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Unraveling uranium induced oxidative stress related responses in *Arabidopsis thaliana* seedlings. Part I: responses in the roots

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ABSTRACT

When aiming to evaluate the environmental impact of uranium contamination, it is important to unravel the mechanisms by which plants respond to uranium stress. As oxidative stress seems an important modulator under other heavy metal stress, this study aimed to investigate oxidative stress related responses in *Arabidopsis thaliana* exposed to uranium concentrations ranging from 0.1 to 100 μM for 1, 3 and 7 days. Besides analyzing relevant reactive oxygen species-producing and -scavenging enzymes at protein and transcriptional level, the importance of the ascorbate–glutathione cycle under uranium stress was investigated. These results are reported separately for roots and leaves in two papers: Part I dealing with responses in the roots and Part II unraveling responses in the leaves and presenting general conclusions. Results of Part I indicate that oxidative stress related responses in the roots were only triggered following exposure to the highest uranium concentration of 100 μM. A fast oxidative burst was suggested based on the observed enhancement of lipoxygenase (*LOX1*) and respiratory burst oxydase homolog (*RBOHD*) transcript levels already after 1 day. The first line of defense was attributed to superoxide dismutase (SOD), also triggered from the first day. The enhanced SOD-capacity observed at protein level corresponded with an enhanced expression of iron SOD (*FSD1*) located in the plastids. For the detoxification of H_2O_2, an early increase in catalase (*CAT1*) transcript levels was observed while peroxidase capacities were enhanced at the later stage of 3 days. Although the ascorbate peroxidase capacity and gene expression (*APX1*) increased, the ascorbate/dehydroascorbate redox balance was completely disrupted and shifted toward the oxidized form. This disrupted balance could not be inverted by the glutathione part of the cycle although the glutathione redox balance could be maintained.

1. Introduction

Environmental uranium contamination due to processes such as uranium mining and milling, metal mining and smelting and the phosphate industry has occurred in many countries (Vandenhove, 2002). Uranium is a naturally occurring radionuclide and heavy metal with a greater risk of chemical toxicity than radiological toxicity because of its very low specific activity of 12.4 kBq g^{-1} (Sheppard et al., 2005). While uranium toxicity effects are predominantly studied on man and animal species, hardly any information exists on toxicity effects in plants (Ribera et al., 1996). When evaluating the environmental impact of uranium contamination, mechanisms by which plants respond to uranium stress should be unraveled. However, information concerning uranium induced stress response mechanisms at molecular and biochemical level in plants is scant.

Induction of oxidative stress related responses in plants seems an important stress response mechanism for other heavy metals as was reported by several authors (Cuypers et al., 2001, 2002; Van Belleghem et al., 2007; Smeets et al., 2008). The oxidative

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; CSD, copper-zinc superoxide dismutase; DHA, dehydroascorbate; DTT, dithiothreitol; FSD, iron superoxide dismutase; GPX, guaiacol peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; LOX, lipoxygenase; MSD, manganese superoxide dismutase; PX, peroxidase; RBOH, respiratory burst oxydase homolog; ROS, reactive oxygen species; SOD, superoxide dismutase; SPX, syringaldazine peroxidase.

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burst is one of the earliest responses of plant cells under various stress conditions, possibly acting as an alarm signal to alert metabolism and gene expression for possible modifications (Foyer et al., 1994). Plasma membrane NADPH-oxidases can be a source of reactive oxygen species (ROS) during the oxidative burst and the transcriptional activation of certain NADPH-oxidases is an essential intermediate step in the activation or amplification of defense responses (Dat et al., 2000; Mittler et al., 2004). Lipid peroxidation can be due to an increase in lipoxygenase (LOX) activity, which initiates the formation of oxylipins (Porta and Rocha-Sosa, 2002). LOXs are shown to play a role in cadmium induced oxidative stress in *Arabidopsis thaliana* roots (Smeets et al., 2008).

ROS are also produced under normal circumstances and have a dual role both as toxic byproducts of aerobic metabolism and as key regulators of biological processes as growth, cell cycle, biotic and abiotic stress responses (Mittler et al., 2004). These ROS can cause cellular damage by oxidizing biological molecules as DNA, proteins and lipids. To regulate the amount of ROS and thus to allow signal transduction processes but limit oxidative damage under normal circumstances and in stress situations, plants use an antioxidative defense system comprising ROS-scavenging enzymes and metabolites located in different plant cell compartments. Superoxide dismutase (SOD) constitutes the first line of defense against ROS as an $O_2^{\bullet -}$-detoxifying enzyme while enzymes such as catalase (CAT) and peroxidases (PX) have a role in the scavenging of H_2O_2, which can act as a signaling molecule. Alterations in enzyme capacities of relevant enzymes of the antioxidative defense system in plant roots following heavy metal stress have been reported by several authors (Cuypers et al., 2002; Smeets et al., 2005; Van Belleghem et al., 2007). Only recently, studies also include analyses of ROS-producing and -scavenging enzymes at transcriptional level (Smeets et al., 2008). The ascorbate–glutathione pathway also plays an important role in the antioxidative defense mechanism against heavy metal stress as was reported by Cuypers et al. (2001) for *Phaseolus vulgaris* roots exposed to zinc.

Uranium can induce physiological and morphological effects and alter the nutrient profile of plants (Vandenhove et al., 2006; Vanhoudt et al., 2008). In addition, previous studies already suggested a role for the cellular redox balance as a modulator in uranium stress for *A. thaliana* (Vanhoudt et al., 2008) and *P. vulgaris* (Vandenhove et al., 2006) but information remains limited. Vanhoudt et al. (2008) reported an induction of oxidative stress related responses in *A. thaliana* leaves and roots exposed to 100 μM uranium. As in the latter study it was shown that uranium can disrupt the cellular redox balance but only one concentration was used, a more profound research was suggested to further unravel uranium stress response mechanisms.

The aim of this study was to further unravel uranium induced oxidative stress related responses in *A. thaliana* and achieve a better understanding of the importance of the cellular redox balance as a modulator in uranium stress. For this purpose, *A. thaliana* seedlings were exposed to uranium concentrations ranging from 0.1 to 100 μM for 1, 3 and 7 days. We investigated which NADPH-oxidases and LOXs contribute to an enhanced ROS-production while several ROS-scavenging enzymes (SOD, CAT, PX) were analyzed to unravel their role in the protection from and regulation of ROS under uranium stress. In addition, different enzymes and metabolites contributing to the ascorbate–glutathione cycle were analyzed to evaluate their importance under uranium stress. In this Part I, oxidative stress related responses in the roots will be discussed. In Part II, oxidative stress related responses in the leaves are discussed and general conclusions are drawn based on the results from the roots and the leaves (Vanhoudt et al., 2011).

2. Materials and methods

2.1. Plant culture and uranium exposure

Seeds of *A. thaliana* (Columbia ecotype) were spread on moist filter paper at 4 °C for 3 days in order to synchronize germination. Afterward, the seeds were sown on polyethylene plugs filled with 2% agar (Difco). The plugs were placed in a PVC cover capable of holding 81 plugs. Next, the PVC cover was placed on a container filled with 2.9 L of a modified Hoagland solution (macro-elements without phosphate: 1/10 diluted, phosphate solution: 1/20 diluted, micro-elements: 1/10 diluted and iron solution: 1/10 diluted). 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 lamps), with day/night temperatures of 22 °C/18 °C and 65% relative humidity. Roots were aerated during the entire experiment.

Subsequently, 17-day-old seedlings were exposed to 0, 0.1, 1, 10 and 100 μM uranium. Uranium was added as $UO_2(NO_3)_2 \cdot 6H_2O$ (Sigma) to the modified Hoagland solution and the pH was adjusted to ± 5.5 with NaOH. As previous experiments recommended the use of 25 μM phosphate in combination with uranium, a 1/80 diluted phosphate solution was used (Vanhoudt et al., 2008).

Following 1, 3 and 7 days exposure, roots and leaves were harvested separately as ±100 mg samples, snap frozen in liquid nitrogen and stored at −80 °C. With roots the complete root system of a plant is meant and never only a portion of the root. With leaves the full rosette of a plant is meant and never a single leaf or a part of the rosette. Therefore, each ±100 mg sample was made by pooling at random a number of complete roots or leaf rosettes from different plants in different containers.

2.2. Analysis of enzyme capacities

Frozen root tissue (approximately 100 mg) was homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 4% insoluble polyvinylpyrrolidone (2 mL buffer for 100 mg FW), using a mortar and pestle. The homogenate was squeezed through a nylon mesh and centrifuged at 20,000×*g* and 4 °C for 10 min. The enzyme capacities, i.e. potential activity measured in vitro under non-limiting reaction conditions, were measured spectrophotometrically in the supernatant at 25 °C.

Guaiacol peroxidase and syringaldazine peroxidase capacities (GPX, SPX, EC 1.11.1.7) were measured at 436 nm and 530 nm according to Bergmeyer et al. (1974) and Imberty et al. (1984), respectively. Ascorbate peroxidase capacity (APX, EC 1.11.1.11) was measured at 298 nm following the method of Gerbling et al. (1984). 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 (1969). Analyses of the capacities of glutathione reductase (GR, EC 1.6.4.2) and catalase (CAT, EC 1.11.1.6) were performed as described by Bergmeyer et al. (1974).

2.3. Gene expression analysis

Frozen root tissue (approximately 100 mg) was ground thoroughly in liquid nitrogen using a mortar and pestle. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). The RNA quantity was determined spectrophotometrically at 260 nm (Nanodrop, Isogen Life Science). The RNA quality was checked electrophoretically using the Bioanalyzer (Agilent Technologies). Before cDNA synthesis, the RNA sample was incubated during 2 min in gDNA wipeout buffer at 42 °C in order to effectively eliminate genomic DNA. First strand cDNA synthesis was primed with a combination of