Oxidative stress responses induced by uranium exposure at low pH in leaves of *Arabidopsis thaliana* plants

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ABSTRACT

Anthropogenic activities have led to a widespread uranium (U) contamination in many countries. The toxic effects of U at the cellular level have mainly been investigated at a pH around 5.5, the optimal pH for hydroponically grown plants. However, since the speciation of U, and hence its toxicity, is strongly dependent on environmental factors such as the pH, it is important to investigate the effects of U at different environmentally relevant pH levels. Although U is poorly translocated from the roots to the shoots, resulting in a low U concentration in the leaves, it has been demonstrated that toxic effects in the leaves were already visible after 1 day exposure at pH 5.5, although only when exposed to relatively high U concentrations (100 μM). Therefore, the present study aimed to analyse the effects of different U concentrations (ranging from 0 to 100 μM) at pH 4.5 in leaves of *Arabidopsis thaliana* plants. Results indicate that U induces early senescence in *A. thaliana* leaves as was suggested by a decreased expression of *CAT2* accompanied by an induction of *CAT3* expression, a decreased CAT capacity and an increased lipid peroxidation. In addition, miRNA398b/c is involved in the regulation of the SOD response in the leaves. As such, an increased *MIR398b/c* expression was observed leading to a decreased transcript level of *CSD1/2*. Finally, the biosynthesis of ascorbate was induced after U exposure. This can point towards an important role for this metabolite in the scavenging of reactive oxygen species under U stress.

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1. Introduction

Uranium-238 (U) is a naturally occurring radionuclide and heavy metal. Uranium-238, the dominant naturally occurring radionuclide, has a very long decay half-life of 4.47×10^9 years, giving it a low specific activity of 1.25×10^4 Bq g^{-1} (Sheppard et al., 2005).

Therefore, it has a greater risk for chemical toxicity than radiological toxicity. Typical concentrations of U in the soil range from 0.3 to 11.7 mg kg^{-1} soil, while concentrations in surface- or ground-waters vary between 3×10^{-2} and 2.1 µg l^{-1} (Bleise et al., 2003). However, in some minerals (e.g. zircon) and rare earths, the concentration may be elevated up to 800 mg kg^{-1} (Vandenhove, 2002). Uranium contamination has occurred in many countries due to anthropogenic processes such as U mining and milling, metal mining and smelting and the phosphate industry (Vandenhove, 2002). The distribution, mobility and biological availability of U not only depends on its concentration, but is strongly dependent on the physicochemical form of the element, which in turn depends on environmental parameters such as the pH (Bernhard, 2005). Since the pH of pore waters can vary significantly (Nagao et al., 2002), it is important to investigate the environmental impact of U under different ecologically relevant conditions. The pH-dependent speciation of U in Hoagland nutrient solution, the nutrient solution used to grow *Arabidopsis thaliana* plants, has been reported by Saenen et al. (2013). By using the Geochemist's Workbench modelling software, they predicted at low pH (pH 4.5) mainly the presence of the free

uranyl ion (UO_2^{2+}). This chemical species is supposed to be the most toxic to aquatic biota (Markich et al., 1996). At pH 7.5, U carbonate species were mainly present (Saenen et al., 2013). While U accumulation and distribution in plants has been reported by several authors (Ebbs et al., 1998; Laroche, 2005; Laurette et al., 2012; Straczek et al., 2010; Tomé et al., 2009), little information is available on the toxicity of different U species at the cellular level.

It has been demonstrated that U and other heavy metals can induce oxidative stress related responses in plants (Cuypers et al., 2011; Saenen et al., 2013; Smeets et al., 2008; Vandenhove et al., 2006; Vanhoudt et al., 2008, 2011a). Under normal conditions, ROS are produced as second messengers in many processes associated with plant growth and development (Foyer and Noctor, 2005). However, under stress conditions, the production of ROS can be enhanced or the ROS scavenging mechanisms can be impaired, a state that is called oxidative stress (Foyer and Noctor, 2005). Plant NADPH oxidases, also called respiratory burst oxidase homologues (RBOHs), have been identified in plants as a source of ROS by transferring electrons from cytoplasmatic NADPH to molecular oxygen (O_2) to form superoxide ($O_2^{\cdot-}$) (Karuppanapandian et al., 2011; Mittler, 2002). Lipoxygenases (LOX) can also be involved in ROS production. They catalyse the dioxygentaion of polyunsaturated fatty acids (PUFA) such as lonleic acid. When the hydroperoxyderivates of PUFAs degrade, they can produce radicals that, in turn, will initiate lipid peroxidation. In addition, LOX can also mediate the formation of singlet oxygen and $O_2^{\cdot-}$ (Blokhina et al., 2003). To control the concentration of ROS, plants have evolved an antioxidative defence system consisting of enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (Px), and antioxidants such as ascorbate (AsA) and glutathione (GSH) (Mittler et al., 2004). SOD acts as the first line of defence against ROS by dismutating $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2). Catalases and Px subsequently detoxify H_2O_2 (Apel and Hirt, 2004). Concerning the non-enzymatic antioxidative systems, AsA can directly scavenge $O_2^{\cdot-}$, hydroxyl radicals ($OH^\bullet$) and singlet oxygen ($^1O_2$) and can reduce H_2O_2 to H_2O via ascorbate peroxidase (APX). Glutathione can also react non-enzymatically with ROS. However, the central role for GSH is its ability to regenerate AsA via the AsA-GSH cycle (Karuppanapandian et al., 2011).

Vanhoudt et al. (2008, 2011b) and Doustaly et al. (2014) showed before that U mainly accumulates in the roots with limited transfer to the leaves. However, U exposure affects the transcript levels of several antioxidative and ROS-producing genes in leaves of *A. thaliana* plants at pH 5.5. Responses were already visible after 1 day U exposure to 100 μM U, although the internal U concentrations in the leaves were negligible at that time (Vanhoudt et al., 2011a). This indicates a possible role for root-to-shoot signalling in the oxidative stress responses after U exposure (Vanhoudt et al., 2011a). However, the latter study was carried out at pH 5.5 which is the optimal pH of the Hoagland solution normally used to grow plants hydroponically. As such, the influence of the different U species present at different pH values are not taken into account. Saenen et al. (2013) showed that the U uptake and related stress responses are strongly influenced by the pH. Since in that study only one U concentration (25 μM) was applied, the present study will further investigate the U-induced stress responses in *A. thaliana* leaves at low pH. For this purpose, 18-day-old *A. thaliana* plants were exposed to different U concentrations ranging from 0 to 100 μM U at pH 4.5 during 3 days.

2. Materials and methods

2.1. Plant culture and treatment

Arabidopsis thaliana seeds (Columbia ecotype) were surface sterilized and incubated in the dark for 3 day at 4 °C on moist filter paper to synchronize germination. Seeds were sown on plugs from 1.5 ml polyethylene centrifuge tubes filled with 0.6% agar in Hoagland solution with low phosphate content (Vanhoudt et al., 2008). The plugs were positioned in a PVC cover capable of holding 36 plugs. Next, the cover was placed on a container filled with 1.35 l of a modified Hoagland solution with a pH of 5.5 (1 mM KNO_3, 0.3 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM $NH_4H_2PO_4$, 1.62 μM $FeSO_4$, 0.78 μM EDTA, 4.6 μM H_3BO_3, 0.9 μM $MnCl_2$, 32 nM $CuSO_4$, 55.6 nM H_2MoO_4, 76.5 nM $ZnSO_4$). Plants were grown in a growth chamber (Microclima 1000E, Snijders Scientific B.V.) under a 14 h photoperiod (photosynthetic photon flux density of 150 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. After 18 days preculture, the pH of the nutrient solution was adjusted to pH 4.5 with NaOH and HCl. To retain the pH at a constant level, 500 μM MES (2-(N-morpholino)ethanesulfonic acid) and 500 μM TRIS (tris(hydroxymethyl)-aminomethane) were added. Plants were exposed to 0, 6.25, 12.5, 25, 50, 75 or 100 μM U. Uranium was added as $UO_2(NO_3)_2.6H_2O$ (SPI chemicals, USA) from a 100 mM stock solution to the Hoagland nutrient solution. Since roots can exudate organic acids or anions (Akhtar et al., 2009), the pH of the nutrient solution was adjusted twice a day. During the exposure time, a modified Hoagland solution was used with 0.025 mM $NH_4H_2PO_4$ (Vanhoudt et al., 2008). After 3 days of exposure, plants were harvested. Leaf fresh weight was determined and samples were snap frozen in liquid nitrogen and stored at −80 °C. Leaf growth was determined as (fresh weight$_{day\ 21}$−fresh weight$_{day\ 18}$)/(fresh weight$_{control\ leaves\ day\ 21}$−fresh weight$_{control\ leaves\ day\ 18}$)]*100.

2.2. Uranium analysis

The fraction of U in solution was measured in medium samples taken at the beginning of the experiment. Samples were acidified with HCl prior to analysis with ICP-MS (see below). Leaf samples were dried for at least 1 week at 70 °C. The oven-dried samples were calcinated in a muffle furnace at 550 °C. After cooling down to room temperature, the plant material was digested into 1 M HCl. The U-238 concentration in these samples was determined by using a quadrupole inductively coupled plasma - mass spectrometer (ICP-MS) (XSeries II, Thermo Scientific, Bremen, Germany) equipped with a PFA-ST Nebulizer (Elemental Scientific, Omaha, Nebraska, USA) and a peltier cooled (2 °C) cyclonic quartz spray chamber for sample introduction. Calibration curves were established using U standard solutions (0–10 μg l^{-1}) prepared from a single element stock solution (SPEX Industries Inc., Edison, NJ, USA). The instrumental detection limit for U was 2 ng L^{-1}. Typical precision for samples with U concentrations well above the limit of detection was below 5% (relative standard deviation, 10 replicates).

2.3. Determination of lipid peroxidation

Thiobarbituric acid reactive compounds (TBA-rc) were used as a measure for membrane damage. Approximately 120 mg of frozen shoots were homogenized in 1 ml 0.1% trichloroacetic acid (TCA) using an ice-cold mortar and pestle. After centrifugation at 20,000 × g for 10 min, 250 μL supernatant was diluted with 1 ml TBA/TCA solution (0.5% TBA in 20% TCA). The mixture was incubated for 30 min at 95 °C and quickly cooled down in an ice bath. After another centrifugation step of 10 min at 20,000 × g, the absorbance of the supernatant was determined spectrophotometrically at 532 nm and corrected for the non-specific absorbance at 600 nm (Dhindsa et al., 1981). The content of TBA-rc was calculated according to the law of Lambert−Beer ($\varepsilon = 155$ mM^{-1} cm^{-1}) taking into account the fresh weight and the dilutions made.