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Tungsten disrupts root growth in *Arabidopsis thaliana* by PIN targeting

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SUMMARY

Tungsten is a heavy metal with increasing concern over its environmental impact. In plants it is extensively used to deplete nitric oxide by inhibiting nitrate reductase, but its presumed toxicity as a heavy metal has been less explored. Accordingly, its effects on *Arabidopsis thaliana* primary root were assessed. The effects on root growth, mitotic cell percentage, nitric oxide and hydrogen peroxide levels, the cytoskeleton, cell ultrastructure, auxin and cytokinin activity, and auxin carrier distribution were investigated. It was found that tungsten reduced root growth, particularly by inhibiting cell expansion in the elongation zone, so that root hairs emerged closer to the root tip than in the control. Although extensive vacuolation was observed, even in meristematic cells, cell organelles were almost unaffected and microtubules were not depolymerized but reoriented. Tungsten affected auxin and cytokinin activity, as visualized by the DR5-GFP and TCS-GFP expressing lines, respectively. Cytokinin fluctuations were similar to those of the mitotic cell percentage. DR5-GFP signal appeared ectopically expressed, while the signals of PIN2-GFP and PIN3-GFP were diminished even after relatively short exposures. The observed effects were not reminiscent of those of any nitric oxide scavengers. Taken together, inhibition of root growth by tungsten might rather be related to a presumed interference with the basipetal flow of auxin, specifically affecting cell expansion in the elongation zone.

Introduction

Tungsten (W) is a rare transition metal with many industrial and military applications. As a result, it accumulates locally at increased concentrations, raising concerns about its possible harmful action on living organisms (Koutsospyros et al., 2006). Medical research has produced a huge amount of information, generally concluding that it could potentially induce serious adverse health effects in humans and animals (Witten et al., 2012). Moreover, tungsten has lately been characterized as a possible tumorigenic and leukemogenic factor (Kelly et al., 2013). Phytoremediation of polluted sites by tungsten phytoextraction could then be a promising perspective (Johnson et al., 2009) to protect human health and the environment from its potential toxicity. However, the observed inhibition of plant growth by tungsten (Adamakis et al., 2012) posed a significant problem for phytoextraction, rendering the acquisition of information about any effects on plants imperative.

Applied mainly in the form of sodium tungstate (Na_2WO_4), tungsten is hitherto extensively being used in the nitric oxide research, thus knowledge about its potential effects on plants until recently was primarily restricted to its use as an inhibitor of the enzyme nitrate reductase (Adamakis et al., 2012; Xiong et al., 2012). Nitrate reductase is the central enzyme of nitrogen assimilation and has the capacity to generate nitric oxide, both *in vitro* (Yamasaki and Sakihama, 2000) and *in vivo* (Rockel et al., 2002). The enzyme generates nitric oxide from nitrite, with NAD(P)H as an electron donor (Kaiser et al., 2002), which is probably created using the molybdenum cofactor at the site of catalysis, as it was found in xanthine oxidoreductase, another nitric oxide-producing enzyme (Harrison, 2002). Nitrate reductase requires molybdenum for the molybdenum cofactor to be activated (Schwarz and Mendel, 2006). Substitution of molybdenum by tungsten induces the synthesis of an inactive tungsto-protein (Notton and Hewitt, 1971) that inhibits the subsequent nitrate reductase activity in plant cells, leading to nitrogen penury. Recently, the attributes of tungsten as a specific nitrate reductase inhibitor have been questioned (Xiong et al., 2012) and additional potential targets in plants have been identified (Adamakis et al., 2012). It seems that it is a toxic metal, beyond being solely an inhibitor, and it was found that, like other toxic heavy metals, it reduced root and shoot biomass and induced

Abbreviations: CLSM, confocal laser scanning microscopy; cPTIO, carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; DAF-DA, 4,5-diaminofluorescein diacetate; DCF-DA, 2,7-dichlorofluorescein diacetate; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; TEM, transmission electron microscopy; LEH, length of the first epidermal cell with visible root hair bulge; NH_4-medium, ammonium succinate medium.

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several malformations in pea (*Pisum sativum*) root (Adamakis et al., 2008). These morphological alterations were attributed to its effect on cortical microtubules (Adamakis et al., 2010a), which control the morphogenesis of cells and ultimately of the whole plant (Chan, 2012). It caused depolymerization and disorganization of the microtubule arrays in pea root cells and eventually induced endoplasmic reticulum stress-derived programmed cell death (Adamakis et al., 2011). The cortical microtubule array has been identified as a universal target of tungsten among different land plant species, including *Arabidopsis thaliana* (Adamakis et al., 2010b). However, in contrast to what was observed in pea, tungsten-affected microtubules in *A. thaliana* were not depolymerized but disoriented. This difference could be indicative of a potential divergence in resistance to tungsten toxicity (Adamakis et al., 2010b), with potential application to phytoremediation techniques.

The response of *A. thaliana* to tungsten treatment was so far restricted to a strong inhibition of nitrate reductase-mediated nitric oxide production and hindering of root elongation (Kolbert et al., 2008; Chen et al., 2010). Any further effects of the metal in *A. thaliana* are currently unknown. Experimentation on *A. thaliana* plants with the commonly used nitric oxide scavenger carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (cPTIO) showed that nitric oxide inhibition blocked auxin-mediated growth and formation of lateral roots (Pagnussat et al., 2002). According to Fernández-Marcos et al. (2011, 2012), cPTIO reduced cell elongation in the primary root meristem but did not affect root growth and cell length in the elongation zone nor the distribution of auxin transporters (pin-formed) PIN1 and PIN2. Tungsten could either mimic the effects of nitric oxide scavengers, as an inhibitor of nitrate reductase, or its attributes as a heavy metal might result to a different response.

In accordance, in the present study the effects of tungsten on the primary root of *A. thaliana* were investigated. Light and transmission electron microscopy (TEM) was applied, along with morphometric analysis, in order to evaluate any cell structure and growth disturbance due to tungsten. Microtubule and actin filament organization was examined after immunostaining and by live imaging with confocal laser scanning microscopy (CLSM). Cell vitality as well as the levels of nitric oxide and hydrogen peroxide were determined, while the activity of auxin and cytokinin and the distribution of auxin transporters PIN1, PIN2 and PIN3 under tungsten application were also examined. The results indicate that root growth inhibition by tungsten should not be attributed to depletion of nitric oxide but rather to a putative interference of tungsten with the basipetal auxin flow, particularly affecting cell expansion in the elongation zone.

Materials and methods

Plant material and preliminary experiments

Seeds of *Arabidopsis thaliana* L. (Heynh) wild type (ecotype Columbia) and of Columbia expressing PIN1-GFP, PIN2-GFP, PIN3-GFP, DR5-GFP (purchased from the NASC European Arabidopsis Stock Centre), TCS-GFP (a generous gift from Dr. Konstantinos Vlachonasios; Department of Botany, School of Biology, Aristotle University, Thessaloniki, Greece) and ABD2-GFP (a generous gift from Prof. Tobias Baskin; Biology Department, University of Massachusetts, Amherst, MA, USA), were surface sterilized with a 30% (v/v) bleach solution and kept at 4 °C for 72 h. Afterwards the seeds were germinated and the seedlings were grown in Petri dishes on a solid agar medium containing 1% (w/v) phytoagar (Duchefa, Haarlem, Netherlands) and 2% (w/v) sucrose in modified Hoagland's solution [2 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 2 mM KH_2PO_4, 0.09 mM Fe-EDTA]. To rule out whether tungsten stops the first step of the assimilatory reduction of nitrogen to ammonium through inhibition of the nitrate reductase activity, experiments were also conducted in seedlings grown in a medium supplemented with 6 mM ammonium succinate (NH_4-medium, for brevity).

The Petri dishes were placed vertically in a growth chamber under a constant 16 h day/8 h night regime at an ambient temperature of 21 ± 1 °C, with light intensity set at 120 μmol m^{-2} s^{-1}. Four-day-old seedlings were transplanted in Petri dishes containing the above medium supplemented with 1, 5, 10, 20, 50, 100 mg/L, Na_2WO_4. The length of the developing roots was measured every 24 h and it was found that the roots stopped growing at 100 mg/L tungstate, so this concentration was further adopted. The above concentration was used also taking into account the fact that in some cases of W pollution, its concentration was found to vary between 135 and 337 mg/L (review by Koutsospyros et al., 2006). Treatments lasted for 3, 6, 12, 24 and 48 h, while prolonged exposures of 4, 6 and 10 days were also conducted. Moreover, seedlings directly grown in the presence of tungsten for 6 consecutive days were also studied. In all experiments, untreated seedlings of the same age were considered as control.

In this study, the terms "rootward" and "shootward" were adopted to determine cell polarity (Baskin et al., 2010). In accordance with Baluška et al. (2010), *A. thaliana* growing region of the root tip was considered to consist of three zones: the meristematic, the transition and the elongation zone, all of which constituted the undifferentiated zone. The region where root hair formation took place was considered as the maturation zone.

All chemicals and reagents were purchased from Sigma (Taufkirchen, Germany), Merck (Darmstadt, Germany) and Applichem (Darmstadt, Germany), unless otherwise stated, while all digital images were optimized for contrast and colour with Adobe Photoshop CS2 software with only linear settings.

Cytoplasmic streaming analysis. Light and transmission electron microscopy

All procedures applied in this study were performed at room temperature, unless otherwise stated. The roots of variously treated seedlings were examined with a Zeiss AxioImager.Z2 light microscope equipped with a digital AxioCam MRc 5 camera under differential interference contrast (DIC) optics. The same microscope and camera were used for all light microscopy observations. The length (i) from the root tip till the first visible root hair bulge, (ii) of the first epidermal cell with visible root hair bulge (LEH) (Le et al., 2001), and (iii) of the epidermal cell located "rootward" were determined. Measurements were done with the AxioVision SE64 4.8.3 software according to the manufacturer's instructions and were statistically processed with the Graph Pad software (San Diego, CA, USA). Cytoplasmic streaming was recorded with DIC optics using the same camera and software.

Seedling roots were also prepared for light and electron microscopy. A fixative [2% (v/v) glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.0) supplemented with 5 mM $CaCl_2$] was poured on the seedlings in the Petri dishes, which were gently rocked for 3 h. After rinsing in buffer (3 × 15 min changes), the root tips (2–3 mm long) were post-fixed in 1% (w/v) OsO_4 in the same buffer for 2 h. After washing in buffer overnight, the root tips were dehydrated through an acetone series at 5 °C, then embedded in Durcupan ACM (Fluka, Chemie AG, Buchs, Switzerland) and sectioned with a Reichert-Jung Ultracut E ultramicrotome with a diamond knife. Semi-thin sections (0.5–2 μm) were stained with 0.5% (w/v) toluidine blue O and observed by light microscopy.

Ultrathin sections (80–90 nm) were double-stained with uranyl acetate and lead citrate and examined with a JEOL JEM 1011 electron microscope equipped with a Gatan ES500W digital