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Cadmium speciation in *Arabidopsis thaliana* as a strategy to study metal accumulation system in plants

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Abstract

The strategy to identify cadmium deactivation mechanism in *Arabidopsis thaliana* has been developed using selective and sensitive hyphenated techniques. Cadmium concentrations, in main parts of the plant, were determined by ICP-MS and total amount was found as 0.43–0.44 $\mu g\,g^{-1}$ in leaves and 3.3–3.4 $\mu g\,g^{-1}$ in roots. Speciation of the metal complexes in cells was investigated by SEC–ICP-MS in order to estimate the accumulation process. Phytochelatins, desglycyl-phytochelatins and phytochelatins homologues lacking the N-terminal γ-linked glutamic acid were extracted from plant and were identified by RPLC–ESI-MS. Two-dimensional chromatography allowed to link the metal complexes separated by SEC with isoforms of phytochelatins analyzed by high resolution RPLC and confirm their significant responsibility for metal accumulation. The potential of the cadmium complexes speciation indicates that obtained results could be reliable source of knowledge to confirm the information coming from the well-known genomic sequence of *Arabidopsis* and to estimate the role of γ-glutamyl transpeptidase in metabolism of glutathione.

Keywords: Cadmium accumulation; Phytochelatins; Reversed phase liquid chromatography; Size exclusion chromatography; ICP-MS; Electrospray MS; Hyphenated techniques

1. Introduction

Cadmium is a widespread heavy metal, released to the environment by power stations, heating systems, metal-working industries, waste incinerators, urban traffic, cement and phosphate fertilizers factories. Cadmium is classified as an intermediate in point of toxicity, however, in high concentrations it can be carcinogenic, mutagenic or teratogenic to large number of animal species and human being. It can be partially explained by the competitive character of cadmium uptake for nutrients like potassium, calcium, magnesium, iron, manganese, copper, zinc and nickel [1]. In spite of that, there are species of higher plants that can grow in highly contaminated soil [2]. The question remains how plants can regulate its metabolism to allow safe uptake of essential metals such as Zn, Cu to cytosol and organelles and simultaneously deactivate toxic metals such as Cd, Hg and Ag [3]. It is known, that in response to Cd stress, the plant cell can generate different systems to defend itself: immobilization and exclusion, synthesis of phytochelatins, compartmentalization, synthesis of metallothioneins and stress proteins or production of stress ethylene [4].

The decisive contribution in deactivation of metal ions has synthesis of phytochelatins (PCs), which form intracellular metal complexes [5]. Phytochelatins are synthesised from glutathione (GSH) and consist of only three amino acids: glutamic acid (Glu), cysteine (Cys) and glycine (Gly). The general formula of PC is $(\gamma\text{Glu-Cys})_n$-Gly, where $n = 2$–11, in which glutamic acid is linked to cys-

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teine through a γ-peptide linkage. The synthesis of this peptide starts with activation of phytochelatin synthase enzyme (PCS) which is strictly dependent on the presence of metal thiolates with ions like: Cd^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Sb^{3+}, Ag^{+}, Hg^{2+} and As^{5+} [3,6–9]. Different plant species synthesise analogous families of PCs, which analogically bind metals creating chelate complexes. They can be divided into six groups: homo-phytochelatins $(\gamma Glu\text{-}Cys)_n$-β-Ala [10]; hydroxymethyl-phytochelatins $(\gamma Glu\text{-}Cys)_n$-Ser [11]; *iso*-phytochelatins $(\gamma Glu\text{-}Cys)_n$-Glu [12]; desglycyl-phytochelatins $(\gamma Glu\text{-}Cys)_n$ [13]; recently discovered *iso*-phytochelatins with the C-terminal glutamine $(\gamma Glu\text{-}Cys)_n$-Gln [14] and phytochelatins homologues lacking the N-terminal γ-linked glutamic acid $\gamma Glu\text{-}(\gamma Glu\text{-}Cys)_{n-1}$-Gly [15]. Similar deactivation system is dedicated to cysteine-rich metallothioneins with amino acids motifs like Cys-$(Xaa)_{1-2}$-Cys and Cys-Cys. However, there is no indication in the literature showing the identification of metallothioneins, induced by Cd and isolated from higher plants, except amino acids sequences in proteins predicted from gene sequences in *Arabidopsis* [16].

Various instrumental techniques have been proposed to study metal speciation in plants. Size exclusion chromatography (SEC) became one of classical and most useful approaches for separation of individual metal species [17]. The speed and elegance of this technique was improved by coupling of SEC method to ICP-AES or ICP-MS detection creating most widely used technique for multi-elemental screening for heavy metals in plants and monitoring the induction of PCs [18]. The poor resolution of this separation technique, lack of the molecular specificity and poor recovery due to metal sorbing on the stationary phase requires stabilisation of the complexes by modification of the stationary or mobile phase [19,20].

Additional method for understanding the detoxification mechanism is the characterisation of the bio-induced ligands. The reversed phase liquid chromatography (RPLC) with spectrometric detection is the classical approach for separation of peptides and proteins, offering high resolution and reproducibility of the results but requiring post-column derivatization of the sulphhydryl groups prior specific detection of PCs [21,22]. However, the method does not allow to differentiate PCs from their isomers and other bioligands containing sulphhydryl groups. Mass spectrometers (MSs) with fast atom bombardment (FAB) and electrospray ionization (ESI) were proposed as an adequate method for identification and quantification of phytochelatins after chromatographic purification [14,23–25]. The coupling of RPLC to molecule-specific ESI-MS improves the selectivity of the separation method and sensitivity in comparison to UV–vis or FAB-MS detector and creates new, fast and efficient method for fingerprinting of phytochelatins in plants [26].

The objective of this work was to develop a strategy to study the model genetic plant (*Arabidopsis thaliana*) response to cadmium stress by monitoring of metal complexes and identification of bioligands using sensitive and selective hyphenated techniques for custom prepared samples. The results obtained by speciation analysis can be complementary source of information to study the metal accumulation mechanism by genetic and proteomic analysis [3,9,27].

2. Experimental

2.1. Instrumentation

Chromatographic separations were performed using a Model HP 1100 gradient HPLC pump (Agilent Technologies, Waldbronn, Germany) as the sample delivery system. Injections were carried out using a Model 7725 injection valve with injection loops: 100 μL for SEC and 10 μL for RPLC (Rheodyne, Cotati, CA, USA). All the connections were made of PEEK tubing (0.17 mm i.d.). An Agilent Model 7500a ICP mass spectrometer (Tokyo, Japan) was used as an element-specific detector for quantification of metal content in plants and for on-line HPLC metal monitoring. Sample or HPLC eluate was introduced to ICP-MS through Babington nebulizer fitted in a commercial Scott's spray chamber. The peptide profiles were obtained by ESI-MS detection Model LC-MSD 1100 (Agilent Technologies, Wilmington, NC, USA) with quadrupole mass analyzer. Metal quantification and chromatographic data were processed using an Agilent ICP-MS software and Agilent ChemStation software for LC-MSD. A Branson Model 1210 ultrasonic bath (Danbury, CT, USA) was used for metal species extraction from plant tissues and MPW Model 210 centrifuge (Warsaw, Poland) was used for the separation of supernatant after leaching.

2.2. Reagents, solutions and materials

All reagents used were of analytical-reagent grade purchased from Sigma–Aldrich (Sigma–Aldrich, Buchs, Switzerland). The acetonitrile (ACN) of HPLC gradient grade was used for RPLC separation method (LabScan Analytical Science, Dublin, Ireland). Water (18 mΩ) prepared with a Mili-Q system (Milipore Elix 3, Milipore, Saint-Quentin, France) was used throughout.

Plants of *Arabidopsis thaliana* L., Columbia 4 (NASC, N 933) were cultivated in continuously aerated hydroponics culture with Hoagland's solution nutrient that was weekly renewed. Five-week old plants were divided into two groups (five plants each) and exposed during 14 days to 25 or 50 μM of Cd^{2+} added at nutrient renewal. To evaluate the form of Cd^{2+}, plants collected after 14 days of Cd^{2+} treatment were divided into leaves and roots, immediately frozen and lyophilised.

2.3. Analytical procedures

2.3.1. Sample preparation

Weighted sample of 40 mg of freeze-dried material was frozen in liquid nitrogen (−196 °C) to break the cell walls.