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Physiological characterization of *Chlamydomonas reinhardtii* acclimated to chronic stress induced by Ag, Cd, Cr, Cu and Hg ions

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ABSTRACT

Acclimation to heavy metal-induced stress is a complex phenomenon. Among the mechanisms of heavy metal toxicity, an important one is the ability to induce oxidative stress, so that the antioxidant response is crucial for providing tolerance to heavy metal ions. The effect of chronic stress induced by ions of five heavy metals, Ag, Cu, Cr (redox-active metals) Cd, Hg (nonredox-active metals) on the green microalga *Chlamydomonas reinhardtii* was examined at two levels – the biochemical (content of photosynthetic pigments and prenyllipid antioxidants, lipid peroxidation) and the physiological (growth rate, photosynthesis and respiration rates, induction of nonphotochemical quenching of chlorophyll fluorescence). The expression of the genes which encode the enzymes participating in the detoxification of reactive oxygen species (*APX1*, *CAT1*, *FSD1*, *MSD1*) was measured. The other gene measured was one required for plastoquinone and α-tocopherol biosynthesis (*VTE3*). The application of heavy metal ions partly inhibited growth and biosynthesis of chlorophyll. The growth inhibition was accompanied by enhanced lipid peroxidation. An increase in the content of prenyllipid antioxidants was observed in cultures exposed to $Cr_2O_7^{2-}$, Cd^{2+} (α- and γ-tocopherol and plastoquinone) and Cu^{2+} (only tocopherols). The induction of nonphotochemical quenching was enhanced in cultures exposed to Cu^{2+}, $Cr_2O_7^{2-}$ and Cd^{2+}, as compared to the control. Chronic heavy metal-induced stress led to changes in gene expression dependent on the type and concentration of heavy metal ions. The up-regulation of antioxidant enzymes was usually accompanied by the up-regulation of the *VTE3* gene.

1. Introduction

In recent centuries, anthropogenic activities have led to the ever increasing release of heavy metal ions into the environment. Nowadays, heavy metals are the major environmental pollutants, posing a threat to water and soil ecosystems, as well as to human health (Nagajyoti et al., 2010). The essential heavy metals, such as Cu, Fe, Zn or Ni are micronutrients necessary for living organisms, but toxic at higher concentrations. Non-essential heavy metals are not known to have physiological functions. Among them are such elements as Pb, Hg and Cd. In the last few years, Cd has been found to be a cofactor of the carbonic anhydrase in diatoms, although this is rather a rare case (Lane and Morel, 2000).

The mechanism underlying the toxicity of heavy metals is complex. Exposure to enhanced concentrations of heavy metal ions causes multiple harmful effects related to the disturbance of the cell metabolism and ultrastructure. Among these effects, one of the most important is the induction of oxidative stress (Nagajyoti et al., 2010; Yadav, 2010). Some heavy metals, such as Cu, Cr or Fe can occur in cells in multiple oxidation states. They were called redox-active metals and are thought to directly react with reactive oxygen species (ROS). The other elements, such as Cd, Pb or Hg, which usually do not undergo redox-cycling in cells, are known as nonredox-active (or redox-inactive) metals (Elbaz et al., 2010; Stoiber et al., 2013; Wang et al., 2015; Zheng et al., 2011). It should be noted here that these terms are used to describe the relative potential of participating in redox reactions in living cells. It does not mean that nonredox-active metals are unable to undergo redox reactions, only that such cases occur rarely (Stoiber

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; Chl, chlorophyll; FeSOD, iron-containing superoxide dismutase; F_v/F_m, maxium quantum yield of photosystem II; GSH, glutathione; LOOH, lipid hydroperoxides; MnSOD, manganese-containing superoxide dismutase; MPBQ MT, 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase; NPQ, nonphotochemical quenching of chlorophyll fluorescence; PQ, plastoquinone; PQH_2, plastoquinol; PQ_{tot}, sum of plastoquinol and plastoquinone; PS II, photosystem II; ROS, reactive oxygen species; Tocs, tocopherols; α-Toc, α-tocopherol; γ-Toc, γ-tocopherol

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et al., 2013). Nonredox-active metals can also induce oxidative stress, although they participate in the generation of ROS indirectly. The indirect impact of heavy metals on the induction of oxidative stress occurs mostly via the depletion of the important cellular antioxidant, glutathione (GSH), and by the inhibition of photosynthesis and respiration (Pinto et al., 2003; Yadav, 2010).

Exposure to toxic concentrations of heavy metal ions leads to the inhibition of growth and a decrease in chlorophyll (Chl) content (DalCorso, 2012; Nagajyoti et al., 2010; Yadav, 2010). However, during prolonged exposure to pollutants, organisms are able to acclimate to the consequent stress by the induction of a wide array of mechanisms enabling heavy metal tolerance. These include the precipitation of heavy metal ions inside and outside the cell, active efflux, bioconversion into less harmful forms and the enhancement of the antioxidant defense (Gaur and Rai, 2001; Wood and Wang, 1983). Some of these mechanisms, such as the induction of phytochelatin synthesis, binding by cell wall components and an increase in the expression and activity of antioxidant enzymes, have been widely studied (Gaur and Rai, 2001; Pinto et al., 2003).

The participation of low molecular weight antioxidants, especially lipophilic ones, such as α-tocopherol (α-Toc), in the response to heavy metal induced stress has been studied to a lesser extent (Collin et al., 2008). α-Toc is a potent antioxidant able to quench and scavenge singlet oxygen ($^1O_2$), scavenge the superoxide radical ($O_2^{\bullet -}$), and inhibit lipid peroxidation (Mène-Saffrané and Della-Penna, 2010). This compound participates in the response of plants to the different environmental stress factors, which are known to generate oxidative stress, such as high light, high or low temperature and osmotic stress (Munne-Bosch , 2005). It has been observed that during the response to chronic stress induced by enhanced concentrations of Cu^{2+}, Cd^{2+}, Pb^{2+} and Ni^{2+}, the α-Toc content increased in different species of higher plants and in *Chlamydomonas reinhardtii* (Gajewska and Skłodowska, 2007; Kumar et al., 2012; Luis et al., 2006; Zengin and Munzuroglu, 2005). Transgenic *Brassica juncea* with an over-expressed γ-tocopherol methyltransferase gene from *Arabidopsis thaliana* contained more α-Toc and was more tolerant to Cd^{2+} than control plants (Yusuf et al., 2010). The increase in α-Toc content was observed in Cu^{2+} and Cd^{2+}-stressed *A. thaliana*, whereas an α-Toc-deficient mutant displayed an enhanced sensitivity towards both metal ions, as compared to the control (Collin et al., 2008).

Other important prenyllipid antioxidants are the prenylquinones, whose most recognized function is participating in electron and proton transfer chains (Nowicka and Kruk, 2010). Among them, plastoquinone (PQ) is mostly recognized as a compound essential for photosynthetic electron transport and a cofactor needed for carotenoid biosynthesis in plants and green algae (Nowicka and Kruk, 2010). What is more, plastoquinone, especially in its reduced, quinol form (plastoquinol, PQH_2), is an effective antioxidant, able to quench and scavenge $^1O_2$, scavenge radicals, and inhibit lipid peroxidation, both in vitro and in vivo (Gruszka et al., 2008; Nowicka and Kruk, 2010, 2012; Nowicka et al., 2013). An increase in PQ content during the response to high light was observed in *C. reinhardtii* (Nowicka and Kruk, 2012). To our knowledge, there have been no articles concerning PQ content in heavy metal stressed organisms up to date.

It is known that heavy metal ions are inhibitors of photosynthesis, acting at different sites, such as the donor and acceptor sides of PS II or the enzymes of the Calvin cycle (Nagajyoti et al., 2010; Parmar et al., 2013; Shah et al., 2010). The inhibition of photosynthesis by heavy metals was reported for microalgae in numerous experiments in the past (Cid et al., 1995; Juneau et al., 2002; Rodríguez et al., 2007; Samson et al., 1988; Vavilin et al., 1995; Wang and Dei, 2006; Wang et al., 2013). Any inhibition of photosynthesis by heavy metals means that in photosynthetic organisms exposed to heavy metal-induced stress the amount of absorbed light energy which can be used for photosynthetic reactions is reduced. In such a case the level of excessive, potentially harmful energy is higher (Demmig-Adams and Adams, 1996). The up-regulation of the mechanisms of light energy dissipation was observed in plants exposed to environmental stresses leading to a decrease in the efficiency of light energy usage, such as a low temperature (Demmig-Adams and Adams, 1996).

Microalgae are important in research into heavy metal toxicity due to their important role as primary producers in water ecosystems, and because of the usage of selected species for bioremediation (Monteiro et al., 2011; Pinto et al., 2003; Rawat et al., 2011). The common freshwater green microalga *C. reinhardtii* is often used in environmental studies, as it is easy to grow, metabolically profiled and its genome has been sequenced. *C. reinhardtii* is also thought of as a model unicellular photosynthetic organism (Hanikenne, 2003; Jamers et al., 2013).

There have been several studies concerning heavy metal toxicity, where the experiments have been performed on several species, grown in different conditions. The major trends are visible, however sometimes it is difficult to compare the results obtained in various experiments, in example even for *C. reinhardtii* the inhibition of growth reported for the same Cu^{2+} concentration range was different (Boswell et al., 2002; Luis et al., 2006; Macfie et al., 1994; Prasad et al., 1998). One of the aims of the present study was to examine the effect of five heavy metals applied in wide range of concentrations in one species grown in certain medium, light regime and temperature. We wanted to compare the response of *C. reinhardtii* to redox-active (Ag^+, Cu^{2+}, $Cr_2O_7^{2-}$) and nonredox-active (Cd^{2+}, Hg^{2+}) metals. The impact of heavy metal ions on growth and pigment synthesis, as well as the rates of photosynthesis and respiration in heavy metal-stressed cultures were studied. We also measured the induction of non-photochemical quenching of chlorophyll fluorescence (NPQ) and the epoxidation ratio. The extent of lipid peroxidation was monitored using a fluorescent probe Spy-LHP for the detection of lipid hydroperoxides (LOOHs).

The second aim was to observe the impact of different heavy metal ions on the prenyllipid antioxidants. The content of tocopherols (α and γ forms) and plastoquinone in heavy metal-stressed cultures was measured. Also the expression of five genes was analysed. Four of them encode enzymes participating in ROS detoxification, ascorbate peroxidase (APX1), catalase (CAT), manganese-containing superoxide dismutase (MnSOD) and iron-containing superoxide dismutase (FeSOD). The other gene encodes enzyme participating in PQ and α-Toc biosynthesis, 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanesyl-1,4-benzoquinone methyltransferase (MPBQ MT). It was interesting to check if the expression of the gene necessary for synthesis of prenyllipid antioxidants would be regulated in similar manner to the genes encoding ROS detoxifying enzymes.

2. Materials and methods

2.1. Culture growth

In the present experiment a *C. reinhardtii* strain 11-32b obtained from the SAG collection (Goettingen, Germany) was used. The basis for each medium was a modified Sager-Granick medium: 3.75 mM NH_4NO_3, 0.73 mM KH_2PO_4, 0.57 mM K_2HPO_4, 1.22 mM $MgSO_4 \cdot 7H_2O$, 0.36 mM $CaCl_2 \cdot 2H_2O$, 3.5 μM $ZnSO_4 \cdot 7H_2O$, 16.2 μM H_3BO_4, 2.02 μM $MnCl_2 \cdot 4H_2O$, 0.84 μM $CoCl_2 \cdot 6H_2O$, 0.24 μM $CuSO_4 \cdot 5H_2O$, 0.83 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 37 μM $FeCl_3$, 0.2 mM sodium acetate, 5 mM HEPES pH 6.8 (diluted from a stock 100 mM