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# Endocytosis is a significant contributor to uranium(VI) uptake in tobacco (Nicotiana tabacum) BY-2 cells in phosphate-deficient culture

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## 20 Abstract

21 Endocytosis of metals in plants is a growing field of study involving metal uptake from 22 the rhizosphere. Uranium, which is naturally and artificially released into the rhizosphere, is 23 known to be taken up by certain species of plant, such as Nicotiana tabacum, and we 24 hypothesize that endocytosis contributes to the uptake of uranium in tobacco. The endocytic 25 uptake of uranium was investigated in tobacco BY-2 cells using an optimized setup of culture 26 in phosphate-deficient medium. A combination of methods in biochemistry, microscopy and 27 spectroscopy, supplemented by proteomics, were used to study the interaction of uranium and 28 the plant cell. We found that under environmentally relevant uranium concentrations, 29 endocytosis remained active and contributed to 14% of the total uranium bioassociation. 30 Proteomics analyses revealed that uranium induced a change in expression of the clathrin heavy 31 chain variant, signifying a shift in the type of endocytosis taking place. However, the rate of 32 endocytosis remained largely unaltered. Electron microscopy and energy-dispersive X-ray 33 spectroscopy showed an adsorption of uranium to cell surfaces and deposition in vacuoles. Our 34 results demonstrate that endocytosis constitutes a considerable proportion of uranium uptake 35 in BY-2 cells, and that endocytosed uranium is likely targeted to the vacuole for sequestration, 36 providing a physiologically safer route for the plant than uranium transported through the 37 cytosol.

Keywords: plant cell; proteomics; radionuclide transport; heavy metal interaction; vesicleuptake

## 40 **1. Introduction**

41 Uranium is a prime component of radionuclide waste emanating from the energy sector 42 (Neill et al., 2018). This necessitates research into how this radionuclide can be safely stored, 43 as well as possible worst-case scenarios in repositories such as ground- and pore-water ingress, 44 which may result finally in leaching of radionuclides into soil and groundwater, thereby 45 bringing it into contact with the environment. Compounded to that, naturally occurring uranium, of which <sup>238</sup>U is the most abundant isotope (99.3%), has lately been growing in 46 47 infamy due to soil contamination resulting from natural processes, but more so from phosphate 48 fertilization in agriculture as well as from mining activity (Schnug and Lottermoser, 2013; 49 Wetterlind et al., 2012). Consequently, uranium in polluted ecosystems can enter the food chain 50 through its uptake and translocation in certain plant species, and the ensuing bioaccumulation 51 in entities at the top of the food chain can reach harmful and potentially lethal levels (Raskin 52 et al., 1994). Since human beings are at the highest levels of the food chain, they are the most 53 at risk. Although natural uranium has negligible radiotoxicity, it gives rise to potent chemical 54 toxicity and poses serious threats to health (Craft et al., 2004). Nephrotoxicity has been 55 observed to be the most prevalent outcome of uranium ingestion in humans and animals, for 56 instance (Ribera et al., 1996).

57 Investigations into radioecological contamination and identifying potential long-term 58 storage areas for radioactive waste are far from trivial, since such studies would have to account 59 for many factors which affect the migration of radionuclides through soil. Uranium as an 60 actinide element has a rather complex chemistry and therefore interaction with biological 61 entities has to take into account the form in which it exists in solution, also referred to its 62 chemical speciation because it impacts its bioavailability (Wall and Krumholz, 2006). The 63 metal exists primarily in its hexavalent oxidation state in oxic soils and water bodies, i.e. U(VI), in the form of the uranyl(VI) ion  $UO_2^{2+}$  (Laroche et al., 2005; Wall & Krumholz, 2006). The 64 65 uranyl(VI) ion has the propensity to form both soluble and insoluble complexes. Under acidic/near neutral conditions, the uranyl(VI) ion can form complexes with hydroxide and 66 phosphate, and under alkaline conditions, with carbonate (Ebbs et al., 1998). Of the various 67 68 complexes of U(VI), those with phosphate are the most stable and generally precipitate out of 69 solution (Gorman-Lewis et al., 2009). Under reducing conditions, U(IV) is the predominant 70 oxidation state and enters into stable complexes and often precipitates as uraninite (Langmuir, 71 1978).

72 Biological factors play a vital role in radionuclide migration through soil. Plants 73 comprise a considerable proportion of the biomass of an ecosystem and are generally the entry 74 points of uranium into the food chain. They require certain heavy metals such as Mn, Fe, Cu, 75 Zn and Co in trace quantities for enzyme function and to act as cofactors for electron transport 76 in photosynthesis (Andresen et al., 2018; Jeong and Connolly, 2009; Nicholas, 1975). Studies 77 have revealed that the transport mechanisms used for these essential minerals are also 78 employed by toxic metals and radionuclides such as uranium to enter the plant tissue (Song et 79 al., 2017). Broad-spectrum ion channel transporters, such as the iron-regulated transporter 1 80 (IRT1) protein, regulate the influx of a range of metals (Nishida et al., 2011), making them 81 vulnerable entry points for radionuclides. In the presence of uranium, metabolic pathways 82 involved in iron uptake and homeostasis, for instance, have been observed to be affected 83 (Doustaly et al., 2014). Moreover, there is evidence pointing towards calcium ion channels 84 being responsible for uranium uptake (Rajabi et al., 2021; Sarthou et al., 2022).

85 Aside from transporter-mediated uptake, there has been mounting evidence of 86 endocytosis-mediated uptake of metals. This has been observed in plants particularly for non-87 essential metals such as aluminium (Illéš et al., 2006; Wu et al., 2015), lanthanum (Ben et al., 88 2021; Wang et al., 2014), cadmium (Wang et al., 2014), lead (Hübner et al., 1985) and a host 89 of other metals (Dragwidge and van Damme, 2020). Metal endocytosis is not only a reported 90 feature in plant tissue but has also been observed in algae (Arunakumara and Zhang, 2007) and 91 rodent kidneys (Zavala-Guevara et al., 2021). Just as in animal cells, endocytosis in plants is 92 mainly a clathrin-mediated process and has so far been linked to the uptake and efflux of 93 materials as well as the regulation of protein and lipid components of the plasma membrane 94 (Murphy et al., 2005; Pérez-Gómez and Moore, 2007). The clathrin coat protein helps in initial 95 invagination and subsequent budding of the lipid bilayer, resulting in the internalization of 96 membrane-bound and extracellular constituents. Finally, dynamin-related GTPases complete 97 the fission process to form a clathrin-coated vesicle (Fujimoto and Ueda, 2012). It is ultimately 98 an energy-driven process, requiring guanosine-5'-triphosphate (GTP) for vesicle formation and 99 subsequent trafficking, which is thought to be aided by a myriad of Rab-GTPases as 100 demonstrated by Nielsen et al. (2008). The trafficking of an endocytic vesicle depends on the 101 cargo it is carrying. A new vesicle fuses with early endosomes or the trans-Golgi network where 102 it is then either transported back to the plasma membrane for recycling or targeted to the 103 vacuole through late endosomes or multi-vesicular bodies for degradation or storage 104 (Ekanayake et al., 2019; Wang et al., 2020). To investigate endocytosis in plant cells, styryl

dyes such as FM4-64<sup>™</sup> have been well-established due to their ability to bind to plant cell
membranes and be internalized, while not conferring major toxicity (Rigal et al., 2015).

107 Currently, there is a wealth of information concerning the uptake of uranium by various 108 species of plants (Chen et al., 2021; Ebbs et al., 2000; Jessat et al., 2021; Ratnikov et al., 2020; 109 Rodriguez-Freire et al., 2021; Saenen et al., 2013; Shahandeh and Hossner, 2002; Soudek et 110 al., 2014; Stojanović et al., 2012; Viehweger and Geipel, 2010). The majority of these studies 111 deal principally with uptake rates of the radionuclide for the purpose of phytoremediation, and 112 a meagre, albeit growing, number attempt to shed light on mechanisms. So far, the most 113 investigated mechanisms of uranium uptake pertain to ion-channels (Berthet et al., 2018; 114 Doustaly et al., 2014; Gupta et al., 2020; Rajabi et al., 2021; Sarthou et al., 2022). In this light, 115 the aforementioned evidence arguing for an endocytosis-mediated uptake of metals, led us to 116 hypothesize that vesicular uptake is one mechanism of uranium transport into plant cells.

117 Notwithstanding, investigating interactions of plants with uranium is a challenging feat 118 due to the use of inorganic phosphate in cultures, which would cause uranium to precipitate 119 out of solution and render it unavailable to the plant (Ebbs et al., 1998). Phosphate is a crucial 120 component of cellular growth and development, and is needed for plasma membrane integrity, 121 energy, nucleic acid biosynthesis, signalling, *etc.* (Mimura, 1995). Deficiencies in phosphate 122 availability would therefore have detrimental effects to cell growth and replication (Sano et al., 123 1999).

124 In this study, we devised an experimental setup to circumvent the adverse effects of 125 phosphate deficiency in tobacco (Nicotiana tabacum) bright yellow 2 (BY-2; Nagata et al., 1992) cell suspensions in order to investigate the uptake of uranium via endocytosis into these 126 127 cells. The model organism tobacco has been a reputable workhorse in heavy metal research 128 (Rehman et al., 2019) and is also known to have pronounced accumulation rates of uranium 129 (Rajabi et al., 2021; Soudek et al., 2014; Stojanović et al., 2012). The undifferentiated BY-2 130 cell line was selected due to its ease of handling and the easier visualization of cellular 131 processes taking place. With the help of proteomics, combined with methods in classical 132 molecular biology involving microscopy, we ascertained that endocytosis remains active in the 133 presence of uranium and is involved in its uptake into the cell.

## 134 **2. Materials and Methods**

#### 135 2.1. Cell culture

Tobacco BY-2 cells were grown as outlined in Rajabi et al. (2021) in modified 136 Murashige-Skoog (MS) medium (4.3 g  $l^{-1}$  MS basal salt, 200 mg  $l^{-1}$  KH<sub>2</sub>PO<sub>4</sub>, 30 g  $l^{-1}$  sucrose, 137 myo-inositol,  $1 \text{ mg } l^{-1}$  thiamine hydrochloride and  $0.2 \text{ mg } l^{-1}$  2,4- $100 \text{ mg } l^{-1}$ 138 139 dichlorophenoxyacetic acid; 2,4-D, pH 5.8) in the dark at 25°C on an IKA®KS 260 basic orbital shaker (IKA Labortechnik, Staufen, Germany) set to 150 rpm. Consult appendix for 140 141 details of chemicals and reagents. pH values were measured with a pH meter (inoLab pH Level 1, WTW, Weilheim, Germany) using a BlueLine 16 pH electrode (SI Analytics, Mainz, 142 143 Germany). Sub-cultivation was performed on a weekly basis, with 1 ml of culture being added 144 to 30 ml of MS medium in 100-ml sterile Erlenmeyer flasks.

#### 145 2.2. Exposure of BY-2 cells to uranyl(VI) nitrate

146 Cells were first cultured in MS medium for 96 h according to the standard culture conditions as mentioned in the preceding section. An appropriate volume, based on the 147 experiment, of this 96-h old culture was then added into 30 ml of phosphate-deficient MS 148 (MS<sub>red</sub>) medium (0.026 mg  $l^{-1}$  CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.025 mg  $l^{-1}$  CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.25 mg  $l^{-1}$ 149 Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 331.9 mg l<sup>-1</sup> CaCl<sub>2</sub>, 1900 mg l<sup>-1</sup> KNO<sub>3</sub>, 1650 mg l<sup>-1</sup> NH<sub>4</sub>NO<sub>3</sub>, 6.2 mg l<sup>-1</sup> 150 H<sub>3</sub>BO<sub>3</sub>, 0.83 mg  $l^{-1}$  KI, 16.9 mg  $l^{-1}$  MnSO<sub>4</sub>·H<sub>2</sub>O, 8.6 mg  $l^{-1}$  ZnSO<sub>4</sub>·7H<sub>2</sub>O, 180.54 mg  $l^{-1}$ 151 MgSO<sub>4</sub>·7H<sub>2</sub>O, 9.3 mg  $l^{-1}$  KCl, 36.7 mg  $l^{-1}$  FeNaEDTA, 1.7 mg  $l^{-1}$  KH<sub>2</sub>PO<sub>4</sub>, 30,000 mg  $l^{-1}$ 152 153 sucrose, 100 mg  $l^{-1}$  myo-inositol, 1 mg  $l^{-1}$  thiamine hydrochloride and 0.2 mg  $l^{-1}$  2,4D; pH adjusted to 5.8 with KOH) in 100-ml Erlenmeyer flasks. Cells were cultured in MS<sub>red</sub> medium 154 155 with reduced phosphate in order to limit the formation of uranyl(VI) phosphate precipitates. 156 Sterile uranyl(VI) nitrate from stock solutions of approximately 10 mM was then added to predetermined concentrations, depending on the experiment, and left to incubate for a 157 158 designated period under culture conditions. The experimental conditions and concentrations of 159 uranium used were based on previous toxicological analyses of uranium in tobacco BY-2 by 160 Rajabi et al. (2021) except without any preconditioning of BY-2 cells in MS<sub>red</sub> medium. Uranium in natural isotope composition is a radioactive  $\alpha$  emitter. It should be handled in 161 162 dedicated facilities with appropriate equipment for radioactive materials to avoid health risks 163 caused by radiation exposure.

#### 164 2.3. Determining phosphate content in BY-2 culture in MS medium

165 In triplicate, cells were sub-cultivated into 30 ml of fresh MS medium and incubated 166 under culture conditions. 1.5 ml of the culture were sampled from the flask after 0, 24, 48, 72 167 and 96 h of incubation and cells were separated by centrifuging at 16,000 g for 10 min with a 168 5804R table-top centrifuge (Eppendorf AG, Hamburg, Germany). 1 ml of each supernatant was 169 acidified with 10 µl of distilled HNO<sub>3</sub> in order to solubilize and stabilize all ions. Phosphorus 170 content was then determined by inductively coupled plasma mass spectrometry (ICP-MS). For 171 ICP-MS measurements, a NexION 350x spectrometer (Perkin Elmer, Rodgau, Germany) with 172 an operating radio frequency power (RFP) of 1300 W or an iCap RQ spectrometer (Thermo 173 Fisher Scientific, Dreieich, Germany) with an RFP of 1550 W, were used. Both machines were 174 equipped with quadrupole mass analysers. Elements were analysed using either helium mode 175 with kinetic energy discrimination or standard mode. Three measurements were performed per 176 sample using argon plasma gas and internal standards of rhodium, scandium and lutetium.

#### 177 2.4. Cell growth and viability in phosphate-deficient culture

5 ml of a 96-h culture in MS medium were added to three Erlenmeyer flasks containing
30 ml of MS<sub>red</sub> medium and one flask containing 30 ml of MS medium. All four flasks were
left to incubate under culture conditions. Phase contrast images of cells were acquired with an
Axiovert 40 CFL light microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

182 Cell viability experiments were performed according to Rajabi et al. (2021). Cells were 183 separated from culture using 37-µm pore size nylon membranes (Bückmann, Mönchengladbach, Germany) placed in CellCrown<sup>™</sup> inserts (Sigma-Aldrich, Taufkirchen, 184 185 Germany) to function as filters in 12-well plates (Greiner, Frickenhausen, Germany) and then 186 stained with a 2.5% (w/v) solution of Evans Blue reagent in MilliQ water for 3 min. Destaining 187 was done by rinsing the cells at least three times in MS<sub>red</sub> medium. Using a Fuchs-Rosenthal counting chamber (Brand GmbH & CO KG, Wertheim, Germany), up to 500 cells were 188 189 counted, with six individual counting events per sample, and the ratio of non-stained cells to 190 the total number of cells was calculated in order to determine viability. The standard error of 191 the mean (SEM; Eq. 1) was calculated for each testing condition and was represented according 192 to Altman & Bland (2005) and a two-tailed student's t-test was used to evaluate statistical 193 significance between measurements at 0 and 24 h.

194 
$$standard \ error \ of \ the \ mean = \frac{sample \ standard \ deviation}{\sqrt{number \ of \ samples}}$$
 Eq. (1)

195 Cell growth was determined by measuring dry biomass. 1.5 ml of each culture were 196 spun down using a 5415R table-top centrifuge (Eppendorf AG, Hamburg, Germany) for 10 min 197 at 16,000 g in pre-weighed 1.5-ml centrifuge tubes. Cells were washed twice with MilliQ water 198 and centrifugation was used to remove each wash. The cell masses were subsequently freeze-199 dried using an Alpha 2-4 LSCbasic lyophilizer (Martin Christ Gefriertrocknungsanlagen 200 GmbH, Osterode am Harz, Germany) and the dry cell mass in each tube was determined by 201 weighing the centrifuge tubes on an ultra-fine balance. SEM was calculated for each testing 202 condition and significance between measurements at 0 and 24 h was calculated using a 203 two-tailed student's t-test.

204 2.5. Visualization of endocytosis

205 2 ml of a 96-h culture in MS medium were added to three Erlenmeyer flasks, each 206 containing 30 ml of MS<sub>red</sub> medium. To one flask, wortmannin was added to a final 207 concentration of 5  $\mu$ M to inhibit endocytosis (Jelínková et al., 2015) and allowed to incubate 208 for 40 min under culture conditions to serve as a positive control for endocytic blocking. To 209 this flask and a flask without wortmannin, uranyl(VI) nitrate was added to a concentration of 200  $\mu$ M. The third flask was maintained as a negative control with the absence of both U and 211 wortmannin, and all flasks were allowed to incubate for 5 h under culture conditions.

212 Following incubation, all cultures were placed on ice for 15 min to temporarily inhibit 213 endocytosis and FM4-64<sup>TM</sup> dye was added to a working concentration of 1.65 µM to stain lipid 214 membranes. Live staining was done by shaking the cells at 25°C in the dark for 90 min. 215 Fluorescence microscopy was subsequently performed on this suspension with an 216 AxioObserver Z1 microscope using a 530-nm Colibri excitation source and an HE60 filter set 217 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) to visualize the FM dye. General 218 physiology was examined using transmitted light with phase contrast. Acquisition was 219 performed with the AxioVision (v4.8) software using a fixed camera exposure time for the FM 220 dye.

221 Quantification of intracellular FM4-64 signals was performed using ImageJ (National 222 Institutes of Health) by measuring mean intensities of drawn regions of interest, which only 223 constituted the inside of the cell and excluded any peripheral staining. Cells were counted from 224 one biological replicate from each testing condition, with two to three locations being 225 considered. The number of cells counted for the control was 61, for the U-treated sample, 43, and for the U+WM-treated sample, 42. These mean intensities were normalized to the
background mean intensities in each image. Statistical analysis was then done in the form of a
two-tailed student's t-test to show significance.

#### 229 2.6. Proteomics analysis

5 ml of a 96-h culture in MS medium were added to 30 ml MS<sub>red</sub> medium in six flasks. To three of these flasks, uranyl(VI) nitrate was added to a concentration of 10  $\mu$ M and the remaining three flasks served as negative controls (i.e. three biological replicates per testing condition). All six cell suspensions were incubated for 24 h under culture conditions.

234 For protein extraction, a modified method based on that by Laing & Christeller (2004) 235 was used. 10 ml of each cell suspension were washed once with ice-cold 3-(Nmorpholino)propanesulfonic acid (MOPS) buffer (50 mM, pH 7.5). The cells were 236 237 resuspended in 1 ml of ice-cold lysis buffer (1% Triton<sup>™</sup> X-100, 2 mM dithiothreitol, 5% 238 polyvinylpolypyrrolidone, 50 mM MOPS, cOmplete<sup>™</sup> protease inhibitor according to 239 manufacturer's instructions, pH 7.5) and ground to a fine powder using liquid nitrogen. Protein 240 extract was obtained by centrifuging at 16,000 g for 20 min at 4°C. A small aliquot of the 241 extract was mixed with four parts of ice-cold acetone in order to precipitate and concentrate 242 proteins. The protein concentration was determined using a Pierce<sup>™</sup> Coomassie Plus 243 (Bradford) Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's 244 instructions.

245 Proteins in solution were digested and desalted according to Majovsky et al. (2014). 246 Consult appendix for details on protein digestion. Dried peptides were dissolved in 5% 247 acetonitrile (ACN), 0.1% trifluoroacetic acid and were injected into an EASY-nLC 1000 liquid 248 chromatography system (Thermo Fisher Scientific). Peptides were separated using liquid 249 chromatography C18 reverse phase chemistry employing a 550 min gradient increasing from 250 5% to 40% ACN in 0.1% formic acid, and a flow rate of 250 nl min<sup>-1</sup>. Eluted peptides were 251 electrosprayed on line into a QExactive Plus mass spectrometer (Thermo Fisher Scientific). 252 The spray voltage was 1.9 kV, the capillary temperature 275°C and the Z-Lens voltage 240 V. 253 A full mass spectrometry (MS) survey scan was carried out with chromatographic peak width set to 15 s, resolution set to 70,000, automatic gain control (AGC) at  $3x10^6$  and a max injection 254 255 time (IT) of 100 ms. MS/MS peptide sequencing was performed using a Top10 data dependent 256 acquisition (DDA) scan strategy with high collision dissociation (HCD) fragmentation. MS scans with mass to charge ratios (m/z) between 400 and 1850 were acquired. MS/MS scans 257

were acquired with a resolution of 17,500, AGC of  $5 \times 10^4$ , IT of 50 ms, isolation width of 1.6 m/z, normalized collision energy of 28, under fill ratio of 3%, dynamic exclusion duration of 20 s, and an intensity threshold of  $3 \times 10^4$ .

261 MS/MS spectra were used to search the Nicotiana tabacum database from UniProt with 262 the Mascot software v.2.5 linked to Proteome Discoverer v.2.1 (Orsburn, 2021). The enzyme 263 specificity was set to trypsin and lysyl endopeptidase, and two missed cleavages were tolerated. 264 Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine 265 as a variable modification. The precursor tolerance was set to 7 ppm and the product ion mass 266 tolerance was set to 0.8 Da. A decoy database search was performed to determine the peptide 267 spectral match (PSM) and peptide identification false discovery rates (FDR). PSM, peptide and 268 protein identifications surpassing respective FDR thresholds of q < 0.01 were accepted. MS 269 raw data were imported into the Progenesis software v4.2 (Waters Corportation, Milford, MA, 270 USA) which performs alignment of liquid chromatography-mass spectrometry (LC-MS) 271 measurements, peptide ion signal peak picking in the aligned data, normalization and peak 272 integration to quantify peptide ion signals across LC-MS measurements. Peptide ion signal 273 abundance was used to quantify cognate proteins using the non-conflicting peptides protein 274 quantification index (PQI). Only peptides that showed an in-group coefficient of variance of 275 < 30% and cognate proteins with an ANOVA multiples testing corrected q-value < 0.05 were 276 retained and considered differentially expressed. The mass spectrometry proteomics data have 277 been deposited to the ProteomeXchange Consortium via the Proteomics Identification 278 Database (PRIDE; Perez-Riverol et al., 2019) partner repository with the dataset identifier 279 PXD028677 and 10.6019/PXD028677.

#### 280 2.7. Endocytosis-mediated uranium bioassociation

281 4.5 ml of a 96-h culture in MS medium were added to three Erlenmeyer flasks, each 282 containing 30 ml MS<sub>red</sub> medium. To one of these cultures, wortmannin was added to a final 283 concentration of 33 µM and this flask was cultured for 30 min. To the wortmannin-treated 284 culture and a second flask of MS<sub>red</sub> culture, uranyl(VI) nitrate was added to a concentration of 285 20 µM. The third flask served as a control. All flasks were incubated for 6 h under culture 286 conditions. Cells were subsequently separated from the culture medium through vacuum 287 filtration and rinsed on the filter with 30 ml of MilliQ water. In triplicate, 100 µg of the washed 288 cell residue were weighed out into 15-ml centrifuge tubes and digested in 3 ml of a solution of 289 10% H<sub>2</sub>O<sub>2</sub> and 43% HNO<sub>3</sub> by heating to 80°C for 4 h. Once cooled, the volume was topped up 290 to 5 ml with MilliQ water and elemental analysis in both the culture medium and cell extracts

was carried out by ICP-MS. SEM was calculated for each testing condition and significance
between the wortmannin-treated and -untreated samples was evaluated using a two-tailed
student's t-test.

#### 294 2.8. Preparation for transmission electron microscopy

295 BY-2 cells exposed to 0, 20 and 200-µM concentrations of uranyl(VI) nitrate in MS<sub>red</sub> 296 medium for 24 h were embedded in 2% agar. Preparations for transmission electron 297 microscopy (TEM) were done using modified methods of those outlined in Mulisch & Welsch 298 (2010) with ethanol substitution and Spurr's resin infiltration (Spurr, 1969). Consult appendix 299 for details. The resin blocks were then trimmed and thin sections (< 100 nm) were obtained 300 using an EM UC7 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). The thin 301 sections were mounted on carbon-coated copper grids and contrasted using lead citrate as 302 described by Reynolds (1963). The thin sections containing cells unexposed to uranium were 303 additionally contrasted with 2.5% uranyl(VI) acetate.

#### 304 2.9. Transmission electron microscopy and spectrum imaging analysis

305 For all TEM-based analyses, the section-containing grids were placed in a high-306 visibility low-background holder and decontaminated for 2 s in a Model 1020 Plasma Cleaner 307 (Fischione, Export, PA, USA). A Titan 80-300 electron microscope (FEI, Eindhoven, 308 Netherlands) operating at an accelerating voltage of 300 kV was used to acquire bright-field 309 TEM micrographs. For selected positions on each section, high-angle annular dark-field 310 scanning transmission electron microscopy (HAADF-STEM) and spectrum imaging analysis 311 based on energy-dispersive X-ray spectroscopy (EDX) were performed with a Talos F200X 312 microscope equipped with a high-brightness X-FEG electron source and a Super-X EDX 313 detector system (FEI) at an accelerating voltage of 200 kV. Using the ESPRIT software version 314 1.9 (Bruker), two-dimensional element distributions were obtained after Bremsstrahlung 315 background correction based on the physical TEM model and series fit peak deconvolution. 316 For representation, selected element maps were overlaid using ImageJ and the Correlia plug-317 in (Rohde et al., 2020).

#### 318 **3. Results**

#### 319 3.1. Alleviating stress caused by phosphate deficiency

320 The standard method of BY-2 cell culture was in MS medium. However, in order to 321 properly expose BY-2 cells to bioavailable uranium, it was necessary to keep the formation of 322 insoluble uranium complexes in culture medium to a minimum. Hence, the cells were cultured in phosphate-deficient  $MS_{red}$  medium, containing only 1.7 mg l<sup>-1</sup> phosphate (as opposed to 323 200 mg  $l^{-1}$  in MS medium), when they were exposed to uranyl(VI) nitrate. Moreover, it had 324 to be ensured that the phosphate content of the MS medium culture had dropped below a 325 threshold of 1.7 mg  $l^{-1}$  to avoid a carryover of phosphate during the transfer of the culture into 326 327 MS<sub>red</sub> medium. ICP-MS measurements revealed that phosphate content of the cell-free MS medium after 96 h of BY-2 culture was  $238 \pm 45 \ \mu g \ l^{-1}$  and thus well-below the threshold (see 328 329 Appendix A Fig. A.1). A previous study showed that cells are still in a late-exponential phase 330 of growth after 96 h of culture in MS medium (Srba et al., 2016), which was also confirmed 331 through our own experiments by analysing cell growth over one week in MS medium (data not 332 shown). Therefore, the following exposures of BY-2 cells to uranyl(VI) nitrate were performed 333 with cultures grown for 96 h in MS medium transferred to fresh MS<sub>red</sub> medium.

334 A lack of phosphate, however, is not conducive to the healthy growth of plant cells. 335 Accordingly, further inquiry was warranted to ensure that uranium-exposed cells were not 336 being additionally stressed as a result of a deficiency of phosphate in the culture. To confirm 337 this, the effects of a 24-h incubation of cells in phosphate-deficient conditions were assessed 338 by cell viability and cell growth (measured by dry biomass). After 24 h of growth in fresh MS<sub>red</sub> 339 culture compared to a fresh MS culture, the difference in cell viability was minor and cultures 340 still consisted of populations with over 90% viable cells (Fig. 1a). Furthermore, cell mass 341 approximately doubled within 24 h of phosphate deficiency, showing that cells continued to replicate (Fig. 1b). Sano et al. (1999) showed that the mitotic index decreases to 0% when 342 343 BY-2 cells are kept in phosphate-deficient conditions for 72 h. Therefore, the progress of the 344 cell cycle over a 24-h time frame in this instance argues for cells that were not yet experiencing 345 high stress due to a lack of phosphate. This was additionally confirmed by light microscopy, 346 revealing the physiology of cells in MS<sub>red</sub> for 24 h to be similar to those in MS medium (Fig. 347 1c, d) However, cells grown for upwards of 72 h in MS<sub>red</sub> had greater populations of larger 348 cells and showed a more adverse appearance, with what appeared to be aggregations of large 349 vesicles, usually representative of cell death processes (Fig. A.2; Hiraga et al., 2010). It was





350

Fig 1: Cell viability (a) and dry biomass (b) of a 96-h old tobacco BY-2 culture in MS medium sub-cultivated into  $MS_{red}$  and grown for 24 h. Error bars represent standard errors of the mean for one biological replicate with three counting events for MS medium and three biological replicates each with three counting events for  $MS_{red}$  medium. Statistical significance is denoted by \* (p < 0.05) and \*\*\* (p < 0.001) between corresponding 0-h and 24-h samples. Micrographs of BY-2 cells before (c) and after (d) 24 h in  $MS_{red}$  medium. Scale bar: 20 µm.

351 thus determined that cells cultured for up to 24 h in phosphate-deficient conditions were 352 sufficiently vital for further investigations with uranium exposure.

#### 353 3.2. Endocytosis remains active in the presence of uranium

Endocytosis in the presence of uranium was firstly examined using fluorescence microscopy. The styryl dye FM4-64<sup>TM</sup> is a compound that intercalates within moieties of the plasma membrane, causing it to fluoresce, and is therefore a useful marker for vesicular uptake as it migrates over time into the cell during live staining (Bolte et al., 2004). Cells were exposed to 20  $\mu$ M uranyl(VI) nitrate for 5 h and subsequently stained with FM4-64<sup>TM</sup>. An exposure to 20  $\mu$ M uranium was chosen because, firstly, it resembles uranium concentrations in soil that is mildly contaminated (Vandenhove, 2002), and secondly, it induces a level of toxicity over 24 h without completely killing the cell population (Fig. **A.3**). The relatively short exposure meant that cells were within the limits of experiencing stress due to phosphate deficiency and also to the presence of uranium. The viability of these cells was still relatively high at  $80 \pm 3\%$  with exposure to  $20 \,\mu$ M uranium for 6 h and thus meant that there was minimal compromise to plasma membrane integrity caused by uranium.

It was evident that in both the presence and absence of uranium, the dye was taken up (Fig. **2a-d**). After 90 min of live staining, an intracellular staining pattern emerged, proving that endocytosis was still in play in both conditions. Consistent with these findings, Bolte et al. (2004) reported a staining of numerous organelle membranes in BY-2 cells within 1 h of dye incubation. The observation that both the uranium-treated and control experiments showed staining under 90 min would imply that the rate of vesicular uptake remained largely unhindered in the presence of uranium.

373 Wortmannin is a phosphoinositol-3-kinase (PI3K) inhibitor and has been widely used 374 in applications to artificially block endocytosis in plant cells (Jelínková et al., 2015). To test 375 the effects of actual endocytosis impairment on the staining pattern of FM4-64<sup>™</sup>, cells were treated with wortmannin for 30 min prior to staining. In this case, even after 90 min of 376 377 incubation with FM4-64<sup>TM</sup>, there was only peripheral staining (Fig. 2e, f), indicating that the 378 dye was unable to migrate into the cell via vesicular uptake. A quantification of the intracellular 379 staining pattern, discounting any peripheral signals, showed that cells treated with wortmannin 380 showed a strongly reduced mean intensity signal within the cells compared to the other testing 381 conditions, which also showed strong statistical significance between the wortmannin-treated 382 and -untreated samples. (Fig 3). This demonstrates that unlike other metals, which have been 383 seen to inhibit endocytosis (Fan et al., 2011; Wang et al., 2014), the presence of uranium at a 384 concentration of 20 µM does not have an inhibitory effect on endocytosis.

385 To verify this observation, abundance levels of proteins responsible for vesicular uptake 386 via clathrin-mediated endocytosis (CME) were analysed using a proteomics approach. Relative 387 protein abundance was analysed in cells exposed to uranyl(VI) nitrate in fresh MS<sub>red</sub> medium 388 and compared to a control experiment in MS<sub>red</sub> without uranium. To elicit a distinct protein response to the presence of uranium in culture, experiments were conducted using a longer 389 390 24 h lower uranium exposure time of and, thus. a



Fig 2: Internalisation of the FM4-64<sup>TM</sup> stain after 90 min of dye exposure in untreated tobacco BY-2 cells (Ctrl), cells treated with 20  $\mu$ M uranyl(VI) nitrate and cells treated with both 20  $\mu$ M uranyl(VI) nitrate and 5  $\mu$ M wortmannin (WM). Cell physiology is shown by phase contrast images (a, c and e), and endocytosis is shown by FM4-64<sup>TM</sup> fluorescence (b, d and f). Scale bar: 50  $\mu$ m.



Fig 3: Signal intensities within the cell after 90 min of FM4-64<sup>TM</sup> in untreated tobacco BY-2 cells (Ctrl; n=61), cells treated with 20  $\mu$ M uranyl(VI) nitrate (U; n=43) and cells treated with both 20  $\mu$ M uranyl(VI) nitrate and 5  $\mu$ M wortmannin (U+WM; n=42). Measurements are taken from at least two sampling events from one biological replicate for each testing condition. Statistical significance is denoted by \*\*\* (p < 0.001) between U and U+WM and n represents the number of cells per field of view.

392 uranium concentration of  $10 \,\mu\text{M}$  to minimize the effects of prolonged toxicity resulting in 393 excessive protein degradation. See Appendix B for a list of all proteins. To be able to easily 394 distinguish between changes in abundance, fold changes are given as logarithmic values to the 395 base of two, where a  $\log_2$  fold change (FC) of +1.00 indicates a doubling of protein expression 396 in the uranium-treated sample and an FC value of -1.00 would indicate the control had double the amount of protein than the uranium-treated sample. A q-value less than 0.05 was used to 397 398 account for false discovery and determine significance between the three replicates of each 399 testing condition (Karp et al., 2007).

400 Out of the 21 identified proteins directly responsible for CME and vesicular trafficking, 401 only seven showed statistically significant differential expression between uranium-treated and 402 control conditions (Table 1). One of these was a clathrin light chain variant, which showed an 403 increase in abundance, with an FC value of 0.44. Additionally, there were several clathrin 404 heavy chain variants showing FC values of -0.84, -0.73 and 0.41 with uranium treatment, as 405 well as an EPSIN 2-like protein showing a uranium-induced increase in expression of 0.22 FC 406 units. Another significantly affected protein was a putative clathrin assembly protein showing 407 an FC value of 0.27, albeit having a considerably low confidence score. Aside from these seven

408 proteins, there were other variants of clathrin heavy and light chains being expressed with no 409 statistically significant differences in the presence of uranium. Other CME-related proteins 410 expressed included clathrin assembly adaptor proteins and proteins guiding vesicular traffic, 411 all showing no significant change in abundance.

412 One would assume that since clathrin heavy and light chains are mainly responsible for 413 the formation of endocytic vesicles in CME, regardless of cargo and destination, they would 414 both be good indicators for quantifying vesicle uptake. In this light, recent evidence has shown 415 that clathrin heavy chain expression may not be linearly tied to clathrin vesicle formation. 416 Tahara et al. (2007) and Dhonukshe et al. (2007) claim that an overexpression of the clathrin 417 heavy chain leads to an inhibition of endocytosis. This would imply that solely quantifying the 418 abundance of the clathrin light chain, rather than that of both clathrin heavy and light chains, 419 would be a more accurate indicator for changes in the rate of endocytosis, which Wang et al. 420 (2013) and Tahara et al. (2007) also advocate. Therefore, the results in Table 1 would argue 421 that there was no major change in the amount of CME in uranium-exposed BY-2 cells, and if 422 anything, the observed slight increase in clathrin light chain expression would hint towards an 423 increase in endocytosis. Remarkably however, the cells seem to be considerably altering the 424 expression of some heavy chain variants of clathrin, suggesting that a specific heavy chain 425 variant could be involved in heavy-metal-related (or uranium-related) CME.

Table 1: Relative protein abundances (log<sub>2</sub> fold changes) involved in clathrin-mediated endocytosis comparing tobacco BY-2 cells exposed to  $10 \,\mu$ M uranyl(VI) nitrate for 24 h vs. unexposed cells. Statistical significance (\*) is denoted by q  $\leq$  0.05; TAIR: The Arabidopsis Information Resource.

UniProt Accession	Gene ID	Protein	Functional Description	Description source	Confidence score	Mass (Da)	q Value	Log 2 FC
A0A1S3Z926; A0A1S4B7G0	LOC107784283	clathrin interactor EPSIN 2- like	Transport via clathrin-coated vesicles from the trans-Golgi network to endosomes. Stimulates clathrin assembly.	UniProt	136	97,935	0.02*	0.22
A0A1S4C6G3; A0A1S4ALF5	LOC107815448	clathrin heavy chain	Heavy chain of clathrin coat protein.	UniProt	2838	193,579	0.02*	-0.84
A0A1S3ZLQ4; A0A1S3WZ44	LOC107788165	putative clathrin assembly protein At2g25430	Clathrin coat assembly, clathrin-dependent endocytosis, vesicle budding from membrane	TAIR	47	67,872	0.02*	0.27
A0A1S4D5H1	LOC107826230	clathrin heavy chain	Heavy chain of clathrin coat protein.	UniProt	2540	194,011	0.03*	-0.73
A0A1S3ZY26	LOC107791617	clathrin heavy chain	Heavy chain of clathrin coat protein.	UniProt	2421	193,993	0.04*	0.41
A0A1S3X3V1; A0A1S4C6L6	LOC107760966	clathrin light chain	Light chain of clathrin coat protein.	UniProt	311	35,504	0.04*	0.44
A0A1S3XNB8; A0A1S4BVK7; A0A1S4BJ02; A0A1S4CD03	LOC107767042	AP-2 complex subunit mu-like	Involved in clathrin-dependent endocytosis in which cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin- coated vesicles, CCVs) which are destined for fusion with the early endosome.	UniProt	169	49,781	0.05*	-0.51
A0A1S3XCL3	LOC107763511	clathrin light chain	Light chain of clathrin coat protein.	UniProt	113	29,685	0.08	-0.35

UniProt Accession	Gene ID	Protein	Functional Description	Description source	Confidence score	Mass (Da)	q Value	Log 2 FC
A0A1S4D5T7; A0A1S4DNU5	LOC107826292	AP-2 complex subunit alpha	Involved in clathrin-dependent endocytosis in which cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin- coated vesicles, CCVs) which are destined for fusion with the early endosome.	UniProt	402	114,588	0.09	-0.53
A0A1S4A500; A0A1S4A526; A0A1S4CT74	LOC107793819	clathrin interactor EPSIN 3- like isoform X1	Transport via clathrin-coated vesicles from the trans-Golgi network to endosomes. Stimulates clathrin assembly.	UniProt	105	98,034	0.09	-0.43
A0A1S4C4M9	LOC107815033	clathrin heavy chain 1-like	Heavy chain of clathrin coat protein.	UniProt	2338	134,959	0.13	0.27
A0A1S3YES2	LOC107775474	AP-2 complex subunit alpha	Involved in clathrin-dependent endocytosis in which cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin- coated vesicles, CCVs) which are destined for fusion with the early endosome.	UniProt	382	114,477	0.19	0.34
A0A1S3X3B5; A0A1S4A7M9	LOC107760733	protein TPLATE	Acts in concert with AP2 in endocytosis.	Gadeyne et al., 2014	446	130,702	0.21	-0.12
A0A1S3Z4B7	LOC107782807	putative clathrin assembly protein At5g35200	Vesicle budding from membrane.	TAIR	23	61,151	0.24	-0.20
A0A1S3Z9A1; A0A1S4AXR4	LOC107784340	putative clathrin assembly protein At2g01600	ANTH domain-containing protein which functions as adaptor protein for clathrin- mediated endocytosis (CME) of the secretory vesicle-associated longintype R- SNARE VAMP72 group. Interacts with the SNARE domain of VAMP72 and clathrin at the plasma membrane. Required for recycling of R-SNARE proteins.	TAIR	124	62,887	0.30	-0.05

UniProt Accession	Gene ID	Protein	Functional Description	Description source	Confidence score	Mass (Da)	q Value	Log 2 FC
A0A1S4A121; A0A1S4DR69; A0A1S3XSG8	LOC107792655	AP-2 complex subunit alpha	Involved in clathrin-dependent endocytosis in which cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin- coated vesicles, CCVs) which are destined for fusion with the early endosome.	UniProt	429	115,182	0.32	-0.13
A0A1S4BEG0; A0A1S4DNZ0	LOC107807398	clathrin interactor EPSIN 1- like	Transport via clathrin-coated vesicles from the trans-Golgi network to endosomes. Stimulates clathrin assembly.	UniProt	253	63,993	0.36	-0.02
A0A1S4CC44	LOC107817476	probable clathrin assembly protein At4g32285	Putative clathrin assembly protein, component of TPLATE complex that functions in clathrin-mediated endocytosis.	TAIR	117	48,373	0.37	-0.05
A0A1S4B3W2; A0A1S4BTL4	LOC107804273	clathrin interactor EPSIN 1- like	Transport via clathrin-coated vesicles from the trans-Golgi network to endosomes. Stimulates clathrin assembly.	UniProt	220	59,105	0.39	-0.07
A0A1S4DK63	LOC107830669	clathrin interactor EPSIN 1- like isoform X1	Transport via clathrin-coated vesicles from the trans-Golgi network to endosomes. Stimulates clathrin assembly.	UniProt	242	64,243	0.40	-0.08
A0A1S4ASV3	LOC107801015	clathrin heavy chain 1-like	Heavy chain of clathrin coat protein.	UniProt	1971	147,946	0.40	-0.02

#### 427 3.3. Inhibition of endocytosis leads to reduced uranium bioassociation

428 To better gauge the contribution of endocytosis to uranium uptake, the total amount of 429 bioassociated uranium was quantified in the presence and absence of the endocytosis blocker, 430 wortmannin. Cells were initially treated with 33 µM wortmannin for 30 min, after which, they 431 were exposed to 20 µM uranyl(VI) nitrate for 6 h. A 6-h exposure was chosen here for two 432 reasons. Firstly, to keep the experimental conditions comparable to the visualization of endocytosis (Fig. 2). Secondly, to cause as little plasma membrane leakage as possible due to 433 434 uranium-conferred toxicity (Chen et al., 2021), thereby inhibiting the amount of uranium that 435 could move freely into the cell as opposed to uranium taken up by transporters and/or 436 endocytosis. Cells were washed to remove loosely-bound uranium and uranium content was 437 determined in the resulting biomass. The wortmannin and uranium-treated cells exhibited a significant decrease in bioassociated (surface-bound and internalized) uranium compared to 438 439 uranium-treated cells without wortmannin (Fig. 4). Thus, an inhibition of endocytosis by 440 wortmannin led to a 14% decrease in bioassociated uranium during 6 h of exposure.



Fig 4: Uranium bioassociation to untreated BY-2 cells (control), cells treated with 20  $\mu$ M uranyl(VI) nitrate (+U) and cells treated with both 20  $\mu$ M uranyl(VI) nitrate and 33  $\mu$ M wortmannin (+U +WM) for 6 h. Error bars represent standard errors of the mean from three measurements of one biological replicate. \*\*\* represents statistical significance between +U and +U+WM where *p* < 0.001.

3.4. Uranium localizes to vacuolar spaces and adsorbs to cell surface components 441 EDX is an established analytical method based on the fingerprints of an element's X-ray 442 443 emissions excited by an energetic source, such as an electron beam, and the possibility of 444 coupling EDX measurements in electron microscopy has opened up avenues of research 445 exploring metal interaction with biological systems (Colpan et al., 2018). Hence, TEM and 446 EDX analysis were used to study the localization of uranium in BY-2 cells. Conventional 447 bright-field TEM (Fig. A.4) along with STEM-based EDX measurements were performed on 448 BY-2 cells exposed to 20 µM uranium for 24 h. These micrographs revealed a formation of 449 dense needle-like entities in what seem to be vacuolar/late endosomal compartments (Fig. 5a, 450 A.5, A.6). EDX-based spectrum imaging analysis of these objects was performed and 451 evaluation of the emission at 13.612 keV, which is the  $L_{\alpha 1}$  peak specific to uranium, showed 452 that they were indeed composed of uranium (Fig. 5b). Overlaying the EDX maps of the other 453 elements intriguingly showed that the uranium signal distinctly colocalized with two elements 454 in particular, namely, phosphorus and oxygen, which was substantiated by the presence of a 455 white signal (Fig. 5c, A.7a). Additionally, the uranium signal showed colocalization with those 456 of sulphur and nitrogen (Fig. 5d, A.7b) but not as densely as with oxygen and phosphorus.

457 Upon investigation of uranium adsorption to cell surfaces, i.e. the plasma membrane 458 and cell wall, after exposure to 20 µM uranyl(VI) nitrate, there were no uranium signals 459 detected at these cell components. A conceivable explanation for this would be that much of 460 the passively adsorbed uranium was washed away by the TEM preparation process, thus 461 making any adsorbed uranium too little to detect. Therefore, a 24-h exposure to a 10-fold higher 462 concentration of 200 µM uranium was tested. Here, a clear signal was found in defined parts 463 of the cell wall and cell membrane (Fig. 6). The spectra in Fig. 6 were obtained from selected 464 regions of the electron micrographs containing noticeable quantities of uranium. A reference 465 spectrum was also obtained from an area devoid of uranium (Fig. A.8) to make clear the 466 presence of the uranium  $L_{\alpha 1}$  peak in areas where it was found. Curiously, the uranium was not 467 ubiquitously distributed along these surfaces as we had anticipated, suggesting an accumulation 468 of specific binding ligands in defined parts of the cell wall and plasma membrane.

Furthermore, the same needle-like objects in vacuolar compartments were found in the high-exposure treatment. The formation of vacuolar precipitates in both 20- and 200- $\mu$ M uranium exposures, which contained a much greater amount of uranium than present at the plasma membrane or cell wall, would suggest an accumulation of uranium in the vacuole and argue for an active uptake mechanism resulting in a deposition of uranium in vacuoles.



Fig 5: Bright-field transmission electron micrograph (a) of uranium-containing needle-like structures in tobacco BY-2 cells exposed to  $20 \,\mu$ M uranyl(VI) nitrate for 24 h. EDX-based elemental mapping (b, c and d) is shown for the structures in a, with c and d containing overlays of various signals. CW: cell wall; N: nitrogen; O: oxygen; P: phosphorus; S: sulphur; U: uranium; scale bar in element distributions: 100 nm.



Fig 6: Uranium adsorption and precipitation in tobacco BY-2 cells exposed to 200  $\mu$ M uranyl(VI) nitrate for 24 h. EDX sum spectra in panel d are from selected regions in the electron micrographs in a, b and c, labelled A1, B1 and C1, respectively, and the sum spectra from f are from selected regions in panel e, labelled E1-E4, to show the presence of uranium. CW: cell wall; M: mitochondria; PM: plasma membrane; V: vacuole; scale bars: 500 nm in a, 200 nm in b and e. The dotted lines in the spectra of d and f point to the position of the L<sub>a1</sub> peak of uranium (13.612 keV).

## 475 **4. Discussion**

#### 476 4.1. The effect of phosphate deficiency

477 While transporter-mediated uptake of heavy metals has been extensively investigated 478 and seems to be one of the main modes of metal transport into cells, the endocytic uptake of 479 metals cannot be easily disregarded and should receive more attention, in particular, for 480 non-essential toxic heavy metals. Endocytosis, being an active mode of uptake, requires the 481 use of guanosine-5'-triphosphate (GTP) and therefore requires adequate phosphate reserves 482 within the cell (Gadeyne et al., 2014; Jelínková et al., 2015). Furthermore, vesicular trafficking, 483 which follows the formation of clathrin vesicles, is also guided by a series of Rab-GTPases to 484 ensure that vesicular cargo reach their intended destinations (Nielsen et al., 2008). It was 485 therefore imperative that the BY-2 cells in suspension culture had sufficient access to inorganic 486 phosphate in order to carry out vesicular uptake and trafficking efficiently.

Cell growth and viability experiments proved that cells within 24 h in 487 488 phosphate-deficient medium showed high viability and vitality, as was seen by an increase in 489 biomass. Since cells would otherwise be arrested in their cell cycle during severe phosphate 490 starvation (Sano et al., 1999), our observations could confirm that a 24-h incubation period in 491 phosphate-deficient medium provided adequately healthy cells for uranium exposure. This 492 would consequently give rise to a more accurate assessment of the cell's response to uranium. 493 Additionally, this would more closely represent a natural encounter with uranium, in contrast 494 to prolonged culture in phosphate deficiency, since whole plants have greater reserves of 495 phosphate, which they are able to mobilize and translocate under low soil phosphate 496 concentrations (Lin et al., 2009; Mimura, 1995).

#### 497 4.2. The fate of uranium

498 Studies of uranium interaction in plants are particularly cumbersome due to the many 499 complex and often undesirable interactions of uranium with various constituents of biological 500 experiments, such as phosphate, as well as the multitude of uranyl(VI) complexes that exist in 501 solution and their unique interaction mechanisms with biological entities. This would explain 502 why detailed investigations into uranium-plant interactions on a molecular level are scarce. The 503 experiments described herein have proven that endocytosis is still in play in the presence of 504 uranium and that blocking endocytosis reduces the amount of bioassociated uranium by 14% 505 over the course of 6 h in tobacco BY-2 cells in phosphate-deficient culture. Few other studies 506 investigating metal endocytosis present more than descriptive evidence. Aluminium

507 internalization is one mechanism that has been explored in greater detail, and this metal has 508 been found to localize to vacuolar compartments within 3 h in meristematic cells and cells in 509 the distal portion of the transition zone in Arabidopsis roots (Illéš et al., 2006). Therefore, the 510 observed localization of uranium in vacuolar spaces within 24 h can also be expected. 511 Nevertheless, it has to once again be stressed that our experiments were conducted with 512 dedifferentiated cells, which might have differing rates and types of endocytosis when 513 compared to various regions of root tissue.

514 As a plant cell first encounters uranium in solution, it is highly likely that adsorption to 515 the cell wall and plasma membrane would be the fastest and most intense form of 516 bioassociation. This has been observed by Moll et al. (2020) in callus cultures of Brassica 517 *napus.* Various ligands such as phosphate and carboxylic groups from surface proteins, 518 phospholipids and sugar moieties, would be prospective binding partners for metals such as 519 uranium and cause immobilisation at these cell surfaces (Koban and Bernhard, 2007; 520 Krzesłowska, 2011). In former studies involving lead, it has been shown that there is an 521 endocytic uptake of lead deposits on low-methylesterified pectins on the cell wall 522 (Krzesłowska, 2011; Krzesłowska et al., 2010). There was an immobilization of uranium to the 523 cell walls of BY-2 cells (Fig. 6e), which would thus make plausible a scenario of an endocytic 524 uptake of such cell wall components during cell wall remodelling as a response to heavy metal 525 stress.

526 In terms of sorption processes, we observed uranium bound not only to the cell wall but 527 also at the plasma membrane. Since endocytosis is still active in the presence of uranium, it 528 could very well be that a considerable amount of plasma-membrane-bound uranium is also 529 internalized. Wheeler & Hanchey (1971) were the first to report the very prominent presence 530 of needle-like crystals in oat roots exposed to uranium and postulated an endocytic uptake of 531 these precipitates. They were however unable to experimentally verify, as they would today, if 532 this was indeed endocytosis. For instance, all heavily contrasted areas of their electron 533 micrographs were automatically assumed to be uranium without any elemental analysis, and 534 vesicles in root tissue were assumed to arise due to endocytosis/pinocytosis, while exocytosis 535 to expel uranium from vacuoles was not considered. Moreover, the concentrations of uranium 536 they used were far higher (1 mM) than what would be environmentally relevant. Unlike in their 537 study, we did not observe the formation of such crystals at cell surfaces, instead only saw trace 538 amounts and uranium localized in pockets along the membrane. Modern spectroscopy 539 techniques using EDX (including our own analyses) have now shown that these crystals are

540 mostly comprised of uranyl(VI) phosphate (Jessat et al., 2021; Laurette et al., 2012; Rodriguez-541 Freire et al., 2021). The excessive formation of crystals in their study could be best explained 542 by the immediate transfer of their roots from a phosphate-rich environment into highly 543 concentrated uranyl(VI) acetate solutions, which would cause uranium to precipitate in the 544 form of uranyl(VI) phosphate complexes with a needle-like appearance. The presence of such 545 crystals, caused by the high artificial phosphate concentrations generally not observed in soil, 546 could lead to a wholly different tissue response that what the plant would undergo when in 547 moderately uranium-contaminated soil, where crystals do not exist in such abundance. This 548 could be in the form of triggering or inhibiting endocytosis since vesicle uptake is also 549 dependent on the cargo stimulus (Reynolds et al., 2018). Nevertheless, considering the 550 observations in their study and in ours, we are now able to confirm an endocytic uptake of 551 uranium at environmentally relevant exposure levels.

552 Many studies involving uranium-exposed plant tissue have consistently discovered the 553 formation of needle-like precipitates, in what appear to be vacuolar and late-endosomal 554 compartments of salt cedar plants (Rodriguez-Freire et al., 2021) and suspension cultures of 555 rapeseed (Jessat et al., 2021). Compared to those studies, we used five-fold lower uranium 556 exposure levels (20  $\mu$ M), albeit, with dedifferentiated suspension cultures instead of tissue, and 557 still observed an accumulation of these precipitates. This would suggest that, one, uranium is 558 actively targeted to the vacuole for sequestration, and two, this is a likely scenario for most 559 plant cell types. The colocalization of the uranium signal with four other EDX signals, namely, 560 phosphorus, oxygen, sulphur and nitrogen, showed that these were not simply precipitates of 561 uranyl(VI) phosphate. The presence of sulphur and nitrogen point to thiol-based chelation 562 mechanisms. Uranium is known to induce cellular production of glutathione (Lai et al., 2021; 563 Viehweger et al., 2011), for instance, which could bind to uranium as it enters the cell, 564 preventing its interaction with susceptible biomolecules, and end up in the vacuole as uranium-565 glutathione chelates.

It is difficult to say how much of this vacuole sequestration is a result of endocytosis and vesicle trafficking, as opposed to transporter-based mechanisms. There is already evidence for a transporter-mediated uptake of uranium into BY-2 cells (Rajabi et al., 2021; Sarthou et al., 2022), however, an endocytic uptake of the metal would provide a completely different pathway as opposed to one that is transporter-mediated. It would provide greater control over where the metal is compartmentalized, as opposed to freely loading metal into the cytosol, thereby reducing contact with susceptible biomolecules. One of the main roles of endocytosis is receptor recycling for plasma membrane remodelling, which involves changing the proteins being expressed on the plasma membrane to adapt to a specific situation. This would help the cell to cope in the presence of toxic metals, by removing proteins on the membrane, which might inadvertently cause metal transport into the cell, and replacing them with such as are needed to prevent and combat the effects of metal toxicity (De Caroli et al., 2020; Wang et al., 2020). The removed proteins would subsequently be targeted initially to multi-vesicular bodies (or endosomes) and eventually to the vacuole for degradation.

580 Thus, one could imagine that endocytosed uranium would be trafficked to the vacuole 581 as part of the receptor recycling process. The question remains as to whether the cell does this 582 intentionally or whether it is an unintended effect of receptor recycling and protein degradation. 583 An unintentional uptake of uranium could stem from autophagy-driven events, for instance, 584 which are known to also be linked to endocytosis (Birgisdottir and Johansen, 2020). The stress 585 conferred to these cells by the presence of the radionuclide could initiate autophagy in an effort 586 to recycle damaged proteins as a result of oxidative stress and would therefore promote events 587 of endocytosis as well (Hasan et al., 2017).

588 An intentional uptake would likely involve a change in binding ligands on the plasma 589 membrane to cause as much adsorption of the toxic metal, followed then by vesicular uptake 590 and sequestration in the vacuole. There are two pieces of evidence presented in this study that 591 would argue for this. One, there is a change in the expression of clathrin heavy chain variants 592 in the presence of uranium, suggesting that there is a shift in the type of endocytosis taking 593 place in terms of cargo and destination, and two, uranium is not evenly distributed along the 594 plasma membrane but concentrated in pockets, alluding to clusters of uranium-ligand 595 complexes. Although these observations could very well imply an intentional vesicular uptake 596 and sequestration of the metal, more thorough investigation is needed to confirm this.

## 597 **5. Conclusions and outlook**

The herein described results present a case for endocytosis as an uptake mechanism of 598 599 uranium into plant cells along with its trafficking to and storage in vacuolar compartments. The 600 successful implementation of a plant cell culture in temporary phosphate deficiency has now 601 made it possible to tackle important research questions, not only studying uranium interaction 602 but also interaction with other relevant metals. As a result, we could conclude that endocytosis 603 remains active in the presence of uranium, contributing to 14% of the bioassociated uranium, 604 and there was significant deposition of uranium precipitates in the vacuole. The phenomenon 605 of uranium adsorption to cell surfaces (cell wall and plasma membrane) was clearly visually 606 demonstrated for the first time despite being observed more than 30 years back and remained 607 unverified until now (Wheeler and Hanchey, 1971). These observations bring us a step closer 608 to elucidating mechanisms of radionuclide uptake in plants, which have remained long elusive. 609 Nevertheless, some questions remain that are yet to be answered. Are there similar levels of 610 uranium endocytosis in specialized plant tissue? Does endocytosis contribute more than 611 transporter-based mechanisms towards uranium uptake from the rhizosphere? Does the vacuole 612 remain the final resting place for sequestered uranium in BY-2 cells or are there mechanisms 613 of exudation at play?

A clearer understanding of these events would greatly advance strategies for nuclear risk assessment and phytoremediation, especially since there are many biological aspects and factors that are yet to be considered in modelling studies involving radionuclide uptake. These results verify the uptake of uranium into plant cells via endocytosis, thereby consolidating the evidence for non-transporter mediated metal uptake, hence underscoring the importance of investigating the role of endocytosis in the uptake of radionuclides such as uranium.

## 620 6. References

- Altman, D.G., Bland, M.J., 2005. Standard deviations and standard errors. BMJ 331, 903.
  https://doi.org/10.1136/bmj.331.7521.903
- Andresen, E., Peiter, E., Küpper, H., 2018. Trace metal metabolism in plants. J. Exp. Bot. 69,
  909–954. https://doi.org/10.1093/jxb/erx465
- Arunakumara, K.K.I.U., Zhang, X., 2007. Heavy metal bioaccumulation and toxicity with
  special reference to microalgae. J. Ocean Univ. China 7, 60–64.
  https://doi.org/10.1007/s11802-008-0060-y
- Ben, Y., Cheng, M., Wang, L., Zhou, Q., Yang, Z., Huang, X., 2021. Low-dose lanthanum
  activates endocytosis, aggravating accumulation of lanthanum or/and lead and disrupting
  homeostasis of essential elements in the leaf cells of four edible plants. Ecotoxicol.
  Environ. Saf. 221, 112429. https://doi.org/10.1016/j.ecoenv.2021.112429
- Berthet, S., Villiers, F., Alban, C., Serre, N.B.C., Martin-Laffon, J., Figuet, S., Boisson, A.M.,
  Bligny, R., Kuntz, M., Finazzi, G., Ravanel, S., Bourguignon, J., 2018. *Arabidopsis thaliana* plants challenged with uranium reveal new insights into iron and phosphate
  homeostasis. New Phytol. 217, 657–670. https://doi.org/10.1111/nph.14865
- Birgisdottir, Å.B., Johansen, T., 2020. Autophagy and endocytosis interconnections and
  interdependencies. J. Cell Sci. 133, 228114. https://doi.org/10.1242/jcs.228114
- Bolte, S., Talbot, C., Boutte, Y., Catrice, O., Read, N.D., Satiat-Jeunemaitre, B., 2004. FMdyes as experimental probes for dissecting vesicle trafficking in living plant cells. J.
  Microsc. 214, 159–173. https://doi.org/10.1111/j.0022-2720.2004.01348.x
- Chen, L., Liu, J., Zhang, W., Zhou, J., Luo, D., Li, Z., 2021. Uranium (U) source, speciation,
  uptake, toxicity and bioremediation strategies in soil-plant system: A review. J. Hazard.
  Mater. 413, 125319. https://doi.org/10.1016/j.jhazmat.2021.125319
- Colpan, C.O., Nalbant, Y., Ercelik, M., 2018. Fundamentals of fuel cell technologies, in:
  Dincer, I. (Ed.), Comprehensive Energy Systems. Elsevier Inc., pp. 1107–1130.
  https://doi.org/10.1016/B978-0-12-809597-3.00446-6
- 647 Craft, E.S., Abu-Qare, A.W., Flaherty, M.M., Garofolo, M.C., Rincavage, H.L., Abou-Donia,
  648 M.B., 2004. Depleted and natural uranium: Chemistry and toxicological effects. J.

- 649 Toxicol. Environ. Heal. Part B Crit. Rev. 7, 297–317.
  650 https://doi.org/10.1080/10937400490452714
- De Caroli, M., Furini, A., DalCorso, G., Rojas, M., Di Sansebastiano, G. Pietro, 2020.
  Endomembrane reorganization induced by heavy metals. Plants 9, 482.
  https://doi.org/10.3390/plants9040482
- Dhonukshe, P., Aniento, F., Hwang, I., Robinson, D.G., Mravec, J., Stierhof, Y.D., Friml, J.,
  2007. Clathrin-mediated constitutive rndocytosis of PIN auxin efflux carriers in
  Arabidopsis. Curr. Biol. 17, 520–527. https://doi.org/10.1016/j.cub.2007.01.052
- 657 Doustaly, F., Combes, F., Fiévet, J.B., Berthet, S., Hugouvieux, V., Bastien, O., Aranjuelo, I.,

658 Leonhardt, N., Rivasseau, C., Carrière, M., Vavasseur, A., Renou, J.P., Vandenbrouck,

- Y., Bourguignon, J., 2014. Uranium perturbs signaling and iron uptake response in
   *Arabidopsis thaliana* roots. Metallomics 6, 809–821. https://doi.org/10.1039/c4mt00005f
- Dragwidge, J.M., van Damme, D., 2020. Visualising endocytosis in plants: past, present, and
   future. J. Microsc. 280, 104–110. https://doi.org/10.1111/jmi.12926
- Ebbs, S.D., Brady, D.J., Kochian, L. V., 1998. Role of uranium speciation in the uptake and
  translocation of uranium by plants. J. Exp. Bot. 49, 1183–1190.
  https://doi.org/10.1093/jxb/49.324.1183
- Ebbs, S.D., Brady, D.J., Norvell, W.A., Kochian, L. V., 2000. Uranium speciation, plant
  uptake, and phytoremediation, in: National Conference on Environmental and Pipeline
  Engineering. pp. 466–475. https://doi.org/10.1061/40507(282)51
- Ekanayake, G., LaMontagne, E.D., Heese, A., 2019. Never walk alone: clathrin-coated vesicle
  (CCV) components in plant immunity. Annu. Rev. Phytopathol. 57, 387–409.
  https://doi.org/10.1146/annurev-phyto-080417-045841
- Fan, J.L., Wei, X.Z., Wan, L.C., Zhang, L.Y., Zhao, X.Q., Liu, W.Z., Hao, H.Q., Zhang, H.Y.,
  2011. Disarrangement of actin filaments and Ca<sup>2+</sup> gradient by CdCl<sub>2</sub> alters cell wall
  construction in *Arabidopsis thaliana* root hairs by inhibiting vesicular trafficking. J. Plant
  Physiol. 168, 1157–1167. https://doi.org/10.1016/j.jplph.2011.01.031
- Fujimoto, M., Ueda, T., 2012. Conserved and plant-unique mechanisms regulating plant postGolgi traffic. Front. Plant Sci. 3, 197. https://doi.org/10.3389/fpls.2012.00197
- 678 Gadeyne, A., Sánchez-Rodríguez, C., Vanneste, S., Di Rubbo, S., Zauber, H., Vanneste, K.,

679 Van Leene, J., De Winne, N., Eeckhout, D., Persiau, G., Van De Slijke, E., Cannoot, B., 680 Vercruysse, L., Mayers, J.R., Adamowski, M., Kania, U., Ehrlich, M., Schweighofer, A., 681 Ketelaar, T., Maere, S., Bednarek, S.Y., Friml, J., Gevaert, K., Witters, E., Russinova, E., 682 Persson, S., De Jaeger, G., Van Damme, D., 2014. The TPLATE adaptor complex drives 683 endocytosis Cell 156, 691–704. clathrin-mediated in plants. https://doi.org/10.1016/j.cell.2014.01.039 684

- 685 Gorman-Lewis, D., Shvareva, T., Kubatko, K.A., Burns, P.C., Wellman, D.M., McNamara, B., Szymanowski, J.E.S., Navrotsky, A., Fein, J.B., 2009. Thermodynamic properties of 686 687 autunite, uranyl hydrogen phosphate, and uranyl orthophosphate from solubility and Technol. 43, 7416–7422. 688 calorimetric measurements. Environ. Sci. 689 https://doi.org/10.1021/es9012933
- Gupta, Dharmendra K., Chatterjee, S., Mitra, A., Voronina, A., Walther, Clemens, 2020.
  Uranium and plants: elemental translocation and phytoremediation approaches, in: Gupta,
  D. K., Walther, C. (Eds.), Uranium in Plants and the Environment. Springer Nature
  Switzerland AG, pp. 149–161. https://doi.org/10.1007/978-3-030-14961-1\_7
- Hasan, M.K., Cheng, Y., Kanwar, M.K., Chu, X.Y., Ahammed, G.J., Qi, Z.Y., 2017. Responses
  of plant proteins to heavy metal stress—a review. Front. Plant Sci. 8, 1492.
  https://doi.org/10.3389/fpls.2017.01492
- Hiraga, A., Kaneta, T., Sato, Y., Sato, S., 2010. Programmed cell death of tobacco BY-2 cells
  induced by still culture conditions is affected by the age of the culture under agitation.
  Cell Biol. Int. 34, 189–196. https://doi.org/10.1042/cbi20090003
- Hübner, R., Depta, H., Robinson, D.G., 1985. Endocytosis in maize root cap cells Evidence
  obtained using heavy metal salt solutions. Protoplasma 129, 214–222.
  https://doi.org/10.1007/BF01279918
- 703 Illéš, P., Schlicht, M., Pavlovkin, J., Lichtscheidl, I., Baluška, F., Ovečka, M., 2006. 704 Aluminium toxicity in plants: Internalization of aluminium into cells of the transition zone 705 in Arabidopsis root apices related to changes in plasma membrane potential, endosomal 706 behaviour. and nitric oxide production. J. Exp. Bot. 57. 4201-4213. 707 https://doi.org/10.1093/jxb/erl197
- Jelínková, A., Müller, K., Fílová-Parezová, M., Petrášek, J., 2015. NtGNL1a ARF-GEF acts in
   endocytosis in tobacco cells. BMC Plant Biol. 15, 272. https://doi.org/10.1186/s12870-

710 015-0621-3

- Jeong, J., Connolly, E.L., 2009. Iron uptake mechanisms in plants: Functions of the FRO family
  of ferric reductases. Plant Sci. 176, 709–714.
  https://doi.org/10.1016/j.plantsci.2009.02.011
- Jessat, J., Sachs, S., Moll, H., John, W., Steudtner, R., Hübner, R., Bok, F., Stumpf, T., 2021.
  Bioassociation of U(VI) and Eu(III) by plant (*Brassica napus*) suspension cell cultures a spectroscopic investigation. Environ. Sci. Technol. 55, 6718–6728.
  https://doi.org/10.1021/acs.est.0c05881
- Karp, N.A., McCormick, P.S., Russell, M.R., Lilley, K.S., 2007. Experimental and statistical
  considerations to avoid false conclusions in proteomics studies using differential in-gel
  electophoresis. Mol. Cell. Proteomics 6.8, 1354–1364.
  https://doi.org/10.1074/mcp.M600274-MCP200
- Koban, A., Bernhard, G., 2007. Uranium(VI) complexes with phospholipid model compounds
   A laser spectroscopic study. J. Inorg. Biochem. 101, 750–757.
  https://doi.org/10.1016/j.jinorgbio.2007.01.001
- Krzesłowska, M., 2011. The cell wall in plant cell response to trace metals: Polysaccharide
  remodeling and its role in defense strategy. Acta Physiol. Plant.
  https://doi.org/10.1007/s11738-010-0581-z
- Krzesłowska, M., Lenartowska, M., Samardakiewicz, S., Bilski, H., Woźny, A., 2010. Lead
  deposited in the cell wall of *Funaria hygrometrica* protonemata is not stable A
  remobilization can occur. Environ. Pollut. 158, 325–338.
  https://doi.org/10.1016/j.envpol.2009.06.035
- Lai, J., Liu, Z., Li, C., Luo, X., 2021. Analysis of accumulation and phytotoxicity mechanism
  of uranium and cadmium in two sweet potato cultivars. J. Hazard. Mater. 409, 124997.
  https://doi.org/10.1016/j.jhazmat.2020.124997
- Laing, W., Christeller, J., 2004. Extraction of proteins from plant tissues. Curr. Protoc. protein
  Sci. 38, 4.7.1-4.7.7. https://doi.org/10.1002/0471140864.ps0407s38
- Langmuir, D., 1978. Uranium solution-mineral equilibria at low temperatures with applications
  to sedimentary ore deposits. Geochim. Cosmochim. Acta 42, 547–569.
  https://doi.org/10.1016/0016-7037(78)90001-7

- Laroche, L., Henner, P., Camilleri, V., Morello, M., Garnier-Laplace, J., 2005. Root uptake of
  uranium by a higher plant model (*Phaseolus vulgaris*) bioavailability from soil solution.
  Radioprotection 40, S33–S39. https://doi.org/10.1051/radiopro
- Laurette, J., Larue, C., Llorens, I., Jaillard, D., Jouneau, P.H., Bourguignon, J., Carrière, M.,
  2012. Speciation of uranium in plants upon root accumulation and root-to-shoot
  translocation: A XAS and TEM study. Environ. Exp. Bot. 77, 87–95.
  https://doi.org/10.1016/j.envexpbot.2011.11.005
- Lin, W.-Y., Lin, S.-I., Chiou, T.-J., 2009. Molecular regulators of phosphate homeostasis in
  plants. J. Exp. Bot. 60, 1427–1438. https://doi.org/10.1093/jxb/ern303
- Majovsky, P., Naumann, C., Lee, C.W., Lassowskat, I., Trujillo, M., Dissmeyer, N.,
  Hoehenwarter, W., 2014. Targeted proteomics analysis of protein degradation in plant
  signaling on an LTQ-Orbitrap mass spectrometer. J. Proteome Res. 13, 4246–4258.
  https://doi.org/10.1021/pr500164j
- Mimura, T., 1995. Homeostasis and transport of inorganic phosphate in plants. Plant Cell
  Physiol. 36, 1–7. https://doi.org/10.1093/oxfordjournals.pcp.a078724
- Moll, H., Sachs, S., Geipel, G., 2020. Plant cell (*Brassica napus*) response to europium(III)
  and uranium(VI) exposure. Environ. Sci. Pollut. Res. 27, 32048–32061.
  https://doi.org/10.1007/s11356-020-09525-2
- Mulisch, M., Welsch, U., 2010. Romeis Mikroskopische Technik, 18th ed. Spektrum
  Akademischer Verlag, Heidelberg.
- Murphy, A.S., Bandyopadhyay, A., Holstein, S.E., Peer, W.A., 2005. Endocytotic cycling of
  PM proteins. Annu. Rev. Plant Biol. 56, 221–251.
  https://doi.org/10.1146/annurev.arplant.56.032604.144150
- 763 Nagata, T., Nemoto, Y., Hasezawa, S., 1992. Tobacco BY-2 Cell Line as the "HeLa" Cell in 764 Plants. 132, the Cell Biology of Higher Int. Rev. Cytol. 1–30. https://doi.org/10.1016/S0074-7696(08)62452-3 765
- Neill, T.S., Morris, K., Pearce, C.I., Sherriff, N.K., Burke, M.G., Chater, P.A., Janssen, A.,
  Natrajan, L., Shaw, S., 2018. Stability, composition, and core-shell particle structure of
  uranium(IV)-silicate colloids. Environ. Sci. Technol. 52, 9118–9127.
  https://doi.org/10.1021/acs.est.8b01756

- Nicholas, D.J.D., 1975. The functions of trace elements in planta, in: Nicholas, D.J.D., Egan,
  A.H. (Eds.), Trace Elements in Soil-Plant-Animal Systems. pp. 181–198.
  https://doi.org/10.1016/B978-0-12-518150-1.X5001-0
- Nielsen, E., Cheung, A.Y., Ueda, T., 2008. The regulatory RAB and ARF GTPases for
  vesicular trafficking. Plant Physiol. 147, 1516–1526.
  https://doi.org/10.1104/pp.108.121798
- Nishida, S., Tsuzuki, C., Kato, A., Aisu, A., Yoshida, J., Mizuno, T., 2011. AtIRT1, the primary
  iron uptake transporter in the root, mediates excess nickel accumulation in *Arabidopsis thaliana*. Plant Cell Physiol. 52, 1433–1442. https://doi.org/10.1093/pcp/pcr089
- Orsburn, B.C., 2021. Proteome discoverer-a community enhanced data processing suite for
   protein informatics. Proteomes 9, 15. https://doi.org/10.3390/proteomes9010015
- Pérez-Gómez, J., Moore, I., 2007. Plant endocytosis: it is clathrin after all. Curr. Biol. 17,
  R217-219. https://doi.org/10.1016/j.cub.2007.01.045
- Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D.J.,
  Inuganti, A., Griss, J., Mayer, G., Eisenacher, M., Pérez, E., Uszkoreit, J., Pfeuffer, J.,
  Sachsenberg, T., Yilmaz, Ş., Tiwary, S., Cox, J., Audain, E., Walzer, M., Jarnuczak, A.F.,
  Ternent, T., Brazma, A., Vizcaíno, J.A., 2019. The PRIDE database and related tools and
  resources in 2019: Improving support for quantification data. Nucleic Acids Res. 47,
  D442–D450. https://doi.org/10.1093/nar/gky1106
- Rajabi, F., Jessat, J., Garimella, J.N., Bok, F., Steudtner, R., Stumpf, T., Sachs, S., 2021.
  Uranium(VI) toxicity in tobacco BY-2 cell suspension culture A physiological study.
  Ecotoxicol. Environ. Saf. 211, 111883. https://doi.org/10.1016/j.ecoenv.2020.111883
- Raskin, I., Kumar, P.N., Dushenkov, S., Salt, D.E., 1994. Bioconcentration of heavy metals by
  plants. Curr. Opin. Biotechnol. 5, 285–290. https://doi.org/10.1016/0958-1669(94)900302
- Ratnikov, A.N., Sviridenko, D.G., Popova, G.I., Sanzharova, N.I., Mikailova, R.A., 2020. The
  behaviour of uranium in soils and the mechanisms of its accumulation by agricultural
  plant, in: Gupta, D.K., Walther, C. (Eds.), Uranium in Plants and the Environment.
  Springer Nature Switzerland AG, pp. 113–135. https://doi.org/10.1007/978-3-030-149611\_5

- Rehman, M.Z., Rizwan, M., Sohail, M.I., Ali, S., Waris, A.A., Khalid, H., Naeem, A., Ahmad,
  H.R., Rauf, A., 2019. Opportunities and challenges in the remediation of metalcontaminated soils by using tobacco (*Nicotiana tabacum* L.): a critical review. Environ.
- 803 Sci. Pollut. Res. 26, 18053–18070. https://doi.org/10.1007/s11356-019-05391-9
- Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron
  microscopy. J. Cell Biol. 17, 208–212. https://doi.org/10.1083/jcb.17.1.208
- Reynolds, G.D., Wang, C., Pan, J., Bednarek, S.Y., 2018. Inroads into internalization: Five
  years of endocytic exploration. Plant Physiol. 176, 208–218.
  https://doi.org/10.1104/pp.17.01117
- Ribera, D., Labrot, F., Tisnerat, G., Narbonne, J.F., 1996. Uranium in the environment:
  occurrence, transfer, and biological effects. Rev. Environ. Contam. Toxicol. 146, 53–89.
  https://doi.org/10.1007/978-1-4613-8478-6\_3
- Rigal, A., Doyle, S.M., Robert, S., 2015. Live cell imaging of FM4-64, a tool for tracing the
  endocytic pathways in Arabidopsis root cells, in: Estevez, J.M. (Ed.), Plant Cell
  Expansion: Methods in Molecular Biology (Methods and Protocols). Humana Press, New
  York, pp. 93–103. https://doi.org/10.1007/978-1-4939-1902-4\_9
- Rodriguez-Freire, L., DeVore, C.L., El Hayek, E., Berti, D., Ali, A.-M.S., Lezama Pacheco,
  J.S., Blake, J.M., Spilde, M.N., Brearley, A.J., Artyushkova, K., Cerrato, J.M., 2021.
- 818 Emerging investigator series: entrapment of uranium–phosphorus nanocrystals inside root
- cells of *Tamarix* plants from a mine waste site. Environ. Sci. Process. Impacts 23, 73–85.
  https://doi.org/10.1039/d0em00306a
- Rohde, F., Braumann, U.-D., Schmidt, M., 2020. Correlia: an ImageJ plug-in to co-register and
  visualise multimodal correlative micrographs. J. Microsc. 280, 3–11.
  https://doi.org/10.1111/jmi.12928
- Saenen, E., Horemans, N., Vanhoudt, N., Vandenhove, H., Biermans, G., Van Hees, M.,
  Wannijn, J., Vangronsveld, J., Cuypers, A., 2013. Effects of pH on uranium uptake and
  oxidative stress responses induced in *Arabidopsis thaliana*. Environ. Toxicol. Chem. 32,
  2125–2133. https://doi.org/10.1002/etc.2290
- Sano, T., Kuraya, Y., Amino, S., Nagata, T., 1999. Phosphate as a limiting factor for the cell
  division of tobacco BY-2 Cells. Plant Cell Physiol. 40, 1–8.

830 https://doi.org/10.1093/oxfordjournals.pcp.a029464

- 831 Sarthou, M.C.M., Devime, F., Baggio, C., Figuet, S., Alban, C., Bourguignon, J., Ravanel, S.,
  832 2022. Calcium-permeable cation channels are involved in uranium uptake in *Arabidopsis*833 *thaliana*. J. Hazard. Mater. 424, 127436. https://doi.org/10.1016/j.jhazmat.2021.127436
- Schnug, E., Lottermoser, B.G., 2013. Fertilizer-derived uranium and its threat to human health.
  Environ. Sci. Technol. 47, 2433–2434. https://doi.org/10.1021/es4002357
- Shahandeh, H., Hossner, L.R., 2002. Role of soil properties in phytoaccumulation of uranium.
  Water. Air. Soil Pollut. 141, 165–180. https://doi.org/10.1023/A:1021346828490
- Song, Y., Jin, L., Wang, X., 2017. Cadmium absorption and transportation pathways in plants.
  Int. J. Phytoremediation 19, 133–141. https://doi.org/10.1080/15226514.2016.1207598
- Soudek, P., Petrová, Š., Buzek, M., Lhotský, O., Vaněk, T., 2014. Uranium uptake in *Nicotiana*sp. under hydroponic conditions. J. Geochemical Explor. 142, 130–137.
  https://doi.org/10.1016/j.gexplo.2013.10.001
- Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy.
  J. Ultrastruct. Res. 26, 31–43. https://doi.org/https://doi.org/10.1016/S00225320(69)90033-1
- Srba, M., Černíková, A., Opatrný, Z., Fischer, L., 2016. Practical guidelines for the
  characterization of tobacco BY-2 cell lines. Biol. Plant. 60, 13–24.
  https://doi.org/10.1007/s10535-015-0573-3
- Stojanović, M.D., Mihajlović, M.L., Milojković, J. V., Lopičić, Z.R., Adamović, M.,
  Stanković, S., 2012. Efficient phytoremediation of uranium mine tailings by tobacco.
  Environ. Chem. Lett. 10, 377–381. https://doi.org/10.1007/s10311-012-0362-6
- Tahara, H., Yokota, E., Igarashi, H., Orii, H., Yao, M., Sonobe, S., Hashimoto, T., Hussey,
  P.J., Shimmen, T., 2007. Clathrin is involved in organization of mitotic spindle and
  phragmoplast as well as in endocytosis in tobacco cell cultures. Protoplasma 230, 1–11.
  https://doi.org/10.1007/s00709-006-0226-7
- Vandenhove, H., 2002. European sites contaminated by residues from the ore-extracting and processing industries. Int. Congr. Ser. 1225, 307–315. https://doi.org/10.1016/S05315131(01)00525-8

- Viehweger, K., Geipel, G., 2010. Uranium accumulation and tolerance in *Arabidopsis halleri*under native versus hydroponic conditions. Environ. Exp. Bot. 69, 39–46.
  https://doi.org/10.1016/j.envexpbot.2010.03.001
- Viehweger, K., Geipel, G., Bernhard, G., 2011. Impact of uranium (U) on the cellular
  glutathione pool and resultant consequences for the redox status of U. BioMetals 24,
  1197–1204. https://doi.org/10.1007/s10534-011-9478-6
- Wall, J.D., Krumholz, L.R., 2006. Uranium reduction. Annu. Rev. Microbiol. 60, 149–166.
   https://doi.org/10.1146/annurev.micro.59.030804.121357
- Wang, C., Yan, X., Chen, Q., Jiang, N., Fu, W., Ma, B., Liu, J., Li, C., Bednarek, S.Y., Pan, J.,
  2013. Clathrin light chains regulate clathrin-mediated trafficking, auxin signaling, and
  development in Arabidopsis. Plant Cell 25, 499–516.
  https://doi.org/10.1105/tpc.112.108373
- 871 Wang, L., Li, J., Zhou, Q., Yang, G., Ding, X.L., Li, X., Cai, C.X., Zhang, Z., Wei, H.Y., Lu, 872 T.H., Deng, X.W., Huang, X.H., 2014. Rare earth elements activate endocytosis in plant S. 873 U. Α. cells. Proc. Natl. Acad. Sci. 111. 12936-12941. 874 https://doi.org/10.1073/pnas.1413376111
- Wang, X., Gao, Y., Feng, Y., Li, X., Wei, Q., Sheng, X., 2014. Cadmium stress disrupts the
  endomembrane organelles and endocytosis during *Picea wilsonii* pollen germination and
  tube growth. PLoS One 9, e94721. https://doi.org/10.1371/journal.pone.0094721
- Wang, X., Xu, M., Gao, C., Zeng, Y., Cui, Y., Shen, W., Jiang, L., 2020. The roles of
  endomembrane trafficking in plant abiotic stress responses. J. Integr. Plant Biol. 62, 55–
  69. https://doi.org/10.1111/jipb.12895
- Wetterlind, J., Richer De Forges, A.C., Nicoullaud, B., Arrouays, D., 2012. Changes in
  uranium and thorium contents in topsoil after long-term phosphorus fertilizer application.
  Soil Use Manag. 28, 101–107. https://doi.org/10.1111/j.1475-2743.2011.00376.x
- Wheeler, H., Hanchey, P., 1971. Pinocytosis and membrane dilation in uranyl-treated plant
  roots. Science (80-.). 171, 68–71. https://doi.org/10.1126/science.171.3966.68
- Wu, D., Shen, H., Yokawa, K., Baluška, F., 2015. Overexpressing OsPIN2 enhances
  aluminium internalization by elevating vesicular trafficking in rice root apex. J. Exp. Bot.
  66, 6791–6801. https://doi.org/10.1093/jxb/erv385

Zavala-Guevara, I.P., Ortega-Romero, M.S., Narváez-Morales, J., Jacobo-Estrada, T.L., Lee,
W.K., Arreola-Mendoza, L., Thévenod, F., Barbier, O.C., 2021. Increased endocytosis of
cadmium-metallothionein through the 24p3 receptor in an in vivo model with reduced
proximal tubular activity. Int. J. Mol. Sci. 22, 7262. https://doi.org/10.3390/ijms22147262

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## 903 8. Author contributions

Warren John: conceptualization, data curation, investigation, methodology, validation,
visualization, writing – original draft.

906 **Benita Lückel**: investigation, methodology, writing – review & editing.

907 Nicole Matschiavelli: conceptualization, investigation, methodology, writing – review &
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909 René Hübner: formal analysis, investigation, methodology, visualization, writing – review &
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- 911 Susanne Matschi: data curation, formal analysis, investigation, methodology, writing review
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- Wolfgang Hoehenwarter: formal analysis, investigation, methodology, writing review &
  editing.
- 915 Susanne Sachs: conceptualization, funding acquisition, investigation, methodology, project
- 916 administration, resources, supervision, writing review & editing.

# 917 9. Declaration of interests

918 The authors declare that they have no known competing financial interests or personal 919 relationships that could have appeared to influence the work reported in this paper.

# 920 10. Data Availability

The proteomics dataset that supports the findings of this study is openly available on the ProteomeXchange Consortium via the Proteomics Identification Database (PRIDE) partner repository with the dataset identifier PXD028677 and 10.6019/PXD028677. A complete list of identified proteins can be found in Appendix B.

## 925 11. Figure legends

Fig 1: Cell viability (a) and dry biomass (b) of a 96-h old tobacco BY-2 culture in MS medium sub-cultivated into MS<sub>red</sub> and grown for 24 h. Error bars represent standard errors of the mean for one biological replicate with three counting events for MS medium and three biological replicates each with three counting events for MS<sub>red</sub> medium. Statistical significance is denoted by \* (p < 0.05) and \*\*\* (p < 0.001) between corresponding 0-h and 24-h samples. Micrographs

931 of BY-2 cells before (c) and after (d) 24 h in  $MS_{red}$  medium. Scale bar: 20  $\mu$ m.

Fig 2: Internalisation of the FM4-64<sup>TM</sup> stain after 90 min of dye exposure in untreated tobacco BY-2 cells (Ctrl), cells treated with 20  $\mu$ M uranyl(VI) nitrate and cells treated with both 20  $\mu$ M uranyl(VI) nitrate and 5  $\mu$ M wortmannin (WM). Cell physiology is shown by phase contrast images (a, c and e), and endocytosis is shown by FM4-64<sup>TM</sup> fluorescence (b, d and f). Scale

936 bar: 50 μm.

Fig 3: Signal intensities from within the cell after 90 min of FM4-64<sup>TM</sup> in untreated tobacco BY 2 cells (Ctrl; n=61), cells treated with 20  $\mu$ M uranyl(VI) nitrate (U; n=43) and cells treated with both 20  $\mu$ M uranyl(VI) nitrate and 5  $\mu$ M wortmannin (U+WM; n=42). Measurements are taken from at least two sampling events from one biological replicate for each testing condition. Statistical significance is denoted by \*\*\* (p < 0.001) between U and U+WM and n represents

942 the number of cells per field of view.

Fig 4: Uranium bioassociation to untreated BY-2 cells (control), cells treated with 20  $\mu$ M uranyl(VI) nitrate (+U) and cells treated with both 20  $\mu$ M uranyl(VI) nitrate and 33  $\mu$ M wortmannin (+U+WM) for 6 h. Error bars represent standard errors of the mean. \*\*\* represents statistical significance between +U and +U+WM where *p* < 0.001.

Fig 5: Bright-field transmission electron micrograph (a) of uranium-containing needle-like structures in tobacco BY-2 cells exposed to  $20 \,\mu$ M uranyl(VI) nitrate for 24 h. EDX-based elemental mapping (b, c and d) is shown for the structures in a, with c and d containing overlays of various signals. CW: cell wall; N: nitrogen; O: oxygen; P: phosphorus; S: sulphur; U:
uranium; scale bar in element distributions: 100 nm.

Fig 6: Uranium adsorption and precipitation in tobacco BY-2 cells exposed to  $200 \,\mu\text{M}$ uranyl(VI) nitrate for 24 h. EDX sum spectra in panel d are from selected regions in the electron micrographs in a, b and c, labelled A1, B1 and C1, respectively, and the sum spectra from f are from selected regions in panel e, labelled E1-E4, to show the presence of uranium. CW: cell wall; M: mitochondria; PM: plasma membrane; V: vacuole; scale bars: 500 nm in a, 200 nm in

- b and e. The dotted lines in the spectra of d and f point to the position of the  $L_{\alpha 1}$  peak of uranium
- 958 (13.612 keV).