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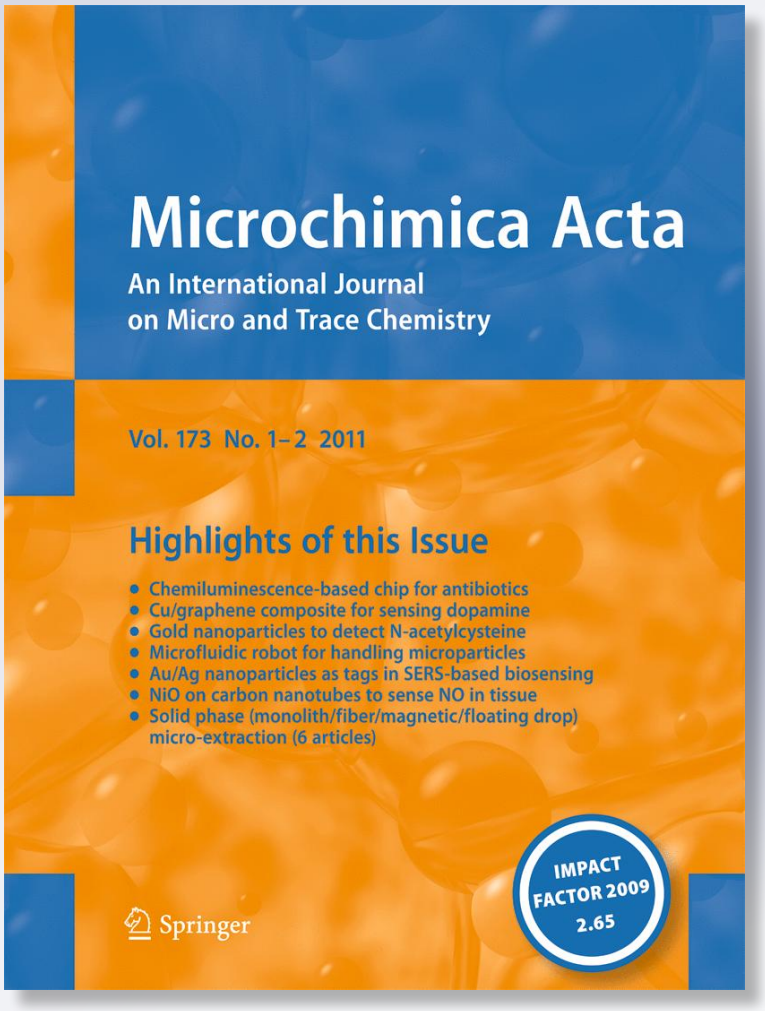

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ORIGINAL PAPER

Microfluidic robotic device coupled with electrochemical sensor field for handling of paramagnetic micro-particles as a tool for determination of plant mRNA

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Abstract The expression of genes responsible for the biosynthesis of stress proteins corresponds to the exposition of an organism to abiotic and/or biotic stress. We utilize two types of paramagnetic particles for isolation of total mRNA from early somatic embryos of Norway Spruce (*Picea abies* /L./ Karst.) and maize plants (*Zea mays* L.) treated with cadmium(II) ions. The paramagnetic particles were evaluated for analysis of real samples, and poly-adenine was used as a model mRNA. Various approaches (from non-automatic to fully automatic) were tested in terms of handling the particles.

Keywords Electrochemical detection · Microfluidics · Environmental stress · Cadmium ions · Paramagnetic particles

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Introduction

An anthropogenic activity directly or indirectly influences not only organism itself, but also the whole populations and/or communities. A technological development contributed to improving of standard living also brought a plenty of negative effects on ecosystems [1]. One of these effects is pollution of air, soil, and water by various types of wastes and undesirable substances such as heavy metals and their compounds. Heavy metals (ρ>5 g.cm^{-3}) are one of the most toxic and undesirable compounds polluting agricultural products [2, 3]. They are also natural components of the Earth's crust and cannot be degraded or destroyed. Heavy metals are dangerous because they tend to bioaccumulate. The soil with high heavy metals content poses a serious threat to plants. Toxic effects of heavy metal ions on plants are related to damaging of photosynthetic system, inhibiting activity of key enzymes for plant metabolism and others [4]. Increasing heavy metal ions concentration in plant tissues results in activation of protective mechanisms, mainly in synthesis of cysteine-rich peptides as glutathione [5, 6] or phytochelatins [7–10] and/or metallothionein like proteins [11–13]. Moreover, plants demonstrating higher tolerance to heavy metals can be employed in phytoremediation technologies [10, 14–16].

All these stress induced processes are closely associated with changes in homeostasis causing subsequent synthesis of signalling compounds e.g. salicylic acid, jasmonic acid, methyljasmonate and others [17–22]. A signalling pathway triggers specific cascades leading to activation of transcription factors. An activated transcription factor is bounded into promoter of a stress gene to start transcription. Transcribed mRNA molecules are transported from nucleus to cytoplasm, where a stress protein stimulating the resistance against stress factor is translated. To investigate the stress cell response, mRNA and/or translated protein detection is needed [23]. The most commonly used method for detection of mRNA is polymerase chain reaction (PCR) [24]. However, prior to detection of mRNA by PCR, an isolation of sufficient amount of mRNA without contamination by other nucleic acids is unavoidable [25–33]. The main aim of this study was to utilize paramagnetic particles for total mRNA isolation and quantification in early somatic embryos of Norway Spruce (*Picea abies* /L./ Karst.) and maize plants (*Zea mays* L.) treated with cadmium(II) ions.

Experimental

Chemicals, material and pH measurements

Cultivation media were prepared using plant cell culture tested chemicals purchased from Duchefa Biochemie BV (The Netherlands, www.duchefa.com). All chemicals of ACS purity used and parafilm were purchased from Sigma Aldrich Chemical Corp. (Sigma-Aldrich, USA, www.sigmaaldrich.com) unless noted otherwise. Synthetic polyadenylic acid (poly(A), Sigma-Aldrich) was used. Stock standard solution of poly(A) (100 $\mu g.mL^{-1}$) was prepared from lyophilized poly(A) (0.5 $mg.mL^{-1}$, Mr=400000) with water of ACS purity and stored in dark at −20 °C. The concentration of poly(A) was determined spectrophotometrically at 260 nm using spectrometer Spectronic Unicam (England, www.spectronic.co.uk). Deionised water underwent demineralization by reverse osmosis using the instrument Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic, www.aquaosmotic.cz) and subsequently purified using Millipore RG (18 MΩ, Millipore Corp., USA, www.millipore.com) called MiliQ water. The pH value was measured using inoLab Level 3 (Wissenschaftlich-Technische Werkstatten GmbH; Weilheim, Germany, www.wtw.com).

Stationary electrochemical measurements

Electrochemical measurements were performed with AUTOLAB PGS30 Analyzer (EcoChemie, Netherlands, www.ecochemie.nl) connected to VA-Stand 663 (Metrohm, Switzerland, www.metrohm.com), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was employed as the working electrode. An Ag/AgCl/3 M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. For smoothing and baseline correction, the software GPES 4.9 supplied by EcoChemie was employed. Square wave voltammetric (SWV) measurements were carried out in the presence of acetate buffer pH 4.6 (35 °C). SWV parameters: potential step 5 mV, frequency 280 Hz, accumulation time 200 s [34]. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 120 s. The temperature of supporting electrolyte was maintained by the flow electrochemical cell coupled with thermostat JULABO F12/ED (Julabo Labortechnik GmbH, Germany, www.julabo.de).

mRNA isolation

Isolation of Poly(A) and mRNA was carried out using paramagnetic particles Dynabeads Oligo (dT)25 (Invitrogen, USA, www.invitrogen.com) or MAGNa (Roche, France, www.roche.fr) and magnetic stand Dynal Magnetic Particle Concentrator-S supplied by Dynal A.S (Norway, www.dynal.no). All experiments with paramagnetic particles were performed in RNA/DNA UV cleaner box UVT-S-AR (Biosan, Latvia, www.biosan.lv). For centrifuging and vortexing of a sample, multi-spin MSC-3000 centrifuge (Biosan) placed in UV cleaner box was used. Denaturation was carried out at 85 °C using the Thermomixer 5355 Comfort/Compact (Eppendorf, Germany, www.eppendorf.com).

The buffers used in our experiments were as follows—a) phosphate buffer I: 0.1 M NaCl+0.05 M Na_2HPO_4+0.05 M NaH_2PO_4; b) phosphate buffer II: 0.2 M NaCl+0.1 M Na_2HPO_4+0.1 M NaH_2PO_4; c) acetate buffer: 0.2 M CH_3COOH+0.2 M CH_3COONa.

Hybridization of nucleic acids

The hybridisation process was optimised previously [34]. Briefly, the optimal composition of hybridization solution was as follows: 100 mM Na_2HPO_4+100 mM NaH_2PO_4, 0.5 M NaCl, 0.6 M guanidinium thiocyanate, 0.15 M Trizma base adjusted by HCl on pH of 7.5. Time of hybridization was 40 min. [34].

Plants cultivation

In vitro cultured maize plants Maize (*Zea mays* L.) F1 hybrid Gila was used in our experiments. After 7 days long germination, maize seedlings were placed into vessels containing Murashige-Skooge medium [35] with (0, 5, 10, 25, 50 and 100 μM) or without addition of $CdCl_2$ and

cultivated in Versatile Environmental Test Chamber (MLR-350 H, Sanyo, Japan, www.sanyo.com) for 5 days. Each time, four plants were harvested at certain time intervals (1, 2, 3, 4 and 5 days) during the experiment, and their roots were rinsed three times in distilled water and 0.5 M EDTA. In addition, each harvested plant was divided into shoots and roots. Fresh weight of the samples was measured immediately after the rinsing by using a Sartorius scale.

Plant embryos The culture of early somatic embryos (ESEs) of Norway spruce (*Picea abies* /L./ Karst.) clone 2/32 [36] was used in our experiments and cultivated according to [37–39]. The ESE clusters of clone 2/32 were cultivated on a modified LP medium with an addition of cadmium chelate (Cd-EDTA [40]) in concentrations of 50, 100, 250 and 500 μM.

Field experiment Maize (*Zea mays* L.) F1 hybrid Gila was used in our experiments. After 7 days long germination, maize seedlings were placed into vessels containing tap water and cultivated in Versatile Environmental Test Chamber (MLR-350 H, Sanyo) for 21 days. Thirty days old plants were placed into pots containing tap water with or without addition of 10 μM $CdCl_2$ in vegetational hall in July 2008. The plants were grown for 21 days. Each time, four plants were harvested at certain time intervals (7, 12, 17 and 21 days) during the experiment, and their roots were rinsed three times in distilled water and 0.5 M EDTA. In addition, each harvested plant was divided into shoots and roots.

Details for cultivation of the biological material can be found in Electronic Supplementary Material (ESM).

Detection of chlorophyll fluorescence

Detection of chlorophyll fluorescence was carried out on homemade instruments from Department of Microelectronics, Brno University of Technology, Czech Republic. The high sensitivity of the measurement is performed by the photon counter as detector, which is the main part of the system. Wavelength of light-emitting diodes (LED) was 560 nm. LEDs grouped around the photon counter window to circle perform the source of the light (50 mm^2). The LEDs are driven by microcontroller, which also counts the pulses from the photon counter. The microcontroller communicates with personal computer through USB. The software with the system does short light exposition followed by counting of pulses from the detector for several seconds. The absolute dark background has to be assured to obtain high sensitivity at level of a few photons.

Fully automated isolation of mRNA

Fully automated isolation was carried out on automated pipetting system epMotion 5075 (Eppendorf, Germany). The position of B4 is a magnetic separator (Promega). The positions of C1 and C4 can be thermostated (Epthermoadapter PCR96). The pipetting provides a robotic arm with adapters (TS50, TS300, TS1000) and Gripper (TG-T). The samples are placed in the position B3 in adapter Ep0.5/1.5/2 mL. Module Reservoir is located in the position B1, where washing solutions and waste are available. The device is controlled by the ep*Motion* control panel. The tips are located in the A4 (ePtips 50), A3 (ePtips 300) and A2 (ePtips 1000) positions. PCR 96 plates are used (Fig. 1).

Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA, www.microsoft.com). Results are expressed as mean ± standard deviation (S.D.) unless stated otherwise.

Results and discussion

mRNA isolation

For studying of gene expression in plants exposed to various stress factors, high quality and chemically unmodified nucleic acids isolation are necessary. Nowadays, the attention is mainly paid to isolation of highly pure nucleic acids. Not only qualitative isolation, but also quantitative determination of nucleic acid makes challenge for bioanalytical chemistry. Using the classic way isolation (phenol:chloroform), all types of RNAs are obtained. The obtained RNA must be converted using reverse transcription to cDNA prior to the consequent experiments. The other way of RNA preparation is addition of the appropriate RNA primers and looking for the target mRNA sequence. Isolation of total mRNA using the standard procedure is practically impossible. Therefore, microparticles allowing isolation of total mRNA or even a specific sequence bring new possibilities to monitor environmental stress or diagnosis of serious illness.

In this study, two methods of mRNA isolation from biological samples were compared. Plant tissues were homogenised according to procedure published by us, which was based on deep-freezing of the tissues by liquid nitrogen and pulverization of them in grinding mortar with addition of guanidinium thiocyanate as inhibitor of RNAse [41]. Sample prepared like this was divided into two equal parts; each part was used for mRNA isolation by different procedure. Process of mRNA micro-isolation (total volume did not exceed 30 μL). To processed sample, paramagnetic

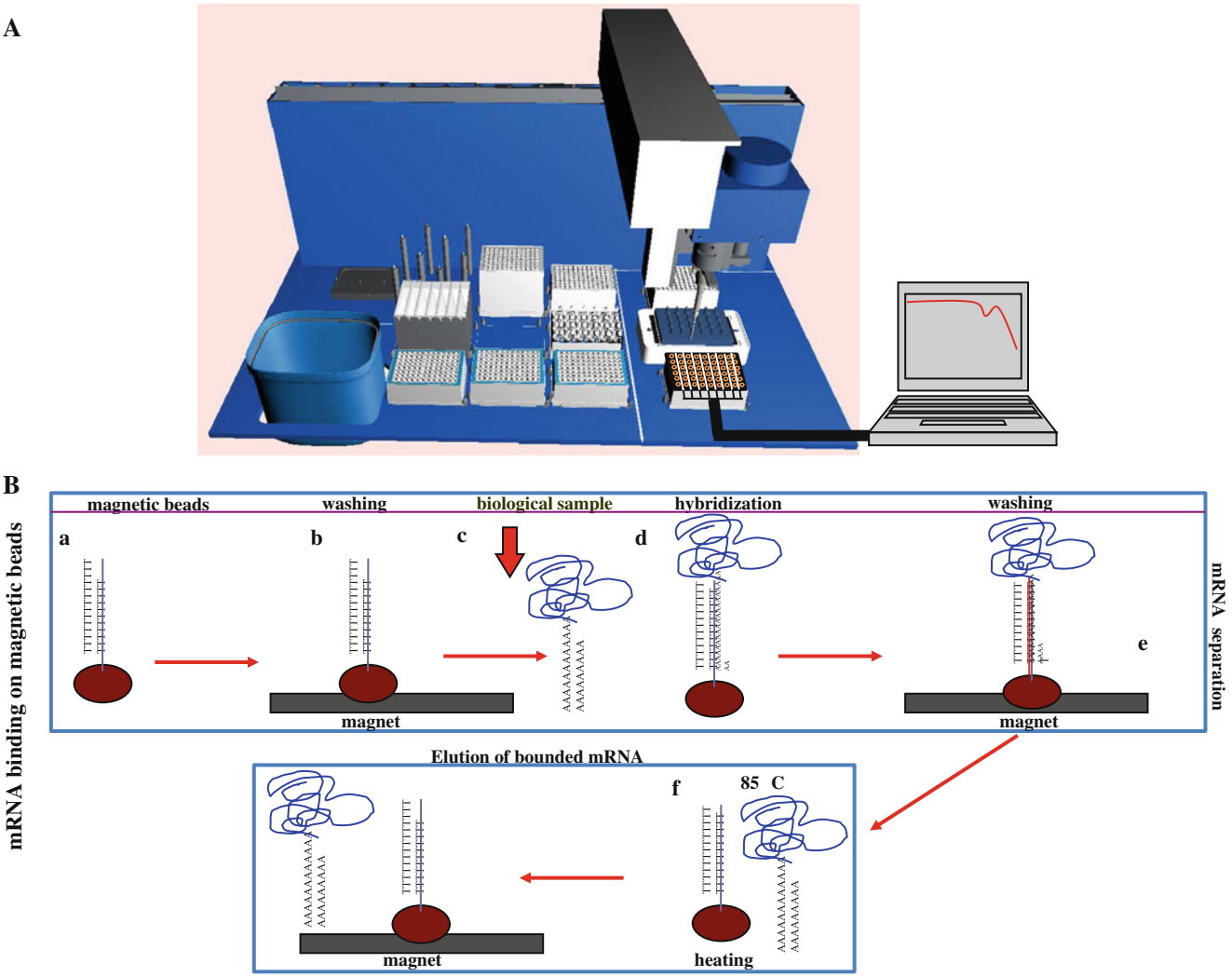

Fig. 1 Simplified scheme of mRNA isolation from plant sample. **a** Automated process of isolation of mRNA from a biological sample using paramagnetic particles. **b** mRNA isolation using paramagnetic microparticles with bound dT25. **a–b** The washing step, **c** adding a biological sample, **d** hybridization, **e** washing and **f** release of isolated mRNA [34]

particles were added. mRNA was captured on the surface of paramagnetic particles. In the following step, paramagnetic particles with captured mRNA were forced by magnet and rinsed. Finally, mRNA was released from the particles. The particles were again forced by magnet and mRNA containing solution was analysed [34]. We obtained tens of microlitres of the solution. Such small volumes could not be analysed by spectrometry to determine nucleic acids. Due to this fact, we suggested and utilized square wave voltammetry coupled with adsorptive transfer stripping technique for mRNA determination.

The effect of type of paramagnetic particles on mRNA isolation

For mRNA isolation from biological sample in connection with electrochemical quantification, we utilized two commercial paramagnetic particles DBT (Dynal) and MAGNa. Poly(A) as model of mRNA was used. To prepared paramagnetic particles, poly(A) (1, 5, 10, 20 and 30 $\mu g.mL^{-1}$) was added. Further, poly(A) was hybridized on the surface of the particles for 10 min at 25 °C. Captured nucleic acid was released from the paramagnetic particles surface by heat treatment and shaking (85 °C, 1450 rpm, 10 min.). The other experimental details of semi-automated isolation of nucleic acids are introduced in Ref. No. [34]. Obtained solution was analysed electrochemically. MAGNa microparticles are of less specific working surface, which is used for isolation of DNA or, RNA. The main purpose of MAGNa particles utilization is to capture still constant amount of RNA and its application to PCR. The increased electrochemical signal with the increasing concentration of poly(A) isolated by two types of paramagnetic particles is shown in Fig. 2. The results

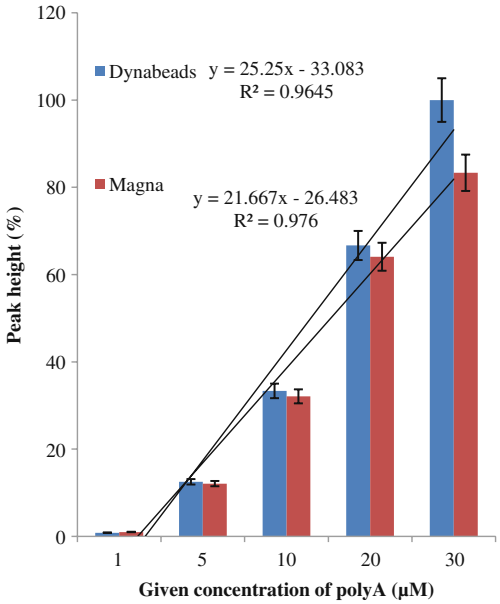

Fig. 2 Changes in cytosine-adenine peak height of poly(A) captured by MAGNa and DBT paramagnetic particles

demonstrate good affinity of poly(A) to both types of paramagnetic particles. However, DBT has higher yield of captured adenine chains (slope 25.25-DBT in comparison with 21.66-MAGNa).

In the consequent experiments, we carried out fully automated isolation of poly(A) by using a robotic device epMotion. The working sequence was as follows: i) paramagnetic microparticles (10 μL) are pipetted into the PCR plate; ii) a washing cycle is three times repeated (50 μL); iii) the sample (poly(A), 10 μL) and hybridization solution (30 μL) are pipetted into PCR plates with paramagnetic microparticles; iv) 15 min. long hybridization at 25 °C is carried out; v) a washing cycle is three times repeated (50 μL). The PCR plate is later moved on thermostated position, where the denaturation of bound nucleic acid chain takes place. We tested the yield of DBT and MAGNa paramagnetic particles. Higher yield gave DBT paramagnetic particles.

mRNA detection in ESEs

Early somatic embryos (ESEs) are suitable experimental model for monitoring of effect of heavy metals on plants. As it follows from the previously published results, ESEs exhibit considerable resistance to increased concentrations of heavy metals [14, 15, 42–49]. The exact mechanism of such higher resistance remains unclear. Photos of ESEs are shown in Fig. S-1A (ESM). ESE is consisted of (1) compact embryonic group, embryonic tubes, and suspensor cells. ESEs form clusters on the solid medium (bottom inset in Fig. S-1A). Automated procedure of mRNA isolation using DBT particles and epMotion instrument was applied to quantitative mRNA isolation from plant samples.

Increased concentration of cadmium(II) ions results in growth depression and structural changes in cluster structure at the end of 14 days long experiment. We determined that total mRNA level gradually enhanced with increasing cadmium(II) ions concentration. The highest concentration was determined in ESEs treated with 250 μM of cadmium(II) ions (Fig. 3a). Clusters cultivated on media contained higher cadmium(II) ions

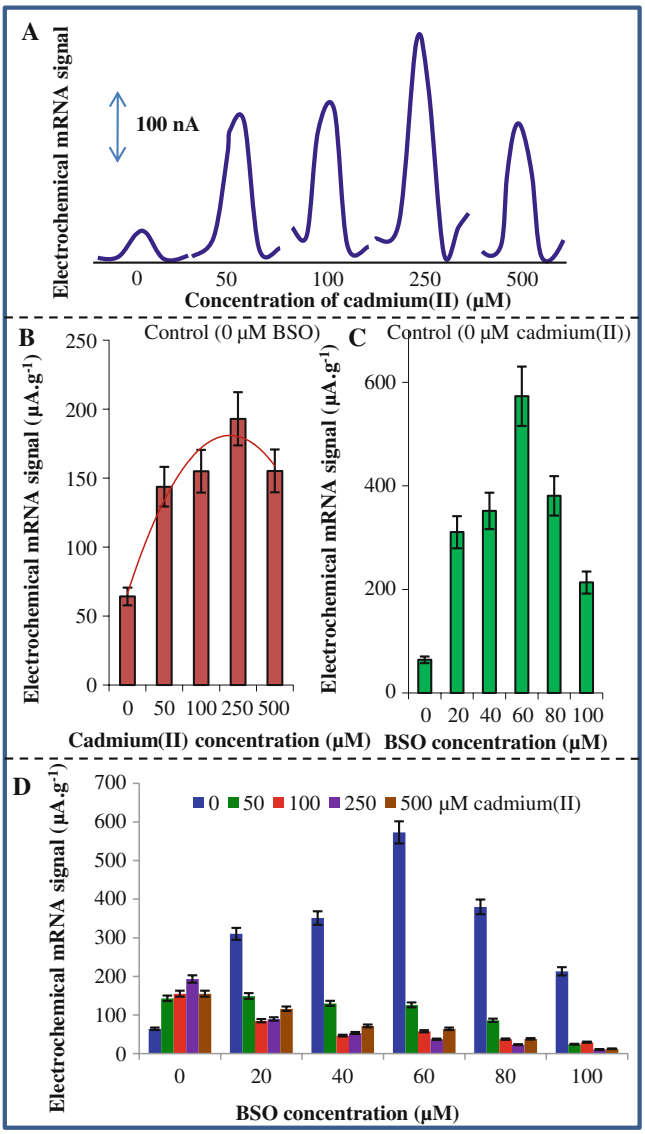

Fig. 3 **a** Electrochemical signal of mRNA in ESEs treated with 0, 50, 100, 250 and 500 μM cadmium(II) ions in 14th experimental day. Volume of isolated sample was 10 μL. Electrochemical signals of mRNA isolated from ESEs treated with **b** cadmium(II) ions or with **c** buthionine sulphoximine (BSO). **d** Electrochemical signals of mRNA isolated from ESEs treated with cadmium(II) ions with or without addition of BSO. All results were obtained at the end of fourteen days long experiment

concentrations demonstrated lower mRNA level. The decline of mRNA level in these clusters is probably associated with inhibition of metabolic activity due to presence of heavy metal ions [15, 44, 47]. The results are shown in Fig. 3b. The electrochemical signal of the isolated mRNA gradually enhanced with increasing concentration of cadmium(II) ions. The highest signal was detected in extract from ESEs treated with 250 μM. All measurements were carried out in triplicates with R.S.D. app. 10 %. Furthermore, we were interested in the question whether using of our suggested procedure will be efficient in monitoring of changes of mRNA level during treatment of ESEs with buthionine sulphoximine (BSO) as an inhibitor of biosynthesis of reduced glutathione. mRNA levels in ESEs after 5 days long exposure to BSO are shown in Fig. 3c. BSO markedly enhanced the level of mRNA more than five times compared to control. Moreover, BSO stimulated synthesis mRNA more than three times intensively compared with cadmium(II) ions. If we simultaneously tested effect of both stress substances, BSO and cadmium(II) ions, we observed significant decrease of mRNA synthesis with increasing concentration of both compounds (Fig. 3d). BSO itself blocks pathways leading to the synthesis of glutathione and causes activation of alternative metabolic pathways to overcome the stress conditions due to BSO. In the event of negative influence of two stress factors (heavy metal ions and the inhibitor), plant metabolism is overburden and therefore mRNA transcription is decreased.

mRNA detection in maize plants

Maize plants were exposed to cadmium(II) ions in concentrations 0, 5, 10, 25, 50 and 100 μM for 5 days. As it is obvious from the growth dependences, increasing concentration of the cadmium(II) ions as well as time of exposition leads to enhanced plant growth inhibition. A typical growth curve is shown in Fig. 4a. Changes in growth depending on the concentration of cadmium(II) ions in the fifth day of the experiment are well evident in Fig. 4b. The highest concentration of cadmium(II) ions (100 μM) resulted in significant growth inhibition from the third day of the exposition. These plants had one or two small leaves at the end of the experiment compared to control plants with four or five large leaves without morphological changes. Moreover, we determined changes in the content of chlorophyll in leaves of treated plants with increasing concentration of cadmium(II) ions at fifth day of the exposition (Fig. 4c). Content of chlorophyll did not change in plants treated with 5, 10 and 25 μM of cadmium (II) ions compared to control plants. Two highest applied concentration of cadmium(II) ions enhanced chlorophyll content. Besides this, we were interested whether content

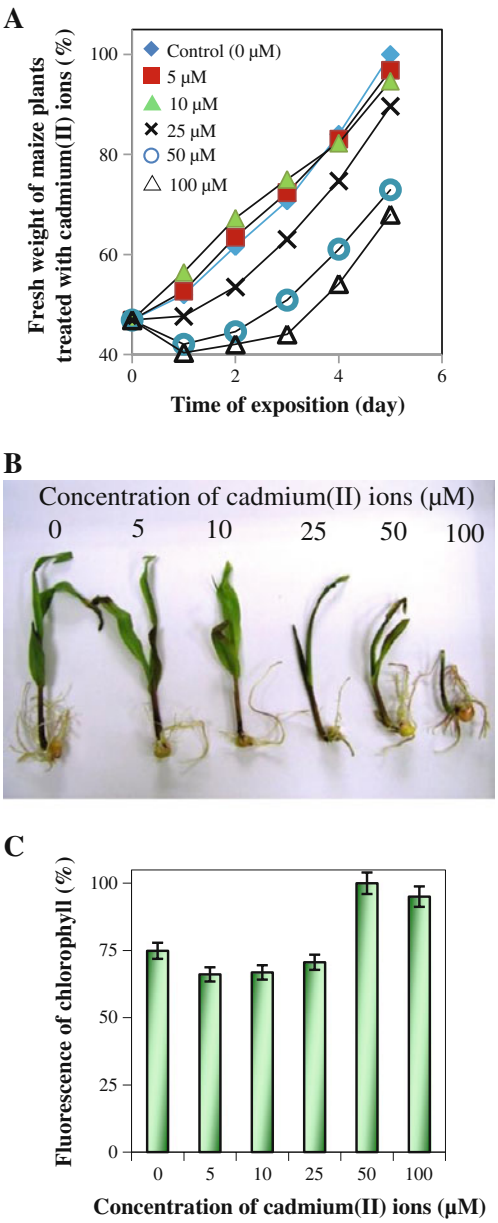

Fig. 4 **a** Fresh weight of *in vitro* cultivated treated and untreated maize plants. **b** Photos of maize plants on fifth day of cadmium(II) ions exposition. **c** Changes in chlorophyll content. Signal of 100% height corresponds to 872 mAU

of primary and secondary metabolites is changed due to presence of cadmium(II) ions. The changes in the roots were determined histochemically using $K_3[Fe(CN)_6]$, which is reduced in tissues with free-SH moieties on the blue colored reduction products [50] (arrows, Fig. S-1B, ESM). In control plants, free-SH moieties were detected in rhizodermis only. The enhanced biosynthesis of compounds containing free-SH groups were observed in plants treated with 10 μM of cadmium(II) ions, namely in rhizodermis as well as cortex. In contrast, almost no

compounds with free-SH moieties were detected in plants treated with two highest concentration of cadmium(II) ions. However, especially in cortex, which is formed by metabolically active cells, synthesis of red colored compounds marked as "X" in these root sections was observed. The substances are polyphenols (probably anthocyanidins) demonstrating high antioxidant capability (Fig. S-1B). The presence of these substances is closely associated with oxidation stress in plants due to cadmium(II) ions, which are able to generate free oxygen radicals; these radicals are scavenged by the mentioned polyphenols.

Plants exposed to stress are forced to increase the synthesis of various biologically active molecules, such as compounds containing free-SH group (reduced glutathione, phytochelatins, metallothionein like proteins), as we determined and discussed above (Fig. S-1B). Numerous of such molecules are synthesized by chemical reactions, but the enzymes involved in these reactions are gene-regulated. For these reasons, it is possible to observe the increased expression of certain groups of genes that lead to the increase in levels of total mRNA (transcriptome) in plants. In all analyzed samples, mRNA was obtained using paramagnetic microparticles and detected electrochemically. The presence and quality of mRNA obtained was verified on gel electrophoresis on chip. Using SWV, we determined well-developed signals at −1.65 V (Fig. S-2A, ESM). As it clearly follows from the Fig. S-2A, CA signal corresponding to isolated mRNA changes with increasing time of exposition and concentration of cadmium(II) ions. The quantity of isolated mRNA ranged from 0.1 to 2.5 $\mu g.g^{-1}$ (poly(A) was used as a standard). In the first 3 days of the experiment, there was the increase in mRNA content in the shoot and roots of plants treated with cadmium(II) ions compared to control ones. Then, the content of mRNA markedly decreased (Fig. S-2B, ESM). At the end of the experiment, only control plants synthesized mRNA. To better understanding the effect of cadmium(II) ions, we attempted to average mRNA content according to concentration of cadmium(II) ions (upper part of Fig. S-2C) or day of the exposition (bottom part of Fig. S-2C, ESM). The highest concentration of cadmium(II) ions resulted in the highest enhancing of mRNA synthesis in average. In spite of the such metabolic activity, plants exposed to highest cadmium(II) ions concentration necrotized and died at the end of the experiment, which is well evident in average content of mRNA per day of the exposition. Moreover, we attempted to mathematically treat these data (Tab. S-1, ESM). Level of mRNA measured in maize plants treated with certain concentration of cadmium(II) ions in individual experimental days was linearly regressed with the following value of mRNA level. Thus, the slope of such regression shows rate of increase or decrease in the mRNA level.

Field experiments

Besides the above discussed experiments with plants cultivated under well-defined laboratory conditions, we performed field experiment. Thirty days old plants were placed into cultivation vessels with tap water and 10 μM cadmium(II) ions only. Each four plants were harvested at strictly defined time intervals (7, 12, 17, and 21 days) during the experiment. DBT particles were used for mRNA isolation from tissues (leaves and roots) of treated as well as untreated maize plants. The content of mRNA was enhanced with increasing time of the treatment and varied from sub-units to units of μg. At the end of the treatment (21st day), maize plants were exhausted and mRNA content significantly reduced. Exhaustion of the plants was also demonstrated by decrease of chlorophyll content, as well as morphological changes involved in reduction of aerial plant parts as well as root system.

Conclusion

Environmental analytical chemistry covers development of new analytical methods or improvement of existing ones useful for the monitoring of pollutants or trace amounts of naturally occurring active chemicals in the environment. Nanotechnology brings new possibilities in this scientific branch. In the introduced study, we use paramagnetic particles for isolation of mRNA as a marker of heavy metal stress. Our work demonstrates the possibility of mRNA isolation from plant cells, tissues, and organs by using of paramagnetic particles and correlation between concentration of cadmium(II) ions, time of exposition and mRNA levels.

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