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Accumulation of Cadmium by Transgenic Tobacco Plants (*Nicotiana tabacum* L.) Carrying Yeast Metallothionein Gene Revealed by Electrochemistry

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Phytoremediation of heavy metals includes phytoextraction and phytoaccumulation, i.e. accumulation of pollution from soils or water into the plants. Inorganic pollutants like heavy metals cannot be chemically degraded and thus it has to be accumulated in roots and leaves of the plants in non-toxic forms. In this study, the influence of cadmium on transgenic *Nicotiana tabacum* plants carrying yeast metallothionein was investigated. Tobacco plants were exposed to 0, 50, 100 and 250 μM $CdCl_2$ for 96 hours. Growth parameters as well as changes in content of cysteine, oxidized/reduced glutathione, phytochelatins (PC2, PC3 and PC4) were monitored using high performance liquid chromatography with electrochemical detection. Moreover, we exploited differential pulse voltammetry Brdicka reaction for determination of metallothionein and verified the results obtained by dot blot assay. In addition, all results were compared with results obtained using non-transgenic tobacco plants. Our results indicate protective role of metallothionein as well as its role in cadmium accumulation in roots of transgenic plants.

Keywords: Metallothionein, Tobacco, *Nicotiana tabacum*, Thiols, Phytoremediation, Electrochemical detection, Brdicka reaction, Voltametry, Liquid chromatography, Heavy metal

1. INTRODUCTION

Heavy metal contamination of the environment still represents topical field of the interest [1-13]. In addition to some natural occurrence of heavy metals (ores, volcanic activity), there is a wide spectrum of anthropogenic sources exemplified by wastes from ore processing or by release from landfills containing industrial waste. The burden of such pollution is dramatically high in some regions and the possibilities for removal of its sources in near future are quite limited. Thus, there is a requirement for application of feasible methods for removal of contaminants from the environment. Phytoremediation technologies using green plants to remove heavy metals from soil and water are now being identified as promising and non-destructive methods [14]. The idea of using plants able to diminish uptake, detoxify or sequester heavy metal/metals (so-called hyperaccumulators) to selectively remove and recycle excessive soil metals has been introduced already in 1983. There are two economically interesting possibilities of the use of plant with metal tolerance as heavy metal extraction (phytomining) and soil/water decontamination with the help of metal accumulating plants [15,16]. Phytoremediation of pollutants includes i. phytoextraction – extraction of the pollutant from the soil and its accumulation in the organs to be harvested, ii. rhizofiltration – absorption of pollutants from water and sewage by the roots, iii. phytodegradation – decomposition of pollutants by plants and associated microorganisms, iv. phytostabilization – binding the pollutants in the form of complexes or by precipitation, and v. phytovolatilization – detoxification of pollutants by the production of volatile compounds [12,17-20]. Considering the fact that heavy metals cannot be metabolized and chemically degraded, thus, they are accumulated in roots, stems and leaves or generative organs of the plants in non-toxic forms. It is not surprising that the mechanisms of transport of heavy metals, both essential and non-essential, are intensively investigated [21-24]. There are two intensively investigated levels of metal ions uptake. From soil to a plant is the first one. Metal ions availability is a question of the solubility of heavy metals or complexes with low-molecular compounds excreted by roots, pH of soil and redox potential of soil. Association of roots with mycorrhizal fungi plays crucial role in heavy metals availability in many plants [25-29]. The second level is represented by both active and passive transport intracellular and extracellular mechanisms, which include selective and nonselective ion channels and special transporters located in cytoplasmatic membranes.

There have been identified three-step mechanisms of metal ions transporting inside a plant (Fig. 1A). The first is apoplastic transport through the intercellular spaces and cell walls outside the plasma membrane, and the second is the synplastic transport through protoplast inside of the plasma membrane through the continuous connection of cytoplasm though plasmodesmata without participation of any organelles [30]. Cell walls play crucial role in the interactions with heavy metals due to presence of carboxylic groups [31]. Finally, the third mechanism involves special cellular transporters including the ion pumps and different types of transporters (Fig. 1A). The further transport of heavy metals is connected with transpiration flow via xylem (water, chelate complexes of heavy metals) into aerial parts, or with sap flow via phloem. Active transport is important for absorption of essential heavy metals and allows their highly selective acceptance. Thanks to active transport, plants can uptake substances from the environment even when their concentrations are very low. The active transport depends on the presence of specific transporters for individual metal ions or for the group of

metal ions [33-36] and the activity of these transporters is related to ATP hydrolysis processes (Fig. 1B).

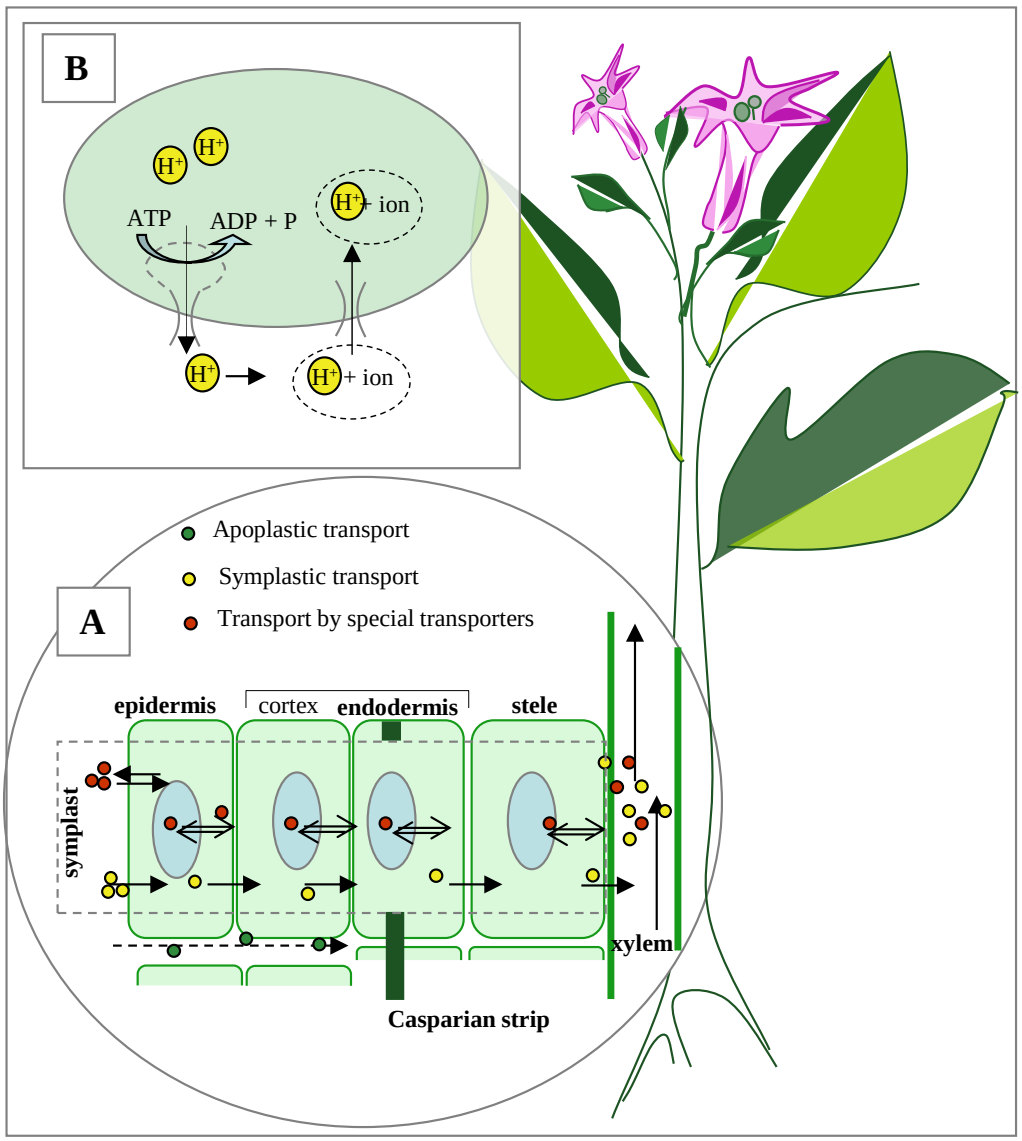

Figure1. Scheme of transport of metal ions in plants. (**A**) Apoplastic/symplastic transport in roots and xylem. (**B**) Active transport based on ATP pump system at cell level. The scheme was adopted according to the following papers [30-32].

From the point of view of content of metal ions in soil or in other part of the environment, their excess is toxic for plants [37]. Plants are protected against negative effects of heavy metals at the cellular level by the following mechanisms: i. activation of antioxidant mechanisms, both enzymatic (catalase, peroxidase, superoxide dismutase) and non-enzymatic (tocopherols, carotenoids, polyphenols); ii. enhanced production of osmolytes as a response to water stress caused by heavy metal/metals; iii. physico-chemical changes of cell walls and plasma membranes; iv. changes in physiological processes including changes in phytohormones levels and v. synthesis of metal-binding peptides and proteins such reduced glutathione (GSH), phytochelatins and metallothionein-like

proteins [9,38-51]. These metal-chelating peptides and proteins are characterized by the presence of thiol groups in their chemical structure [46,52-55]. These groups allow to bind metals and free-radicals, but the mechanisms differ significantly. GSH belongs to the most abundant intracellular thiol-peptides, reaching up to 10^{-3} molar concentrations in certain tissues and organelles [56,57]. As an important antioxidant, GSH plays role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by glutathione-S-transferases and glutathione peroxidases [58-60]. In addition, GSH is highly reactive and is often found conjugated to other molecules via its sulfhydryl moiety such as NO (S-nitrosoglutathione) [61-63]. The synthesis of GSH from its constituent amino acids, L-glutamate, L-cysteine, and L-glycine, involves two ATP-requiring enzymatic steps [64]. GSH serves several vital functions, including *1)* detoxifying electrophiles; *2)* maintaining the essential thiol status of proteins by preventing oxidation of -SH groups or by reducing disulphide bonds induced by oxidative stress; *3)* scavenging free radicals; *4)* providing a reservoir for cysteine; and *5)* modulating critical cellular processes such as DNA synthesis, microtubular-related processes, and immune function [64,65]. Moreover GSH can be used for synthesis of phytochelatins (a basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2 to 11)) participating in the detoxification of heavy metals in plants. Phytochelatins have the ability to bind heavy metal ions via SH groups of cysteine units and consequently transport them to vacuole, where an immediate toxicity does not menace yet [66-70]. Complex of PC and a metal ion is called as low molecular weight metal-phytochelatin complex (LMW M-PC). After transporting of this complex through tonoplast to vacuole low molecular complex is transformed to high molecular weight M-PC complex (HMW M-PC) via –S–S– groups [66,71]. The synthesis PC itself involves the transpeptidation of the γ-Glu-Cys moiety of GSH onto initially a second GSH molecule to form PC_2 or, in later stages of the incubation, onto a PC molecule to produce an $n + 1$ oligomer. The reaction is catalysed by γ-Glu-Cys dipeptidyl transpeptidase (EC 2.3.2.15), which has been called as phytochelatin synthase [72,73]. *In vitro* the purified enzyme was active only in the presence of metal ions. Cadmium was the best activator of phytochelatin synthase followed by Ag, Bi, Pb, Zn, Cu, Hg, and Au cations. Recently, high performance liquid chromatography with electrochemical detection method for determination of this enzyme activity was optimized [48,74]. The role of plant metallothionein-like proteins is not fully understood in plant protective mechanisms against metal ions Metallothioneins (MTs) are low molecular mass (from 2 to 16 kDa) proteins with unique abundance of cysteine residues (more than 30 % from all aminoacids). Other interesting structural property is the lack of aromatic amino acids. However, as discovered recently, there is an exception: a group of certain yeast and bacterial species rarely containing histidine [75]. The main function of MTs in organism is a metal ion transport, maintenance of the oxidative-reducing conditions and regulation of gene expression. MTs are also known as effective radical scavengers creating optimal oxidative-reducing conditions. MTs regulate free radical level also indirectly by binding of metal ions which are potential radical producers, e.g. Cu [76]. As confirmed by several studies [77,78], MT expression in cells is induced also by superoxide and hydroxyl radicals generated by γ-radiation. It is supposed that MT acts either as a scavenger of radicals or zinc donor for enzymes participating in repairing processes. MT exhibits the highest affinity for Cu^+ (stability constant $10^{-19} – 10^{-17}$), followed by Cd^{2+} ($10^{-17} – 10^{-15}$) and Zn^{2+} ($10^{-14} – 10^{-11}$); however it is not capable of binding Cu^{2+}. Generally, 18 metal ions suitable to be bound by MT are known but only Cu^+, Cd^{2+}, Pb^{2+}, Hg^{2+}, Ag^+ and Bi^{2+} can replace

Zn^{2+} in MT structure. Binding capacity of MT is 7 and 12 atoms for divalent and monovalent ions, respectively. MT`s tertiary structure consists of two domains: more stable α (C-terminal), containing 4 ion binding sites, and β (N-terminal) capable to incorporate 3 ions [79].

The growing knowledge of the factors important for the phytoremediation can provide a basis for targeted genetic modifications of plants for improving of phytoremediation potential [80]. The use of genetically engineered plants allows multiplying metal accumulation capacity, especially for Cd and Cu by the overproduction of metal-chelating molecules including PCs and MTs. The introduction of animal MT genes to improve the ability of the plants to tolerate heavy metals has been already demonstrated [20,81-84]. In this study, we employed both stationary and flow electrochemical methods for analyses of extracts obtained from transgenic tobacco plants (*Nicotiana tabacum*) carrying yeast metallothionein gene treated with cadmium(II) ions (0, 50, 100 and 250 μM) for 96 hours. Electrochemical methods were used for determination of reduced and oxidized glutathione, phytochelatins and yeast metallothionein. The results were correlated with cadmium(II) ions treatment and the potential usage of such modified plants for phytoremediation technologies was discussed.

2. EXPERIMENTAL PART

2.1 Chemicals and pH measurement

Rabbit liver MT (M_W 7143), containing 5.9 % Cd and 0.5 % Zn, was purchased from Sigma Aldrich (USA). Tris(2–carboxyethyl)phosphine(TCEP) was acquired from Molecular Probes (Evgen, Oregon, USA). Phytochelatin2 (PC2, (γ-Glu–Cys)$_2$–Gly), phytochelatin3 (PC3, (γ-Glu–Cys)$_3$–Gly) and phytochelatin4 (PC4, (γ-Glu–Cys)$_4$–Gly) were synthesized in Clonestar Biotech (Brno, Czech Republic) with a purity higher than 90 %. HPLC-grade methanol (99.9%, *v*/*v*) from Merck (Dortmund, Germany) was used. $Co(NH_3)_6Cl_3$ and other chemicals were purchased from Sigma-Aldrich (USA) with an ACS grade purity unless noted otherwise. Stock standard solutions were prepared using ACS water (Sigma-Aldrich) and stored in the dark at -20 °C. Working standard solutions were prepared daily from stock solutions. All solutions were filtered through a 0.45 μm Nylon filter discs (Millipore, Billerica, Mass., USA) prior to electrochemical analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by the personal computer program MultiLab Pilot (Weilheim, Germany). The pH electrode (SenTix-H, pH 0–14/3 mol/dm^3 KCl) was calibrated by a set of WTW buffers (Weilheim, Germany).

2.2 Transgenic plants

Clones derived from *Nicotiana tabacum* L. cv. Wisconsin 38 were used in experiments. Clones from the first line (T-XHis) carried gene encoding a peptide containing six histidin´ moieties (additional metal binding domain) combined with a *CUP1* metallothionein gene originating from *Saccharomyces cerevisiae*, which was firstly introduced into *Nicotiana tabacum* using *Agrobacterium tumefaciens* transformation. More details are summarized in [85].

2.3 Plants cultivation conditions

Plants (non-transgenic, transgenic) were maintained under the strictly defined and controlled conditions in cultivation box (Sanyo, Japan): photoperiod 12-h light/dark cycle (25 ± 1°C (light), and 23.5 ± 1°C (dark)) in Hoagland hydroponic medium. The 3 weeks old plants representing homogenous material of the same morphological appearance were transferred into a new Hoagland medium supplemented with 0, 50, 100, and 250 μM cadmium(II) ions in the form of $CdCl_2$. Experimental plants were cultivated for 96 hours, samples were taken in strictly defined time intervals of 12, 24, 48, 72 and 96 hours and immediately processed. Cultivation conditions were as follows: temperature 23±2°C, photoperiod 16 hours (6 – 22 h), light intensity 140 $\mu E/m^2s$. Samples of harvested plants were three-times rinsed by distilled water and divided into root and shoot parts. The fresh weight of the samples was measured on a Sartorius R160P scales (Sartorius GmbH, Goettingen, Germany) immediately after rinsing. Parts of tobacco plants were dried at 45 °C for 24 hours in thermostat (UNB 300, Memmert, Germany) and then dry weight was determined on a Sartorius R160P scales (Sartorius GmbH, Goettingen, Germany).

2.4 Preparation of biological samples

2.4.1 Electrochemical determination of metallothionein

The transgenic and control tobacco plant samples were divided into leaves and roots (app. 0.2 g of fresh weight each) followed by homogenization using deep-freezing in liquid nitrogen. One ml of 0.2 M phosphate buffer pH 7.2 was added to the homogenate. The homogenate was subsequently treated for 30 min at 99 °C using a thermomixer (Eppendorf 5430, USA). The solid particles and denatured proteins were removed and the process repeated twice, followed by centrifugation (Eppendorf 5402, USA) at 16,000 × g for 30 min at 4°C [86]. The supernatant was transferred to a new test-tube and shaken on a Vortex-2 Genie (Scientific Industries, New York, USA) at 4 °C for 30 min. The homogenate was centrifuged (16,000 × g) for 30 min at 4°C using a centrifuge (Eppendorf 5402, USA). Prior to analysis, the supernatant was filtered through a membrane filter (0.45 μm Nylon filter disc, Millipore, Billerica, Mass., USA).

2.4.2 Electrochemical determination of cadmium

Leaf and root tissues of maize were dried at 45 °C for 24 hours in thermostat (UNB 300, Memmert, Germany). Samples of homogenized roots (0.1 g) or leaves (0.5 g) were used for microwave digestion in concentrated nitric acid. To prepare the samples microwave digestion was used according to recently published papers [19,87,88]. Briefly, the mineralization of samples took place in a microwave system Multiwave 3000 (Anton-Paar GmbH, Austria). A sample (100 mg) was placed into glass vials MG5 and 900 μl of nitric acid (65 %, w/w) were added. Prepared samples were sealed and placed into the rotor 64MG5 (Anton-Paar GmbH, Austria). Rotor with the samples was inserted into the microwave system and the microwave digestion was carried out under the following

conditions: power 50 W – 10 min., power 100 W – 30 min., cooling (power 0 W) – 10 min., maximum temperature 80 °C. Sample preparation for subsequent electrochemical measurements was as follows: 15 µl mineralized sample was pipetted into Eppendorf tubes with 985 µl acetate buffer (pH = 5.00). A blank digestion was simultaneously carried out in the same way.

2.4.3 Chromatographic analysis

Approximately 0.5 g of leaf/root tissue was frozen using liquid nitrogen and subsequently homogenized with liquid nitrogen and with 0.5 ml of potassium buffer (pH 7.0). In addition, 0.5 ml of potassium buffer was added. The homogenate was centrifuged (15 000 × g) for 15 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). Supernatant was collected and used for chromatographic analysis.

2.5 Differential pulse voltammetry Brdicka reaction – Metallothionein content

Electrochemical measurements were performed on an AUTOLAB analyzer (EcoChemie, The Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), which used a standard cell with three electrodes. The three-electrode system consisted of a hanging mercury drop electrode with working electrode surface 0.4 mm^2 as a working electrode, an Ag/AgCl/3 M KCl reference electrode and a glassy carbon auxiliary electrode. For smoothing and baseline correction, the software GPES 4.4 supplied by EcoChemie (EcoChemie, The Netherlands) was employed. The Brdicka reaction supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. Surface-active agent was not added. Adsorptive transfer stripping technique in connection with differential pulse voltammetry AdTS DPV - Brdicka reaction parameters were as follows: initial potential of -0.6 V, end potential -1.6 V, modulation time 0.057 s, time interval 0.2 s, step potential of 1.05 mV, modulation amplitude of 250 mV, E_{ads} = 0 V. Temperature of the supporting electrolyte was 4 °C [7,9,19,53,55].

2.6 Electrochemical determination of cadmium in plant tissues

Electrochemical measurements were performed using Autolab (EcoChemie, Netherland) with VA-Stand 663 (Metrohm, Switzerland). Three-electrode system, which consisted of hanging mercury drop electrode (HMDE) with working electrode surface 0.4 mm^2, silver-chloride electrode (Ag/AgCl/3 mol/l KCl) as reference electrode and platinum wire auxiliary electrode was used. GPES software (EcoChemie, Netherland) was used for data processing. Acetate buffer (pH 3.6, 0.2 M CH_3COOH + CH_3COONa) was used as the supporting electrolyte. Electrolyte with samples was deoxygenated by argon (99.999%) for 120 s. The concentration of cadmium was measured using differential pulse anodic stripping voltammetry (DPASV). Anodic scan started at -0.7 V and finished at -0.4 V. Cadmium was accumulated on HMDE at 0.7 V with a 120 s accumulation at room temperature. The

solution was mixed at 1450 rev min-1. Other parameters were as follows: modulations time 0.02 s, step potential 1.05 mV, and modulation amplitude 49.5 mV.

2.7 Dot-immunobinding assay – Metallothionein content

Chicken antibodies were prepared by HENA (Czech Republic). Two hens were immunized by commercially available metallothionein (1 mg of the mixture of rabbit MT 1 and MT 2, Sigma-Aldrich, USA). IgY fractions with reactivity to MT were obtained from the chicken yolk. The obtained IgY from egg yolks were purified by immunoaffinity chromatography and the fraction with the highest reactivity to MT was used. Protein concentration was 54.7 mg/ml in the isolated fraction. The antibodies in PBS were stabilized with 0.1% sodium azide [89-91].

For analyses of the biological samples 0.2 μm nitrocellulose blotting membrane from Bio-Rad was used and the samples analyses were performed in triplicates. All incubation steps were carried out using the Bio-dot apparatus from Bio-Rad. A sample volume of 10 μl was applied on membrane wetted in PBS. Further, the membrane was blocked in 1 % BSA in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 4.3 mM Na_2HPO_4, pH 7.4) for 30 min. The incubation with chicken primary antibody in dilution of 1:500 PBS with 0.1 % of BSA was carried out for 2 h at room temperature. After the three times repeated washing with PBS containing 0.05 % (*v/v*) Tween-20 (PBS-T) for 5 min the membrane was incubated in the presence of secondary antibody (rabbit anti-chicken labelled with horseradish peroxidase, Sigma - Aldrich, USA in dilution 1:5000 for 1 h at room temperature. Then, the membrane was washed three times with PBS-T for 5 min and incubated with chromogenic substrate (0.4 mg ml^{-1} AEC - 3-aminoethyl-9-carbazole in 0.5 M acetate buffer with 0.1 % H_2O_2, pH 5.5), after the adequate development the reaction was stopped by rinsing with water. Finally, the membranes were dried and scanned. Function optical density (OD) of Biolight software (Vilber-Lourmat, France) was used for calculation of dot volumes by contour recognition. The signals were recalculated to dot area [89-91].

2.8 High performance liquid chromatography coupled with electrochemical detector (HPLC-ED) – Low molecular mass thiols

HPLC-ED system consisted of two solvent delivery pumps operating in the range of 0.001-9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA), Zorbax eclipse AAA C18 (150 × 4.6; 3.5 μm particles, Agilent Technologies, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes one flow cell (Model 6210, ESA, USA). Each cell consists of four working carbon porous electrodes, each one with auxiliary and dry Pd/H_2 reference electrodes. Both the detector and the reaction coil/column were thermostated. The sample (20 μl) was injected using autosampler (Model 542 HPLC, ESA, USA). Samples were kept in the carousel at 8 °C during the analysis. The column was thermostated at 32 °C. Mobile phase consisted of 80 mM TFA (A) and methanol (B). The compounds of interest were separated by the following linear gradient: 0 → 1 min. (3% B), 1 → 2 min. (10% B), 2 → 5 min. (30% B), 5 → 6 min (98% B). Mobile phase flow

rate was 1 ml/min, working electrode potential was 900 mV [48,74,92-94]. Time of analysis was 30 min.

2.9 Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences between markers levels were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one way ANOVA test (particularly Scheffe test), which was applied for means comparison.

3. RESULTS AND DISCUSSION

3.1 The effect of cadmium(II) ions on tobacco plants growth

Untransformed *Nicotiana tabacum* (*Solanaceae*) plants are able to accumulate relatively high levels of cadmium compared to other plants. Due to intense progress of phytoremediation technologies, there are trends to increase heavy metal accumulation abilities of plants. There are two different approaches. The first comprises techniques, which lead to increase availability and uptake of heavy metals. These techniques are based on use of different chelators. In addition, plants are able to exclude different low-molecular mass compounds serving as chelators of metal ions [95]. However, exploitation of these compounds in phytoremediation technologies may represent potential risk to the environment due to toxicity and food chain entry. The second approach is based on construction of genetically modified plants with high tolerance to heavy metal/metals and great accumulation properties [81]. Genetic engineering allows to multiply the metal accumulation capacity and may effectively provide the means for phytoremediation developing into a viable industry for practical use. Establishment of genetically modified plants for heavy metals remediation is very long and complicated process, which includes many stages and steps controlled by different analyses. Recently, tobacco was engineered to accumulate higher levels of Cd in the leaves [96]. The *Saccharomyces cerevisiae* CUP1 MT gene in fusion with additional metal binding domain encoding a peptide with six histidine moieties, under the control of the CaMV 35S promoter, was introduced into tobacco plants (Wisconsin 38) [26]. In our previous paper, we showed the possibility to determine protein product of genetic modification of tobacco plants [27]. In this study, we were interested in the issue how cadmium treatment could influence content of low molecular mass thiols as glutathione and phytochelatins and metallothionein. There were no significant differences in growth and morphological appearance between transgenic tobacco plants treated with cadmium(II) ions up to 100 μM and control untreated group. The highest applied cadmium concentration led to formation of small pale spots on lower leaves; however, the area of these spots was insignificant. Darkening of roots of untransformed tobacco plants was evident after five days of the treatment with the highest cadmium concentration (250 μM). This fact was probably connected with formation of precipitates of heavy

metals ions with anions in intercellular spaces under formation of insoluble salts. Similar effects were observed in paper focused on members of the *Brassicaceae* family in connection with the treatment with heavy metal ions, especially lead [97]. Significant changes were noted in the fresh weight of both roots and aerial parts (shoots) of untreated and treated plants as well as between non-transgenic and transgenic plants (not shown). The dependencies of fresh weights of roots and shoots of transgenic tobacco plants on applied cadmium(II) ions concentrations are shown in Figs. 2A and B, respectively. The fresh weight of the roots cadmium-treated transgenic plants roots was reduced for more than 48 % in comparison with control plants after 96-hour treatment in the highest cadmium concentration (Fig. 2C). In the case of the lowest cadmium concentration (50 μM), fresh weight of roots of treated plants was reduced for about 26 % in comparison with control plants after 96-hour treatment (Fig. 2D).

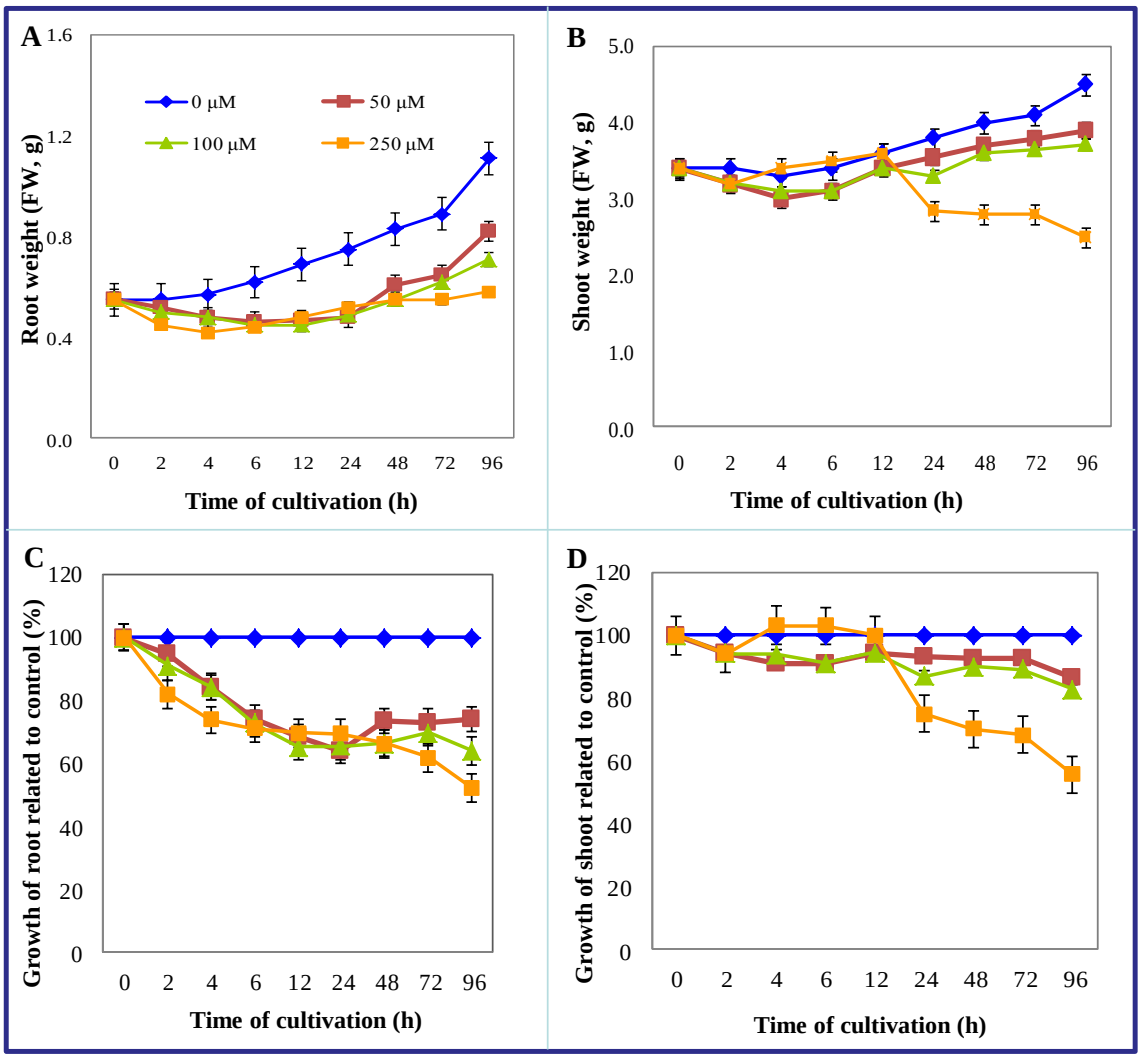

Figure 2. Changes of fresh weight of roots (**A**) and shoots (**B**) of transgenic *Nicotiana tabacum* plants exposed to different cadmium(II) concentrations (0, 50, 100 and 250 μM). Fresh weights of (**C**) roots and (**D**) shoots related to control plants for each time interval.

However, the differences in fresh weights between transgenic and non-transgenic plants were well evident with significantly more toxic effects of cadmium ions on non-transgenic plants. Whereas the fresh weight of roots of transgenic plants treated with the highest cadmium concentration was reduced for 43 % compared to control, non-transgenic plants proved 59 % reduction of roots fresh weight after 96-hour treatment. In addition, massive necrosis of outermost root tissues of non-transgenic plants was well evident. This fact was connected with growth of shoots. Fresh weight of shoots was reduced to 86 % (50 μM) and 55 % (250 μM) in transgenic plants, whereas in non-transgenic plants was fresh weight reduction more conspicuous. In conclusion, cadmium(II) ions plants in the same concentrations proved more toxic effect on non-transgenic plants compared to transgenic plants.

3.2 Phytochelatins and metallothionein content

In the following part of this study, we were focused on determination of glutathione (GSH), phytochelatins and inserted yeast metallothionein (MT) in tobacco tissues treated with various concentrations of cadmium(II) ions. The electrochemical method, differential pulse voltametry Brdicka reaction, was used for the determination of yeast metallothionein content. MT content in exposed plants was determined also immunochemically employing anti-rabbit MT 1, 2 chicken antibodies. High performance liquid chromatography with electrochemical detection was used for determination of cysteine (Cys), glutathione (GSH, GSSG) and phytochelatins (PC2, PC3 and PC4). Glutathione itself plays a crucial role in the control of the cell redox state. Stress conditions caused by heavy metals lead to the alterations in glutathione content including changes in the cell redox state. In addition, glutathione depletion may cause reactive oxygen species (ROS) production, which can be generated by heavy metal ions [98]. Glutathione plays not only detoxification function (glutathione-ascorbate cycle as the well-known metabolic pathways of hydrogen peroxide detoxification), but it is very important building unit for phytochelatins synthesis catalysed by phytochelatin synthase [74]. Its level correlates with PCs levels in plants. The content of reduced glutathione (GSH) in untreated non GM plants was generally higher in shoots (383 nmol/g- 430 nmol/g FW) compared to roots (285 nmol/g – 310 nmol/g FW) during experiment. Contrariwise, the levels of oxidized glutathione (GSSG) were approximately five times lower (98 nmol/g – 142 nmol/g FW in roots and 160 nmol/g – 254 nmol/g in shoots) during the experiment. Reciprocal rates (GSH/GSSG) in control untreated plants were as follows: time 0 – 3.22 (roots) and 2.69 (shoots), 12 h – 3.05 (roots) and 2.12 (shoots), 24 h – 2.98 (roots) and 2.15 (shoots), 48 h – 3.11 (roots) and 2.21 (shoots), 72 h – 3.31 (roots) and 2.52 (shoots), and 96 h – 3.35 (roots) and 2.55 (shoots). Higher values of GSH/GSSG were recorded at the beginning and at the end of experiment (0, 72 and 96 hours of treatment). This fact is probably connected with transfer of plants into fresh cultivation medium containing low concentration of the metal ions. Application of cadmium(II) ions in higher concentrations (100 and 250 μM) led to the depletion of reduced and enhancement of oxidized glutathione in shoots as well as roots, whereas in low concentration (50 μM) the treatment led to the enhancement of GSH. Reciprocal GSH/GSSG rates were significantly changed at all cadmium concentrations. At the highest cadmium concentration (250 μM) after 96-h treatment,

these rates were as follows: 1.56 (roots) and 1.12 (shoots). These changes show evidence of the ability of cadmium to generate ROS as well as involvement of GSH in maintenance of homeostasis. Amino acid cysteine serves as crucial substrate for GSH synthesis (enzymes γ-glutamylcysteine synthetase, which catalyses biosynthesis of γ-glutamylcysteine and glutathione synthetase, which catalyses biosynthesis of glutathione from γ-glutamylcysteine and glycine) and subsequently for PCs synthesis, which represents oligomers of glutathione. We analysed content of cysteine in roots and leaves of both transgenic and non-transgenic plants. Our results demonstrated fact that cysteine does not represent a limiting factor in PCs synthesis. In addition, treatment of plants with cadmium did not lead to the alteration in cysteine content neither in roots nor shoots (data not shown). The concentrations of individual PCs (PC2, PC3, and PC4) during 96-hour treatment of transgenic plants by cadmium of concentration of 50, 100, and 250 μM are shown in Figs. 3A and B. Their content was determined in roots and stems in 12-, 24-, 48-, 72- and 96-hour intervals.

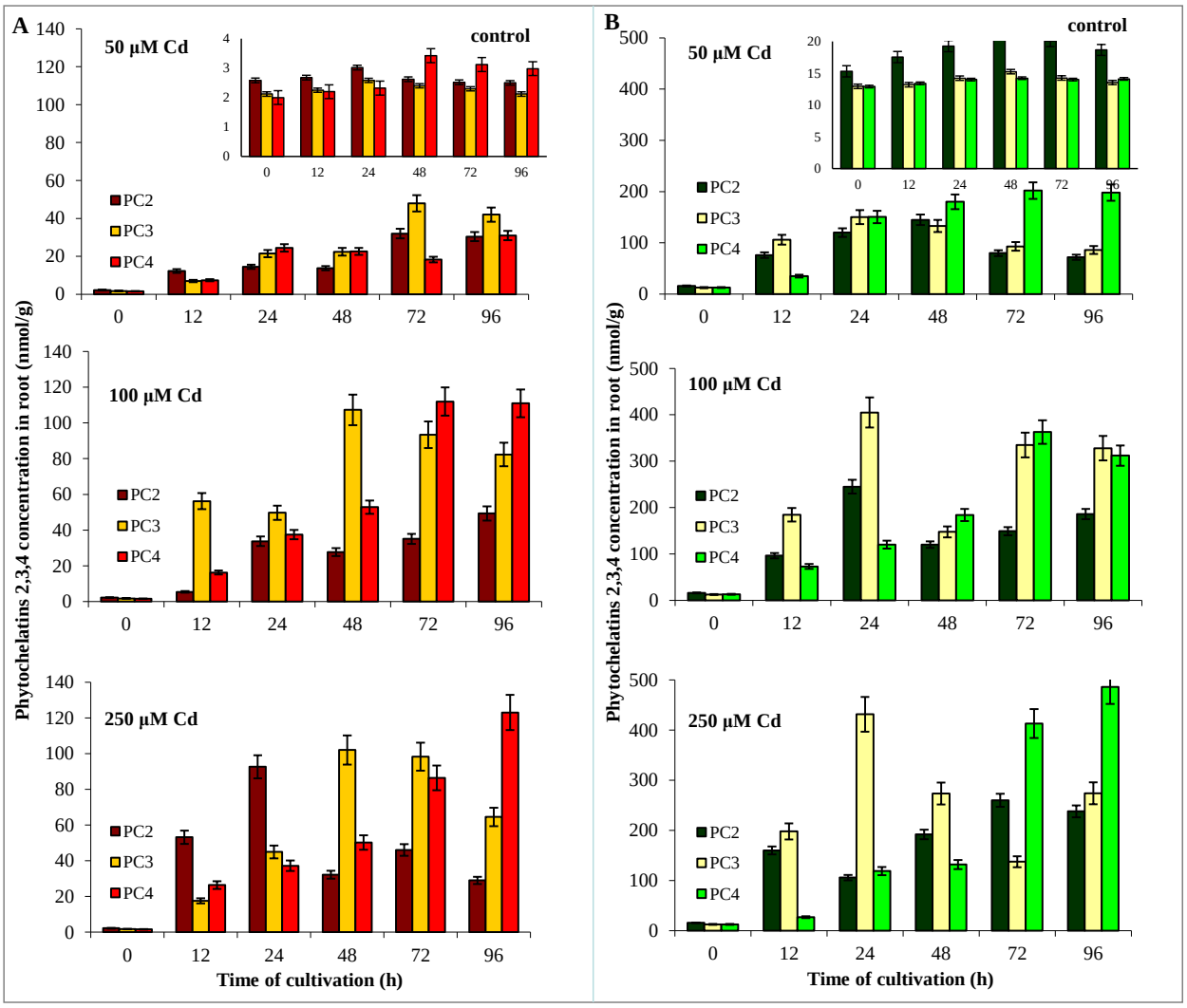

Figure 3. Levels of phytochelatins 2, 3, and 4 (PC2, PC3, PC4) in (**A**) roots and (**B**) shoots in transgenic *Nicotiana tabacum* plants (line T-XHis) exposed to different cadmium(II) concentrations (0, 50, 100 and 250 μM).

Control untreated plants did not demonstrate significant changes in PCs content. Content of all three analysed PCs was very low in both roots and shoots of control plants (units of nmol/g FW – up to 3.42 for PC4 in roots and up to 21.5 for PC2 in shoots). However, levels of PCs were higher in shoots compared to roots. This fact is in agreement with work of Zhang et al., who detected significantly higher levels of PCs in shoots of *Sedum alfredii* Hance [99]. Application of cadmium(II) ions led to the enhancement of PCs levels in all concentrations, whereas these changes were comparable with those observed in non-transgenic plants (not shown). The prevalent PC form in the lowest cadmium concentration (50 μM) was PC3 in roots and PC4 in shoots (Figs. 3A and B).

Under higher concentrations PC4 dominated in both roots and leaves with one exception – PC3 was predominating PC form in 100 μM concentration in shoots. It clearly follows from the results obtained that the increasing PCs levels are closely connected with extending time of treatment. 22.5-, 35- and 66-fold enhancement for PC2, PC3 and PC4 were detected in roots after 96-hour treatment in the highest cadmium concentration compared to time 0 h. In the case of shoots these levels were 11.6-, 18.7- and 27.0-fold higher compared to time 0 h. Significant differences were recorded in representation of individual PCs forms – PC2 – 13 %, PC3 – 29 % and PC4 – 58 % in roots and PC2 – 24 %, PC3 – 28 % and PC4 – 48 % in shoots after 96-hour cadmium treatment. At the beginning of the treatment, the rates were as follows – PC2 – 44 %, PC3 – 30 % and PC4 – 26 % in roots and PC2 – 36 %, PC3 – 34 % and PC4 – 30 % in shoots. The obtained results are in well agreement with the fact that phytochelatins are actively associated with the protection of plants against adverse effects of metal ions [100]. In addition, the role of PCs in the transport of metal ions from roots to aerial parts and further complexing of heavy metal ions is still discussed. Possible role of PCs in cadmium transport was clearly evident in our experiment, where increasing cadmium concentration together with time of treatment led to enhanced PCs synthesis. Similar results were demonstrated by Garg et al., where treatment of *Cajanus cajan* (L.) Millsp. with cadmium(II) ions led to the increase of GSH levels as well as levels of phytochelatins [101].

Further, we measured ´the content of transgenic metallothionein in tobacco plants. The content of metallothionein in transgenic plants with cadmium(II) ions (0, 50, 100 and 250 μM) during 96-hour long treatment is shown in Fig. 4. Control untreated plants did not demonstrate significant changes in MT content. Average MT content in roots was 65 ± 9 μg/g FW and in shoots the average MT content was 92 ± 17 μg/g FW. After initial 10 % increase, MT content decreased gradually for the whole time of the experiment.

On the contrary, MT content in shoots of the treated plants increased markedly during the first 12 hours. The increase in MT content for 40, 100 and 200 % was observed after the treatment of the plants with 50, 100 and 250 μM Cd. In roots of the exposed plants the increase in MT content was 40, 100 and 120 % after application of the same concentrations of cadmium(II) ions. In spite of the fact that the content of low molecular mass thiols in treated plants exhibited marked increase in roots than in shoots, the trend in the increase of yeast MT was opposite. Possible explanation of this phenomenon is that MT was induced by Cd, which was not chelated by PCs and GSH. This indicates that expression of yeast MT in plants is a functional mechanism, which supports the ability of the transgenic plants to tolerate higher Cd concentration.

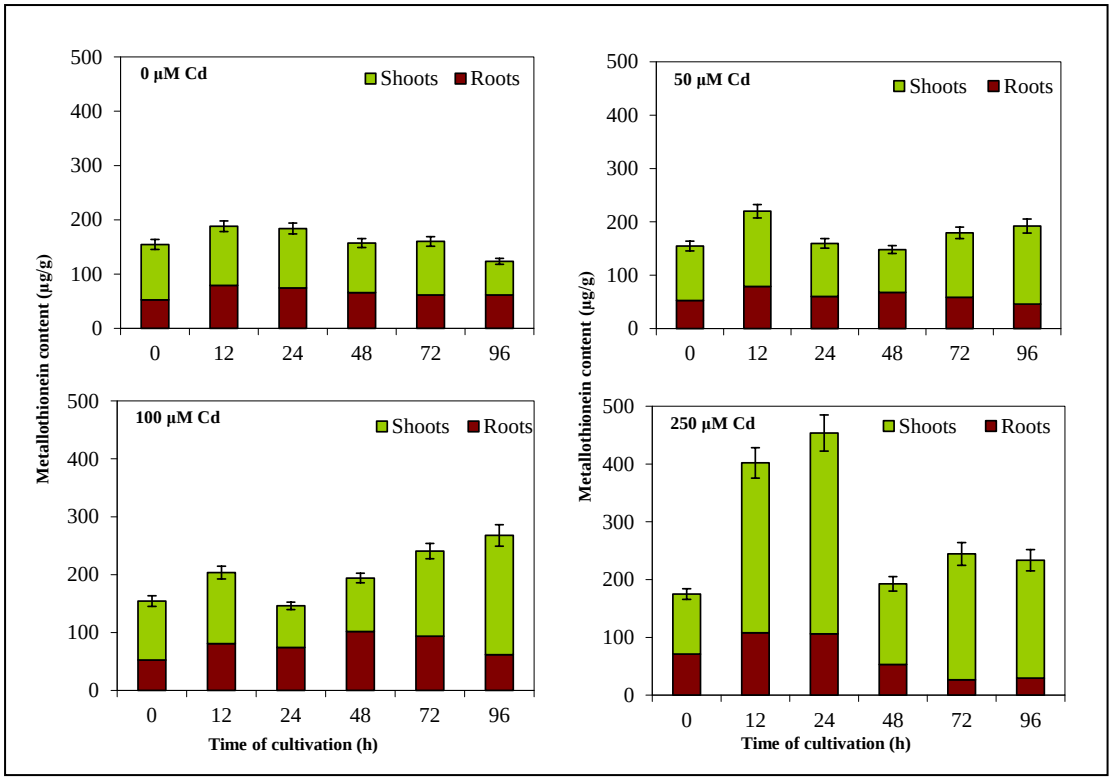

Figure 4. MT levels in roots and shoots in transgenic *Nicotiana tabacum* plants exposed to different cadmium concentrations (0, 50, 100 and 250 µM). Cultivation time 0, 12, 24, 48, 72 and 96 h.

It clearly follows that there is synergy between low molecular mass thiols and production of transgenic metallothionein. The next experiment aimed to characterise the dynamic of the stress reaction of tobacco plants on the presence of cadmium(II) ions. Contents of PCs (PC2, PC3, PC4), yeast MT and GSH were determined in both transgenic and non-transgenic plants. Samples were taken each 2 hours after cadmium application in concentrations of 0, 50 and 100 µM. We chose only two cadmium concentrations (50 and 100 µM) due to negative effect of cadmium in the highest concentration (250 µM) on non-transgenic plants. The harvested plants were divided into roots, stems and leaves.

Due to the fact that we were interested in the dynamics of stress response, leaves and stems were analysed separately. The total time of treatment was 12 hours. All results were recalculated to control, which represents in graphs value of 0. Changes of monitored compounds are expressed by negative values (decrease compared to control) or by positive values (increase compared to control). Primarily we analysed leaves of non-transgenic and transgenic tobacco plants. Contents of MT, determined by (A) Brdicka reaction and (B) dot blot assay, (C) GSH, (D) PC2, (E) PC3 and (F) PC4, are shown in Fig. 5. The results obtained from analyses of stems and roots are shown in Figs. 6 and 7. Treatment of tobacco plants with cadmium(II) ions led to the rapid response. Changes detected in non-transgenic plants included decrease of GSH, PC2 and PC4 and enhancement of PC3 in stems and increase of all above-mentioned phytochelatins in roots (not shown).

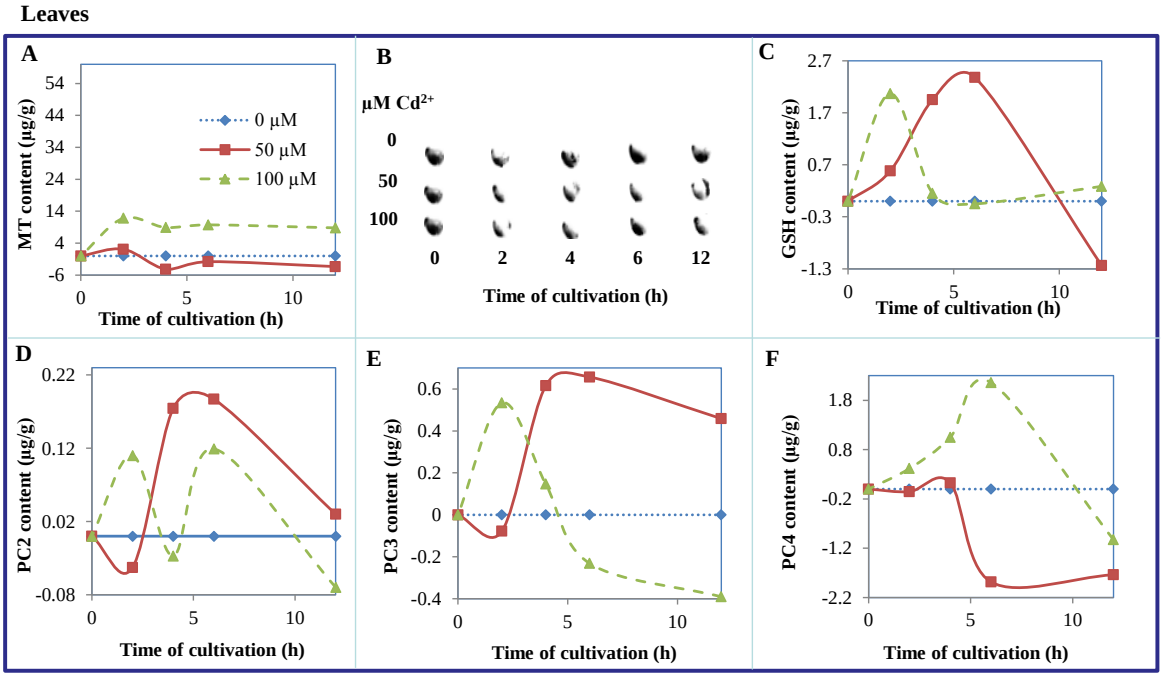

Figure 5. Changes of MT, GSH, PC2, PC3, and PC4 in leaves of the transgenic plants exposed to cadmium(II) ions in concentrations 0, 50 and 100 µM. All results were recalculated to control, which represents in graphs value of 0. MT content was determined by (**A**) Brdicka reaction and (**B**) dot blot assay, (**C**) GSH, (**D**) PC2, (**E**) PC3 and (**F**) PC5 were determined by HPLC-ED.

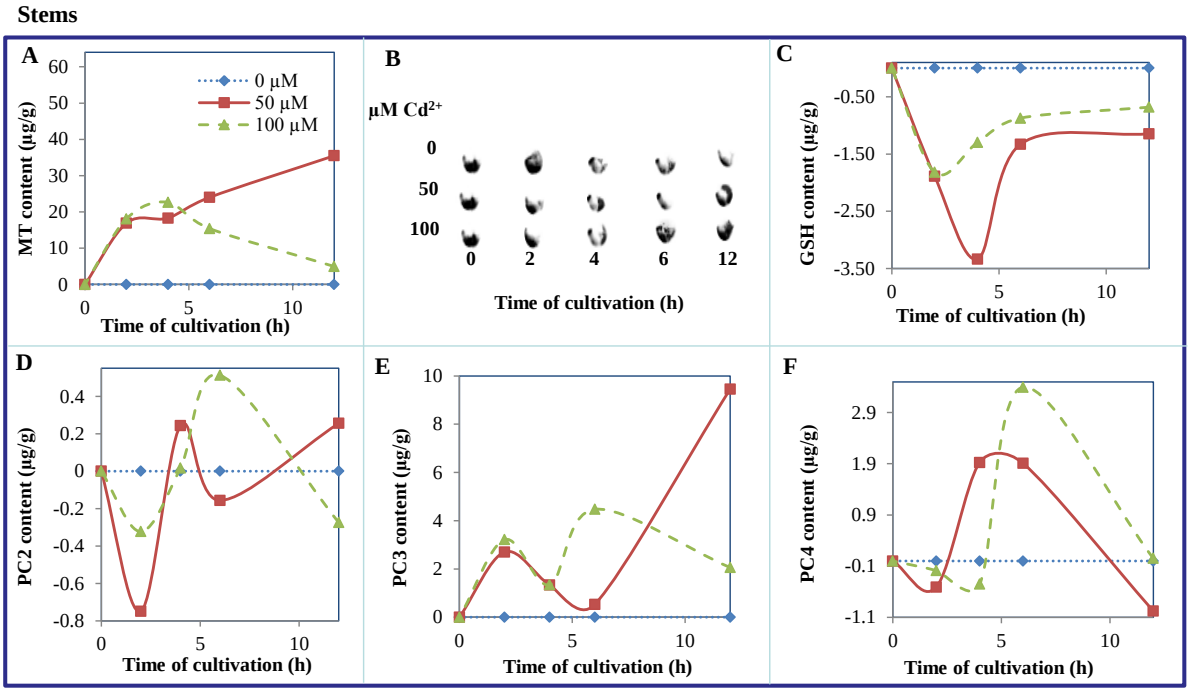

Figure 6. Changes of MT, GSH, PC2, PC3, and PC4 in stems of the transgenic plants exposed to cadmium(II) ions in concentrations 0, 50 and 100 µM. All results were recalculated to control, which represents in graphs value of 0. MT content was determined by (**A**) Brdicka reaction and (**B**) dot blot assay, (**C**) GSH, (**D**) PC2, (**E**) PC3 and (**F**) PC5 were determined by HPLC-ED.

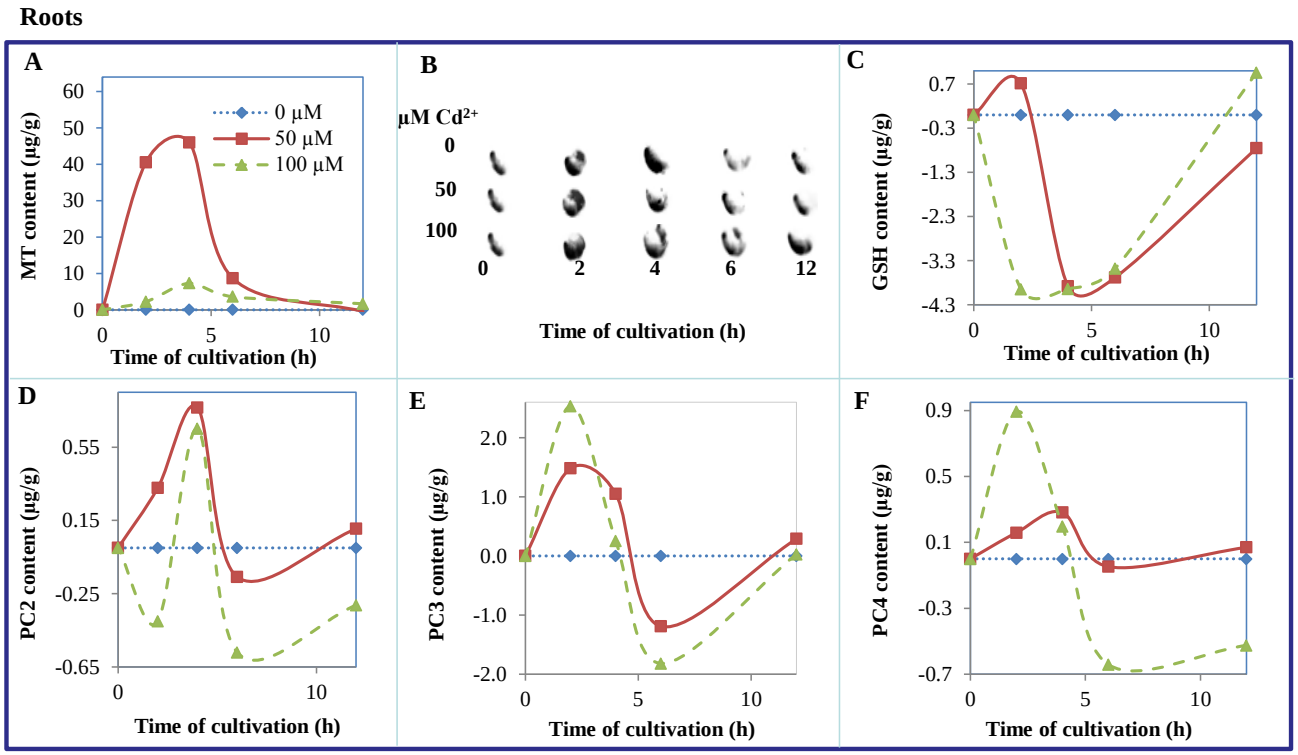

Figure 7. Changes of MT, GSH, PC2, PC3, and PC4 in roots of the transgenic plants exposed to cadmium(II) ions in concentrations 0, 50 and 100 µM. All results were recalculated to control, which represents in graphs value of 0. MT content was determined by (**A**) Brdicka reaction and (**B**) dot blot assay, (**C**) GSH, (**D**) PC2, (**E**) PC3 and (**F**) PC5 were determined by HPLC-ED.

In the case of leaves, changes in PCs comprised increase of PC2 and PC3 (the most evident 6th hour after cadmium application). Compared to PC2 and PC3 values detected in roots and stems, they increased during the experiment (50 µM of cadmium(II) ions). Significant decrease of PC4 (for more than 90 %) was observed 6th hour after cadmium application at the same concentration. All above-mentioned changes were marked at cadmium concentration of 100 µM. Increase of PCs in roots followed by decrease of PCs in stems and leaves is closely connected with their role in transport and further complexing of heavy metals. PCs together with glutathione represent the most potent tool in protection of plants against cadmium. Some published papers showed rapid response of phytochelatin synthase on presence of cadmium ions, however, biosynthesis of PCs may be limited by limited amount of glutathione. *Arabidopsis thaliana* mutant lacking phytochelatin synthase demonstrated great sensitivity to cadmium ions [102]. Introduction of phytochelatin synthase gene (TcPCS1) from hyperaccumulator *Thlaspi caerulescens* led to the increased accumulation of cadmium in both roots and shoots of transformed tobacco plants. In addition, higher antioxidant enzymes activities and reduced production of malondialdehyde was observed compared to wild tobacco type [102]. On the other hand, transgenic tobacco plants demonstrated not so distinctive changes in PCs content in our experiments, so, we can assume that the expression of yeast MT gene does not cause alterations in glutathione and phytochelatins synthesis. The trends as well as levels of GSH and PCs were

comparable with trends and levels of GSH and PCs determined in non-transgenic plants. The most significant changes in transgenic tobacco plants between control and treated ones were observed in content of yeast metallothionein – for almost 20 % after 4 hours of treatment and for 36 % after 12-h treatment in stems (50 μM). In the case of roots, rapid enhancement of MT content (for almost 48 % after 4-h treatment) was followed by its rapid decrease, which was comparable with control plants after 12 hours of treatment. Only minimal changes in comparison with control transgenic plants were detected in leaves. Treatment with cadmium ions in the concentration of 50 μM led to the reduction of MT in leaves in comparison with the control. Those results were confirmed by dot immunobinding assay. The change in MT content exhibited similar trend and the correlation between the results obtained by Brdicka reaction and dot blot assay was 0.83 (Fig. 8).

The observed changes in MT level are probably connected with the role of MT in metal binding. However, transport of MT and formation of complexes with cadmium(II) ions must be carefully considered. There is one important question. What can we say about effectiveness of yeast metallothionein in cadmium binding and detoxification? This question may be clarified by the fact that transgenic tobacco plants demonstrated no darkening of roots in the presence of cadmium(II) ions, which is caused by formation of precipitates in different root structures, especially in symplast. In addition, growth of transgenic tobacco plants was significantly less affected by cadmium(II) ions even at the highest concentration (250 μM), where necroses of outermost root tissues were observed. The role of yeast metallothionein in cadmium transport and complexing of metal ions may be elucidated by other finding as accumulation abilities of transformed plants compared to non-transformed ones. Therefore, we focused our interest to determination of cadmium content in roots and shoots of both transgenic and non-transgenic plants treated with these ions (Fig. 9).

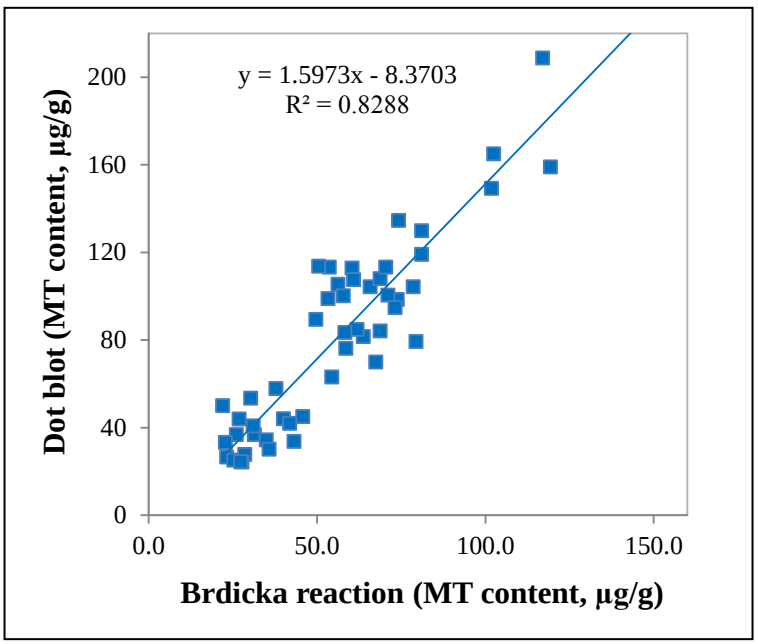

Figure 8. Correlation between the contents of MT determined by Brdicka reaction and dot blot analysis.

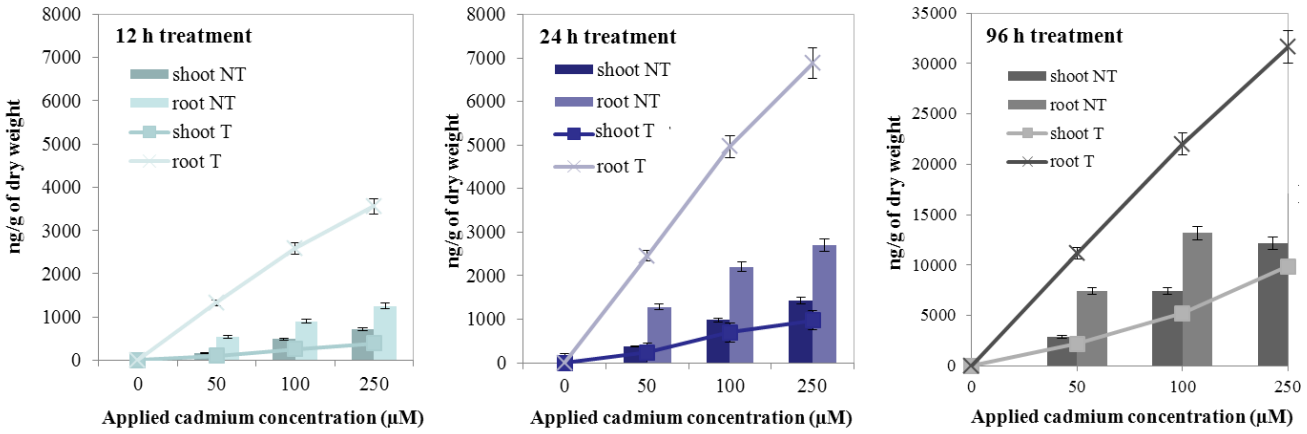

Figure 9. Accumulation of cadmium in transgenic and non-transgenic tobacco plants treated with 0, 50, 100 and 250 µM of cadmium(II) ions. Treatment time: (**A**) 12 h, (**B**) 24 h and (**C**) 96 h.

The highest cadmium content was determined in roots of transgenic tobacco plants (54 % higher compared to non-transgenic plants) after 96 h treatment. On the other hand, amount of cadmium was higher in shoots of non-transgenic plants (23 % at the highest concentration compared to transgenic plants) after 96 h treatment. This fact may be explained by the role of PCs in transport of heavy metal ions for long distances, i.e. roots to shoots. On the other hand, phytochelatins may be rapidly transported to the roots; their transport is closely associated with detoxification of heavy metals directly in roots as well as with translocation of heavy metals to the aerial parts [103-105]. *Arabidopsis thaliana* transgenic plants with expression of *TAPCS1* gene (gene for phytochelatin synthase) demonstrated increased transport of cadmium(II) ions from roots to aerial parts and, in addition, reduced accumulation of cadmium ions in roots [103]. This fact is in agreement with our results (higher amount of cadmium in shoots of non-transgenic plants) despite the fact, that our transgenic tobacco plants expressed gene for yeast metallothionein - *CUP1*. We can conclude that expression of *CUP1* reduces toxic effects of cadmium on plants and contribute to accumulation of cadmium(II) ions in roots and its limited transport into aerial parts in transgenic plants [106]. In our previous paper it was found, that the antioxidant activity of extracts form transgenic tobacco plants carrying human or yeast MT fragments was higher than in control non-transgenic plants [54]. On the other hand, levels of GSH and PCs were not changed and were comparable in both transgenic and non-transgenic plants.

4. CONCLUSION

Cadmium represents one of the most important contaminants of the environment. Transgenic tobacco plants with *CUP1* gene (*Nicotiana tabacum* L. cv. Wisconsin 38) and non-transgenic *Nicotiana tabacum* plants were used for comparison of the effect of cadmium(II) ions with special focus to thiol compounds including cysteine, GSH/GSSG, phytochelatins and yeast metallothionein (in transgenic plants) and accumulation abilities. Transgenic plants demonstrated higher tolerance to cadmium(II) ions compared to non-transgenic plants. In addition, alterations connected with transport

and cadmium(II) ions accumulation were observed. We can conclude that yeast metallothionein is responsible for higher cadmium accumulation in roots of transgenic plants and its limited transport to aerial parts. On the other hand, changes in GSH/GSSG and PCs in transgenic and non-transgenic plants were insignificant.

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