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Induction of oxidative stress related responses in *Arabidopsis thaliana* following uranium exposure

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Abstract. The reactive oxygen species (ROS)-signaling pathway is very important in heavy metal toxicity. Induction of the antioxidative defense mechanism, comprising ROS-scavenging enzymes and metabolites, in plants after environmental uranium contamination has been insufficiently studied in the past. This study aimed to analyze oxidative stress related responses in *Arabidopsis thaliana* after uranium exposure. Seventeen-day-old seedlings were exposed to 0, 0.1, 1, 10 and 100 μM uranium for 3 days. After exposure to 100 μM uranium, a decrease in fresh weight for leaves and roots was observed, leaves colored anthocyanous and roots were stunted and yellow. To reveal the importance of oxidative stress in uranium toxicity, alterations in ROS-scavenging enzymes were studied at protein and transcriptional level. Superoxide dismutase (SOD) capacities increased in leaves and roots after exposure to 100 μM uranium but no differences were observed for catalase (CAT) capacities. Transcript levels of different SODs located at various cellular compartments were affected depending on the place of action. Gene expression of CAT in leaves and roots was also affected after uranium exposure. Results indicate that oxidative stress plays an important role in uranium toxicity but suggest that plant responses differ for leaves and roots.

1. INTRODUCTION

Uranium mining and milling, metal mining and smelting and industrial activities exploiting materials containing naturally occurring radionuclides (*e.g.* phosphate industry) have caused radioactive contamination of the environment in many countries [1]. Uranium toxicity effects are predominantly studied on man and animal species [2] but little information is available for plants. Especially information on responses induced in plants after exposure to low uranium concentrations is scant. Notwithstanding, information on the contamination impact could be of great importance for risk assessment and derivation of clean-up standards.

Plants exposed to environmental stress situations (*e.g.* heavy metals, drought ...) can experience oxidative stress. Reactive oxygen species (ROS) are produced in stressed and unstressed cells and have a dual role as toxic byproducts of aerobic metabolism and key regulators in growth, development and defense pathways [3]. To regulate the steady-state level of ROS, cells have developed an antioxidative defense system comprising ROS-scavenging enzymes (*e.g.* superoxide dismutases (SODs), catalase (CAT) ...) and antioxidants (*e.g.* ascorbic acid, glutathione ...) [3, 4]. Exposure to heavy metals can disrupt the cellular redox balance, resulting in an increase/decrease of the capacity of the antioxidative defense mechanism as was shown by several authors [5–7].

In this study, responses on the development of *Arabidopsis thaliana* seedlings were investigated after exposure to different uranium concentrations for 3 days. The importance of oxidative stress related responses in uranium toxicity was evaluated by analyzing relevant enzymes of the antioxidative defense system on protein and transcriptional level.

2. MATERIALS AND METHODS

2.1 Plant culture and uranium exposure

Seeds of *Arabidopsis thaliana* (Columbia ecotype) were placed on moist filter paper at 4 °C for 3 days in order to synchronize germination. Afterwards, seeds were placed on plugs from 1.5 ml polyethylene centrifuge tubes filled with agar. The plugs were positioned in a PVC cover capable of holding 81 plugs. The PVC cover was placed on a container filled with 2.9 l of a modified Hoagland solution. Plants were grown in a growth chamber (Microclima 1000E, Snijders Scientific B.V.) under a 14 h photoperiod (photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} at the leaf level, supplied by Sylvania BriteGro F36WT8/2084 and F36WT8/2023), with day/night temperatures of 22 °C/18 °C and 65% relative humidity.

Seventeen-day-old plants were then exposed for 3 days to 0, 0.1, 1, 10 and 100 μM uranium. Uranium was supplied as $UO_2(NO_3)_2 \cdot 6H_2O$ (Sigma) and the pH was adjusted to $\pm$5.5 with NaOH.

2.2 Plant sampling and biometric measurements

At harvest, fresh weight of leaves and roots was determined and samples were snap frozen in liquid nitrogen and stored at −80 °C.

Samples for uranium analyses were dried for 1 week at 70 °C. Leaves were rinsed with distilled water and roots were washed twice for 10 minutes with 10 mM $Pb(NO_3)_2$ at 4 °C to exchange surface-bound uranium.

2.3 Uranium analyses

After dry-ashing using a muffle furnace, dried plant material was digested in 0.1 M HCl for uranium determination. The ^{238}U concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS, Perkin-Elmer).

2.4 Enzyme capacities

Frozen leaf or root tissue (approximately 100 mg) was homogenized in 2 ml ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 4% insoluble polyvinylpyrrolidone, using a blender. The homogenate was squeezed through a nylon mesh and centrifuged at 20000 × g and 4 °C for 10 minutes. The enzyme capacities were measured spectrophotometrically in the supernatant at 25 °C.

Analysis of the capacity of catalase (CAT, EC 1.11.1.6) was performed as described by Bergmeyer et al. [8]. Analysis of superoxide dismutase capacity (SOD, EC 1.15.1.1) was based on the inhibition of cytochrome C at 550 nm according to McCord and Fridovich [9].

2.5 Gene expression

Frozen leaf or root tissue (approximately 100 mg) was ground thoroughly in liquid nitrogen using a mortar and a pestle. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). The RNA concentration was determined spectrophotometrically at 260 nm (Nanodrop, Isogen Life Science).

First strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) and equal amounts of starting material were used (1 μg).

Quantitative real time PCR was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems), using Sybr® Green chemistry.

Gene expression data were normalized against multiple housekeeping genes according to Vandesompele et al. [10] and represented relative to the control treatment (untreated leaves).

2.6 Statistical analyses

For the statistical analysis of the fresh weight of leaves and roots, the one way non parametric Kruskall Wallis test was applied to see if there were differences between the treatments. Subsequently, the Wilcoxon Mann Whitney test with normal approximation, adjusting by multiple comparisons using Bonferroni correction, was used to find which treatments were different by performing pair wise comparisons [11].

For the statistical analysis of the uranium content, enzyme capacities and gene expression in leaves and roots, a one way parametric analysis of variance with Tukey's multiple comparison test was used to see if there were differences between the treatments [12].

3. RESULTS AND DISCUSSION

Exposure of plants to environmental stress situations (*e.g.* heavy metals, radiation, drought ...) can enhance the production of ROS as was described by several authors [3, 4, 13, 14]. As a response to the disruption of the cellular redox balance, the antioxidative defense system, comprising ROS-scavenging enzymes and metabolites, can be activated. While oxidative stress related responses induced in plants after exposure to other heavy metals are well investigated [6, 7, 14–16], alterations in the antioxidative defense system after uranium contamination are understudied. In this study early responses on growth and development and the importance of the antioxidative defense system after uranium stress were investigated for *Arabidopsis thaliana*.

After exposure to 100 μM uranium, anthocyanous-colored leaves were observed and the fresh weight was significantly reduced (Fig. 1). An increase in fresh weight for roots was observed after exposure to 1 and 10 μM uranium (Fig. 1) which could be explained by a hormesis effect. But after exposure to 100 μM uranium roots were stunted, turned yellow and a significant reduction in fresh weight was observed (Fig. 1). Vandenhove et al. [5] also reported an increasing trend in growth parameters for *Phaseolus vulgaris* after application of 0–10 μM uranium, and a decrease (not significant) after exposure to 1000 μM uranium. Inhibition of leaf expansion and/or growth was also reported by several authors after exposure of *Phaseolus vulgaris* to cadmium [6], zinc [14] and copper [15].

The uranium content in the roots increased significantly when exposed to a uranium concentration range (table 1). Only exposure to 100 μM uranium resulted in an enhancement of the uranium content in the leaves (table 1). Transfer to the leaves was limited, a root-to-shoot transfer factor of $\pm 1.5 \times 10^{-4}$ was observed for *Arabidopsis thaliana* plantlets exposed for 3 days to 100 μM uranium.

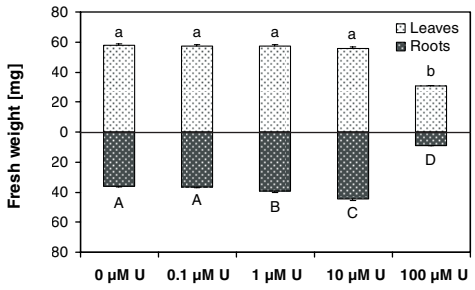

Figure 1. Fresh weight [mg] of leaves and roots of *Arabidopsis thaliana* seedlings exposed for 3 days to 0, 0.1, 1, 10 and 100 μM uranium. Each point represents the mean $\pm$ S.E. of 162 biological replicates. Values with different letters are significantly different ($p < 0.05$).

Exposure of plants to biotic or abiotic stresses can enhance the production of ROS, which can potentially lead to damage [3, 4]. The SODs constitute the first line of defense against ROS; they

Table 1. Uranium concentration [μg g^{-1} DW] in leaves and roots of *Arabidopsis thaliana* seedlings exposed to 0, 0.1, 1, 10 and 100 μM uranium for 3 days. Presented is the mean $\pm$ S.E. of at least 3 biological replicates. Values with different letters are significantly different ($p < 0.05$).

	Uranium concentration [μg g^{-1} DW]	
	Leaves	*Roots*
0 μM U	1.1 ± 0.3^{ab}	4 ± 0.4^{A}
0.1 μM U	0.7 ± 0.1^{ab}	162 ± 7^{B}
1 μM U	0.6 ± 0.2^{a}	915 ± 244^{C}
10 μM U	2.1 ± 0.7^{b}	8425 ± 1646^{D}
100 μM U	10.2 ± 2.4^{c}	67582 ± 3640^{E}

transform $O_2^{\bullet-}$ to H_2O_2. As $O_2^{\bullet-}$ is formed at locations where an electron transport chain is present (*e.g.* mitochondria, chloroplasts, cytosol...), it is important that SODs are present in all these compartments [17]. Depending on the metal co-factor used and the location in the cell, these SODs can be classified into 3 groups: copper-zinc SOD (CuZnSOD) which is found throughout the plant cell, iron SOD (FeSOD) located in the plastids including the chloroplast, and manganese SOD (MnSOD) located in the mitochondria and peroxisomes [17]. To investigate the importance of SOD in the defense against ROS produced after uranium exposure, transcript levels of *csd1*, *fsd1* and *msd1* were analyzed.

Csd1 (CuZnSOD located in the cytosol) was significantly down-regulated for leaves and roots after exposure to 10 and 100 μM uranium (Fig. 2A) suggesting antioxidative defense via this pathway was limited. Concerning *fsd1* (FeSOD located in the plastids) expression, a different pattern was observed. Transcript levels were significantly down-regulated in leaves exposed to 100 μM uranium as compared with the exposure to 10 μM uranium (Fig. 2B). Roots on the other hand showed an increased expression of *fsd1* after exposure to 100 μM uranium (Fig. 2B). The results for *fsd1* indicate that the antioxidative defense against $O_2^{\bullet-}$ is enhanced in the plastids of the roots but falls down in the chloroplasts of the leaves

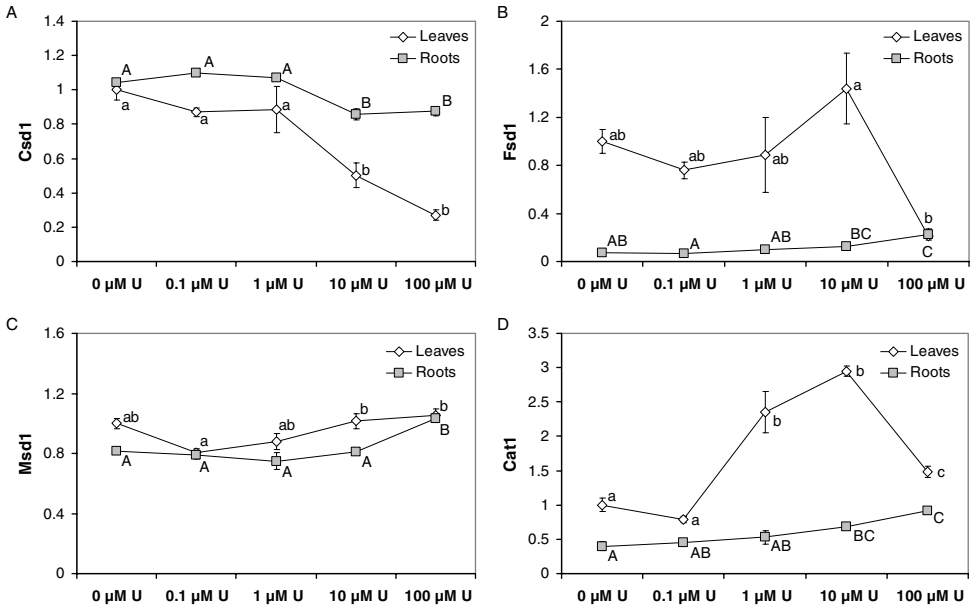

Figure 2. Gene expression of different ROS-scavenging enzymes. Expressions in leaves and roots of *Arabidopsis thaliana* seedlings exposed for 3 days to 0, 0.1, 1, 10 and 100 μM uranium are relative to the control treatment (leaves untreated). Values are the mean $\pm$ S.E. of 3 biological replicates. Results with different letters are significantly different ($p < 0.05$).

after exposure to 100 μM uranium. Transcript levels of *msd1* (MnSOD located in the mitochondria) in leaves were least altered (Fig. 2C) suggesting mitochondria in leaves are doing well under uranium stress. A significant increase in *msd1* transcript levels was observed for roots exposed to 100 μM uranium (Fig. 2C) indicating the antioxidative defense is triggered in mitochondria of the roots. The difference in expression for CuZnSOD, FeSOD and MnSOD can be explained by their location within the cell and the site of action for uranium induced oxidative stress as was also stated by Alscher et al. [17] for *Arabidopsis thaliana* exposed to different stressors. At the protein level an increase in SOD capacities was observed for leaves exposed to 10 and 100 μM uranium and roots exposed to 100 μM uranium (Fig. 3A). These results indicate an enhanced detoxification of $O_2^{\bullet -}$. Reported effects by other authors on SOD capacities depend on the applied metal and plant species used [14–16].

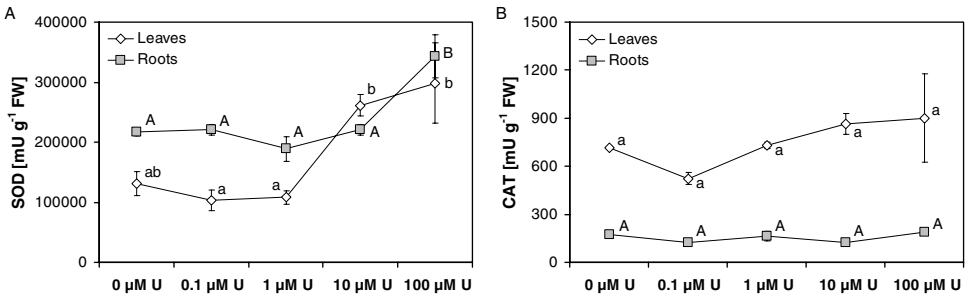

Figure 3. Enzyme capacities [mU g^{-1} FW] for superoxide dismutase (SOD) (A) and catalase (CAT) (B) in leaves and roots of *Arabidopsis thaliana* seedlings exposed for 3 days to 0, 0.1, 1, 10 and 100 μM uranium. Values represent the mean $\pm$ S.E. of at least 3 biological replicates. Data points with different letters are significantly different ($p < 0.05$).

After conversion of $O_2^{\bullet -}$ into H_2O_2, several enzymes (*e.g.* CAT, ascorbate peroxidase ...) regulate the transformation of H_2O_2 into H_2O. Transcript levels for *cat1* (CAT located in the peroxisomes) in the leaves were up-regulated after exposure to 1 and 10 μM uranium but down-regulated after exposure to 100 μM uranium (Fig. 2D). In the roots only a small enhancement was observed after exposure to 100 μM uranium (Fig. 2D). These results suggest that CAT plays a role in the antioxidative defense pathway triggered after uranium exposure. But on the protein level, CAT capacities were not affected by uranium exposure for leaves or roots (Fig. 3B). Similar results were reported by Weckx and Clijsters [15]: zinc exposure had no effect on CAT capacities in *Phaseolus vulgaris* seedlings.

4. CONCLUSIONS

This study aimed to analyze early effects on growth and development of *Arabidopsis thaliana* seedlings and investigate the importance of the antioxidative defense system after uranium stress. Results indicate important toxicity effects on growth and plant development after exposure to 100 μM uranium. Furthermore, the study indicates that the induction of oxidative stress related responses plays a role in uranium stress but responses differ for leaves and roots.

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