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Thorium impact on tobacco root transcriptome

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HIGHLIGHTS

- Thorium in 200 μM concentration up-regulated 152 and down-regulated 100 genes.
- Genes involved in response to abiotic and biotic stress were up-regulated.
- Changes in gene expression indicate disturbed phosphate and iron uptake.
- Negative regulation of intrinsic proteins indicated affected water homeostasis.
- *ZIF2*, *PCR2*, and *ABCG40* are suggested as thorium transporters.

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ABSTRACT

Thorium is natural actinide metal with potential use in nuclear energetics. Contamination by thorium, originated from mining activities or spills, represents environmental risk due to its radioactivity and chemical toxicity. A promising approach for cleaning of contaminated areas is phytoremediation, which need to be based, however, on detail understanding of the thorium effects on plants. In this study we investigated transcriptomic response of tobacco roots exposed to 200 μM thorium for one week. Thorium application resulted in up-regulation of 152 and down-regulation of 100 genes (p-value <0.01, fold change ≥ 2). The stimulated genes were involved in components of jasmonic acid and salicylic acid signaling pathways and various abiotic (e.g. oxidative stress) and biotic stress (e.g. pathogens, wounding) responsive genes. Further, up-regulation of phosphate starvation genes and down-regulation of genes involved in phytic acid biosynthesis indicated that thorium disturbed phosphate uptake or signaling. Also expression of iron responsive genes was influenced. Negative regulation of several aquaporins indicated disturbance of water homeostasis. Genes potentially involved in thorium transport could be zinc-induced facilitator *ZIF2*, plant cadmium resistance *PCR2*, and ABC transporter *ABCG40*. This study provides the first insight at the processes in plants exposed to thorium.

1. Introduction

Thorium is a radioactive actinide metal, present in small amounts in the environment. The concentration of thorium is around 6 ppm in the earth crust. More than 99% of thorium occurs as a radioisotope ^{232}Th with half-life 14 billion years, emitting especially alpha particles. It can be found in higher concentrations in some rocks (e.g. monazite sand), which are considered as a source of thorium [1].

The thorium mining has recently attracted enhanced attention due to its potential use in the nuclear industry (power-plants). Thorium content is three up to four times higher than that of the other natural actinide – uranium. This is the reason why some countries, such as India, prefer this element as potential fuel for nuclear power-plants [2]. Such use can enhance the risk for the environment, as thorium may be released during mining, processing or by an occasional accident. The hazard lays in radiological and chemical toxicity. Irradiation increases the probability of the occurrence of lung and pancreatic cancer, changes in the genetic material of somatic cells, liver damage and failure of haematogenesis [1]. Although most studies have been focused on long-term toxicity caused by irradiation, thorium is also substantially toxic

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element [3]. When Th exposure is sufficiently high, chemical toxicity exceeds the radiological one [4].

Plants can be used for the phytoremediation of areas contaminated by Th. The necessary prerequisite is, however, understanding of Th effect on the plants. Up-to-now, there is a lack of knowledge about this topic. Published studies mostly deal with the accumulation of thorium in the human food [5], medical plants [6], mushrooms [7], fern [8] or crops such as wheat [9]. Nowadays most investigations have been focused on the distribution of thorium in plants with the aim to investigate their ability to bind this metal. Zhou et al. [10] described the localization of thorium on molecular level but they did not determinate chemical structure of the precipitated thorium compound in the cell wall. Selected plants were tested *in situ* on uranium [11–14] or thorium mill tailings [15] and also on artificially supplied contaminated soils [16] or in liquid media [16–18] in laboratory conditions. Thorium transfer into the shoots was very low and most of it was accumulated in the root system. Presence of phosphate decreased Th as well as uranium uptake, while other modifications of cultivation substrates (e.g. addition of organic acids and polyamines) did not significantly influence Th accumulation in plants [17–20].

Exposition of plants to Th or uranium induced formation of reactive oxygen species (ROS) and subsequent activation of antioxidant defense system [21,22]. Recently, the effect of uranium on root transcriptome of *Arabidopsis thaliana* was studied [23]. Similar study on plants exposed to Th is, according to our knowledge, missing. Only transcriptomic response of *Saccharomyces cerevisiae* exposed to Th was explored [4]. Therefore we decided to investigate the effect of Th on the gene transcription in tobacco roots with the aim to reveal how this metal affects processes at molecular level in plants.

2. Material and methods

2.1. Plant material and cultivation conditions

Tobacco seeds were sown in Perlite and cultivated for two months. All seedlings were watered by Hoagland medium [24] every three days. The hydroponic medium with pH adjusted to 5.0 contained 4 mM $CaCl_2$, 2 mM K_2SO_4, 2 mM NH_4NO_3, 2 mM NaH_2PO_4, 1.5 mM $MgSO_4$, 4 mM $NaNO_3$, 4 mM NH_4Cl, 0.2 mM $FeSO_4$, 138.8 μM H_3BO_3, 20.8 μM $MnSO_4$, 2.3 μM $ZnSO_4$, 3.3 μM $CuSO_4$ and 0.2 μM Na_2MoO_4. Eight-week old plants were placed for one week into Araponics boxes (Araponics SA, Belgium) supplemented with 2 L of Hoagland hydroponic medium to acclimate to the hydroponic conditions. The plants were kept at 20° C under 16 h light period, humidity about 60%, average irradiation 72 μmol m^{-2} s^{-1} [17].

2.2. Plant treatment with thorium

Tobacco plants (*N. tabacum* L. cv. La Burley 21) grown in hydroponics were transferred into the Hoagland hydroponic medium containing 0.2 mM $Th(NO_3)_4$ (Lachema n.p., Brno, Czech Republic). Plants were exposed to $Th(NO_3)_4$ for seven days. The Th concentration and exposure time were selected according to previous toxicity tests [17]. During sampling for gene expression studies, roots and shoots were separated. The roots were then rinsed in demineralized water and dried on filtrated paper. The fresh weight of the samples was determined before freezing in liquid nitrogen and storing at −80° C until RNA isolation.

2.3. Microarray analysis

The RNA was isolated from the roots of *N. tabaccum* plants that were treated with $Th(NO_3)_4$ and of control, untreated ones using the Plant RNA Isolation Mini Kit (Agilent Technologies, CA, USA). RNA was then labeled using LowInput QuickAmp Labeling Kit (Agilent Technologies) by Cyanine 3 and Cyanine 5 using a dye swap design to avoid dye-based bias. Labeled cRNA was purified by RNeasy Plant Mini Kit (Qiagen, Germany), fragmented and hybridized on the Nicotiana (V4) Gene Expression Microarray (Agilent Technologies) according to the manufacturer's instructions. After a 17-h hybridization at 65 °C, slides were washed in GE Wash Buffers (Agilent Technologies), acetonitrile and Stabilization and Drying Solution (Agilent Technologies). Microarrays were scanned using a GenePix 4000 B scanner controlled by GenePix Pro Microarray Analysis Software (Molecular Devices, CA, USA). Experiments were repeated three times with root cRNA prepared independently from individual plants. The data acquired from the scanner were processed in R scripting environment using software package LIMMA according to Smyth and Speed [25], Smyth [26], and Smyth et al. [27]. The LOESS normalization method was used to balance the mean fluorescence intensities between the green and red channels in the frame of single arrays, and the Aquantile method was used to normalize signals among arrays. The background intensity was not subtracted from the overall spot intensities. The statistical analyses were performed without spots with zero weights. The false discovery rate (FDR) method was used for statistical evaluation. Genes showing ≥2-fold change in gene expression (p-value <0.01) were selected. Tobacco chip was reannotated using recently sequenced *N. tabacum* data [28]. Probes sequences were blasted against *N. tabacum* TN90 mRNA database (solgenomics.net) and annotation was assigned to those with resulting E-value $<10^{-6}$. Functional classification of up- and down-regulated transcripts was done by Classification Super-Viewer (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi) [29].

2.4. Quantitative real-Time PCR analysis

The transcription levels of selected genes obtained from microarrays were verified by the quantitative real-time PCR (RT-qPCR). RNA was treated with Ambion DNA-free kit (Thermo Fisher Scientific Inc., MA, USA) to eliminate the traces of genomic DNA. Complementary DNA (cDNA) was prepared from the total RNA by the M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant, Promega, WI, USA). The protocol for the first strand cDNA synthesis with oligo dT primers was used and the Protector RNase Inhibitor (Roche Applied Science, Mannheim, Germany) was added. 2.5 μL of 20 x diluted cDNA was mixed with the LightCycler 480 DNA SYBR Green I Master (Roche Applied Science, Mannheim, Germany) and 500 nM of respective primers to final volume 10 μL. The qPCR cycle included initiation (95 °C, 10 s), annealing (60 °C, 10 s) and elongation (72 °C, 10 s) steps and the reaction was performed by the Light Cycler 480 (Roche Applied Science, Mannheim, Germany). Relative content of RNA in sample was calculated according to Hellemans et al. [30]. *RPS4A* and *GTPbEFTu* genes were used for normalization. qPCR efficiency estimated for each primer pair from the calibration curve was used for the calculation. The list of primers is in Table 1.

3. Results and discussion

3.1. Transcriptomic response to thorium

Thorium (200 μM) up-regulated up-regulated 152 and down-regulated 100 genes (p-value <0.01, fold change ≥2) in the tobacco roots after 7-day exposure (complete list of up- and down-regulated genes is in Supplementary Table 1).