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ORIGINAL PAPER

Determination of content of metallothionein and low molecular mass stress peptides in transgenic tobacco plants

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Abstract Phytoremediation is a process that utilizes plants to remove, transfer, stabilize, or destroy pollutants in soil, sediment, and groundwater. Plants used for such purposes have several requirements. Genetic engineering these plants could be an effective tool used to acquire features needed for such purposes within a substantial amount of time. This paper aims to utilize electrochemical techniques to analyze transgenic tobacco and, thus, to reveal their heavy metals phytoremediation potential. Total thiol and metallothionein (MT) quantities were determined in the control and transgenic tobacco plants. The total content of thiols in transgenic plants varied within the range of 561 to 1,671 μg g^{-1}. Furthermore, the determination of MT was done on transgenic tobacco plants. The level of human MT in transgenic tobacco plants varied between 25 and 95 μg g^{-1}. However, a plant cell protects itself by synthesizing low molecular mass thiols such as reduced glutathione and phytochelatins to protect itself against heavy metals toxicity. The most important thiols, cysteine (Cys), glutathione (GSH), oxidised glutathione (GSSG) and phytochelatin 2 (PC2), were determined in the non-transgenic and transgenic tobacco plants by high performance liquid chromatography with electrochemical detection. Tobacco plants synthesizing the highest amount of metallothionein have the highest basal level of phytochelatin 2 as well as reduced glutathione and free cysteine. It clearly follows from the results obtained that the biosynthesis of particular thiols is mutually linked, which contributes to a better protection of a transgenic plant against heavy metals effects.

Keywords Metallothionein · Tobacco · *Nicotiana tabacum* · Thiols · Phytoremediation · Electrochemical detection · Brdicka reaction · Voltammetry · Liquid chromatography

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Abbreviations

MT	Metallothionein
DPV	Differential pulse voltammetry
AdTS	Adsorptive transfer stripping
PC	Phytochelatins
Cys	Cysteine
GSH	Reduced glutathione
GSSG	Oxidised glutathione

Introduction

Toxic heavy metal ions are natural elements present (Volesky and Holan 1995) throughout the world. In some regions, such as industrial sectors, they are present in much higher concentrations, consequently causing potential health risks. Of the many toxins present in such areas, contaminants comprised of Cd^{2+}, Hg^{2+}, Pb^{2+} are more atrocious in bringing about serious, life threatening conditions than other toxins. Plants, due to their sessile living, must have effective but diverse protective means (Clemens 2006; Leopold et al. 1999; Meister and Anderson 1983; Supalkova et al. 2007b) of harbouring themselves from such conditions. In spite of the presence of such mechanisms, heavy metals can alter the plant's biochemical pathways (Athar and Ahmad 2002; Daniel and Gyori 2000; Shanker et al. 2005; Stroinski 1999). However, higher doses of biogenic heavy metals in addition to foreign toxics can result in death of an exposed plant. The concentration of free metal ions in plant tissues is regulated by several molecules, particularly the phytochelatins (PCs) and glutathiones (GSH) (Cobbett and Goldsbrough 2002; Grill et al. 1985; Meister and Anderson 1983). These low molecular mass peptides possess an abundant amount of free sulfhydryl groups (–SH). The heavy metals ions can be chelated to these independent –SH residues. In animals heavy metals monitoring is maintained by a group of proteins called metallothioneins (MTs) (Henry et al. 1994; Palmiter 1994). These proteins are more effective than plant protective compounds and belong to a group of intracellular, low molecular mass and cysteine-rich proteins with molecular weights ranging from 6 to 10 kDa. MTs consist of two binding domains (α, β) that are assembled from cysteine clusters. The N-terminal part of the protein is marked as α-domain, which has three binding places for divalent ions. β-Domain (C-terminal part) has the ability to bind four divalent ions of heavy metals. In the case of univalent ions of heavy metals, MT is able to bind twelve metal ions. Because of its capacious heavy metal binding capabilities the gene encoding MT production was introduced into tobacco plant genome aimed to test the accumulative potential for heavy metals (Macek et al. 2002). The hyperaccumulation of heavy metal ions was demonstrated in these transgenic plants (Francova et al. 2001; Macek et al. 2002; Pavlikova et al. 2004a, b). Nevertheless the presence of the transgene was demonstrated using different molecular methods, but the expressed protein as well as the content of other plant low molecular mass thiols has not yet been measured. Therefore, the main aim of this paper is to utilize electrochemical techniques for analysis of transgenic tobacco and, thus, to reveal their heavy metals phytoremediation potential. Specifically total content of thiols and MT is measured using Brdicka reaction as an electrochemical method due to its sensitivity to –SH groups (Kizek et al. 2004; Mikelova et al. 2007; Petrlova et al. 2006b; Trnkova et al. 2002). For the determination of plant low molecular mass thiols, high performance liquid chromatography coupled with electrochemical detector is carried out (Petrlova et al. 2006a; Potesil et al. 2005; Supalkova et al. 2007a).

Material and methods

Chemicals and pH measurements

Rabbit liver MT (MW 7143), containing 5.9% Cd and 0.5% Zn, was purchased from Sigma Aldrich (St. Louis, USA). Tris(2–carboxyethyl)phosphine (TCEP) was acquired from Molecular Probes (Evgen, Oregon, USA). Phytochelatin 2 (PC_2, (γ-Glu–Cys)$_2$–Gly was synthesized at Clonestar Biotech (Brno, Czech Republic) with a purity greater than 90%. HPLC-grade methanol (>99.9%; *v*/*v*) from Merck (Dortmund, Germany) was used. $Co(NH_3)_6Cl_3$ and other chemicals used were purchased from Sigma Aldrich (Sigma-Aldrich, USA) with an ACS grade purity unless noted otherwise. Stock standard solutions were prepared with ACS water (Sigma-Aldrich, USA) 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 disc (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).

Transgenic plants

The tobacco clones derived from *Nicotiana tabacum L.*, var. Wisconsin 38 and their genetically modified lines labelled 1A, 1B, 1F, 2A and 2B were used. Genetically modified plants carrying the transgene for α chain of human metallothionein was of particular interest and study. The vector plasmid pBI121, containing the CaMV 35S promoter, from the cauliflower mosaic virus was used. For additional details see Macek et al. 2002.

Sample preparation

The transgenic and controlled tobacco plant samples were divided into leaves and roots (app. 0.2 g of fresh weight) followed by homogenization using deep-freezing in liquid nitrogen. One ml of 0.2M phosphate buffer pH 7.2 was added to the homogenate. The homogenate was then treated for 30 min at 99°C using a thermomixer (Eppendorf 5430, USA). The solid particles and denatured proteins were removed and repeated twice followed by centrifugation (Eppendorf 5402, USA) at 16,000*g* for 30 min at 4°C. 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 centrifuge (Eppendorf 5402, USA). Prior to analysis the supernatant was filtered through a membrane filter (0.45 μm Nylon filter disk, Millipore, Billerica, Mass., USA).

Stationary electrochemical analyser: adsorptive transfer stripping differential pulse voltammetry Brdicka reaction: MT content

Electrochemical measurements were performed on AUTOLAB analyser (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 as the working electrode, a 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 was employed. The Brdicka 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. 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. For additional experimental conditions see Petrlova et al. 2006b.

Stationary electrochemical analyser coupled with autosampler: differential pulse voltammetry Brdicka reaction: total thiols content

Electrochemical measurements were performed on 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and a cooled sample holder (4°C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. A Ag/AgCl/3M KCl reference electrode and a glassy carbon auxiliary electrode was used. The supporting electrolyte (1 mM $[Co(NH_3)_6]Cl_3$ and 1 M ammonium buffer; $NH_3(aq)$ and NH_4Cl, pH 9.6) was changed after five measurements. The DPV parameters were as follows: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude −250 mV, E_{ads} = 0 V. All experiments were carried out at a temperature of 4°C (Julabo F12, Germany). For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed.

High performance liquid chromatography coupled with electrochemical detector: content of low molecular mass plant thiols

HPLC-ED system consisted of two solvent delivery pumps operating in the range of 0.001-9.999 ml min^{-1}

(Model 582 ESA Inc., Chelmsford, MA), Metachem Polaris C18A reverse-phase column (150.0 × 2.1 mm, 5 µm particle size; Varian Inc., CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector included three flow cells (Model 6210, ESA, USA). Each cell consisted of four analytical cells containing a working carbon porous electrode, two auxiliary and two reference electrodes. Both the detector and the reaction coil/column were thermostated. The sample (5 µl) was injected using an autosampler (Model 540 Microtiter HPLC, ESA, USA). HPLC-ED experimental conditions were as follows–mobile phase: trifluoroacetic acid (80 mM): methanol 98:2 (v/v); flow rate of the mobile phase: 0.4 ml min^{-1}; the working electrode potential: 900 mV.

Mathematical processing of the data obtained

Data were processed using MICROSOFT EXCEL® (USA). Results are expressed as mean ± standard deviation (SD) unless noted otherwise.

Results and discussion

Total content of thiols in transgenic tobacco plants

The electrochemical analysis of the transgenic clones (1A, 1B, 1F, 2A and 2B) of *Nicotiana tabacum* carried out by differential pulse voltammetry Brdicka reaction was utilized for the determination of thiol content (cysteine, reduced glutathione, phytochelatins and metallothionein). Typical DP voltammograms of the leaves and roots from non-transgenic tobacco cultivar WSC are shown in Fig. 1a. The height of the catalytic peak, labelled Cat 2, is proportional to total thiol concentration. Other peaks observed in the voltammogram were also associated with of the presence of -SH groups in the analysed electrolyte, but their exact origin is not clear (Petrlova et al. 2006b; Trnkova et al. 2002). The heights of the Cat2 peaks indicate the concentration of compounds with –SH groups are higher in leaves than in roots. The total content of thiols was 43 ± 37 µg g^{-1} in roots, but the level of thiols in leaves was approximately twenty times higher (830 ± 410 µg g^{-1}). The variability in the results is apparently associated with the ability of the clones to produce thiols. More details will be published. The typical DP voltammogram of leaf of a transgenic tobacco plant carrying the gene T-α MT1A is shown in Fig. 1b. Similar voltammograms were measured for the leaves and roots of non-transgenic tobacco plants (Fig. 1a and b). The average level of thiols in the roots and leaves of transgenic tobacco plants were 151 ± 78 µg g^{-1} (enhance in thiol content about 3.5 times in comparison with control) and 920 ± 450 µg g^{-1} (enhance in thiol content about 1.1 times in comparison with control) respectively. The results clearly show the expression of the inserted gene in the roots has an obvious influence on the total thiol content, but the expression of thiols in leaves of transgenic plants did not differ from leaves of the control plants.

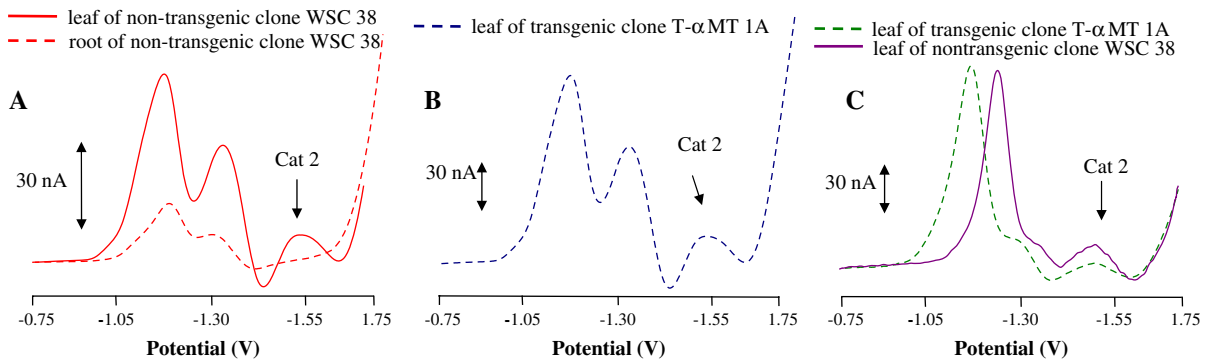

Fig. 1 DP voltammograms of (**a**) control non-transgenic tobacco plants (roots and leaf) and (**b**) leaf of transgenic tobacco plant clone T-α MT 1A measured by DPV Brdicka reaction. (**c**) DP voltammograms of leaves from control and transgenic tobacco plants measured by AdTS DPV Brcicka reaction

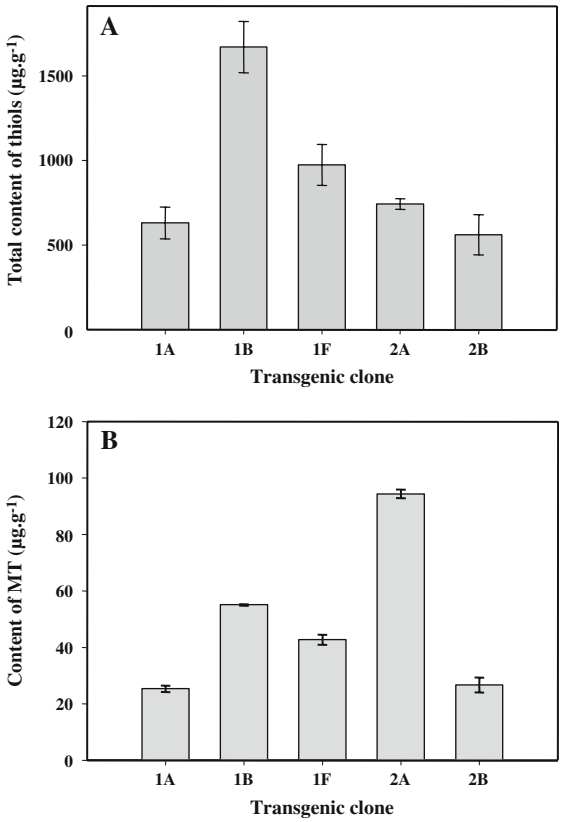

Fig. 2 (**a**) Total content of thiols and (**b**) content of metallothionein measured by DVP Brdicka reaction and AdTS DPV Brdicka reaction, respectively

The total content of thiols in leaves of a clone is shown in Fig. 2a. The content varied from 561 to 1,671 μg g^{-1}. The highest content of thiols was determined in the samples obtained from clones 1B and 1F. Transgenic as well as nontransgenic plants have similar levels of protective thiols, mainly in leaves. Therefore it can be concluded that the inserted gene for human metallothionein production probably does not interfere with the metabolism of thiols; however, the effect of the inserted gene was prominent during the presence of heavy metal ions on the investigated transgenic and non-transgenic tobacco plants (Macek et al. 2002). Data suggest the ability of transgenic plants to withstand high doses of heavy metal compared to non-transgenic plants is associated with a corresponding level of metallothionein. Therefore, determination of MT level was studied only in transgenic tobacco plants.

Human metallothionein expression in transgenic tobacco plants

To determine MT levels in transgenic plants an AdTS DPV Brdicka reaction (Petrlova et al. 2006b) was carried out Differences between transgenic and non-transgenic tobacco clone voltammograms were apparent (Fig. 1c). A peak at a potential of −1.5 V called Cat 2 was detected in transgenic as well as non-transgenic tobacco clones. Although the AdTS DPV Brdicka reaction is primarily sensitive to MT, the Cat 2 peak present and measured in non-transgenic plants is due to metallothionein-like proteins that cannot be completely removed from the samples. The levels of metallothionein-like proteins in non-transgenic plants were determined to be approximately 5 μg g^{-1}. Subtraction of metallothionein-like proteins content yield MT levels in transgenic tobacco plants between 25 and 95 μg g^{-1} (Fig. 2b). Based on obtained results, the highest levels of MT existed in clones 1B and 2A. The variance in metallothionein expression at individual clones may be associated with somaclonal selection in the process of transformants regeneration as well as unequal process of heterogeneous DNA integration in individual plant cells. In addition to electrochemical analysis, the presence of MT in transgenic tobacco plants was confirmed by using dot-blot assay with monoclonal antibodies (E9); more experimental details will be published. The highest metallothionein levels were found in clones 1B and 2A, which is in good agreement with the electrochemical results. Based on the electrochemical and immunohistochemical results, clones 1A and 2B should have had the highest tolerance to the presence of heavy metals; however, under non-stress conditions the metallothionein synthesis was present at low levels.

Content of low molecular mass plant thiols

As mentioned above, a plant cell protects itself against heavy metal toxicity by synthesizing low molecular mass thiols. The level of such thiols shows the natural ability of a plant to survive in environments polluted by heavy metals. Thiols, Cysteine (Cys), Glutathione (GSH), oxidised Glutathione (GSSG) and Phytochelatin 2 (PC2), were determined in non-transgenic and transgenic tobacco plants by high performance liquid chromatography with

electrochemical detection (HPLC-ED). A HPLC-ED chromatogram of a transgenic tobacco sample (clone 2A) is shown in Fig. 3. The highest PC2 content (1.45 μmol g^{-1}) was determined in clone 2A followed by clone 1B, clone 1A and clone 2B where PC2 levels were 0.35 μmol g^{-1}.

Although Glutathione, has a significant role in heavy metal detoxification processes, because of its antioxidant properties, its major role is to scavenge reactive oxygen species. The consumption of GSH and its conversion into GSSG may be an important indicator of oxidative stress in cells. The levels of reduced glutathione varied from 0.68 μmol g^{-1} to 4.80 μmol g^{-1} while oxidised glutathione levels ranged from 0.10 μmol g^{-1} to 0.59 μmol g^{-1}. Relatively high levels of GSSG were detected in clones 2A and 1A followed by higher content of GSH (Fig. 4).

The importance of independent cysteine residues for heavy metals accumulation involves a series of steps. Cysteine can bind heavy metals and form complexes (chelates) (Zimmermann et al. 2005), but these chelates most likely do not play a key role in the detoxification process. More importantly, the cysteine serves as a precursor for the biosynthesis of glutathione and phytochelatins. The lowest level of cysteine (0.95 μmol g^{-1}) was determined in clone 2B and the highest one (3.4 μmol g^{-1}) in clone 2A. We found tobacco plants synthesising the highest amount of metallothionein have the highest basal level of

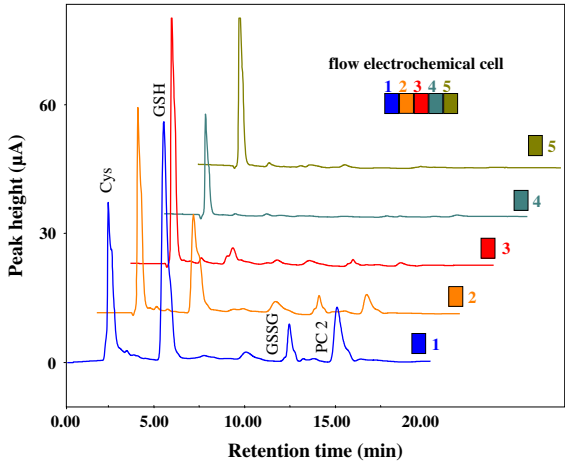

Fig. 3 HPLC-ED chromatogram of transgenic clone of tobacco (MT-α-2A) with marked signals corresponding to cysteine, reduced and oxidized glutathione and phytochelatin 2

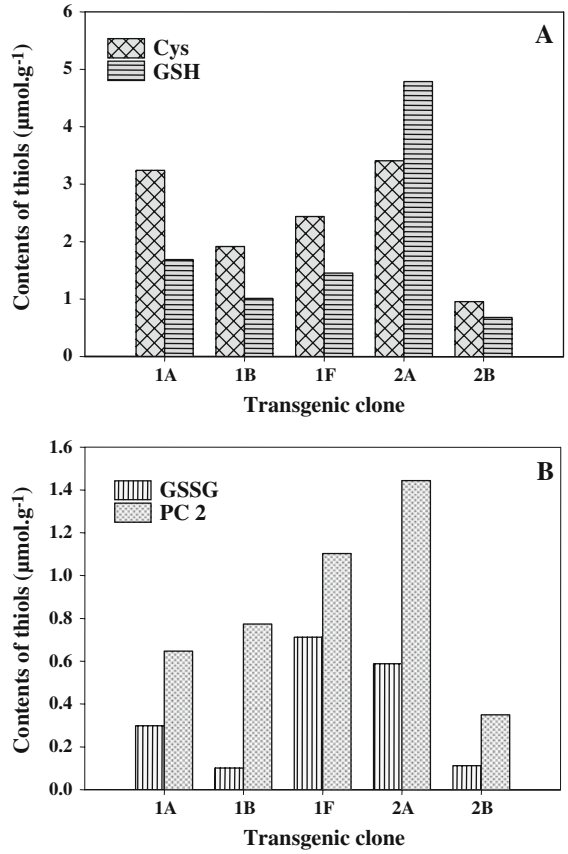

Fig. 4 Content of low molecular mass plant thiols, (**a**) Cys and GSH; (**b**) GSSG and PC2, in transgenic tobacco clones 1A, 1B, 1F, 2A and 2B determined using HPLC-ED

phytochelatin 2 as well as reduced glutathione and free cysteine (Fig. 4). The results obtained clearly demonstrate the biosynthesis of particular thiols is mutually linked, which contribute to a better protection of a transgenic plant against heavy metals effects.

Conclusion

Heavy metal pollution is of great concern due to its prominence throughout many parts of the world. Utilizing plants as a mode for decontaminating heavy metal infested sites would be of great and further interest Since phytoremidetation requires selected plants have a high-level of tolerance as well as the capacity to withstand large assimilation of heavy metals, obtaining plants that fulfil all requirements for successful remediation of polluted areas using

classical procedures such as plant selection is time consuming (Salt et al. 1995, 1998). A possible alternative solution would be to genetically engineer the plants so they have the necessary features for phytoeremedation in a short period of time. (Kotrba et al. 1999; Pavlikova et al. 2004a, b). Since MTs are very effective molecules in heavy metal detoxification one potential modification is to insert a MT transgene into a plant. It is believed that plants with the highest MTs production will be best suited for phytoremediation. In spite of the fact that plants contain an identical transgene, they may not show identical phytoremediation abilities because of diverse point of insert integration, number of integration events and somaclonal selection. Therefore, in addition to characterizing the detection of the MT gene, determining MT content is also necessary. In the present paper the content of various thiols involved in plant heavy metal detoxification processes were determined in transgenic plants carrying the gene for MT synthesis. Plants with the highest content of MT also have the highest content of other naturally occurring heavy metals induced thiols.

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