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Originally published:

February 2022

Science of the Total Environment 823(2022), 153700

DOI: <https://doi.org/10.1016/j.scitotenv.2022.153700>

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1 **Endocytosis is a significant contributor to uranium(VI) uptake in**
2 **tobacco (*Nicotiana tabacum*) BY-2 cells in phosphate-deficient**
3 **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.

38 **Keywords:** plant cell; proteomics; radionuclide transport; heavy metal interaction; vesicle
39 uptake

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
46 uranium, of which ^{238}U is the most abundant isotope (99.3%), has lately been growing in
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),
64 in the form of the uranyl(VI) ion UO_2^{2+} (Laroche et al., 2005; Wall & Krumholz, 2006). The
65 uranyl(VI) ion has the propensity to form both soluble and insoluble complexes. Under
66 acidic/near neutral conditions, the uranyl(VI) ion can form complexes with hydroxide and
67 phosphate, and under alkaline conditions, with carbonate (Ebbs et al., 1998). Of the various
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

105 dyes such as FM4-64TM have been well-established due to their ability to bind to plant cell
106 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.,
126 1992) cell suspensions in order to investigate the uptake of uranium via endocytosis into these
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

136 Tobacco BY-2 cells were grown as outlined in Rajabi et al. (2021) in modified
137 Murashige-Skoog (MS) medium (4.3 g l⁻¹ MS basal salt, 200 mg l⁻¹ KH₂PO₄, 30 g l⁻¹ sucrose,
138 100 mg l⁻¹ myo-inositol, 1 mg l⁻¹ thiamine hydrochloride and 0.2 mg l⁻¹ 2,4-
139 dichlorophenoxyacetic acid; 2,4-D, pH 5.8) in the dark at 25°C on an IKA®KS 260 basic
140 orbital shaker (IKA Labortechnik, Staufen, Germany) set to 150 rpm. Consult appendix for
141 details of chemicals and reagents. pH values were measured with a pH meter (inoLab pH Level
142 1, WTW, Weilheim, Germany) using a BlueLine 16 pH electrode (SI Analytics, Mainz,
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
147 conditions as mentioned in the preceding section. An appropriate volume, based on the
148 experiment, of this 96-h old culture was then added into 30 ml of phosphate-deficient MS
149 (MS_{red}) medium (0.026 mg l⁻¹ CoCl₂·6H₂O, 0.025 mg l⁻¹ CuSO₄·5H₂O, 0.25 mg l⁻¹
150 Na₂MoO₄·2H₂O, 331.9 mg l⁻¹ CaCl₂, 1900 mg l⁻¹ KNO₃, 1650 mg l⁻¹ NH₄NO₃, 6.2 mg l⁻¹
151 H₃BO₃, 0.83 mg l⁻¹ KI, 16.9 mg l⁻¹ MnSO₄·H₂O, 8.6 mg l⁻¹ ZnSO₄·7H₂O, 180.54 mg l⁻¹
152 MgSO₄·7H₂O, 9.3 mg l⁻¹ KCl, 36.7 mg l⁻¹ FeNaEDTA, 1.7 mg l⁻¹ KH₂PO₄, 30,000 mg l⁻¹
153 sucrose, 100 mg l⁻¹ myo-inositol, 1 mg l⁻¹ thiamine hydrochloride and 0.2 mg l⁻¹ 2,4D; pH
154 adjusted to 5.8 with KOH) in 100-ml Erlenmeyer flasks. Cells were cultured in MS_{red} medium
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
157 predetermined concentrations, depending on the experiment, and left to incubate for a
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_{red} medium.
161 Uranium in natural isotope composition is a radioactive α emitter. It should be handled in
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 HNO₃ 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

178 5 ml of a 96-h culture in MS medium were added to three Erlenmeyer flasks containing
179 30 ml of MS_{red} medium and one flask containing 30 ml of MS medium. All four flasks were
180 left to incubate under culture conditions. Phase contrast images of cells were acquired with an
181 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,
184 Mönchengladbach, Germany) placed in CellCrown™ inserts (Sigma-Aldrich, Taufkirchen,
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_{red} medium. Using a Fuchs-Rosenthal
188 counting chamber (Brand GmbH & CO KG, Wertheim, Germany), up to 500 cells were
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.

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

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_{red} medium. To one flask, wortmannin was added to a final
207 concentration of 5 μ 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
210 20 μ 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 FM4-64TM dye was added to a working concentration of 1.65 μ M 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,

226 and for the U+WM-treated sample, 42. These mean intensities were normalized to the
227 background mean intensities in each image. Statistical analysis was then done in the form of a
228 two-tailed student's t-test to show significance.

229 2.6. Proteomics analysis

230 5 ml of a 96-h culture in MS medium were added to 30 ml MS_{red} medium in six flasks.
231 To three of these flasks, uranyl(VI) nitrate was added to a concentration of 10 μ M and the
232 remaining three flasks served as negative controls (i.e. three biological replicates per testing
233 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-(N-
236 morpholino)propanesulfonic acid (MOPS) buffer (50 mM, pH 7.5). The cells were
237 resuspended in 1 ml of ice-cold lysis buffer (1% TritonTM X-100, 2 mM dithiothreitol, 5%
238 polyvinylpolypyrrolidone, 50 mM MOPS, cOmpleteTM 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 PierceTM 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⁻¹. 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
254 set to 15 s, resolution set to 70,000, automatic gain control (AGC) at 3x10⁶ and a max injection
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
257 scans with mass to charge ratios (m/z) between 400 and 1850 were acquired. MS/MS scans

258 were acquired with a resolution of 17,500, AGC of 5×10^4 , IT of 50 ms, isolation width of 1.6
259 m/z, normalized collision energy of 28, under fill ratio of 3%, dynamic exclusion duration of
260 20 s, and an intensity threshold of 3×10^4 .

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 Corporation, 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_{red} 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_{red} 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% H₂O₂ and 43% HNO₃ 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

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

294 2.8. Preparation for transmission electron microscopy

295 BY-2 cells exposed to 0, 20 and 200- μ M concentrations of uranyl(VI) nitrate in MS_{red}
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
323 in phosphate-deficient MS_{red} medium, containing only 1.7 mg l⁻¹ phosphate (as opposed to
324 200 mg l⁻¹ in MS medium), when they were exposed to uranyl(VI) nitrate. Moreover, it had
325 to be ensured that the phosphate content of the MS medium culture had dropped below a
326 threshold of 1.7 mg l⁻¹ to avoid a carryover of phosphate during the transfer of the culture into
327 MS_{red} medium. ICP-MS measurements revealed that phosphate content of the cell-free MS
328 medium after 96 h of BY-2 culture was 238 ± 45 µg l⁻¹ and thus well-below the threshold (see
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_{red} 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_{red}
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
342 replicate (Fig. 1b). Sano et al. (1999) showed that the mitotic index decreases to 0% when
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_{red} 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_{red} 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

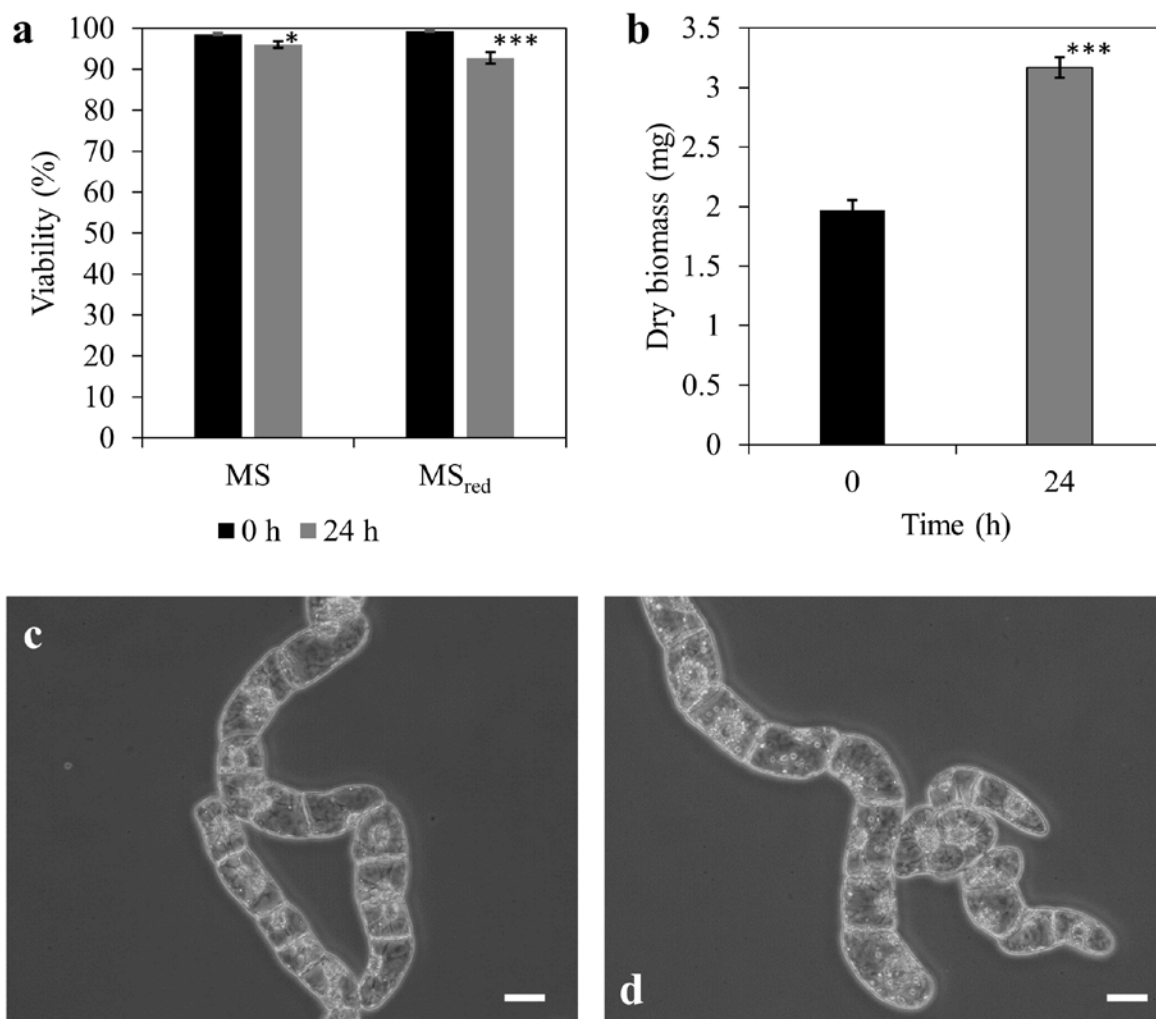


Fig 1: Cell viability (a) and dry biomass (b) of a 96-h old tobacco BY-2 culture in MS medium sub-cultivated into MS_{red} and grown for 24 h. Error bars represent standard errors of the mean for one biological replicate with three counting events for MS medium and three biological replicates each with 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 μ m.

350

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

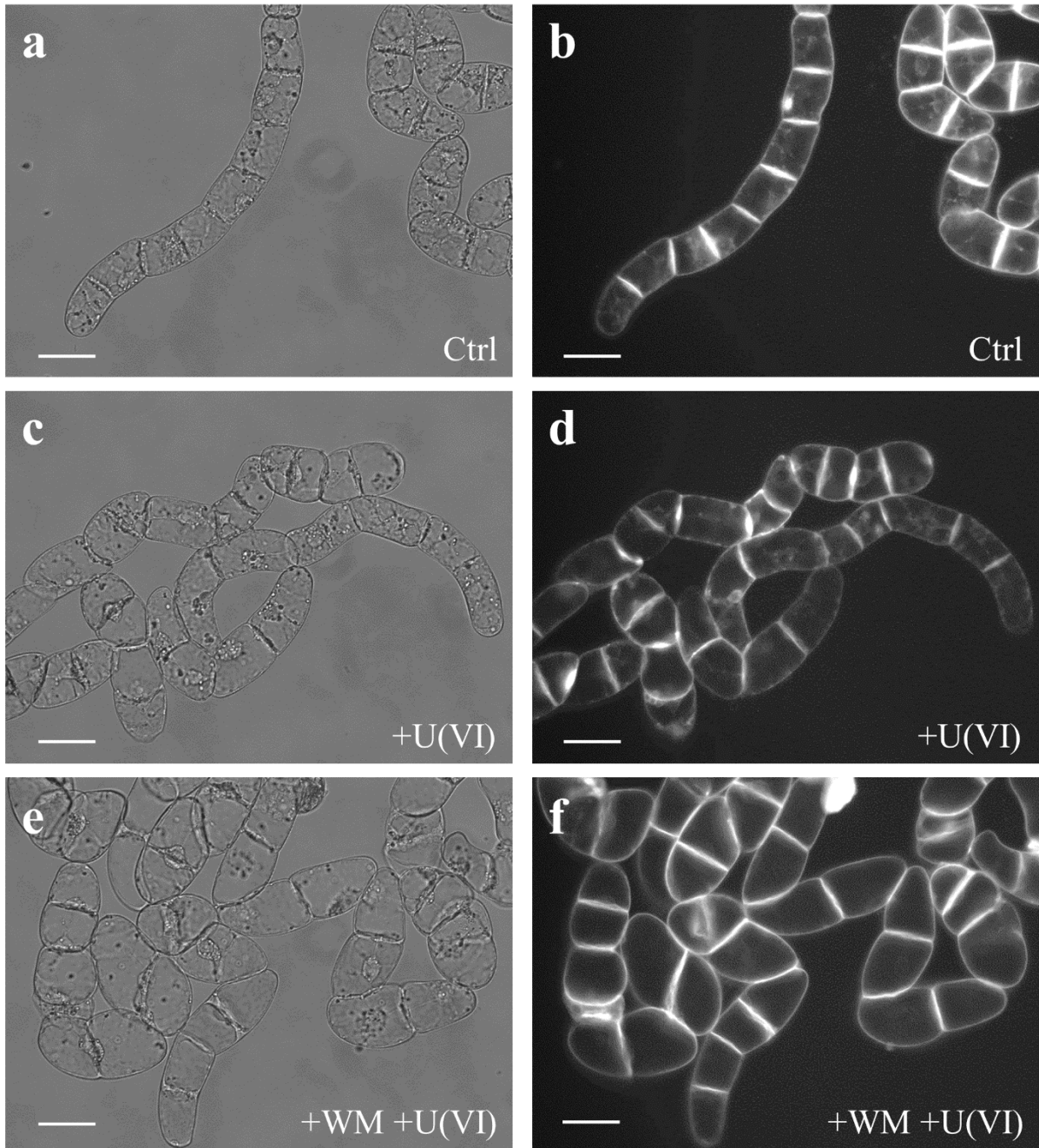
354 Endocytosis in the presence of uranium was firstly examined using fluorescence
 355 microscopy. The styryl dye FM4-64TM is a compound that intercalates within moieties of the
 356 plasma membrane, causing it to fluoresce, and is therefore a useful marker for vesicular uptake
 357 as it migrates over time into the cell during live staining (Bolte et al., 2004). Cells were exposed
 358 to 20 μ M uranyl(VI) nitrate for 5 h and subsequently stained with FM4-64TM. An exposure to
 359 20 μ M uranium was chosen because, firstly, it resembles uranium concentrations in soil that is

360 mildly contaminated (Vandenhove, 2002), and secondly, it induces a level of toxicity over 24 h
361 without completely killing the cell population (Fig. **A.3**). The relatively short exposure meant
362 that cells were within the limits of experiencing stress due to phosphate deficiency and also to
363 the presence of uranium. The viability of these cells was still relatively high at $80 \pm 3\%$ with
364 exposure to $20 \mu\text{M}$ uranium for 6 h and thus meant that there was minimal compromise to
365 plasma membrane integrity caused by uranium.

366 It was evident that in both the presence and absence of uranium, the dye was taken up
367 (Fig. **2a-d**). After 90 min of live staining, an intracellular staining pattern emerged, proving
368 that endocytosis was still in play in both conditions. Consistent with these findings, Bolte et al.
369 (2004) reported a staining of numerous organelle membranes in BY-2 cells within 1 h of dye
370 incubation. The observation that both the uranium-treated and control experiments showed
371 staining under 90 min would imply that the rate of vesicular uptake remained largely
372 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 FM4-64TM, cells were
376 treated with wortmannin for 30 min prior to staining. In this case, even after 90 min of
377 incubation with FM4-64TM, 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 $20 \mu\text{M}$ 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_{red} medium
388 and compared to a control experiment in MS_{red} without uranium. To elicit a distinct protein
389 response to the presence of uranium in culture, experiments were conducted using a longer
390 uranium exposure time of 24 h and, thus, a lower



391

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

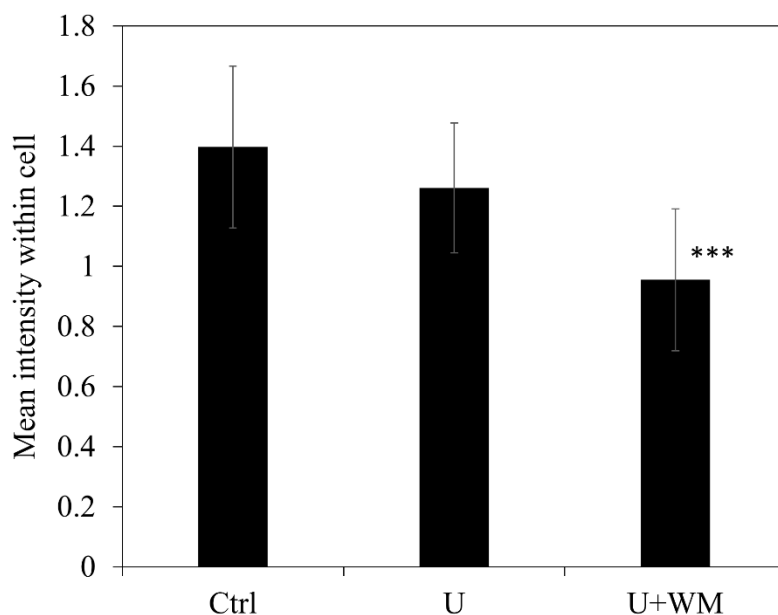


Fig 3: Signal intensities within the cell after 90 min of FM4-64TM in untreated tobacco BY-2 cells (Ctrl; n=61), cells treated with 20 μ M uranyl(VI) nitrate (U; n=43) and cells treated with both 20 μ M uranyl(VI) nitrate and 5 μ M wortmannin (U+WM; n=42). Measurements are taken from at least two sampling 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 μ 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
 397 the amount of protein than the uranium-treated sample. A q-value less than 0.05 was used to
 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_2 fold changes) involved in clathrin-mediated endocytosis comparing tobacco BY-2 cells exposed to 10 μM 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
433 endocytosis (Fig. 2). Secondly, to cause as little plasma membrane leakage as possible due to
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
438 significant decrease in bioassociated (surface-bound and internalized) uranium compared to
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.

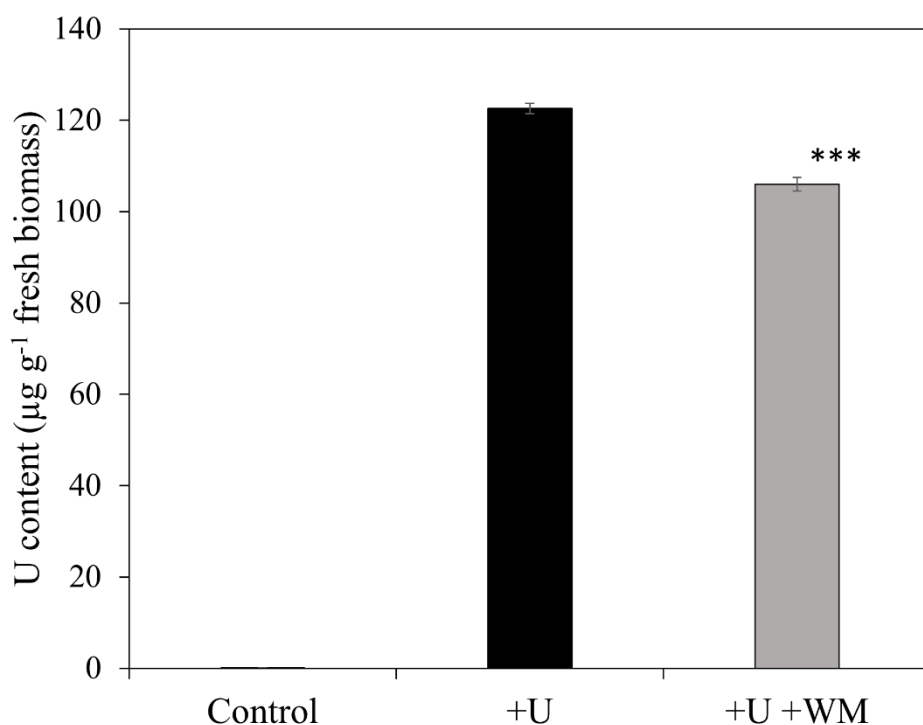


Fig 4: Uranium bioassociation to untreated BY-2 cells (control), cells treated with 20 μM uranyl(VI) nitrate (+U) and cells treated with both 20 μM uranyl(VI) nitrate and 33 μM wortmannin (+U +WM) for 6 h. Error bars represent standard errors of the mean from three measurements of one biological replicate.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

441 3.4. Uranium localizes to vacuolar spaces and adsorbs to cell surface components

442 EDX is an established analytical method based on the fingerprints of an element's X-ray
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.

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

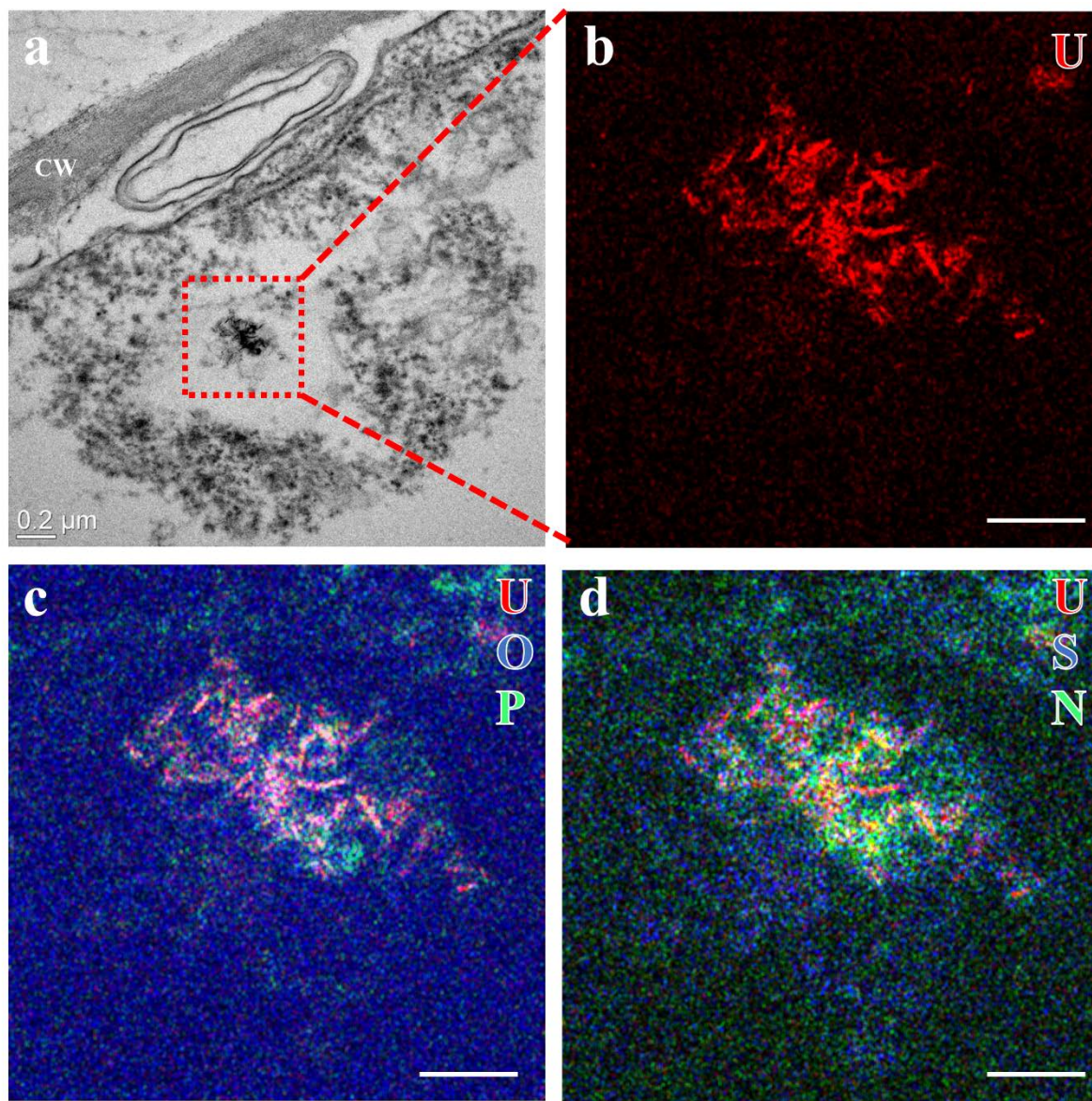


Fig 5: Bright-field transmission electron micrograph (a) of uranium-containing needle-like structures in tobacco BY-2 cells exposed to 20 μ M uranyl(VI) nitrate for 24 h. EDX-based elemental mapping (b, c and d) is shown for the structures in a, with c and d containing overlays of various signals. CW: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

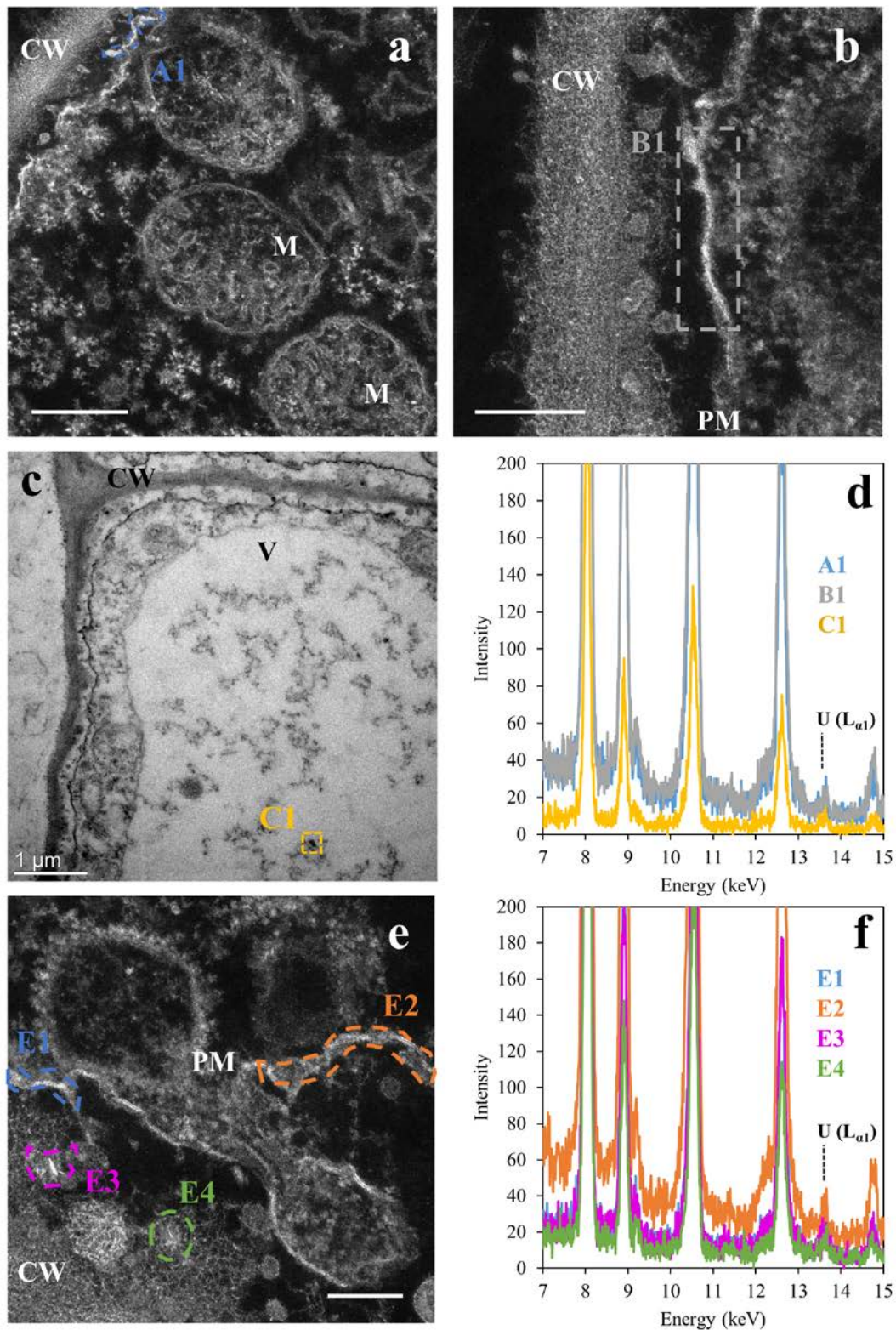


Fig 6: Uranium adsorption and precipitation in tobacco BY-2 cells exposed to 200 μ M uranyl(VI) nitrate for 24 h. EDX sum spectra in panel d are from selected regions in the electron micrographs in a, b and c, labelled A1, B1 and C1, respectively, and the sum spectra from f are from selected regions 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 (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.

487 Cell growth and viability experiments proved that cells within 24 h in
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 than 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 μ 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.

566 It is difficult to say how much of this vacuole sequestration is a result of endocytosis
567 and vesicle trafficking, as opposed to transporter-based mechanisms. There is already evidence
568 for a transporter-mediated uptake of uranium into BY-2 cells (Rajabi et al., 2021; Sarthou et
569 al., 2022), however, an endocytic uptake of the metal would provide a completely different
570 pathway as opposed to one that is transporter-mediated. It would provide greater control over
571 where the metal is compartmentalized, as opposed to freely loading metal into the cytosol,
572 thereby reducing contact with susceptible biomolecules. One of the main roles of endocytosis

573 is receptor recycling for plasma membrane remodelling, which involves changing the proteins
574 being expressed on the plasma membrane to adapt to a specific situation. This would help the
575 cell to cope in the presence of toxic metals, by removing proteins on the membrane, which
576 might inadvertently cause metal transport into the cell, and replacing them with such as are
577 needed to prevent and combat the effects of metal toxicity (De Caroli et al., 2020; Wang et al.,
578 2020). The removed proteins would subsequently be targeted initially to multi-vesicular bodies
579 (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**

598 The herein described results present a case for endocytosis as an uptake mechanism of
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?

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

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893 **7. Acknowledgments**

894 The first batch of BY-2 cells was kindly provided by Prof. P. Nick from the Botanical
895 Institute, Karlsruhe Institute of Technology. The authors are also grateful for experimental
896 assistance from S. Beutner and S. Bachmann with respect to ICP-MS measurements, and J.
897 Seibt for cell culture. The authors also thank Dr. F. Rajabi for her insight and contributions to
898 wortmannin treatment of BY-2 cells. The use of the Ion Beam Center TEM facilities and the
899 funding of TEM Talos by the German Federal Ministry of Education and Research (BMBF,
900 Grant No. 03SF0451) in the framework of HEMCP are gratefully acknowledged. This work is
901 part of the project TRANS-LARA, which was funded by the BMBF under contract number
902 02NUK051B.

903 **8. Author contributions**

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

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

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914 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**

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

925 **11. Figure legends**

926 Fig 1: Cell viability (a) and dry biomass (b) of a 96-h old tobacco BY-2 culture in MS medium
927 sub-cultivated into MS_{red} and grown for 24 h. Error bars represent standard errors of the mean
928 for one biological replicate with three counting events for MS medium and three biological
929 replicates each with three counting events for MS_{red} medium. Statistical significance is denoted
930 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 μm .

932 Fig 2: Internalisation of the FM4-64TM stain after 90 min of dye exposure in untreated tobacco
933 BY-2 cells (Ctrl), cells treated with 20 μM uranyl(VI) nitrate and cells treated with both 20 μM
934 uranyl(VI) nitrate and 5 μM wortmannin (WM). Cell physiology is shown by phase contrast
935 images (a, c and e), and endocytosis is shown by FM4-64TM fluorescence (b, d and f). Scale
936 bar: 50 μm .

937 Fig 3: Signal intensities from within the cell after 90 min of FM4-64TM in untreated tobacco
938 BY 2 cells (Ctrl; n=61), cells treated with 20 μM uranyl(VI) nitrate (U; n=43) and cells treated
939 with both 20 μM uranyl(VI) nitrate and 5 μM wortmannin (U+WM; n=42). Measurements are
940 taken from at least two sampling events from one biological replicate for each testing condition.
941 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.

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

947 Fig 5: Bright-field transmission electron micrograph (a) of uranium-containing needle-like
948 structures in tobacco BY-2 cells exposed to 20 μM uranyl(VI) nitrate for 24 h. EDX-based
949 elemental mapping (b, c and d) is shown for the structures in a, with c and d containing overlays

950 of various signals. CW: cell wall; N: nitrogen; O: oxygen; P: phosphorus; S: sulphur; U:
951 uranium; scale bar in element distributions: 100 nm.

952 Fig 6: Uranium adsorption and precipitation in tobacco BY-2 cells exposed to 200 μ M
953 uranyl(VI) nitrate for 24 h. EDX sum spectra in panel d are from selected regions in the electron
954 micrographs in a, b and c, labelled A1, B1 and C1, respectively, and the sum spectra from f are
955 from selected regions in panel e, labelled E1-E4, to show the presence of uranium. CW: cell
956 wall; M: mitochondria; PM: plasma membrane; V: vacuole; scale bars: 500 nm in a, 200 nm in
957 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).