CoulArray electrochemical detector with three Model 6210 analytical flow cells (each with working carbon porous electrodes with working potential of 900 mV against H/Pd reference electrode), and using an analytical Zorbax Eclipse AAA C18 column (150 mm × 4.6 mm, 5 μm particles; Agilent Technologies, Inc., Santa Clara, CA, USA). The methanol (gradient grade, Sigma–Aldrich) proportion in H_2O (both with 80 mM trifluoroacetic acid from Sigma–Aldrich) during linear gradient elution at a flow rate of 1 ml min^{-1} was 0–3% (v/v) from 0 to 1 min, 3–10% from 1 to 2 min, 10–30% from 2 to 5 min and 30–98% from 5 to 15 min. The custom-made PC standards (Clonestar Biotech, Brno, Czech Rep.) were used to produce calibration curve (within the linear detector response range) and the standard addition method was used to identify individual peaks.

2.5. Separation of intracellular Cd complexes

The cell-free extracts were obtained from 1 g of tissues from seedlings grown on MS agar medium supplemented with 100 μM Cd. Tissue was ground in liquid nitrogen with mortar and pestle and disintegrated tissue was extracted with 500 μl ml of 50 mM HEPES (pH 6.5). The cell debris was removed by centrifugation (20,000 × *g*, 20 min, 4 °C). The size exclusion chromatography (SEC) of 1.5 ml of cell-free extract was performed with a Superdex Peptide GL column (1 cm × 30 cm; GE-Healthcare, Uppsala, Sweden), a BioLogic DuoFlow FPLC system (BioRad, Hercules, CA, USA) and 50 mM HEPES, 100 mM NaCl (pH 7.3) as a mobile phase at flow rate of 0.5 ml min^{-1}. Ribonuclease A (GE Healthcare),

ubiquitin (Sigma–Aldrich), synthetic 2.1-kDa peptide and glutathione (Merck, Dortmund, Germany) were used as molecular mass standards. Cd contents in aliquots of 0.5 ml fractions from SEC were determined by AAS.

3. Results

3.1. The effect of Cd on germination and seedling growth and appearance

It has been well documented that cadmium toxicity to plants is exerted especially on roots and manifested by impaired root elongation and morphological changes (Lux et al., 2011). Root length is also considered to be a reliable parameter for cadmium tolerance (Murphy and Taiz, 1995). Seeds of *L. usitatissimum* cv AGT 952 were germinated on MS agar media in the presence of Cd and the lengths of primary roots of 12 days old seedlings grown at 50, 100, 250, or 500 μM Cd were scored against those of control seedlings grown on medium without metal supplement. The elongation of the primary root was inhibited by Cd in a dose-dependent manner, with 51% inhibition observed at 100 μM Cd (Table 1). Reduced length of the primary root was apparently compensated by increased frequency of root branching, which maintained root dry mass proportion in seedlings grown at up to 250 μM Cd within the range of 29–36% (Table 1). The Cd toxicity reduced relative biomass of roots at 500 μM to 18% (the net biomass yield of cotyledons remained relatively constant, resulting in high ratio of shoots versus roots). While Cd at up to 500 μM had no effect on seed germination, 1000 μM Cd impaired germination by 25%, as compared to seeds at lower Cd concentrations (Table 1). Moreover, only cotyledons, which suffered from apparent chlorosis and necrosis (not shown), and which further failed to produce rooting seedlings, germinated from seeds under this condition.

Strongly reduced growth of roots and decrease in their relative water content (RWC) observed with seedlings at 250 and 500 μM Cd had the incidence on decreased RWC in shoots (Table 1). Compared to seedlings grown in the absence of Cd, those at 500 μM Cd showed not only 93% reduction in root elongation (and reduced branching), but also reduced RWC to average 79% in all tissues, which was accompanied by turgor-deficient appearance of cotyledons.

3.2. Cadmium accumulation in seedling tissues

To investigate the Cd distribution and translocation efficiency in seedlings germinated and grown 12 days on Cd-containing media, the metal contents in roots, hypocotyls and cotyledons were compared. The Cd levels in roots of seedlings produced on 50, 100, 250 and 500 μM clearly increased in a dose-dependent manner and contained, respectively, average 3.5, 6.4, 5.6 and 9.6 times higher levels of Cd than corresponding shoots. The Cd levels in hypocotyls and cotyledons produced on a particular medium were essentially the same. Concentrations of Cd in shoots obtained from media supplemented with 50 and 100 μM Cd or 250 and 500 μM Cd did not show significant difference (Fig. 1). Calculated proportion of Cd amounts in shoots versus whole seedlings was similar (ranging from 26 to 33%) irrespective of the metal concentration in media (Fig. 1). This constant proportion of Cd in shoots is a consequence of higher shoot-to-root biomass ratios observed at higher Cd concentrations in medium (Table 1).

3.3. Fluorescence detection of Cd localization in roots

Accumulation of Cd in roots of 12 days old seedlings was further investigated by using fluorescence detection microscopy. Leadmium was used as a Cd-staining marker insensitive to other divalent metals except for Pb. Due to high autofluorescence signal produced by vascular cylinder (not shown), the analysis was restricted to sections through the ground meristem and cortex. While stained controls from roots grown in the absence of Cd produced only weak background fluorescence (Fig. 2A), virtually all cells of roots grown at 100 μM showed intense fluorescent labeling. As documented in Fig. 2B, Cd was consistently localized in discrete areas corresponding to the cell interior, signifying that detected Cd was accumulated in the root symplasm. More detailed view at the subcellular level often identified brightly fluorescent Cd deposits apparently colocalizing with spherical vesicles or inclusion bodies (Fig. 2C). Although the vacuoles within the inspected first 2 mm from the apex are usually small and may occupy about 15–30% of the cell volume (Patel et al., 1990), the actual observations do not justify ascribing distinguished spheres to vacuoles.

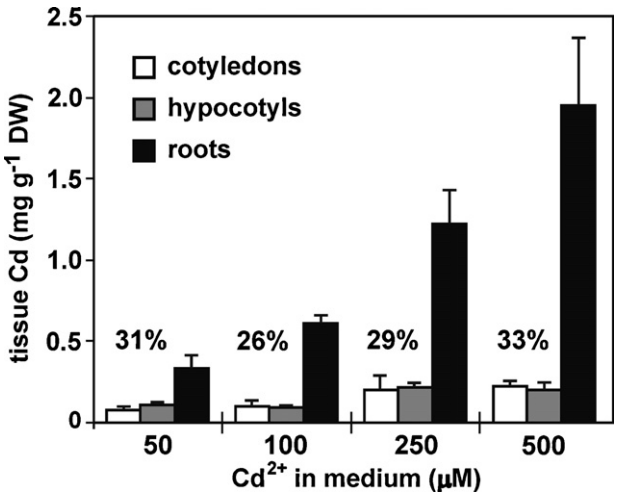

Fig. 1. Cadmium concentration in tissues of *Linum usitatissimum*, cv AGT 952. Seedlings were harvested after 12 days of growth on MS supplemented with Cd^{2+} and the metal content was analyzed in dry-mineralized roots, hypocotyls and/or cotyledons by means of AAS. The percentage of Cd in shoots versus whole seedlings calculated on a dry weight is indicated. Data were produced in four replicate measurements (standard deviation of the mean calculated at $\alpha = 0.05$).

3.4. Phytochelatins in seedling tissues

To gain information concerning the contribution of different PC species to Cd sequestration in roots and shoot organs, the PC contents and identity (up to PC5) were analyzed in tissue extracts by using RP-HPLC with electrochemical detection. As reported in Table 2, Cd induced synthesis of PCs in all seedling organs. The basal levels of PCs observed also in tissues of seedling grown in the absence of added Cd could be attributed to the presence of Zn in MS media (Grill et al., 1989). PC3 was the dominant PC peptide in hypocotyls and in roots of seedlings at 50–250 μM Cd. Exposure to 500 μM Cd resulted in sharp increase in the amount of PC4 in roots (100- and 10-fold compared to the roots grown at 100 and 250 μM Cd, respectively) and apparent onset of PC5 synthesis. While roots and hypocotyls did not accumulate high levels of PC2, the response of cotyledons to Cd involved production of PC2 (the highest at 100 μM Cd) and PC3, with PC3 remaining the prevailing PC species. PC thiol levels, calculated as moles of PC2, PC3 and PC4 (and PC5 in roots at 250 and 500 μM Cd) were the highest in cotyledons. The most pronounced difference was at 100 μM Cd with cotyledon PC thiols 5.6-fold higher than in roots and 3.7-fold higher than in hypocotyls. Thus, PC thiol levels are not proportional to Cd content, signifying different speciation of Cd in seedling organs.

Table 1
Effect of Cd on growth of *L. usitatissimum* seedlings. Seeds were germinated and grown on MS media supplemented with Cd^{2+} at indicated concentrations. Elongation of the main root, mass proportion of seedling organs and relative water content (RWC) were scored in 12-day old seedlings. Data are mean values of three replicate experiments ± standard deviation ($\alpha = 0.05$).

Cd^{2+} (μM)	Germination (%)	Root length (cm)	Organ proportion (%)			RWC (%)		
			Roots	Hypocotyls	Cotyledons	Roots	Hypocotyls	Cotyledons
0	96 ± 2.1	7.4 ± 0.71	30 ± 1.6	20 ± 1.1	50 ± 2.3	84 ± 1.2	88 ± 1.2	86 ± 2.1
50	98 ± 1.3	6.0 ± 0.48	36 ± 1.7	22 ± 1.0	42 ± 1.8	86 ± 1.4	89 ± 0.8	88 ± 1.1
100	93 ± 0.6	3.8 ± 0.63	34 ± 1.1	23 ± 0.8	43 ± 1.4	84 ± 1.2	89 ± 0.7	87 ± 1.0
250	94 ± 0.8	1.5 ± 0.29	29 ± 1.3	18 ± 0.8	53 ± 2.1	80 ± 1.5	86 ± 1.0	84 ± 1.0
500	95 ± 4.1	0.51 ± 0.09	18 ± 0.5	15 ± 0.4	67 ± 1.9	79 ± 0.9	79 ± 0.8	79 ± 0.6
1000	73 ± 2.7	–	–	–	–	–	–	–

3.5. Size distribution of Cd complexes in seedling tissues

The production of PCs in seedlings (Table 2) indicates that the Cd-detoxification mechanism in *L. usitatissimum* involves formation of intracellular Cd–PC complexes. To investigate the intracellular speciation of Cd and inspect the Cd-containing cellular complexes, the disintegrated tissues of seedlings grown at 100 μM Cd were extracted under mild conditions at pH 6.5. The size exclusion chromatography (SEC) of extracts revealed majority of Cd present peaks eluting within the column fractionation range of 20–0.3 kDa (Fig. 3). Association of Cd with fractions of molecular mass >20 kDa has traditionally been ascribed to nonspecific binding of Cd to proteins (Rauser, 2003). It should be noted that the extraction procedure used does not allow clear discrimination between portion of Cd bound in vivo and that adsorbed on proteins during the extraction procedure.

SEC of root extracts revealed the high molecular mass peak of maximum corresponding to 9 kDa, to which approximately 40% of extracted Cd could be attributed, and a lower molecular mass complex of 2-kDa (28% of Cd species) (Fig. 3A). A substantial proportion of root Cd (23%) was found in tailed peak corresponding to ≤0.3 kDa, signifying the presence of non-PC complexes and/or of free Cd ions (Fig. 2A). SEC of the hypocotyl extract showed two resolved Cd peaks within the region of ≤0.3 kDa – one of approximately 0.3 kDa, which represented 23% of extracted Cd, and the other eluting with volume matching that for free Cd ions (31% of Cd) (Fig. 3B). Approximately 27% of hypocotyl Cd was associated with a peak of maximum at 2 kDa. Remaining portion of Cd eluted with fractions corresponding to high molecular mass complex of the root and >20-kDa protein fractions. The majority (65%) of extracted cotyledon Cd was contained in 2-kDa complex, nearly 15% of Cd eluted with 9-kDa peak and the

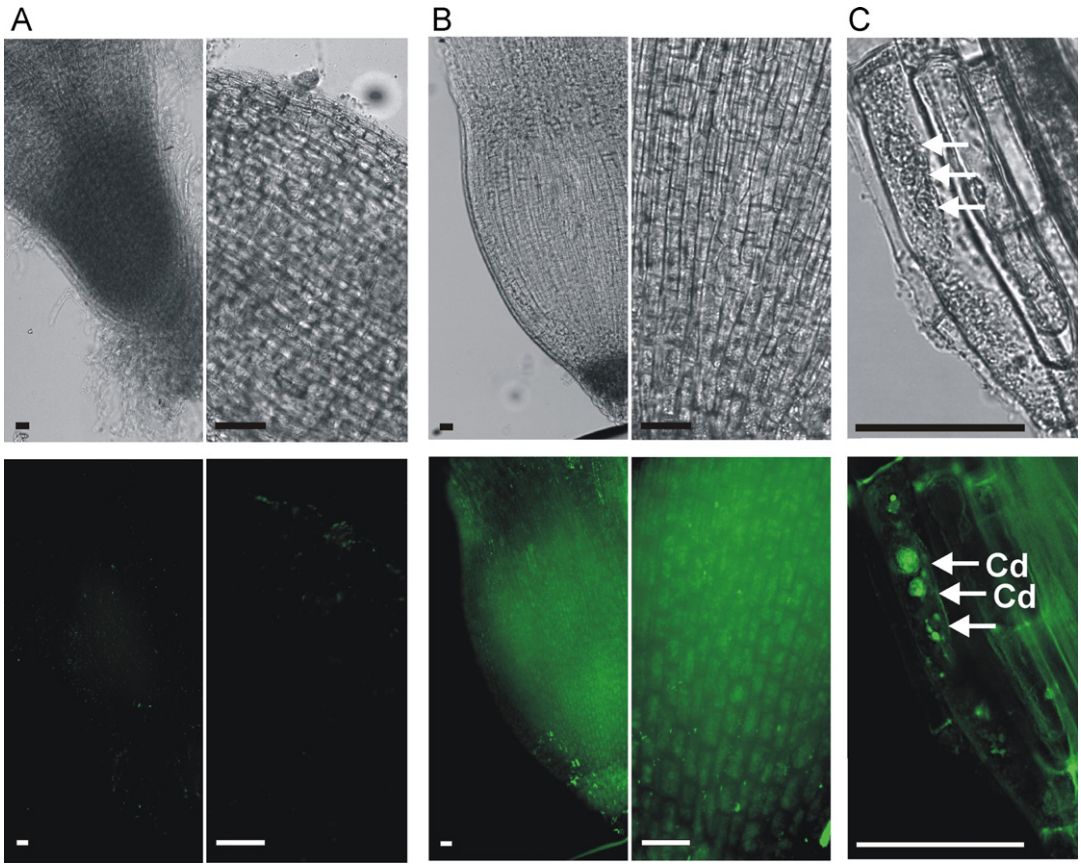

Fig. 2. Visualization of Cd in roots of 12 days old AGT 952 seedlings grown in the absence (A) and presence of Cd (B and C). Longitudinal section from unexposed control and seedlings exposed to 100 μM Cd were stained with Leadmium fluorescent marker and Nomarski interference micrographs (upper panels) and fluorescent micrographs (bottom panels) were recorded (A and B). Fluorescence semiconfocal microscopy mode was used to highlight subcellular deposition of Cd in spheres indicated with arrows (C). Green fluorescence indicates the binding of Leadmium with Cd and bars represent 50 μm.

Table 2
Abundance of PC species in tissues of *Linum usitatissimum* seedlings exposed to Cd. Individual PCs were estimated by RP-HPLC-ED in extracts obtained from 12-day old seedlings grown at indicated concentrations of Cd^{2+}. Data are mean values of three replicate measurements ± standard deviation of the mean ($\alpha = 0.05$).

Organ	Cd^{2+} (μM)	PCn (μmol g^{-1} DW)			
		PC2	PC3	PC4	PC5
Root	0	0.141 ± 0.007	0.052 ± 0.002	0.007 ± 0.001	0.019 ± 0.004
	50	0.424 ± 0.021	1.44 ± 0.072	0.007 ± 0.001	0.013 ± 0.003
	100	0.208 ± 0.010	1.25 ± 0.062	0.024 ± 0.001	0.030 ± 0.002
	250	0.442 ± 0.022	1.48 ± 0.074	0.267 ± 0.013	0.013 ± 0.001
	500	0.409 ± 0.020	2.26 ± 0.113	2.36 ± 0.118	0.122 ± 0.006
Hypocotyl	0	0.499 ± 0.025	0.196 ± 0.010	0.295 ± 0.015	0.013 ± 0.001
	50	0.216 ± 0.010	2.99 ± 0.149	0.206 ± 0.010	0.010 ± 0.001
	100	0.261 ± 0.013	1.74 ± 0.087	0.187 ± 0.009	0.029 ± 0.003
	250	0.155 ± 0.008	1.48 ± 0.074	0.198 ± 0.010	0.019 ± 0.001
	500	0.201 ± 0.010	1.34 ± 0.067	0.151 ± 0.008	0.015 ± 0.001
Cotyledon	0	0.095 ± 0.005	0.016 ± 0.001	0.058 ± 0.003	0.009 ± 0.001
	50	1.21 ± 0.060	2.94 ± 0.147	0.042 ± 0.002	0.007 ± 0.001
	100	3.01 ± 0.150	5.73 ± 0.286	0.132 ± 0.007	0.027 ± 0.003
	250	1.54 ± 0.077	4.50 ± 0.225	0.054 ± 0.002	0.016 ± 0.001
	500	1.11 ± 0.056	3.84 ± 0.192	0.127 ± 0.006	0.037 ± 0.002

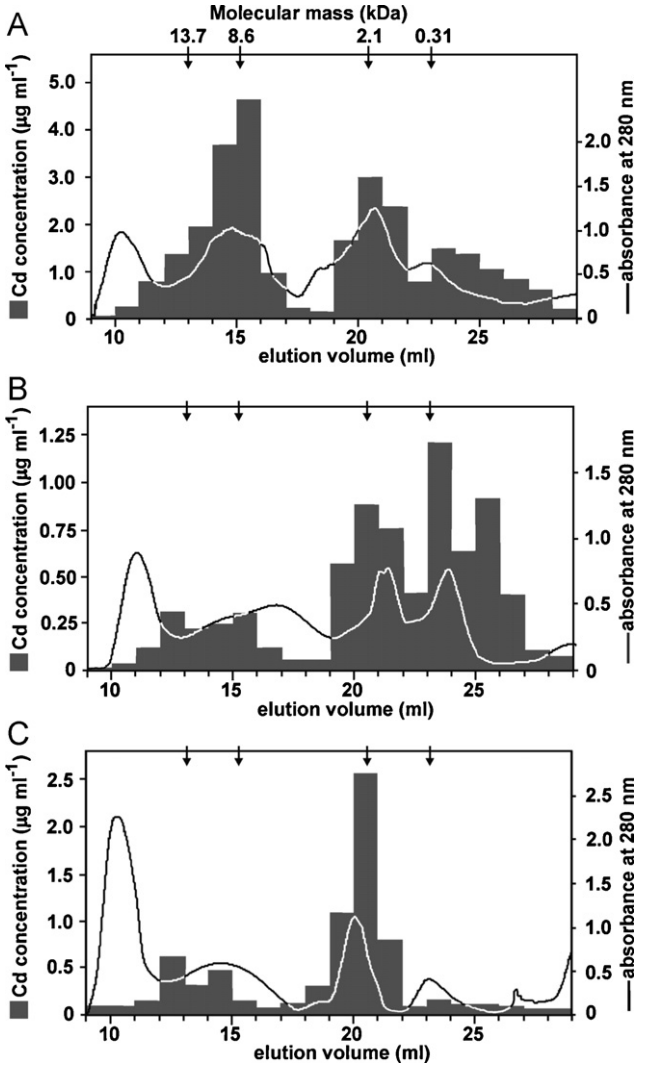

Fig. 3. Size exclusion chromatography (SEC) fractionation of the extracts from roots (A), hypocotyls (B) and cotyledons (C) of 12 days old seedlings of *Linum usitatissimum*, cv AGT 952, cultivated with 100 μM Cd^{2+}. The extracts prepared at pH 6.5 were resolved on Superdex Peptide GL column (exclusion limit of ≥20 kDa) and the Cd concentration of individual fractions was determined by AAS. The elution maxima of the molecular mass standards are indicated by arrows.

remaining portion was associated mainly with excluded proteins (Fig. 3C).

4. Discussion

Although flax received attention for use as heavy metal phytoremediation plant since mid-nineties, the efforts towards the identification of specific ligands and intracellular sequestration of toxic heavy metal species were missing. The data in this study demonstrated Cd-dependent induction of PC synthesis in the model *L. usitatissimum*, cv AGT 952. The total PC thiol levels, number of (γ-Glu-Cys) repeats in PCs as well as sizes of intracellular Cd-complexes showed certain tissue-specific variations reflecting also the tissue Cd content. While PC3 was dominant in all organs, the extension of PC3 to PC4 and PC5 with higher Cd-binding capacity, signifying insufficiency of PC3 to confer Cd protection to the cell (Clemens, 2006), was observed only in roots exposed to the highest Cd concentrations. PC2 accumulated at high levels only in cotyledons (Table 2). In maize and durum wheat, the Cd binding PCs in LMW complex, similar in apparent molecular mass to that shown in Fig. 3C, consist of PC2 and PC3 (Rauser, 2003; Hart et al., 2006). It appears that in cotyledons storage of Cd was in 2 kDa complex, equated to LMW PC-based complex, sufficient for Cd detoxification. Although hypocotyls and cotyledons contained similar concentrations of Cd (Fig. 1), hypocotyls accumulated substantially less PC thiols (Table 2). More than 50% of total hypocotyl Cd detected in fractions corresponding to <0.3 kDa (Fig. 3B) could be accounted to localization of Cd in the xylem apoplasm for translocation. The xylem transport of heavy metal ions, eventually complexed by carboxylates, amines and probably also short-chain PCs, is implicated in the metal translocation in tomato, *A. thaliana*, Indian mustard and tobacco (Pitch et al., 1994; Hussain et al., 2004; Verret et al., 2004; Kramer et al., 2007).

The use of seedlings exposed to high Cd levels also offers a means of deducing the role that PCs play in the partitioning of Cd between root and shoot tissues of mature *L. usitatissimum* at the contaminated site. In most terrestrial plants, Cd concentrations are higher in roots than in shoots (Lux et al., 2011) and this trait has also been confirmed with flax in several studies conducted mainly with plants grown on soils of geogenic or slightly increased Cd content of ≤1 mg kg^{-1} (Baraniecki et al., 2001; Jiao et al., 2004; Angelova et al., 2004). In a recent study, Bjelková et al. (2011) have shown pronounced dose-dependent increase in root Cd concentrations with both flax and linseed grown on soils artificially amended with Cd solution to levels of 10–200 mg Cd kg^{-1},

while Cd levels of above-ground biomass remains 3–6 times lower than those in roots. Consequently, some 60% of the total plant Cd escapes the phytoextraction. Similar proportion of Cd (67–74%) was retained in roots of AGT 952 grown at 50–500 μM (5.6–56 mg kg^{-1}) Cd (Fig. 1). Emergence of 9-kDa complex as the main Cd form in roots at 100 μM Cd (Fig. 3A) suggested that vacuolar sequestration of HMW CdS–PC complex was effective for deposition of Cd in *L. usitatissimum* roots; however, the colocalization of the Cd-derived fluorescence with vacuoles was not conclusively distinguished (Fig. 2C). In *S. pombe*, the LMW complex, similar to that shown in Fig. 3A is produced first, with the vacuolar HMW complex building up later (Clemens, 2006). It involves the transport of cytosolic LMW complex to the vacuole by ABC-type transporters Hmt1 (Ortiz et al., 1995) and recently described Abc2 (Mendoza-Cózatl et al., 2010). Unlike Hmt1 orthologs, which has not yet been identified in plants, Abc2 orthologs are widely distributed among kingdoms. These may represent proteins responsible for vacuolar sequestration of Cd–PCs, which have been shown as ATP-dependent in oat (Salt and Rauser, 1995). In vacuoles, subsequent incorporation of inorganic S^{2-} reduces need for PC thiols and some Cd may also dissociate from PCs to form a vacuolar complex with GSH or carboxylic acids (Ortiz et al., 1995; Clemens, 2006). Deposition of Cd in vacuoles would explain substantially lower levels of root PC thiols compared to those of cotyledons and abundance of Cd in ≤0.3-kDa fractions. The approach used, however, does not allow discrimination between vacuolar and apoplasm portions of Cd in these fractions.

As argued above, the effective Cd-sequestering pathway in *L. usitatissimum* roots is likely to play an important role in the retention of the metal in this organ, rendering the metal unavailable for xylem loading from symplasm. There are also several lines of evidence suggesting positive correlation of transpiration activity and the apoplasmic xylem metal transport to shoots (Barceló and Poschenrieder, 1990; Van der Vliet et al., 2007). The second factor that may thus have influenced Cd passage to shoots would be the perturbation in plant-water relationship reflected by reduction in their RWC (Table 1). Impaired water uptake in plants due to the acute Cd toxicity is being attributed to reduced root biomass, impaired activity or quantity of root aquaporins (Tyerman et al., 2002) or to reduction in the transpiration rate due to the stomatal closure (Haag-Kerwer et al., 1999; Perfus-Barbeoch et al., 2002).

5. Conclusions

Like in cv AGT 952, the preferential retention of Cd in roots is a feature reported in most flax varieties and cultivars (Baraniecki et al., 2001; Jiao et al., 2004; Angelova et al., 2004; Douchiche et al., 2010; Bjelková et al., 2011). However, the desired trait of phytoextraction plant is its efficient translocation to aboveground biomass. The data in this study demonstrate that response of flax to Cd involved induced synthesis of intracellular Cd-binding phytochelatins in all seedling organs and that the storage of intracellular Cd involved formation of LMW and HMW complexes with certain organ specific variations. Dominant abundance of HMW complex as intracellular Cd complex in roots did support the notion that, like in *S. pombe* and certain plants, vacuolar compartmentalization of Cd–PC complexes might be responsible for high capacity of roots to sequester Cd. Further efforts are now needed to delineate the role of vacuoles in protecting flax from Cd toxicity and retention of the metal in roots. Concerning the sequestration of Cd in roots, the mechanism(s) responsible for loading of Cd into xylem are being attributed mainly to P-type family of plasma membrane ATPases (Kramer et al., 2007). Several lines of evidence suggest that overproduction of plasma membrane metal transporters and/or of enzyme activities improving production PCs in shoots of genetically modified plants might support the translocation of accumulated metals to shoots (Kotrba et al., 2009). Cultivar AGT 952, showing high susceptibility to genetic transformations (M. Griga, Agritec Ltd, personal communication) is suitable model target to such genetic modifications.

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