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Investigation of the Antioxidant Properties of Metallothionein in Transgenic Tobacco Plants using Voltammetry at a Carbon Paste Electrode

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Electrochemical detection of DNA damage *in vitro* has been used to evaluate the antioxidant properties of transgenic tobacco plant extracts containing metallothionein (MT). This method is based on assessment of efficiency with which the studied plant extracts are able to reduce to the amount of damage of standard chicken DNA by free OH radicals prepared by the Fenton reaction. We have tested the MT isolated from rabbit liver as a standard reference solution and compared its activity with extracts from wild type tobacco plants, and from twelve clones of transgenic tobacco plants variously carrying human or yeast MT. The present experimental results clearly demonstrate that insertion of a MT gene into the DNA of tobacco plants significantly improves their antioxidant properties in comparison with the non transgenic plants. The method developed for the purpose of this study has been demonstrated to be suitable for rapid screening of various plants with respect to their potential for phytoremediation of heavy metals.

Keywords: Square wave voltammetry, carbon paste electrode, DNA, metallothionein, oxidation signal of DNA

1. INTRODUCTION

1.1. DNA electrochemistry

It has been known for more than four decades that DNA is electrochemically and surface active and exhibits distinct behaviour at different electrodes [1-5]. Depending on the electrode material and other conditions DNA can undergo charge transfer (i.e., reduction and oxidation) and adsorption/desorption processes. DNA bases adenine, cytosine, and guanine yield redox signals at mercury (ME) and silver solid amalgam (AgSAE) electrodes. All purine and pyrimidine bases are detectable at carbon and carbon paste electrodes [6,7]. Among the electrochemical detectors, carbon electrodes demonstrate several unique properties. The extensive potential window in the positive direction allows sensitive electrochemical detection of the oxidative damage caused to DNA by monitoring the appearance of the oxidation peaks of the DNA bases. Additionally, the electrochemical oxidation signal of the components of DNA, such as, nucleotides, nucleosides, purine and pyrimidine bases, can be employed as biological recognition elements for the determination of a more specific interaction. Different DNA-electrochemical biosensors can be prepared from the known selected sequences of the DNA components, in the form of homopolynucleotides or heteropolynucleotides. Thus, multilayer dsDNA-electrochemical biosensors can be a powerful tool in elucidating the nature of DNA–metal ion binding and detecting the conformational changes or oxidative damage resulting from these interactions [8]. Adsorptive transfer stripping technique (AdTS) coupled with square wave voltammetry was employed for DNA measurements avoiding interferences [9]. Such method of analysis of DNA interactions with other molecules appears to be suitable for the rapid examination of molecule–DNA interactions [3,10,11].

1.2. Detecting DNA damage with carbon paste electrode. Fenton reaction

Hydroxyl radical ($OH^{\bullet}$), is one of the most aggressive radicals which can damage the DNA molecules. $OH^{\bullet}$ can attack every part of DNA (deoxyribose, purine and pyrimidine bases) and destroy their structure. But what is worse, this process leads to formation of new free radicals that propagate further damage [12]. Scavenging of the free radicals by antioxidants can stop such chain reaction. Hydroxyl radicals are most often created in living organisms by hydrogen peroxide reaction with metals (so called Fenton reaction) [13,14]. This reaction based on original work by H.J.H. Fenton at the end of XIX century [15], was in detail described by Haber and Weiss [16] and can be easily realized *in vitro* by mixing hydrogen peroxide and bivalent iron in acidic medium. The Fe^{3+} ions produced in this solution further react with H_2O_2 and OH radical, regenerating Fe^{2+} (scheme 1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\text{-} + OH^{\cdot} \quad \textbf{(1)}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H^{+} + OOH^{\cdot} \;//\; Fe^{3+} + OOH\boldsymbol{.} \rightarrow Fe^{2+} + O_2 + H+ \quad \textbf{(2)}$$

Scheme 1. Fenton‘s reaction

Whilst other metals including Cu(II) or Cr(II) can be used in similar reaction schemes, it is most common to use Fe(II) to create OH radicals in biochemical experiments. This is because Fe is widely present in living organisms [13]. The Fenton reaction system is very important mechanism relevant to many scientific investigations of mediators of oxidative stress. It is hypothesized in some studies that the Fenton reaction can be involved in termination of cancer cells mutations [14]. Other use of Fenton reaction is in assessing of antioxidant activity of different molecules [17]. Such experiments are based on inducing damage to immobilised standard DNA by the $OH^{\bullet}$ radicals and measuring the protective effects (if any) of added sample solutions. Voltammetry on carbon paste electrode can be used to detect DNA and also to analyze the degree of its destruction [18-26]. Mello at al. (2006) successfully used the Fenton reaction with biosensor consisted of plants DNA immobilised on carbon screen-printed electrode [27]. We recently applied voltammetry at a carbon paste electrode in investigation of free oxygen radicals influence on signals of DNA protected by some flavonoids [28].

1.3. Metallothionein as a free radical scavenger

As we mentioned above, the reactive oxygen species (ROS) cause cell injury when they are generated in excess or the cellular antioxidant defence is impaired. In the cytosol hydrophilic scavengers such as glutathione are active in sequestering O_2• species among ROS while hydroxyl radicals HO• are preferably scavenged by metallothioneins [29,30]. Metallothioneins has been also implicated in the scavenging of heavy metals, including cadmium, mercury, zinc and copper by forming trimercaptide linkages [31,32].

Metallothioneins (MTs, Fig. 1) are sulphur and metal rich low-molecular weight proteins, which are found in most animal tissues and can be induced by a number of inducers including various oxidative stresses [31,33-36]. It has been proved many times, that metallothionein synthesis is increased by oxidative stress [37,38]. High metallothioneins antioxidant activity has been described for peroxide oxidation of membranes lipids and DNA [39]. The specific mechanism of metallothionein antioxidant activity is not clear yet. It was discovered by Thornalley and Vasak in 1985 [29], that cysteines in metallothionein can catch fast hydroxyl radicals on SH-groups and terminate the oxidation damage of other important biomolecules (DNA, proteins). Chelation of Fe^{2+} is another possibility to stop oxidation. In that case Fe^{2+} is changed to Fe^{3+} non active form (direct termination of the Fenton reaction) [40]. Metallothionein-iron complex has been created *in vitro* [41]. MT can also reverse oxidation processes. It has been proved that MT can donate hydrogen atom to the place damaged by free radicals and so get back DNA to the normal structure [42]. MT can also participate as a co-factor of activation of other antioxidant mechanisms for example synthesis of glutathione peroxidase [43,44]. It is still unclear, which function of MT is dominant for oxidative stress termination, the rates are different for various organisms. What is sure - the role of MT is indispensable for oxidative stress and investigation of it is necessary [33]. Methods for study of this protein has been recently reviewed by Adam et al. [34], from which electrochemistry has many advantages compared to other ones [45-57].

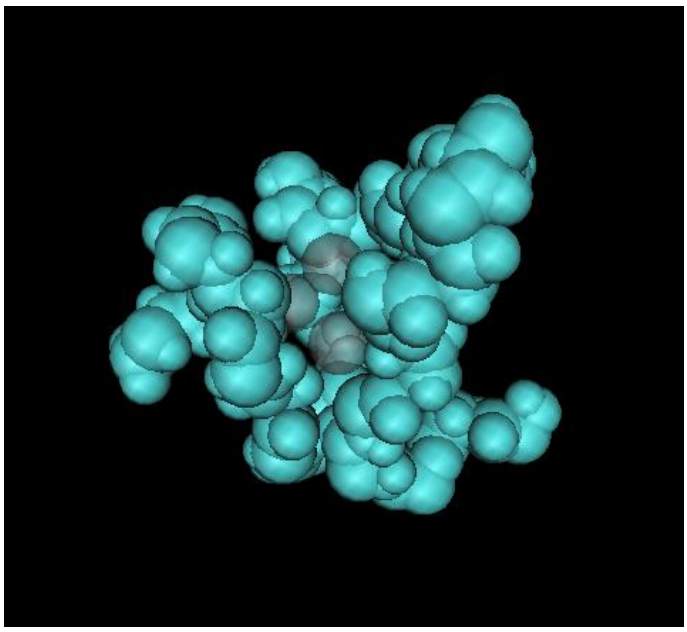

Figure 1. Three-dimensional structure of rabbit liver Cd-7 metallothionein-2a in aqueous solution determined by nuclear magnetic resonance (PDB ID: 2MRB) [58]. The structure is shown with complete protein backbone and ball and stick style. Figure prepared using the program Cn3D of the National Centre for Biotechnology Information.

1.4. Metallothionein in plants

The introduction of animal MT genes significantly enhances the metal accumulation capacity of resulting transgenic plants and therefore their phytoremediation potential [59-62]. Those abilities are important for application in phytoremediation. The gene encoding human and yeast MT production was introduced into tobacco plant genome aimed to test the accumulative potential for heavy metals [63]. The genetically modified tobacco clones derived from *Nicotiana tabacum* L., var. Wisconsin 38 were used. Clones from the first line carried polyhistidine gene in fusion with yeast metallothionein (gene CUP 1). Clones from the second line contained transgene for a chain of the human metallothionein. The hyperaccumulation of heavy metal ions and the presence of the MT were demonstrated in these transgenic plants [62-64]. The presence of MT in plant material could be verified by PCR [63] or by differential pulse voltammetry Brdicka reaction [64].

The process of inserting MT gene into the plant is already quite well mastered methodologically. However fast and precise methods for the assessment phytoremediation abilities of the resulting transgenic plants are still missing. As we will show in this article, electrochemical methods, namely square wave voltammetry, might be very useful for this purpose. The objective of the present study is to quantify antioxidant function of the extracts from transgenic tobacco plants expressing metallothioneins. In order to validate the newly developed method described below in Section 3 (based on voltammetric detection of damaged DNA) we have studied the ability of a reference MT standard (isolated from rabbit kidneys) to prevent the DNA destruction by the free hydroxyl radicals, OH, created by the Fenton reaction. Then we used the same method to assess the antioxidant properties of extract from ten available transgenic tobacco clones [63]. The ultimate

objective is to demonstrate that suggested electrochemical analysis can serve as a quick test revealing potential of different transgenic plants to be used for phytoremediation of heavy metals.

2. MATERIAL AND METHODS

2.1. Chemicals

Working standard solutions were prepared daily by diluting the stock solutions. Lyophilised polymerized DNA (Reanal, Hungary) was isolated from chicken erythrocytes (MW = 400 000 g/mol). Rabbit liver metallothionein MT (MW 7143 g/mol), containing 5.9 % Cd and 0.5 % Zn, was purchased from Sigma Aldrich (St. Louis, USA). All other chemicals used were purchased from Sigma Aldrich unless noted otherwise.

2.2. Preparation of deionised water and pH measurement

The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.3 Preparation of carbon paste electrode

The carbon paste mixture was prepared by hand mixing (about 0.5 g) of 70 % carbon powder (OD 30-50 μm) and 30 % mineral oil (Sigma-Aldrich; free of DNase, RNase, and protease). This paste was packed into a teflon body having a 2.5 mm diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing with a soft filter paper [9,65,66].

2.4. Fenton´s reaction with DNA

Fenton's reaction was carried out in order to study the effects of DNA damaged by oxidation [17]. DNA (100 μg per test tube) was exposed to $FeCl_2$ and H_2O_2 mixed in a stoichiometric ratio of 1:1. Applied concentrations of these reagents were 1, 2 or 4 mM. To investigate the influence of metallothionein or plant thiols on this reaction, metallothionein or plant supernatant samples were added to the test tubes. The reactions were carried out in the presence of 10 mM NaCl and 10 mM phosphate buffer (10 mM NaH_2PO_4 + 10 mM Na_2HPO_4 in ratio 2:8 (*v*/*v*), pH 7.4) at 37 °C for 15 or 30 min and stopped by adding 100 μl of 100 mM EDTA solution. Reaction solutions were then measured by square wave voltammetry with CH Instruments. The DNA oxidation signal was detected (see "Results and Discussion").

2.5. Electrochemical measurements

Electrochemical measurements were performed with CH Instruments Electrochemical Workstation (USA) using a three electrodes cell system. A carbon paste working electrode, a Ag/AgCl/3M KCl reference electrode and a platinum wire counter electrode were used. Phosphate buffer (0.1 M NaH_2PO_4 + 0.1 M Na_2HPO_4 in ratio 4:6 (*v*/*v*), pH 6.98) and/or acetate buffer (0.2 M CH_3COOH + 0.2 M CH_3COONa, pH 6.5 were the supporting electrolytes (for optimization see "Results and Discussion"). Square wave voltammetric parameters were as follows: initial potential 0.0 V, end potential 1.2 V, pulse amplitude 49.85 mV and step potential 1.4 mV. The frequency was optimized. For other details see "Results and Discussion". The instrument was controlled by programme CHI, Version 7.12 (Auxtin USA). For smoothing and baseline correction the GPES 4.9 software supplied by EcoChemie was employed. All experiments were carried out at room temperature.

2.6. Transgenic plants

The genetically modified tobacco clones derived from *Nicotiana tabacum* L., var. Wisconsin 38 were used. Clones from the first line carried polyhistidine gene in fusion connection with yeast metallothionein (gene CUP 1). Clones from the second line carried transgene for a chain of the human metallothionein. Details of preparation of transgenic plants see in Macek et al. 2002 [63].

2.7. 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 by twice repeated 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).

3. RESULTS AND DISCUSSION

3.1. Electrochemical response of DNA molecule and nucleic bases

Some parameters (voltammetry type, potentiostat, available standard of DNA, pH of electrolyte and other) are very important for DNA detection by carbon paste electrode. Because of that, published results of DNA detection from different authors are often different [6,8,67]. We made our pilot optimization of experimental condition for DNA detection on carbon paste electrode. Square wave

voltammograms of standard chicken DNA and nucleic bases standards adenine (A), thymine (T), cytosine (C) and guanine (G) we used to locate potentials of oxidation signals, are shown in Fig. 2.

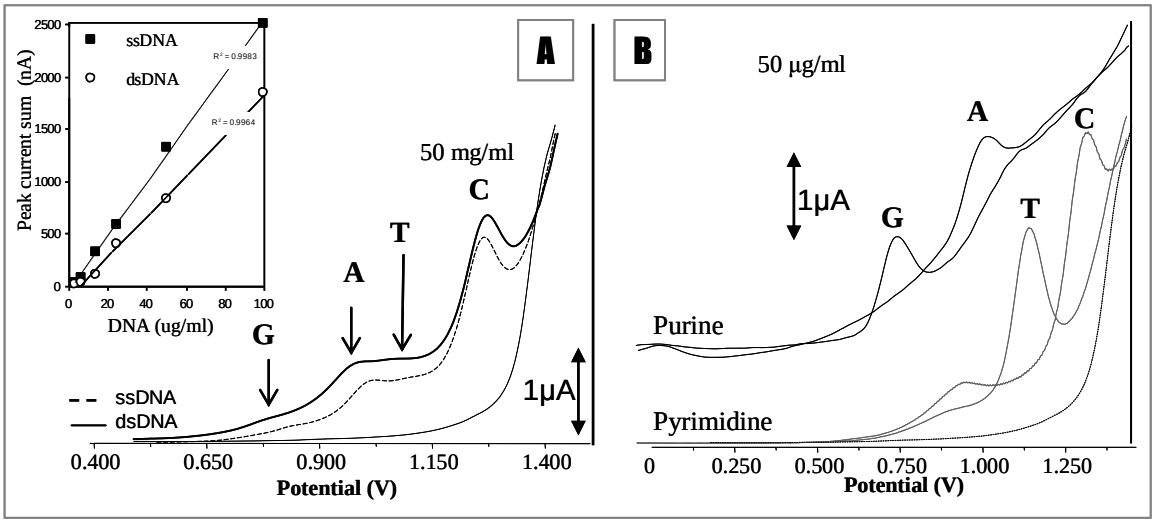

Figure 2. Square wave voltammograms of standard chicken DNA (A) and nucleic bases adenine, thymine, cytosine and guanine (B) in 0.1M phosphate buffer (0.1 mM NaH_2PO_4 + Na_2HPO_4) pH 6.00. Initial potential 0 V, end potential 1.4V, frequency 100 Hz, potential step 1.05mV.

It is clear from Fig. 2B that nucleic bases adenine, thymine, cytosine and guanine are oxidized on carbon paste electrode in phosphate buffer pH 6.00 at potentials: C 1.324 V; T 1.236 V; A 1.044 V; G 0.764 V. For each nucleic base standard, linearity between concentration and SWV signal was verified. The similar oxidation potentials were detected for mixture of standard nucleic bases and for standard chicken DNA molecule (Fig 2.A). The important difference in electrochemical detection between DNA molecule and mixture of nucleic bases was observed. For example cytosine had 10 times lower oxidation signal as a part of DNA molecule then as free nucleic bases Fig.2. Also, guanine gave the lowest electrochemical response when it was bound in DNA. It was evident, that upon the denaturation DNA, double stranded DNA (dsDNA) macromolecules denaturation partly into to single stranded DNA (ssDNA) by the rupture of hydrogen bonds, so free guanine and adenine are formed. Then the bases oxidation could be easily seen by cyclic voltammetry [68]. Also in our measurement on carbon paste electrode, higher electrochemical response for every nucleic base was detected for DNA denatured 30 minutes at 99^0 C (Fig. 2). Therefore for our study of MT antioxidant activity, the use of ssDNA is optimal.

3.2. The influence of pH

pH of electrolyte is very important factor influencing electrochemical detection of DNA [69]. The pH dependence of the square wave peak potential and current of DNA and nucleic bases was studied over a wide pH range between 5 and 8 using by carbon paste electrode (Fig. 3).

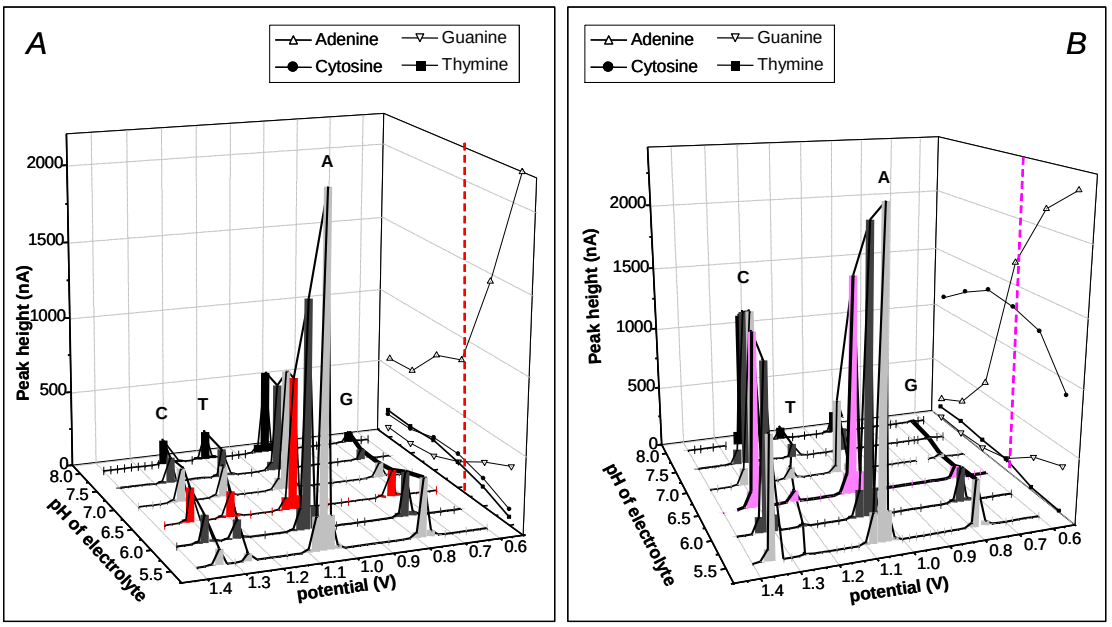

Figure 3. 3D plot of SW voltammograms obtained in DNA and nucleic bases mixture as function of pH in 0.1 M phosphate buffer. A – nucleic bases mixture in concentration 25 μg/ml; B – standard chicken ssDNA in concentration 25 μg/ml.

We were looking for a pH of electrolyte which would be optimal for detection of DNA oxidation. How can be seen in Fig. 3, the optimum of peak current (oxidation peak) for purine and pyrimidine is different. In fact the lower pH increased peak current for adenine and guanine, but for thymine and cytosine the effect was opposite – with lower pH heights of peaks were the lowest. Also important influence pH of electrolyte was observed for oxidation peak potential (Fig. 3 Y axis). For the denatured DNA it was found that at high pH thymine and cytosine became closer, so that makes difficult to detect all DNA bases. Then, the good compromise between peak separation and peak current was obtained at pH 6.5. The limit of detection in optimised conditions where we could see all of four bases DNA was 1.5 μg/ml.

3.3. Detecting DNA damage with carbon paste electrode. Fenton reaction

After the optimization of experimental conditions, the oxidative signals of nucleic acids were measured at the surface of a carbon paste electrode by square wave voltammetry. SW voltammograms of 50 μg/ml ssDNA measured in the presence of phosphate buffer 0.1 M pH 6.5 are shown in Fig. 4A,B. Oxidative signals of guanine, thymine, adenine and cytosine can be easily seen in voltammograms. Then the influence of free oxygen radicals on DNA molecule signals was examined. The oxygen radicals were generated by Fenton reaction (1mM H_2O_2 and 0.5 mM $FeCl_2$ in the presence of 50 μg/ml DNA for 15 min at 37 ◦C). The non-oxidized ssDNA (50 μg/ml) gave well developed signals but after oxidative damaging, observed oxidative signals decreased. The signals of guanine,

adenine and thymine were significantly affected by oxidation Fig. 4 A,B. As it is documented on Fig. 4, most influenced by free radical damaging was signal of guanine (Fig. 4B).

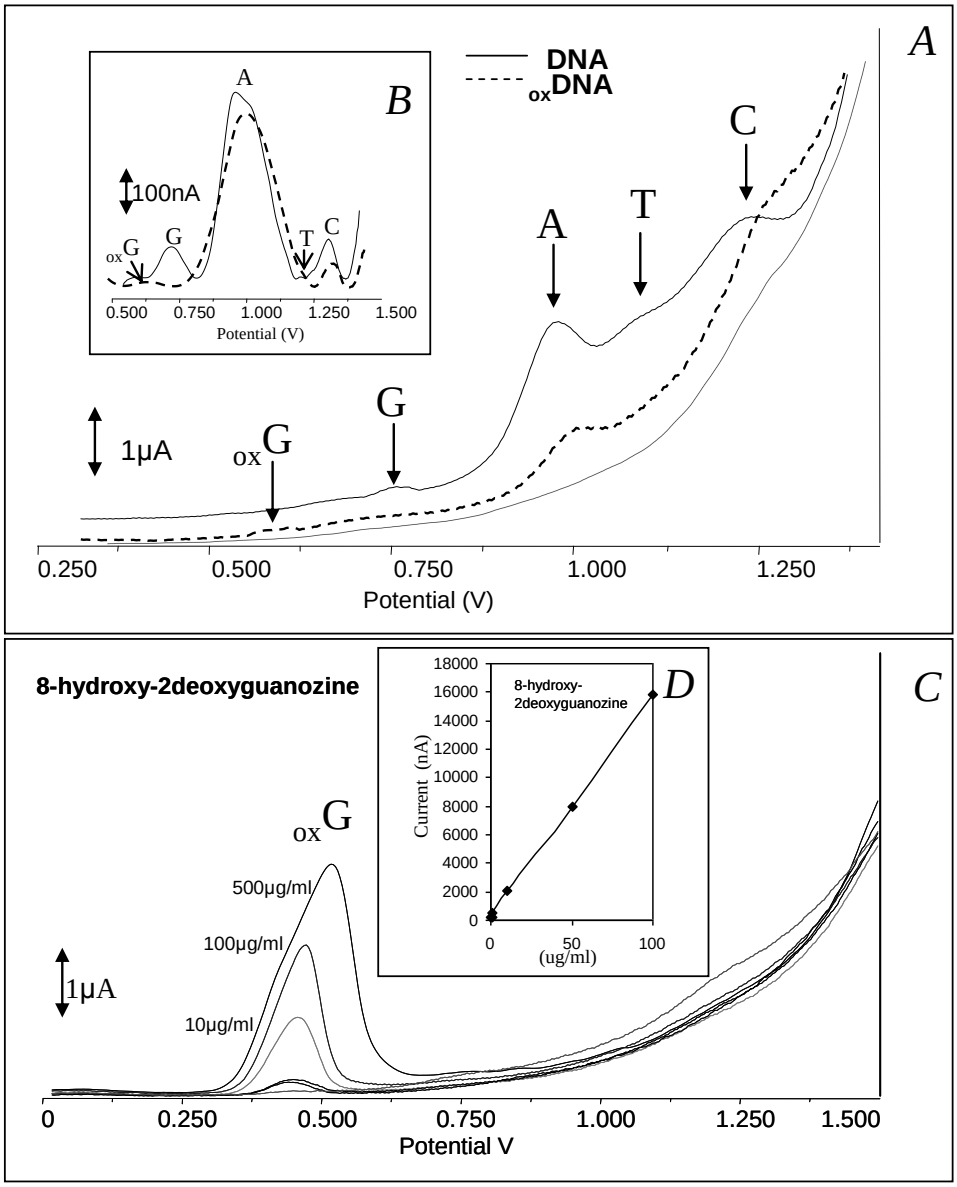

Figure 4. SWV response of DNA bases in phosphate buffer electrolyte (pH 6.5) before and after damaging by Fenton reaction free radicals. A – real SW voltammograms of ssDNA; B – base line corrected (GPES 2.0.) SWV of oxidized and non oxidized ssDNA; C - SW voltammograms of 8-hydroxy-2deoxyguanosine standard solution; D – concentration dependence of SWV peak current for the 8-hydroxy-2deoxyguanine.

Moreover, at the foot of guanine peak, new oxidation peak at 0.530 V was observed, which can be attributed to 8-oxoguanine (8oxG) [70], a product of guanine chemical oxidation. To confirm this we measured standard 8-hydroxy-2deoxyguanosin. Figs. 4 C, D demonstrate SW voltammogram and concentration dependence of 8-hydroxy-2-deoxyguanosine (from 0.25 ug/ml to 1 mg/ml). It is important to note, SW oxidation potential of oxG depends on its concentration. Guanine oxidized form

is easily detectable on electrochemical voltammograms. That is why oxG is often considered as a biomarker of DNA molecule damage [2]. However, generation of oxG peak on voltammogram informs about the damage of just one part of nucleic acid, but every part of DNA is attacked by free radicals. For observing the change of DNA molecule in our experiments, we therefore followed signals of all four nucleic bases (G, C, T, A).

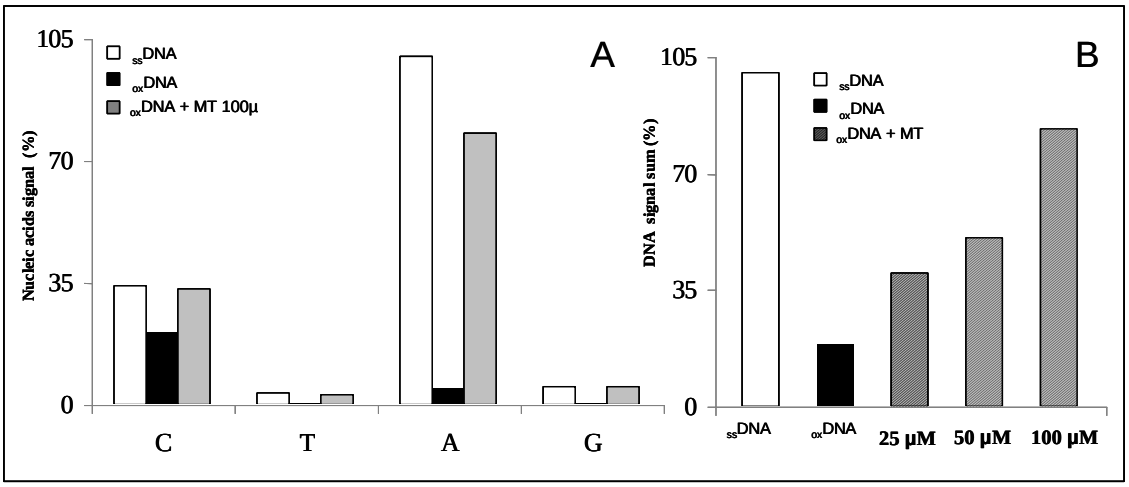

Figure 5. Effect of the antioxidant properties of standard reference metallothionein. A - Column height expressed as all oxidative signal of each nucleic base adenine (A), thymine (T), cytosine (C) and guanine (G). B - Column height expressed as the total sum of all oxidative signals of DNA bases. The highest signal for non damaged ssDNA represents 100 %, concentration of DNA is 50 µg/ml.

3.4. Antioxidant activity of metallothionein

At first we studied the ability of a reference MT standard to prevent the DNA destruction by the free hydroxyl radicals, $OH^{\bullet}$, created by the Fenton reaction. The influence of free oxygen radicals on DNA molecule signals protected and not protected by metallothionein is shown in Fig. 5A. After the oxidative damaging by Fenton reagents (black columns), we observed all nucleic acids oxidative signals decreased in comparison with non-oxidized ssDNA (white columns). But when the 100 µM MT was added to ssDNA exactly before Fenton solution, we observed significant protective effects (gray columns). Influence of different concentration of MT 25, 50 and 100 µM were also tested. The sum of the peak heights for G, T, A, C represents the protective effect of the MT for whole DNA molecule (Fig. 5B). It is shown that MT has high protective effect on DNA free radicals, and developed electrochemical method can be used to observe level of oxidation activity exposed to transgenic plant extracts.

3.5. Antioxidant activity of transgenic tobacco plants extracts

It is clear from the previous experiment, that metallothionein can scavenge oxygen free radicals and prevent the DNA damage. Then we expected enhanced antioxidant activity in transgenic plants

carrying MT gene. We have used this method to quantify antioxidant function of the extracts from 10 clones of transgenic tobacco plants expressing MT, in comparison with nontransgenic tobacco plants (WSC38). In this experiment ssDNA (50µg/ml) and ssDNA oxidized by Fenton solution were used as a standard substance to compare the efficiency of radical scavenging of the plants. The total sum of peak currents of all bases of non-oxidized DNA was considered as 100 %. As it can be seen in Fig. 6, the experiment was verified in the conditions with high damage to DNA (black column), and without its complete damage. When a Fenton reagent is added to the reaction, the DNA signal was 19-23 %. For comparison the column showing the influence of 50 µM standard MT and also column of extract from non-transgenic plant (WSC38) are included. In latter case, only the naturally existing plant thiols are responsible for slight antioxidant activity.

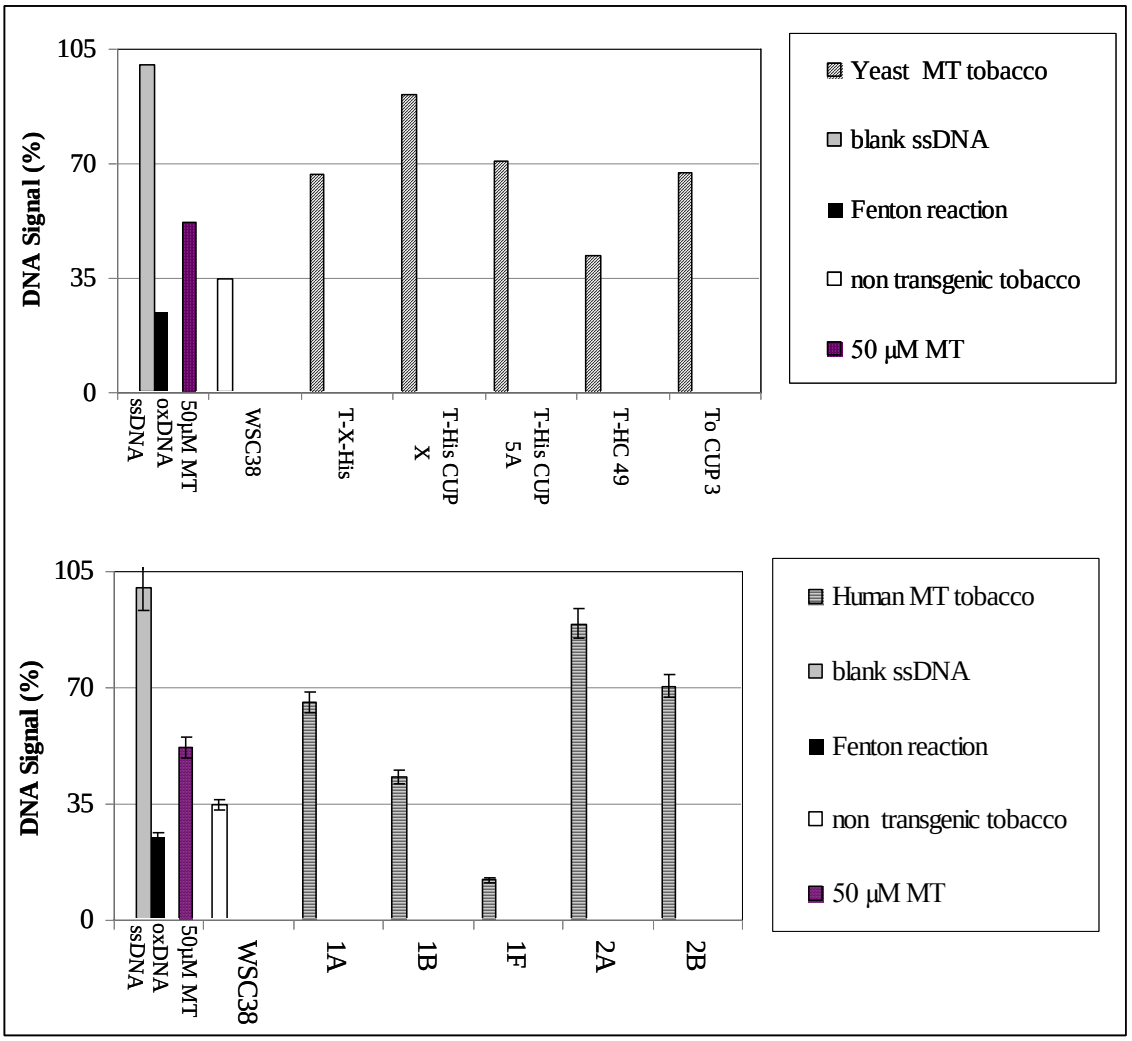

Figure 6. Effect of the antioxidant presence on the signal of ssDNA. Gray column – blank ssDNA; black column – DNA damaged by Fenton reagents; white column - *Nicotiana tabacum* L., var. Wisconsin 38 – nontransgenic plants. Upper part – the genetically modified tobacco clones carried polyhistidine gene in fusion connection with yeast metallothionein (gene CUP 1); lower part - the genetically modified tobacco clones carried transgene for a chain of the human metallothionein.

Capability of protection of DNA damage by transgenic plant extracts is shown in further columns in Fig.6. It follows from the obtained results that the most of clones is able to effectively inhibit oxidation. All clones demonstrated good ability to inhibit DNA oxidation excepting F1, clone with inserted gene for human metallothionein, which demonstrated very low antioxidant activity. Also clones THC-49 (with yeast MT) and 1B (human MT) were practically comparable with control of non-transgenic sample. This result also correlated with MT content in these plants analyzed and published previously [64], where was observed in clones 1F, 1A and 2B low concentration of MT in leaves. The highest antioxidant capacity was recorded in clone 2 A with human metallothionein gene, TX-His, T-His CUP X with yeast MT gene. After comparison of plants with human and yeast MT, we can say that the plants with yeast metallothionein demonstrate better ability to resist to oxidative stress; with this fact is probably connected also better ability to bind heavy metals ions. Comparison implies better function of yeast metallothionein for antioxidant protection and probably also for capturing of metal ions and thereby usage in phytoremediation technologies.

4. CONCLUSIONS

One of the potential approaches to increase ability of heavy metal accumulation in plants is introduction of transgene for MTs. These proteins are very effective in the processes of heavy metals ions binding. It is possible that the plants with high MTs production will be also the best for phytoremediation purposes. With the view of carrying out of time consuming experiments with all new plants, it is possible to use in this work presented method for unpretentious evaluation of plant clone in metallothionein production and for selection for the next experiments. Method which has been developed was used for measurement of the antioxidant activity of metallothionein and transgenic plants for the first time. We have described a simple, fast electrochemical approach for detection DNA damage and studying the ability MT scavenge oxygen free radicals and prevent the DNA damage. Using oxidative damage of DNA, followed by square wave voltammetry, we tested ten transgenic plants and we were able to discriminate between nontransgenic plants and transgenic plants with low or high antioxidant activity. Such electrochemical analysis can serve as a quick test revealing potential of different transgenic plants. This method can be useful method for assessment phytoremediation abilities of plants.

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