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Lead-stress induced changes in the content of free, thylakoid- and chromatin-bound polyamines, photosynthetic parameters and ultrastructure in greening barley leaves

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ABSTRACT

The aim of this study was to determine the impact of lead (Pb) stress as 0.6 mM $Pb(NO_3)_2$ on the content of free, thylakoid- and chromatin-bound polyamines (PAs) and diamine oxidase (DAO) activity in detached greening barley leaves. Additionally, photosynthetic-related parameters, generation of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content and ultrastructural changes under Pb-stress were studied. The level of putrescine (Put) was reduced progressively to 56% at 24 h of Pb stress, and it was correlated with 38% increase of DAO activity. Spermidine (Spd) content was not affected by Pb-stress, while the free spermine (Spm) level significantly increased by about 83% at 6 h, and in that time the lowest level of H_2O_2 was observed. The exogenous applied Spm to Pb-treated leaves caused a decrease in the content of H_2O_2. In greening leaves exposed to Pb an accumulation of chlorophylls *a* and *b* was inhibited by about 39 and 47%, respectively, and photosynthetic parameters of efficiency of electron transport and photochemical reaction in chloroplasts as ΦPSII, ETR and RFd were lowered by about 23–32%. The level of thylakoid-bound Put decreased by about 22%. Moreover, thylakoids isolated from chloroplasts of Pb-treated leaves were characterized with lower Put/Spm ratio as compared to control leaves. In the presence of Pb the significant decrease in the number of thylakoids per granum and cap-shape invaginations of cytoplasmic material were noticed. In Pb-stressed leaves the level of chromatin-bound Spm increased by about 48% and sometimes condensed chromatin in nuclei was observed. We conclude that in greening barley leaves exposed to Pb-stress changes in free, thylakoid- and chromatin-bound PAs play some role in the functioning of leaves or plants in heavy metal stress conditions.

1. Introduction

Recently, metal contamination of soil has increased considerably due to human activities. One of the major consequences of heavy metals in plants is the enhanced production of reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals and H_2O_2, which lead to lipid peroxidation, disturbance in photosynthesis, and increased rates of DNA damage and cell death (Verma and Dubey, 2003; Sytar et al., 2013). Among common pollutants that affect plants, Pb is one of the most toxic and frequently encountered (Hartwig, 1995). Pb continues to be used widely in many industrial processes and occurs as a contaminant in all environmental compartments (soils, water, the atmosphere, and living organisms). This metal impairs plant growth, root elongation, seed germination, seedling development, transpiration, chlorophyll (Chl) production, thylakoid organization in the chloroplast, and cell division (Maestri et al., 2010; Sytar et al., 2013). At the cellular level, Pb induces accumulation of ROS, as a result of imbalanced production and scavenging processes (Gupta et al., 2013). Plants respond to the noxious effects of Pb in various ways, such as selective metal uptake, metal binding to the root surface, binding to the cell wall, and induction of antioxidants (Maestri et al., 2010). It has also been documented that metal excess induced changes in polyamine (PA) metabolism (Weinstein et al., 1986; Groppa et al., 2003; Groppa and Benavides, 2008). Polyamines (PAs) are small ubiquitous polycations involved in many processes of plant growth and development and are well known for their anti-senescence and anti-stress effects due to their acid neutralizing and antioxi-

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dant properties, as well as for their membrane and cell stabilizing abilities (Bouchereau et al., 1999; Alcazar et al., 2010; Sytar et al., 2013). The amount and/or ratio of various PAs may strongly vary during stress adaptation. PAs play an important role in the structural organization and functional activity of thylakoid membranes (Kotzabasis et al., 1993). The electrostatic interaction of PAs' positive charges or their conjugation with protein of PSII may confer some stability of their conformational structure and function under various stresses (Besford et al., 1993; Legocka and Zajchert, 1999; Sfichi et al., 2004). Some of the attributes ascribed to PAs, particularly Spm, also include the stabilization of chromatin (Snyder, 1989; Feuerstein et al., 1990). Several genes coding the enzymes of PA metabolism have been characterized and cloned in different species. More recent studies using either transgenic overexpression or loss-of-function mutants support this protective role of PAs in the plant response to abiotic stress (Alcazar et al., 2010; Wen et al., 2010). Moreover, PAs are components of the cellular antioxidant systems that are regarded as scavengers of oxygen radicals during various types of stresses (Bors et al., 1989; Benavides et al., 2000). One of the manifestations of the antioxidant effect of PAs is their ability to regulate the expression of genes encoding antioxidant enzymes (Groppa et al., 2001; Aronova et al., 2005). In many cases the relationship of plant stress tolerance was noted with the production of conjugated and bound forms of PAs as well as stimulation of PA oxidation (Gill and Tuteja, 2010). PA catabolism generates ROS, and particularly H_2O_2, a signaling molecule that can enter the stress signal transduction chain promoting activation of an antioxidative defense response, but can also act as a prooxidant agent. However, the mechanism of their antioxidant action is still poorly understood. PA metabolism is differently regulated under heavy metal stress (Groppa and Benavides, 2008). Surprisingly, data related to the role of PAs in plant response to heavy metal remain scare, and most studies reported data on free PA concentration, while it may be argued that, as protecting compounds, bound PAs should play a key role. In studies on the action of heavy metals in plants more efforts are needed to identify the physiological and molecular significance of PAs in plant heavy metal tolerance through an analysis of the level of free and bound forms.

The objective of this work was to establish the influence of Pb on: (a) Chl and Chl fluorescence kinetic parameters (b) ultrastructure of chloroplasts and nuclei (c) the levels of free, thylakoid- and chromatin-bound PAs (d) DAO activity and (e) the levels of H_2O_2, MDA in etiolated excised barley leaves light exposed. Additionally, the influence of exogenously added Spm on H_2O_2 and MDA levels in excised greening barley leaves exposed to Pb stress was analyzed.

2. Material and methods

Barley seeds (*Hordeum vulgare* L. 'Nagrad') were surface sterilized for 5 to 10 min by using commercial sodium hypochlorite (10%) and then thoroughly rinsed with sterile water. Seeds were germinated and grown in moist cellulose in the darkness for 6 d at 23 °C. Excised 6 day-old primary leaves represented etiolated control. For experiments with the content of chlorophyll *a* and *b* and photosynthetic parameters, detached etiolated 6 day-old leaves were placed in water (control) or in 0.5, 0.6, 0.7 and 0.8 mM $Pb(NO_3)_2$ and exposed to continuous illumination (150 μmol/m^2/s) for or 24 h. For further experiments 0.6 mM $Pb(NO_3)_2$ which inhibited chlorophyll accumulation by about 45% was used. In the experiments with examination of exogenous Spm on H_2O_2 and MDA content, 1 mM Spm (spermine tetrahydrochloride, Sigma–Aldrich) was added to 0.6 mM $Pb(NO_3)_2$ solution, and these leaves were additionally sprayed three times at 7:00 am, 2:00 pm and 8:00 pm with 1 mM Spm. Spm concentration used for treatments was chosen according to concentration found in literature (Groppa et al., 2001). Four biological replicates (i.e., sample sets collected during four subsequent repeats of the entire time-course of the experiment) were obtained.

2.1. Isolation of thylakoid-enriched fraction

Prothylakoids and thylakoid membranes from etiolated greening leaves were isolated according to the procedure of Sobieszczuk-Nowicka et al. (2009). Leaves were homogenized in a mortar in a buffer for chloroplast isolation containing 0.4 M sucrose. The homogenate was then filtered and centrifuged for 10 min at 1,500 × *g*. A chloroplast pellet was suspended in an isolation buffer and layered on 3 mL of isolation buffer containing 65% sucrose and centrifuged as above. Chloroplasts were collected from the top of the sucrose layer and centrifuged as previously. Then chloroplasts were osmotically shocked in an isolation buffer without sucrose, and chloroplast membranes were collected by centrifugation for 15 min at 12,000 × *g*. The membrane pellet was washed twice in the buffer without sucrose and centrifuged as above. The weighed pellet was used for determination of PA content. The intactness and purity of the isolated plastids was evaluated by phase contrast microscopy as well as by enzymatic and immunological approaches (glutamate dehydrogenase, a mitochondrial marker enzyme, UDP-glucose pyrophosphorylase, cytosolic marker enzyme). They confirmed that the samples can be reliably used for subsequent studies.

2.2. Isolation of chromatin

Chromatin was isolated by the procedure of Huang and Bonner (1962) modified by Schneider et al. (1988). Frozen leaves were powdered in a mortar and mixed with homogenization buffer (100 mM Tris–HCl, pH 6.0, 100 mM MgCl2, 20 mM mercaptoethanol, 250 mM sucrose). The homogenate was filtered through 4 layers of gauze and 2 layers of Miracloth. The filtrate was centrifuged at 400 × *g* for 4 min, and the pellet was discarded. The supernatant containing chromatin was treated with 30% Triton X-100 to a final concentration of 2% to disintegrate the chloroplasts, and centrifuged at 12,000 × *g* for 10 min. The chromatin pellet was suspended in a buffer containing 10 mM Tris–HCl, pH 6.0, 10 mM mercaptoethanol, 350 mM sucrose and 2% Triton X-100 and centrifuged again at 12,000 × *g* for 10 min. To remove Triton, the pellet was washed twice with the above buffer without Triton, each time centrifuging the suspension at 12,000 × *g* for 10 min. After centrifugation, the chromatin pellet was weighed and used for determination of PAs.

2.3. Chlorophyll analysis

The extraction of chlorophyll was carried out according to Hiscox and Israelstam (1979) using dimethylsulfoxide (DMSO). Fresh material (100 mg) was incubated with 5 mL of DMSO at 65 °C for 2 h. The content of Chl a was quantified by a spectrophotometer (Shimadzu UVVis-160) with emission at 665 and Chl b at 649 nm.

2.4. Measurement of chlorophyll fluorescence induction kinetics

Measurements of chlorophyll fluorescence induction kinetics of non-stressed and Pb-incubated barley leaves (24 h) were performed by using a pulse amplitude modulated PAM fluorometer (FMS1, Hansatech) according to Lichtenthaler et al. (2005). A predarkened leaf reveals minimal fluorescence (F_0) under low red light. Measurement of other fluorescence parameters is based on transient fluorescence induction kinetics known as the Kautsky effect. After fluorescence rise, induced by high saturating light pulse, to a maximum intensity level (Fm), the leaf is left for a slow fluorescence