Environmental and Experimental Botany 123 (2016) 98–107

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

# Prenyllipid antioxidants participate in response to acute stress induced by heavy metals in green microalga *Chlamydomonas reinhardtii*

Beatrycze Nowicka*, Bartosz Pluciński[1], Paulina Kuczyńska[1], Jerzy Kruk

*Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland*

ARTICLE INFO

*Article history:*
Received 12 October 2015
Received in revised form 19 November 2015
Accepted 21 November 2015
Available online 24 November 2015

*Keywords:*
*Chlamydomonas reinhardtii*
Heavy metals
Oxidative stress
Plastoquinone
α-Tocopherol

ABSTRACT

The induction of oxidative stress is an important mechanism of heavy metal toxicity. That is why, isoprenoid antioxidants, such as chromanols and prenylquinones, are thought to participate in the response to heavy metal-induced stress. In the present study, we performed a comparative analysis of the prenyllipid and pigment content and lipid peroxides in *Chlamydomonas reinhardtii* during 7.5 h of acute stress induced by Cu, Cr, Cd, Hg and Ag ions. We also measured the expression of genes encoding enzymes participating in the detoxification of reactive oxygen species (*APX1*, *CAT1*, *FSD1*, *MSD1*) and a gene required for α-tocopherol and plastoquinone biosynthesis (*VTE3*). In an $AgNO_3$-treated culture, pigments and prenyllipids were degraded at the same rate. The significant peroxidation of lipids was also observed. For other metals, a different pattern of changes in pigment and prenyllipid content was observed. The significant degradation of pigments was observed during the response to $Cu^{2+}$. The decrease in prenyllipid content occurred in Cu and Cr-stressed algae. Massive oxidation of plastoquinol was observed in the presence of $Cu^{2+}$, $Ag^{+}$ and $Cr_2O_7^{2-}$. The most pronounced increase in the expression of the investigated genes was found in the presence of $Cu^{2+}$ and $Hg^{2+}$ ions. The genes whose expression was the most up-regulated were *APX1*, *MSD1*, *VTE3*.



## 1. Introduction

Heavy metals are important environmental pollutants. Industry and mining have led to a redistribution of heavy metals, leading to the contamination of soil and water. Nowadays, heavy metals pose a severe threat to living organisms (Nagajyoti et al., 2010). Depending on their biological importance these elements can be divided into two groups: essential and non-essential heavy metals. Essential heavy metals, such as Cu, Fe, Zn or Ni, are micronutrients, causing toxicity when present at higher concentrations. Non-essential heavy metals, such as Pb, Cd, Hg, are not known to have physiological functions. The one exception discovered to date is that Cd is a cofactor of the carbonic anhydrase in diatoms (Park et al., 2007; Collin et al., 2008).

Heavy metal toxicity is a very complicated phenomenon, related to disturbance of metabolism and ultrastructure of cells. Among the consequences of exposure to a toxic concentration of heavy metals, one of the most important is oxidative stress (Nagajyoti et al., 2010; Yadav, 2010). Heavy metals can contribute to the generation of reactive oxygen species (ROS) directly or indirectly. Some metals, such as Cu, Cr or Fe can occur in multiple oxidation states and they are known to generate ROS directly *via* Fenton-type reactions and Haber–Weiss cycling. They are known as redox-active heavy metals. Other metals, such as Cd, Pb or Hg do not undergo redox-cycling in cells, so they are often called nonredox active metals. The term “nonredox active” (or “redox-inactive”), is used to describe the relative potential of a metal to undergo redox reactions in cells as compared to other transition metals. It does not mean that these metals do not undergo redox reactions at all (Stoiber et al., 2011). The redox-inactive metals can

*Abbreviations:* APX, ascorbate peroxidase; CAT, catalase; Chl, chlorophyll; FeSOD, iron-containing superoxide dismutase; GSH, glutathione; LOOH, lipid hydroperoxides; MnSOD, manganese-containing superoxide dismutase; MPBQ MT, 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase; PC-8, plastochromanol-8; PC-OH, hydroxy-plastochromanol; PQ, plastoquinone; PQ-C, plastoquinone C; $PQH_2$, plastoquinol; $PQ_{tot}$, sum of plastoquinone and plastoquinol; ROS, reactive oxygen species; Tocs, tocopherols; α-Toc, α-tocopherol; γ-Toc, γ-tocopherol; α-TQ, α-tocopherol quinone.

* Corresponding author.
*E-mail addresses:* beatrycze.nowicka@uj.edu.pl, beatrycze.nowicka@wp.pl (B. Nowicka), cw15@o2.pl (B. Pluciński), kuczynska.paul@gmail.com (P. Kuczyńska), jerzy.kruk@uj.edu.pl (J. Kruk).
[1] These authors contributed equally to the article.

http://dx.doi.org/10.1016/j.envexpbot.2015.11.008
0098-8472/

participate in ROS generation indirectly, mostly by causing depletion of glutathione (GSH), which is an important cellular antioxidant, as well as by disrupting the electron transfer chains (Pinto et al., 2003; Yadav, 2010).

As the induction of oxidative stress is one of the mechanisms of heavy metal toxicity, the ability to detoxify ROS should be an important factor in heavy metal tolerance. The impact of heavy metals on the expression of ROS-detoxifying enzymes has been widely studied (Pinto et al., 2003). The participation of low molecular weight antioxidants has been examined to a lesser extent, especially in the case of lipophilic antioxidants (Collin et al., 2008).

The important lipophilic antioxidants are chromanols and prenylquinones (Mène-Saffrané and DellaPenna, 2010; Nowicka and Kruk, 2010). Among chromanols, the most extensively examined compounds are tocopherols (Tocs) and tocotrienols, collectively known as vitamin E. Due to its importance in medicine, α-tocopherol (α-Toc) has been the subject of intensive research for several years. In a series of *in vitro* and *in vivo* experiments, it has been shown that α-Toc is able to inhibit lipid peroxidation, quench and scavenge singlet oxygen ($^1O_2$) and scavenge the superoxide radical ($O_2^{-\bullet}$). Other forms of vitamin E, as well as plastochromanol-8 (PC-8), a chromanol with a longer isoprenoid side-chain, also display antioxidant properties (Mène-Saffrané and DellaPenna, 2010; Kruk et al., 2014).

Prenylquinones, such as ubiquinone, plastoquinone (PQ) and phylloquinone (vitamin $K_1$) are mostly recognized as crucial electron and proton carriers in electron transfer chains and as enzyme cofactors (Nowicka and Kruk, 2010). What is more, they are effective antioxidants, especially in their reduced, quinol form. They are able to inhibit lipid peroxidation, scavenge radicals, as well as scavenge and quench $^1O_2$ (Gruszka et al., 2008; Nowicka and Kruk, 2010, 2012; Nowicka et al., 2013).

The participation of prenyllipids in the response to stress caused by excessive light, drought, low or high temperature has been widely examined (Mène-Saffrané and DellaPenna, 2010; Munné-Bosch, 2005), whereas their role in the response to heavy-metal induced stress is still rather poorly studied. The importance of α-Toc in the tolerance to $Cu^{2+}$ and $Cd^{2+}$ in *Arabidopsis thaliana* has been proved (Collin et al., 2008). Transgenic *Brassica juncea* with overexpressed γ-tocopherol methyltransferase from *A. thaliana* contained an elevated level of α-Toc and was more tolerant to $Cd^{2+}$ than control plants (Yusuf et al., 2010). There were also observations of an increase in α-Toc level during prolonged exposure to toxic concentrations of $Cu^{2+}$, $Cd^{2+}$, $Pb^{2+}$ and $Ni^{2+}$ (Gajewska and Skłodowska, 2007; Kumar et al., 2012; Luis et al., 2006; Zengin and Munzuroglu, 2005).

In the present study, we have performed a comparative analysis of the following prenyllipids: α and γ-tocopherol (γ-Toc), PC-8, PQ and plastoquinol ($PQH_2$), as well as their oxidation products, such as α-tocopherol quinone (α-TQ), hydroxy-plastochromanol (PC-OH) and plastoquinone C (PQ-C) in cultures of the green alga *Chlamydomonas reinhardtii* during the response to acute stress caused by the application of toxic concentrations of $Cu^{2+}$, $Cr_2O_7^{2-}$, $Cd^{2+}$, $Hg^{2+}$ and $Ag^+$ ions. We have also measured chlorophyll and carotenoid content under heavy-metal exposure. We have monitored lipid peroxidation using a fluorescent probe Spy-LHP for the detection of lipid peroxides (LOOH). Finally, we have analyzed the expression of four genes encoding enzymes participating in ROS detoxification: ascorbate peroxidase (APX), catalase (CAT), manganese-containing superoxide dismutase (MnSOD) and iron-containing superoxide dismutase (FeSOD) and one gene participating in PQ and α-Toc biosynthesis: 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanesyl-1,4-benzoquinone methyltransferase (MPBQ MT) after 7.5 h of exposure to heavy metals.

## 2. Materials and methods

### 2.1. Culture growth and exposure to heavy metals

A *C. reinhardtii* strain 11-32b obtained from the SAG collection (Goettingen, Germany) was grown in a modified Sager-Granick medium: 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 $FeCl_3$, 3.75 mM $NH_4NO_3$, 1.22 mM $MgSO_4 \cdot 7H_2O$, 0.36 mM $CaCl_2 \cdot 2H_2O$, 0.57 mM $K_2HPO_4$, 0.73 mM $KH_2PO_4$, 0.2 mM sodium acetate. A 100 mM HEPES buffer of pH 6.8 was added to the medium to a final concentration of 5 mM. Cultures were grown in Erlenmeyer flasks on a shaker at 22 °C at 70–80 μmol photons $m^{-2}\,s^{-1}$.

For the experiments, four weeks-old cultures were used. Photosynthetic pigments were extracted from centrifuged samples with acetone as described in (Nowicka and Kruk, 2012). Spectrophotometric measurements and calculation of chlorophyll and carotenoid concentration were done according to the procedures and equations given in (Lichtenthaler, 1987). The method described by Lichtenthaler makes it possible to assess total chlorophyll and total carotenoid concentration in μg/ml of extract.

Before the experiment, cultures were diluted with a fresh medium to get a final chlorophyll concentration of 2.5 μg Chl/ml and portioned into a series of smaller flasks. For each metal, a set of 6 flasks was prepared: 3 control flasks and 3 flasks with added heavy metal salt. The following heavy metal stock solutions were used: 50 mM $CuSO_4$, 50 mM $CdCl_2$, 50 mM $K_2Cr_2O_7$, 10 mM $HgCl_2$, 10 mM $AgNO_3$. The final concentrations of metals added to algal cultures were: 200 μM of $CuSO_4$, $CdCl_2$ or $K_2Cr_2O_7$ and 20 μM of $HgCl_2$ or $AgNO_3$. Cultures were incubated with metals for 7.5 h. During that time sets of samples were taken for the spectrophotometric determination of photosynthetic pigments, the determination of prenyllipids using RP-HPLC, the determination of LOOH using the fluorescent probe Spy-LHP, and at the end of incubation for an analysis of gene expression by the real-time PCR method.

### 2.2. Sample extraction and HPLC analysis of prenyllipids

The extraction of prenyllipids and determination of α-Toc, PC-8, PC-OH, $PQH_2$ and PQ using RP-HPLC were preformed as described in (Nowicka and Kruk, 2012). It should be noted here that $PQH_2$ is a compound prone to non-enzymatic oxidation, so to avoid $PQH_2$ oxidation, prenyllipid extraction should be done quickly (2 min of extraction of the centrifuged algal cells with acetone, followed by 90 s × 9000 × *g* centrifugation and evaporation of the supernatant in a stream of nitrogen) and the extracted evaporated sample should be stored in liquid nitrogen and diluted in methanol just before HPLC separation. The HPLC of α-TQ, γ-Toc and PQ-C was performed in the following system: $C_{18}$ reverse-phase column (Tracer Excel 120 ODS-A, 5 μm, 25 cm × 0.4 cm) + platinum post-column (5 wt.% on alumina, powder, 325 mesh, Aldrich), eluent methanol:hexane (340:20, v/v), flow rate of 1.5 ml/min, fluorescence detection at $\lambda_{ex}$ = 290 nm, $\lambda_{em}$ = 330 nm. The concentration of prenyllipids in extracts was assessed by a comparison with respective standards (Dłużewska et al., 2015) and normalized on a dry weight (DW) basis.

### 2.3. Lipid hydroperoxide measurements using Spy-LHP

For the assessment of LOOH in algal cells, a method with the fluorescent probe 2-(4-diphenylphosphanyl-phenyl)-9-(1-hexyl-heptyl)-anthra(2,1,9-def,6,5,10-d′e′f′)-diisoquinoline-1,3,8,10-tetraone, commercial name Spy-LHP (Dojindo, Japan), was used. For each sample, 1.5 ml of the suspension was taken and centrifuged for 5 min × 9000 × *g* . The pellet was resuspended in 496 μl of