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1 **The plasma membrane-associated cation-binding protein PCaP1 of**
2 ***Arabidopsis thaliana* is a uranyl-binding protein**

3

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26

27 Keywords: plant, uranium, radionuclide, metal, binding, fluorescence spectroscopy, solution
28 state NMR, translocation

29

30 **Highlights**

- 31 • Metalloproteomics analysis identified 38 UraBPs from *Arabidopsis* roots and/or shoots.
32 • Recombinant PCaP1 protein (UraBP25) binds up to 2 U(VI) ions with high affinity *in*
33 *vitro*.
34 • Recombinant PCaP1 protein binds about 15 Cu(II) or 60 Fe(III) ions *in vitro*.
35 • U(VI) binding triggers PCaP1 oligomerization.
36 • U(VI) translocation capacity is reduced in an *Arabidopsis pcap1-null* mutant.

37

38 **Abstract**

39

40 Uranium (U) is a naturally-occurring radionuclide that is toxic to living organisms. Given that
41 proteins are primary targets of U(VI), their identification is an essential step towards
42 understanding the mechanisms of radionuclide toxicity, and possibly detoxification. Here, we
43 developed an immobilized metal affinity chromatography procedure to trap protein targets of
44 uranyl from *Arabidopsis thaliana*. This procedure allowed the identification of 38 uranyl-binding
45 proteins (UraBPs) from root and shoot extracts. One of these, UraBP25, previously identified
46 as plasma membrane-associated cation-binding protein 1 (PCaP1), was further characterized
47 for its ability to interact with U(VI) and other metals *in vitro*, by spectroscopic and structural
48 approaches, and *in planta*, by analyzing the fate of U(VI) in Arabidopsis lines affected in *PCaP1*
49 gene expression. Our results showed that recombinant PCaP1 binds U(VI) *in vitro* with affinity
50 in the nM range, as well as Cu(II) and Fe(III) in high proportions, and that Ca(II) competes with
51 U(VI) for binding. U(VI) induced PCaP1 oligomerization through binding at the interface of
52 monomers, both at the N-terminal structured domain and the C-terminal flexible region. Finally,
53 U(VI) translocation in Arabidopsis shoots was affected in a *pcap1* null-mutant, suggesting a
54 role for this protein in ions trafficking *in planta*.

55

56 1. Introduction

57 Uranium (U) is a radionuclide naturally present in the Earth's crust. It is mainly redistributed in
58 the environment by human activities (e.g. industry, agriculture, mining) and can accumulate
59 locally to concentrations that present potential risks for ecosystems, agrosystems, and
60 eventually human health. Indeed, this element is chemotoxic and possibly radiotoxic for all
61 living organisms. In most natural settings, the di-oxo uranyl cation UO_2^{2+} is the most stable
62 form of U, with U in the +VI oxidation state (thereafter referred to as U(VI)). Although U(VI) is
63 not essential for plants, it is absorbed from the soil, incorporated into the biomass, and thus
64 enters the food chain. Consequently, contamination of soils by U(VI) and its absorption by
65 plants represent a substantial health risk for humans. As predicted by the hard and soft acids
66 principle, uranyl cation is a hard acid and has a high propensity to interact with hard oxygen
67 donors, primarily by electrostatic bonding (Pearson, 1963). Therefore, the biological ligands of
68 U(VI) may be as wide as metabolites, nucleic acids and polypeptides (Garai and Delangle,
69 2020). Since uranyl has the ability to bind strongly to biomolecules through carboxylate and
70 phosphate groups (two of the main hard oxygen donors), proteins may be considered as
71 primary targets of U(VI). Thus, identifying protein targets of uranyl is a crucial step in an attempt
72 to decipher the molecular bases of radionuclide toxicity, and perhaps their detoxification, in
73 any living being (Sarhou et al., 2020).

74 In the last decade, various strategies have been developed for the identification of proteins
75 with high-affinity and/or selectivity for uranyl and forming uranyl-protein complexes *in vivo*
76 and/or *in vitro* (Acharya and Blindauer, 2016; Basset et al., 2013; Bucher et al., 2014; Creff et
77 al., 2019; Eb-Levadoux et al., 2017; Gallois et al., 2021; Huynh et al., 2015; Pardoux et al.,
78 2012; Qi et al., 2014; Safi et al., 2013; Vidaud et al., 2019; Xu et al., 2014). Most of them were
79 metalloproteomic approaches combining efficient protein separation techniques with powerful
80 and sensitive protein and metal identification devices, namely protein tandem mass
81 spectrometry (MS/MS) and inductively coupled plasma mass spectrometry (ICP-MS),
82 respectively. These investigations identified a wide range of proteins that bind uranyl in various
83 organisms including archaea, bacteria, animals and humans. Their discovery was an important
84 milestone for understanding the cellular toxicity of the radionuclide (Basset et al., 2013;
85 Cvetkovic et al., 2010; Dedieu et al., 2009; Gallois et al., 2021; Huynh et al., 2016; Xu et al.,
86 2014). Uranyl-binding proteins (UraBPs) are implied in calcium homeostasis (for example
87 calmodulin, fetuin-A and osteopontin), iron homeostasis (ferritin and transferrin), glucose
88 metabolism, oxidative stress response, microtubule and actin cytoskeleton regulation, or
89 proteolysis. Despite the large number of studies published to date on the impact of U(VI)
90 exposure on plant physiology and development, and on its fate upon absorption (Aranjuelo et
91 al., 2014; Berthet et al., 2018; Doustaly et al., 2014; Ebbs et al., 1998; Lai et al., 2020; Laurette
92 et al., 2012; Misson et al., 2009; Saenen et al., 2014; Saenen et al., 2015; Sarhou et al., 2020;

93 Serre et al., 2019; Tewari et al., 2015; Vanhoudt et al., 2014), no plant UraBP has been
94 identified so far. Yet, such discoveries can help to better understand the molecular
95 mechanisms that govern the fate of U(VI), and understanding how plants manage to thrive in
96 contaminated environmental ecosystems. This knowledge is also required for a future
97 sustainable management of U(VI) in polluted soils and waters as well as in the food chain
98 (Berthet et al., 2018).

99 In this study, we developed a successful two-stage enrichment procedure (ion-exchange
100 chromatography prefractionation step followed by affinity capture) to identify protein targets of
101 uranyl in *Arabidopsis thaliana*. This procedure enabled the purification of 38 UraBP from root
102 and/or shoot extracts by shotgun proteomics. One of these proteins, named UraBP25 (25th
103 U(VI)-interacting protein of our list), hitherto-identified as the plasma membrane-associated
104 cation-binding protein 1 (PCaP1), was of particular interest. PCaP1 was further characterized
105 for its ability to interact with U(VI) and other metals *in vitro*, by a combination of biochemical
106 and structural approaches, and *in planta*, by analyzing the fate of U(VI) in *Arabidopsis* lines
107 affected in the expression of the corresponding gene. Taken together, our results showed first,
108 that recombinant PCaP1 binds U(VI) *in vitro* with high affinity and Cu(II) and Fe(III) in high
109 proportions, and that Ca(II) competes with U(VI) for binding. Second, we showed that U(VI)
110 induces PCaP1 oligomerization through binding to both the N-terminal structured protein
111 domain and the C-terminal flexible region. Last, we observed that U(VI) translocation in
112 *Arabidopsis* shoots is affected in a *pcap1* null-mutant suggesting a role *in planta* for this protein
113 in cations mobility.

114

115

116 **2. Materials and Methods**

117 *2.1. Plant growth conditions for protein fractionation studies*

118 Sterilized and stratified seeds of wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) were
119 sown into homemade thermoprinted plates, as described previously (Sarhou et al., 2022).
120 Plants were grown in hydropony for 5 weeks in the 'Gre medium' nutrient solution, as detailed
121 in (Sarhou et al., 2022), with weekly solution changes. Growth was performed in a controlled
122 environment, with alternation of 8 h light period at 22°C (light intensity of 110 μmol of photons
123 $\text{m}^{-2}\text{s}^{-1}$) and 16 h dark period at 20°C.

124 *2.2. Preparation of protein extracts and anion exchange chromatography (AEC)* 125 *fractionation*

126 Total soluble proteins from plant shoot and root tissues were extracted by grinding 15 g
127 powdered samples in 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and a cocktail of protease
128 inhibitors (Roche Applied Science). Samples were clarified by centrifugation, desalted on
129 PD10 Sephadex G25 (M) columns (GE Healthcare), and 30-40 mg soluble proteins were then

130 fractionated by chromatography onto Q-Sepharose High Performance columns (1.6 x 5 cm),
131 as described previously (Sarhou et al., 2020). Stepwise elution was performed using
132 discontinuous increasing NaCl concentrations in the extraction buffer (from 0 to 1 M NaCl;
133 Sub-fractions F0 to F1000). The salt concentrations were chosen to obtain a homogenous
134 repartition of the quantity of proteins in each sub-fraction (Supplementary Figure S1). Collected
135 sub-fractions were stored at -80°C until used for further analyses. Four independent
136 fractionation experiments from independent 8-plant cultures each were performed (Series 1 to
137 4).

138 2.3. Protein determination

139 Proteins from plant extracts were measured by the Bradford method using Bio-Rad protein
140 assay reagent, with bovine serum albumin as a standard (Bradford, 1976). Protein AEC sub-
141 fraction aliquots (30 µg) were analyzed by SDS-PAGE 12% and staining with Coomassie
142 Brilliant Blue R250, as described (Sarhou et al., 2020).

143 2.4. Protein capture by immobilized metal affinity chromatography (IMAC)

144 Anion exchange chromatography (AEC) eluted proteins from leaf and root extracts were
145 divided in a rational way into 2 and 3 main homogenous fractions, respectively, covering the
146 whole polypeptide profiles (herein named leaf LA3, LB3 and LA4, LB4 fractions and root RA3,
147 RB3, RC3 and RA4, RB4 and RC4 fractions, from Series 3 and 4, respectively). Protein fraction
148 aliquots were then adjusted to 0.05 to 0.1 mg/mL with binding buffer (50 mM Hepes, pH 7.4,
149 0.5 M NaCl, 0.05% (w/v) Brij) and extensively dialyzed overnight against the binding buffer.
150 High salt concentration and surfactant supplementation in the binding buffer aimed at
151 minimizing non-specific interactions with the IMAC matrix (Dedieu et al., 2009). After addition
152 of 5 µL/mL of 0.2 M NaHCO₃ (a chelating agent for uranyl cations which acts as a competitor
153 for proteins with low uranyl affinity, when used at low concentration), to prevent weak
154 interactions, 1-2.5 mL fraction aliquots were put in contact with 50 µL freshly prepared and
155 conditioned U(VI)-loaded and U(VI)-free (chromatographic blanks) Duolite C467 beads
156 (Basset et al., 2008; Dedieu et al., 2009), and stirred gently for 1 h at room temperature. The
157 supernatants (flow through) were put aside after centrifugation and the beads washed
158 extensively with binding buffer and dialyzed overnight at 4°C to remove any non-specifically
159 bound proteins. Then, the proteins were eluted with 600 µL of 0.2 M NaHCO₃, for 90 min
160 (Vidaud et al., 2019). The eluates were collected, concentrated to a final volume of 60 to 200
161 µL using a micro-concentrator system (Amicon® Ultra, Ultracel® - 3K, 3-kDa cutoff; Millipore)
162 and stored at -20°C until use for further protein identification. Flow through fractions were
163 concentrated and stored in the same way.

164 2.5. Identification of the captured proteins by high-resolution tandem mass spectrometry

165 Ten µL aliquots of proteins (concentrated flow through or eluted fractions) were supplemented
166 with 20 µL of 1.5X Laemmli LDS buffer (Invitrogen). Samples were heated for 5 min at 99°C,

167 centrifuged for 1 min, and the resulting supernatants loaded onto a NuPAGE 4-12% Bis-Tris
168 gel (Invitrogen). After a short denaturing electrophoresis of 4 min at 200 V, gel bands
169 containing the LDS-soluble proteomes were excised and processed for an in-gel trypsin
170 proteolysis, as previously described (Hartmann et al., 2014). The resulting peptides were
171 analyzed using a LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific) coupled to
172 an Ultimate 3000 nanoRSLC nano LC system (Dionex-LC Packings). A volume of 10 μ L (out
173 of 50 μ L) of peptides was injected and desalted on-line on a reverse-phase Acclaim
174 PepMap100 C18 micro precolumn (5 μ m bead size, 100 Å pore size, 300 μ m internal diameter,
175 5 mm length, Thermo Fisher Scientific) and then separated on an Acclaim PepMap100 C18
176 nano column (3 μ m bead size, 100 Å pore size, 75 μ m internal diameter, 15 cm length, Thermo
177 Fisher Scientific) at a 0.2 μ L/min flow rate with a 4-48% gradient of solvent B (99.9% CH₃CN,
178 0.1% HCOOH) against solvent A (99.9% H₂O, 0.1% HCOOH). Each run was preceded by at
179 least one blank run avoid cross-contamination. Data-dependent acquisition was done on
180 peptides with potential charges of either 2+ or 3+. Full-scan mass spectra were recorded at a
181 resolution of 30,000 from 300 to 1800 m/z with an automatic gain control target set at 5×10^5
182 ions. MS/MS scans were acquired on the three most abundant ions, selecting ions with a
183 dynamic exclusion of 60 s of all previously fragmented peptide ions. MS/MS spectra were
184 assigned to peptide sequences (p value ≤ 0.05) using the MASCOT search engine (version
185 2.5.1, Matrix Science) against the protein sequences of the SwissProt database (SwissProt
186 2015_02 download), selecting *Arabidopsis thaliana* as organism (13,640 protein sequences).
187 MS and MS/MS mass tolerances were set to 5 ppm and 0.5 Da, respectively. The other search
188 parameters were: a maximum of two trypsin miss-cleavages, systematic
189 carbamidomethylation of cysteine, as well as deamidation of glutamine and asparagine and
190 oxidation of methionine as three possible dynamic modifications. Proteins were identified when
191 at least two different peptide sequences were assigned. The abundance of the identified
192 proteins in each fraction was assessed by their spectral counts (number of MS/MS spectra
193 assigned per protein).

194 2.6. Proteomics data deposition

195 The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE
196 (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD033354 and
197 10.6019/PXD033354. [The dataset is available with the Username:
198 reviewer_pxd033354@ebi.ac.uk and Password: 62dQFqjg].

199 2.7. Cloning of PCaP1 cDNA forms for recombinant proteins production

200 Sequences of primers used in this study are listed in Supplementary Table S1. Full-length
201 *PCaP1* (At4g20260) and splicing variant *PCaP1v* cDNA coding sequences were amplified by
202 RT-PCR using total RNA isolated from 3-week-old *Arabidopsis* leaves with the RNeasy plant
203 mini extraction kit (Qiagen) and reverse-transcribed with the ThermoScript RT-PCR system

204 (Invitrogen). The cDNA fragments were amplified by PCR with primers introducing *Nco*I and
205 *Sa*II restriction sites upstream the initiation codon and downstream the stop codon,
206 respectively (Supplementary Table S1). The PCR products were sequenced (Eurofins) and
207 ligated into the pET28b+ plasmid (Novagen) between the *Nco*I and the *Sa*II sites to obtain the
208 pET28-PCaP1 and pET28-PCaP1v recombinant plasmids, respectively. The cDNA encoding
209 the truncated PCaP1 form, PCaP1 Δ , was obtained by PCR amplification using pET28-PCaP1
210 plasmid as a matrix and specific primers (Supplementary Table S1). The PCR product was
211 ligated into the pET28b+ plasmid to obtain the pET28-PCaP1 Δ recombinant plasmid. The
212 resulting pET28-PCaP1 constructs were amplified into *Escherichia coli* DH5 α cells and then
213 introduced into the *E. coli* overexpression host Rosetta 2 (DE3) (Stratagene).

214 2.8. Production and purification of the recombinant PCaP1 forms

215 The transformed cell cultures were grown at 37°C in lysogeny broth (LB) medium
216 supplemented with the appropriate antibiotics until A_{600} was 0.6. Then, isopropylthio β -D-
217 galactoside was added to a final concentration of 0.4 mM and incubations pursued for 15 h at
218 37°C. Cell pellets from 2-L cultures were resuspended in 50 mL of extraction buffer containing
219 20 mM Tris-HCl, pH 8, 10% (w/v) glycerol, 1 mM dithiothreitol and a cocktail of complete
220 protease inhibitors (Roche Applied Science), and then disrupted by sonication with a Vibra-
221 Cell disrupter (Branson Ultrasonics). Cell debris were eliminated by centrifugation at 40,000 x
222 g for 30 min and soluble proteins recovered from supernatants were subjected to ammonium
223 sulphate precipitation at 4°C with crystalline ammonium sulphate to 50% saturation. The
224 resulting precipitates were collected by centrifugation (40,000 x g, 15 min, 4 °C), resuspended
225 in 10 mM potassium phosphate buffer, pH 7 supplemented with protease inhibitors and
226 dialyzed overnight at 4°C against 4 L of 10 mM phosphate buffer, pH 7. Protein samples were
227 applied onto a Bio-Gel HTP hydroxylapatite column (2.6 x 7 cm, Bio-Rad) equilibrated with 10
228 mM potassium phosphate buffer pH 7. Proteins were eluted with a linear gradient of phosphate
229 buffer from 10 to 350 mM potassium phosphate (320 mL) at a flow rate of 0.5 mL/min. Five-
230 mL fractions were collected. Fractions containing recombinant PCaP1 proteins were pooled
231 and desalted by successive concentrations/dilutions with 10 mM Tris-HCl buffer, pH 7.5
232 containing 10% (w/v) glycerol, using Amicon® Ultra-15 centrifugal filtration units (3 kDa cut-
233 off; Millipore). Samples were then loaded onto a Fractogel EMD-DEAE (650 M) column (1.6 x
234 5 cm; Merck) equilibrated with 10 mM Tris-HCl, pH 7.5 buffer containing 10% (w/v) glycerol.
235 After extensive wash of the column with five volumes of buffer, proteins were eluted using a
236 150-mL linear gradient from 0 to 500 mM NaCl in this buffer, at a flow rate of 0.5 mL/min.
237 PCaP1-containing fractions were pooled and concentrated before being applied onto a
238 Hiload® Superdex 75 column (1.6 X 60 cm, Cytiva) equilibrated with 10 mM Tris-HCl buffer,
239 pH 7.5 containing 10% (w/v) glycerol and 150 mM NaCl. Elution was conducted in the same

240 buffer at a flow rate of 1 mL/min. Purified recombinant proteins were finally concentrated and
241 stored aliquoted at -80°C, until used. Protein purity and integrity were monitored by SDS-PAGE
242 and mass spectrometry under denaturing conditions. Purified protein concentration was
243 determined by recording UV absorption spectra, using a NanoDrop 2000 spectrophotometer
244 (Thermo Fischer Scientific) (Mass Extinction Coefficients at 280 nm were $E_{1\%} = 4.66 \text{ Lg}^{-1} \text{ cm}^{-1}$
245 for PCaP1; 5.70 for PCaP1v and 7.32 for PCaP1 Δ , as calculated from the Molar Extinction
246 Coefficient $\epsilon_{280} = 11460 \text{ M}^{-1} \text{ cm}^{-1}$, ProtParam ExPASy).

247 Production and purification of recombinant PCaP1 protein uniformly labelled with ^{15}N and ^{13}C
248 ($U\text{-}^{15}\text{N}, ^{13}\text{C}\text{-PCaP1}$) or labelled with ^{15}N and ^2H ($^2\text{H}, ^{15}\text{N}, \text{-PCaP1}$) was performed similarly as for
249 unlabeled protein, except that growth was conducted in 2 L of a minimal M9 medium,
250 containing antibiotics and supplemented with either $^{15}\text{N}\text{-NH}_4\text{Cl}$ (15N, 99%) and $^{13}\text{C}\text{-glucose}$
251 ($U\text{-}^{13}\text{C6}$, 99%) or $^{15}\text{N}\text{-NH}_4\text{Cl}$ (15N, 99%) and D_2O (99.9%) (Cambridge Isotope Laboratories,
252 Inc.), respectively, after progressive medium acclimatization from pre-cultures conducted in LB
253 medium.

254 The presence of recombinant PCaP1 in eluted fractions during the purification process was
255 assessed by western-blotting, initially using a commercially available polyclonal rabbit antibody
256 raised against KLH-conjugated peptide derived from the Arabidopsis PCaP1 sequence
257 (1:2000 dilution) (Agrisera; Ide et al. (2007)), and subsequently using a more sensitive custom-
258 made rabbit polyclonal PCaP1 antibody raised against the whole purified recombinant protein
259 (1:50000 dilution) (Covalab). Proteins were separated by SDS-PAGE, electro-transferred to
260 nitrocellulose membrane and probed using the PCaP1 antibody and horseradish peroxidase-
261 conjugated anti-rabbit IgGs (Bio-Rad). Protein detection was achieved using the ECL PlusTM
262 Western Blotting detection reagents and a Typhoon 9400 imager (Cytiva).

263 2.9. Native mass spectrometry

264 Mass Spectrometry under native conditions was performed to determine the oligomeric state
265 of PCaP1. Prior to analysis, purified protein (a 100- μL 50 μM aliquot) was extemporaneously
266 chromatographed onto a Superdex 200 Increase 10_300 GL column equilibrated with 250 mM
267 ammonium acetate, pH 8, for buffer exchange. Protein was injected in the spectrometer at a 7
268 μM final concentration. A nanoflow electrospray (nano-ESI) source and Nanoflow platinum-
269 coated borosilicate electrospray capillaries (Thermo Electron SAS, Courtaboeuf, France) were
270 used to generate ions (Boeri Erba et al., 2018; Puglisi et al., 2020). A quadrupole time-of-flight
271 mass spectrometer (Q-TOF Ultima, Waters Corporation, Manchester, U.K.) modified for the
272 detection of high masses was operated with the following parameters: capillary voltage = 1.2–
273 1.3 kV, cone potential = 40 V, RF lens-1 potential = 40 V, RF lens-2 potential = 1 V, aperture-
274 1 potential = 0 V, collision energy = 30–140 V, and microchannel plate (MCP) = 1900 V. The
275 mass spectrometer was calibrated externally with a solution of cesium iodide at 6 mg/mL
276 dissolved in 50% isopropanol. Mass spectra were processed using the Masslynx 4.0 software

277 (Waters Corporation, Manchester, U.K.), Massign software package (Morgner and Robinson,
278 2012) and UniDec (Marty et al., 2015).

279 2.10. Metal-binding affinities measured by fluorescence titration

280 Fluorescence spectroscopy was performed using a MOS450 Bio-Logic spectro-fluorimeter
281 (Bio-Logic-Science Instruments, Claix, France) set at 277 nm for excitation. The slit widths for
282 emission and excitation were set at 8 nm. The spectro-fluorimeter was driven by Bio-Kine 32
283 4.1 software. The measurements were performed at 25°C, in a 1 cm path cell.

284 For fluorescence titrations of PCaP1 in the presence of copper (CuCl₂), ferric iron (Fe(NO₃)₃)
285 or calcium (CaCl₂), we used a 0.8 μM protein solution in 10 mM Tris-HCl pH 7.5 or 10 mM
286 MES pH 6 buffer with 100 mM NaCl. The fluorescence was transmitted through a long pass
287 filter (FF01-300/LP-25, Semrock) and emission was collected at 342-348 nm. The apparent
288 dissociation constant (K_{dapp}) and the stoichiometry of metal binding to PCaP1 (n) were
289 determined using the fluorescence titration curves. The variation of PCaP1 fluorescence
290 intensity as a function of the metal concentration was analyzed by using the following equation
291 (Eq. 1) as described (Nagasaki-Takeuchi et al., 2008):

292 (1)

$$293 (F_0 - F) = \frac{\Delta F_{max} \times [Metal]}{K_{dapp} + [Metal]}$$

294
295 where, F_0 and F are fluorescence intensities measured at 342-348 nm without or with the
296 presence of metal, respectively; ΔF_{max} is the maximum fluorescence change observed when
297 the protein is fully saturated at the specific metal-binding sites with an apparent affinity of K_{dapp}
298 in a binding-site dependent manner; $[Metal]$ and $[PCaP1]$ are concentrations of free metal and
299 PCaP1 in the assay, respectively. We also applied the following equation (Eq. 2) to determine
300 the ligand-binding number (n) with the postulate that PCaP1 has n binding sites with identical
301 affinity (Nagasaki-Takeuchi et al., 2008):

302 (2)

$$303 \frac{1}{F_0 - F} = \frac{2n}{\Delta F_{max}} \times \frac{1}{[Metal]/[PCaP1]} + \frac{1}{\Delta F_{max}}$$

304 Fitting of the data was performed with KaleidaGraph 4.51 software (Synergy Software, PA,
305 USA).

306 For fluorescence titrations of PCaP1 in the presence of U(VI), we used a 0.8 μM protein
307 solution in 10 mM MES pH 6 buffer with 100 mM NaCl. Iminodiacetate (IDA) was added to the
308 protein solutions at a IDA:protein ratio of 12.5:1 to prevent the appearance of hydroxo
309 complexes and to control U(VI) speciation (Pardoux et al., 2012). Fluorescence emission
310 spectra were acquired from 280 to 470 nm. Competition experiments between the PCaP1
311 proteins and IDA were performed to calculate the conditional stability constants of the U(VI)-

312 protein complexes at pH 6. IDA binds uranyl with mild affinity and forms the UO_2IDA ,
313 $[\text{UO}_2(\text{IDA})_2]^{2-}$ and $[(\text{UO}_2)_2(\text{IDA})_2(\text{OH})_2]^{2-}$ complexes. The conditional stability constants of these
314 three species at 25°C and 0.1 M ionic strength were calculated from published global constants
315 (Jiang et al., 2002; Starck et al., 2015). These values were set up in the spectral data analyses,
316 which were performed using the SPECFIT software (Binstead et al., 2003; Gampp et al., 1985).
317 Several models for the UO_2^{2+} -protein complexes were assayed, and only the one predicting a
318 $\text{UO}_2(\text{Protein})_2$ specie only gave a good fit for the experimental data. The model considering a
319 non-luminescent complex was the most reliable. Experiments were repeated to ensure
320 reproducibility.

321 2.11. *Metal quantification in metal-protein complexes by Size Exclusion Chromatography* 322 *(SEC) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)*

323 Metal-protein complexes were prepared by incubating 10 μM purified PCaP1 solutions for 15
324 min at 25°C, in 20 mM Tris-HCl pH 7.5, or 20 mM MES pH 6 buffer containing 150 mM NaCl
325 and 2 mM CuCl_2 , 2 mM $\text{Fe}(\text{NO}_3)_3$, 10 mM CaCl_2 or 50 μM $\text{UO}_2(\text{NO}_3)_2$, as indicated. Formed
326 complexes were separated from unbound metals by SEC, through centrifugation for 2 min at
327 724g of the samples (150 μL), on MicroSpin™ G-25 columns (Cytiva), equilibrated with the
328 appropriate working buffer (Tris-HCl/NaCl or MES/NaCl). Controls without protein were run in
329 parallel for background correction. Binding assays were performed at least in triplicate for each
330 metal. Protein in the eluates was quantified by recording A_{280} . Eluates (50 μL aliquots) were
331 then incubated with 10% (v/v) HNO_3 (Suprapur, Merck) for 2h at 60°C to ensure protein
332 denaturation and release of bound elements. Precipitated proteins were removed by
333 centrifugation and the mineralized samples were then diluted in 0.5% (v/v) HNO_3 and analyzed
334 by ICP-MS using an iCAP RQ quadrupole mass instrument (Thermo Fisher Scientific GmbH,
335 Germany), as detailed previously (Sarhou et al., 2020). Elements (^{44}Ca , ^{56}Fe , ^{57}Fe , ^{63}Cu , ^{65}Cu ,
336 ^{238}U) were analyzed using the standard mode and the collision mode with helium as a cell gas.
337 Elements concentration was determined using standard curves and corrected using a solution
338 of ^{45}Sc , ^{103}Rh and ^{172}Yb , as an internal standard. Data integration was performed using the
339 Qtegra software (Thermo Fisher Scientific GmbH, Germany).

340 2.12. *Solution-state Nuclear Magnetic Resonance (NMR) studies*

341 NMR experiments were performed on Bruker Avance IIIHD spectrometers operating at
342 magnetic field strengths of 16.5, 20, and 22.3 T (^1H Larmor frequency of 700, 850 and 950
343 MHz). All spectrometers were equipped with helium-cooled triple-resonance probes (HCN TCI
344 5mm) and pulsed z-field gradients. NMR assignments were obtained from a set of 3D Band-
345 selective Excitation Short-Transient Transverse Relaxation-Optimized Spectroscopy (BEST-
346 TROSY) (Favier and Brutscher, 2011) type correlation experiments: HNCQ, HNCACO, HNCA,
347 HNCQCA, HNCACB, and HNCOCACB. These experiments were performed on uniformly

348 $^{13}\text{C}/^{15}\text{N}$ labeled PCaP1 samples (in 10 mM Tris-HCl, pH 7.5 buffer, at 300K) and sample
349 concentrations ranging from 100 to 500 μM .

350 Translational diffusion constants of the proteins in solution were measured by 1D ^1H Diffusion
351 Ordered Spectroscopy (DOSY) experiments (Johnson, 1999) focusing either on the methyl or
352 amide spectral region. A series of 1D spectra was recorded with varying gradient strength and
353 a total acquisition time of about 15 min.

354 ^{15}N relaxation experiments (T_1 and T_2) were performed on uniformly ^{15}N -labeled full-length
355 PCaP1 on the free form and in complex with U(VI) using standard NMR pulse sequences
356 (Farrow et al., 1994). The ^{15}N T_1 signal decay was sampled for 12 time points varying between
357 0 to 1.8 s while ^{15}N T_2 relaxation experiments were recorded with relaxation delays ranging
358 from 0 to 280 ms.

359 NMR titration experiments were performed by adding up to 5-fold excess of U(VI) to either full-
360 length PCaP1 or PCaP1 Δ and recording at each titration step a 2D ^1H - ^{15}N BEST-TROSY
361 spectrum.

362 All NMR experiments handled in this study were implemented in the NMRlib pulse sequence
363 library (Favier and Brutscher, 2019) that can be freely downloaded from the IBS website
364 (<http://www.ibs.fr/research/scientific-output/software/pulse-sequence-tools>). The experiments
365 were processed and analyzed using Bruker Topspin 3.5 and CCPNMR V3 software
366 (Collaborative Computing Project for NMR; <https://ccpn.ac.uk/software/downloads/>).

367 2.13. Characterization, genetic complementation and U(VI) treatment of the *Arabidopsis* 368 *pcap1.4* mutant

369 Seeds of the *Arabidopsis pcap1.4* knockout mutant (Li et al., 2011; Nagata et al., 2016) were
370 obtained from the Arabidopsis Biological Resource Center (registration no. SAIL_241_A08).
371 Homozygous status of the T-DNA insertion line was checked by PCR, using gene-specific and
372 T-DNA border primers (Supplementary Table S1). The T-DNA insertion sites within exon 4 of
373 *PCaP1* gene (Li et al., 2011) were confirmed by sequencing. For genetic complementation
374 experiments, the complete coding sequence of *PCaP1* was fused to a double enhanced 35S
375 promoter using the plasmid pFP101 containing a green fluorescent protein (*GFP*) reporter
376 gene (Bensmihen et al., 2004). This construct was introduced into homozygous *pcap1.4* plants
377 by the Agrobacterium-mediated floral-dip transformation method (Clough and Bent, 1998).
378 Transformed seeds expressing the GFP marker were selected by fluorescence detection. All
379 primer sequences are available in Supplementary Table S1. PCaP1 expression in Arabidopsis
380 lines was checked by western blot analysis of total proteins extracted from leaves with 10 mM
381 Tris-HCl, pH 7.5 buffer, containing 1 mM dithiothreitol, 1% SDS and a cocktail of protease
382 inhibitors (Roche Applied Science), using custom-made PCaP1 antibody.

383 For U(VI) treatment, plants were first grown in hydropony for 5 weeks in Gre medium with
384 weekly solution changes until treatment, and transferred to Gre medium depleted with

385 phosphate and complemented with 20 μM uranyl nitrate ($\text{UO}_2(\text{NO}_3)_2$). Plants were harvested
386 1 to 11 days after U(VI) treatment. To remove U(VI) weakly bound on the root surface, the
387 plants were first rinsed once with 10 mM Na_2CO_3 , then twice with distilled water. Photosystem
388 II efficiency in dark-adapted leaves (Fv/Fm) was assessed using a FluorPen (FP100/D, Photon
389 Systems Instruments, Brno, Czech Republic) and used as an indicator of plant health. Roots
390 and shoots were separated, quickly dried on absorbing paper and fresh biomass was then
391 determined. Finally, roots and shoots were dehydrated at 80°C for 24 h and dried samples
392 were mineralized in 0.5-1 ml of 65% (w/v) HNO_3 (Suprapur, Merck), diluted in distilled water
393 and analyzed by ICP-MS, as described previously (Sarhou et al., 2022).
394 Statistical analyses were performed using the R Studio software (RStudio Team, 2015) and
395 the *nparcomp* package (Konietschke et al., 2015). Statistical significance was determined
396 using non-parametric Tukey tests conducted with a confidence level set at 99% ($p < 0.01$).

397

398 **3. Results**

399 3.1. *Identification of 38 UraBPs from Arabidopsis roots and shoots captured by IMAC* 400 *on U(VI)-loaded beads*

401 To isolate UraBPs from plant extracts, we used the efficient uranyl-loaded Duolite C467™
402 matrix previously developed for the identification of UraBPs from human serum, kidney-2 and
403 dopaminergic SH-SY5Y cells (Basset et al., 2008; Dedieu et al., 2009; Vidaud et al., 2019).
404 The experimental design is depicted in Figure 1. Soluble proteomes isolated from roots and
405 leaves from hydroponically grown *Arabidopsis thaliana* plants were first pre-fractionated by
406 anion exchange chromatography (AEC) on High Performance Q-Sepharose column. Protein
407 concentration profiles and SDS-PAGE analysis of the sub-fractions obtained from four
408 independent biological experiments are presented in Supplementary Figure S1. This
409 fractionation procedure reduced the complexity of the protein samples in a very reproducible
410 way and enriched the less abundant proteins facilitating their capture and identification. As an
411 example, in the case of the leaf proteome, the method allowed to separate the very abundant
412 Rubisco protein (present in the LB fraction) from less abundant proteins displayed in the LA
413 fraction (Supplementary Figure S1). In the case of the root proteome, the method led to the
414 separation of three enriched homogenous protein fractions, RA, RB and RC.

415 Two of the four-fractionation experiments conducted with Arabidopsis root and shoot tissues
416 (Series 3 and 4, Supplementary Figure S1) were used as starting biological materials
417 (biological duplicate, experiments 1 and 2) to capture UraBPs by immobilized metal affinity
418 chromatography (IMAC) on U(VI)-loaded beads in a batch system, and to subsequently identify
419 them by mass spectrometry (Figure 1). Fractionation experiments from series 1 and 2 were
420 set aside for analyses unrelated to this work (unpublished data). All the specifically captured
421 proteins obtained from the ten IMAC experiments (five from experiment 1 and five from

422 experiment 2), as well as the corresponding flow through supernatants were submitted to
423 nanoLC-ESI MS/MS analysis for identification (Supplementary Table S2). The IMAC process
424 including the capture conditions and the washing steps was highly efficient and selective since
425 we did not detect any protein in the fractions eluted from the different control runs on U(VI)-
426 free beads, except for the highly abundant large Rubisco subunit from leaf extracts, which was
427 thus not considered as a U(VI)-affine protein (Supplementary Table S2). Taken together, our
428 analyses identified a total of 338 different proteins from leaves and roots, among which 38
429 were specifically captured on U(VI)-loaded beads, and were named UraBP1 to 38
430 (Supplementary Table S2). The overall low number of total proteins identified in our analyses
431 was the consequence of the high dilution and the small amounts of protein fractions exposed
432 to U(VI)-loaded beads, which was a prerequisite for high selectivity and very low background
433 noise. We considered as genuine UraBPs only proteins captured from identical AEC fractions
434 in experiments 1 and 2, or proteins captured from different AEC fractions from the same organ
435 (leaf or root) (highlighted in red and pink, respectively in Supplementary Table S2). Single
436 occurrences were not considered as proteins of interest.

437 Among the 38 UraBPs identified, 30 were specifically captured from leaf protein extracts, 4
438 from root protein extracts and 4 from both tissues (Supplementary Table S2). Interestingly, 10
439 out of the 38 UraBPs (UraBP3, 15, 18, 21, 25, 28-31 and 33) were detected only in captured
440 fractions and not in the flow through fractions, or were highly enriched on the captured fractions
441 (on a spectral count basis) (Supplementary Table S2). The other UraBPs were detected
442 similarly in both fractions and could either correspond to abundant UraBPs saturating U(VI)-
443 loaded beads or to proteins with low uranyl binding affinity with a free/bound equilibrium being
444 established. In order to select the most interesting UraBPs for further analyses, we performed
445 gene ontology (GO) and semantic terms enrichment analyses (biological process and/or
446 molecular function in relation with metals) as well as analyses of some of their physico-
447 chemical characteristics (Supplementary Table S2). This included their content in some amino
448 acid residues known to coordinate with U(VI) (the oxygen of aspartate, glutamate, and tyrosine
449 and the nitrogen of histidine are both hard Lewis bases and are able to bind U(VI)). Also,
450 phosphorylated amino acid residues that are known to interact with U(VI) and have been
451 shown to strengthen the binding of U(VI) to aspartate and glutamate were analysed (Garai and
452 Delangle, 2020; Laporte et al., 2019; Pardoux et al., 2012; Sauge-Merle et al., 2017; Starck et
453 al., 2017). Firstly, these analyses indicated that most of the *Arabidopsis* UraBPs (27/38 *i.e.*
454 71% of the protein data set) are described as metal-binding proteins and/or are metal-
455 responsive proteins. The phosphoproteins were also greatly represented (29/38 *i.e.* 76% of
456 the protein data set), which is quite higher than in global proteomes (about 40-47% in
457 *Arabidopsis*) (Mergner et al., 2020; van Wijk et al., 2014). Among phosphoproteins, 20 out of

458 29 were poly-phosphorylated. Particularly, UraBP17 (PSBP1), UraBP18 (BCA2), UraBP23
459 (RCA), UraBP25 (PCaP1), UraBP28 (PSBQ2), UraBP33 (RBG7) and UraBP38 (CATA3)
460 contain more than five phosphorylation sites (Supplementary Table S2). Noticeably, 38% of
461 the phosphoproteins present one or more phosphotyrosine group, which is much higher than
462 in global *Arabidopsis* phospho-proteome (< 5%) (Mergner et al., 2020; Reiland et al., 2009;
463 van Wijk et al., 2014).

464 With all these criteria taken into consideration, we decided to focus our attention more
465 particularly onto UraBP25 for more detailed characterization. Indeed, UraBP25 is a Ca²⁺
466 regulatory protein that binds Ca²⁺ and Cu²⁺ ions that was previously reported as the Plasma
467 membrane-associated Cation-binding Protein 1 (PCaP1), or the Microtubule Destabilizing
468 Protein of 25 kDa (MDP25) (Ide et al., 2007; Li et al., 2011; Nagasaki-Takeuchi et al., 2008;
469 Nagasaki et al., 2008; Qin et al., 2014). UraBP25 accumulates in response to Cu²⁺ and Mg²⁺
470 ions excess and its abundance is lowered in response to Fe³⁺ ions exposure (Ide et al., 2007;
471 Nagata et al., 2016). Moreover, UraBP25 was isolated from both leaf and root extracts on
472 U(VI)-loaded beads and was highly enriched in captured fractions from both R3A, R3B and
473 L3A, L4B AEC fractions (Supplementary Table S2). Finally, UraBP25 is a poly-phosphorylated
474 protein, with 9 identified sites, and presents a high frequency of glutamate residues (44/225
475 *i.e.* 19.6% of total residues) (Supplementary Table S2), compared to 6.65 % on average in the
476 *Arabidopsis* proteome (Tsuji et al., 2010).

477

478 3.2. PCaP1 binds U(VI) with high affinity and Cu(II) and Fe(III) in high proportions

479 In order to confirm the capacity of PCaP1 from *Arabidopsis thaliana* to bind U(VI) and to further
480 characterize its metal-binding properties, we cloned the cDNA encoding the full length PCaP1
481 protein by RT-PCR and produced the recombinant protein devoid of any tag sequence in *E.*
482 *coli*. We used a three-step chromatographic procedure to purify recombinant PCaP1 protein
483 to near homogeneity (Figure 2). The integrity of the preparations was confirmed by LC-ESI
484 TOF MS under denaturing conditions. The experimental mass of PCaP1 was 24,453.06 Da,
485 matching the amino acidic sequence 2-225 (with loss of the N-terminal methionine)
486 (Supplementary Figure S2A). As previously observed (Ide et al., 2007), the purified protein
487 was detected as a 36 kDa polypeptide on SDS-PAGE instead of 24.5 kDa due to its peculiar
488 amino acid composition (Figure 2A), PCaP1 being particularly rich in glutamate, lysine and
489 valine residues. Also, recombinant PCaP1 protein behaved as an apparent ~90 kDa globular
490 protein by size exclusion chromatography (SEC) onto a Superdex 200 Increase 10/300 GL
491 column, as calculated from a calibration curve obtained by measuring elution volumes of
492 spherical folded calibration proteins (Figure 2B). However, native ESI MS analysis indicated

493 that PCaP1 was essentially a monomer (1mer) in solution under our assay conditions, with a
494 small proportion of dimer (2mer) also being detected (Figure 2C). The unexpected migration
495 of recombinant protein by SEC analysis could be explained by the occurrence of an intrinsically
496 unstructured region in PCaP1 structure, as suggested previously by far-UV circular dichroism
497 spectroscopy analyses (Nagasaki-Takeuchi et al., 2008).

498 In a previous biochemical and biophysical study, Nagasaki-Takeuchi et al. (2008)
499 demonstrated by fluorescence titration and circular dichroism analyses that a recombinant *A.*
500 *thaliana* PCaP1 protein was able to bind Cu(II) ions. These analyses indicated that binding of
501 Cu(II) altered the structure of PCaP1 locally at proximity of aromatic amino acid residues, and
502 particularly of Tryptophan 4 (Trp4), the unique Trp residue within the protein sequence. Here,
503 taking advantage of this property, we checked for U(VI) binding to PCaP1 by Trp fluorescence
504 titration. We observed that the fluorescence emission peak at 342 (typical for Trp residue
505 exposed to a water solution) when excited at 277 nm, was also markedly quenched by the
506 addition of U(VI) ions at low concentrations to recombinant PCaP1 protein (up to 4 equivalents
507 on a monomer basis) buffered at pH 6, suggesting interaction of the metal with the protein
508 (Supplementary Figure S3). Addition of an excess of EDTA restored almost completely the
509 original fluorescence spectrum indicating reversibility of U(VI) binding and testifying of protein
510 integrity maintenance upon U(VI) exposure. Trp fluorescence quenching was followed by
511 titration of a 0.8 μM PCaP1-buffered solution at pH 6 containing a 12.5-fold excess of
512 iminodiacetic acid (IDA), a well-known low affinity competitor for U(VI) binding, to control uranyl
513 speciation (Figure 3A). The stability constants of complexes formed with IDA at pH 6 being
514 known (Jiang et al., 2002), one can infer the conditional stability constant of the uranyl
515 complexes with PCaP1. Under our assay conditions, the fluorescent peak at 342 nm gradually
516 decreased with the increase in the U(VI) concentration from 0.1 to 2 μM and reached less than
517 20% of the original fluorescence (Figure 3A). The evolution of fluorescence intensity with the
518 quantity of uranyl added was characteristic of the formation of a $(\text{UO}_2^{2+})\text{-(Protein)}_2$ complex,
519 since the extrapolation of the initial slope converged to 0.5 uranyl equivalent in the complex.
520 This was clearly confirmed in the fitting procedure (Figure 3B). Indeed, the best fit to the
521 experimental data was obtained with a model considering a non-fluorescent $(\text{UO}_2^{2+})\text{-(Protein)}_2$
522 complex only, involving two proteins for one single uranyl cation. The calculated conditional
523 stability constant for the formation of this species deduced from the deconvolution of
524 fluorescence data was $\log\beta_{12} = 14.5 \pm 0.4$. This constant was defined for a 1:2 complex so its
525 value has not the same order of magnitude than a $1/K_d$ value. Thus, this value cannot be
526 compared directly with literature data reporting β_{11} constants (1:1 complex) or K_d values. To
527 make such a comparison possible, we simulated competitions with a $(\text{UO}_2^{2+})\text{-P'}$ complex with
528 a theoretical competing P' protein of 1 nM affinity ($\log\beta_{11} = 9$), 10 nM ($\log\beta_{11} = 8$) or 100 nM
529 ($\log\beta_{11} = 7$). This simulation indicated that the protein has an affinity for uranyl similar to a

530 protein having a 10 nM affinity ($\log\beta_{11} = 8$) in a $(\text{UO}_2^{2+})\text{-P}'$ complex, using a similar approach
531 (Supplementary Figure S4). Trp fluorescence quenching was also followed by titration of a
532 PCaP1-buffered solution at pH 7.5 (Supplementary Figure S5A). The evolution of fluorescence
533 intensity with the quantity of uranyl added could not be easily interpreted since there was no
534 plateau with an addition of a large amount of U(VI) up to 20 μM (Supplementary Figure S5B).
535 So, fits were not appropriate under these conditions.

536 To study the stoichiometry of U(VI) complexation to PCaP1 at pH 6 and 7.5, we determined
537 U(VI) content by ICP-MS in U(VI)-PCaP1 complexes formed after incubation of 10 μM PCaP1-
538 buffered samples in the presence of 50 μM uranyl nitrate (5 U(VI) equivalents). Separation of
539 complexes from unbound metal was achieved through centrifugation onto Sephadex
540 MicroSpin G-25 columns. We did not perform such analyses at higher U(VI) to protein ratios
541 since under large U(VI) excess conditions, protein samples were somewhat affected, as
542 monitored by UV-Vis absorption, and a slight precipitate was observed, particularly when
543 titration was done in MES buffer at pH 6 (not shown). Under optimized conditions, data
544 obtained revealed a ligand-binding number of 0.50 ± 0.03 U(VI) ions per PCaP1 monomer at
545 pH 6 (Supplementary Table S3), in line with fluorescence spectroscopy studies (Figure 3B).
546 Since PCaP1 was found to be mainly a monomer in solution (Figure 2C), these data could
547 suggest dimerization of the protein upon U(VI) exposure under our experimental conditions,
548 with one uranyl cation located at the interface of two monomers (compatible with formation of
549 a 1:2 complex, in line with fluorescence titration experiments; Figure 3B). When performed at
550 pH 7.5 in Tris-HCl buffer, binding experiments revealed a U(VI) content of the resulting U(VI)-
551 protein complex of 2.11 ± 0.07 U(VI) ions per PCaP1 monomer (Supplementary Table S3).

552 As mentioned before, Nagasaki-Takeuchi et al. (2008) characterized Cu(II)-binding properties
553 of PCaP1 by fluorescence titration of Trp4 with CuCl_2 increasing concentrations and monitoring
554 of changes in fluorescence spectra. The obtained values of apparent K_d (K_{dapp}) and ligand-
555 binding number, as determined by indirect fluorescence and absorption spectroscopy
556 methods, were 10 to 16 μM and 6 to 7 Cu(II) ions per monomer, respectively (Nagasaki-
557 Takeuchi et al., 2008). In our hands, under similar assay conditions, the fluorescence emission
558 peak at 342 nm was also markedly quenched when Cu(II) was added in the protein solution,
559 at concentrations up to 300 μM (Supplementary Figure S6A). From the collected data we
560 calculated a K_{dapp} for Cu(II) binding of 29 μM , and a ligand-binding number of 13 ions per
561 monomer (Supplementary Table S4).

562 PCaP1 has been shown to interact with calcium and calmodulin in a calcium-dependent
563 manner and has both *in vitro* and *in vivo* calcium-dependent microtubule destabilizing and actin
564 severing activities (Li et al., 2011; Nagasaki et al., 2008; Qin et al., 2014). Despite PCaP1
565 being a genuine Ca(II)-binding protein, thus requiring calcium for its activities, we observed
566 that the addition of CaCl_2 has a limited effect on fluorescence emission, at pH 7.5. However,

567 fluorescence changes were significant at pH 5.5, yielding a K_{dapp} of 241 μM (Supplementary
568 Figure S6B and Supplementary Table S4). Interestingly, Fe(III) but not Fe(II) ions also affected
569 Trp fluorescence emission in a similar way as Cu(II) ions (K_{dapp} of 26 μM and ligand-binding
570 number of about 19 ions per monomer; Supplementary Figure S6C and Supplementary Table
571 S4). Finally, none of the other metal ions tested had any impact on fluorescence emission
572 (Supplementary Table S4). In order to confirm the ability of PCaP1 to bind Cu(II), Ca(II) and
573 Fe(III) ions, we used SEC on MicroSpin G-25 columns and ICP-MS analyses to measure
574 metals in protein complexes resulting from the incubation of 10 μM PCaP1 with large excess
575 of any of the metal ions. Data obtained revealed an apparent stoichiometry for Cu(II)-PCaP1
576 protein complexes of 15:1, consistent with the values estimated by fluorescence spectroscopy.
577 Surprisingly, much higher values were observed by direct ICP-MS determination for Fe(III)-
578 PCaP1 protein complexes (stoichiometry of 60:1 compared to 19:1 by fluorescence
579 spectroscopy; Supplementary Table S4). Unfortunately, we were unable to measure
580 accurately the calcium content of Ca(II)-PCaP1 protein complexes because of unexplained
581 high background values of unbound metal ion in MicroSpin G-25 column eluates.
582 PCaP1 is known to bind the physiological ion Ca(II) both *in vivo* and *in vitro* (Li et al., 2011;
583 Nagasaki et al., 2008; Qin et al., 2014). However, we have shown that at physiological pH (pH
584 7.5), Ca(II) alone did not significantly quench PCaP1 fluorescence (Supplementary Table S4),
585 therefore a competition between UO_2^{2+} and Ca(II) could be evidenced by an increase in Trp
586 fluorescence intensities of U(VI)-PCaP1 protein complexes upon addition of increasing Ca(II)
587 concentrations (Supplementary Figure S7). Thereby, addition of 8 mM Ca(II) fully restored Trp
588 fluorescence intensity of PCaP1 protein initially quenched to half its initial value by 20 μM
589 U(VI), which suggested identical or close binding-sites for both metals and indicated a much
590 higher affinity of the protein for U than for calcium, even at physiological pH.

591

592 3.3. *U(VI)-binding to PCaP1 induces oligomerization*

593 In order to obtain information about the *in vitro* interaction of U(VI) with PCaP1 at the molecular
594 level, we initiated structural studies of the complex. The recombinant protein was predicted to
595 be an instable protein (Instability Index of 53.55 using ProtParam tool; Supplementary Table
596 S2) due to the presence of a long intrinsically disordered C-terminal domain (residues 140-
597 225, as identified by computational disorder prediction tools) (Supplementary Figure S8A). All
598 our attempts to crystallize PCaP1 were unsuccessful, most likely because of this high degree
599 of flexibility. Consequently, structure determination based on X-ray crystallographic data was
600 not pursued. As an alternative strategy, we have undertaken solution-state NMR spectroscopic
601 analyses of this protein. Solution-state NMR spectroscopy is a well-adapted technique for low
602 molecular weight proteins (< 30 kDa), which is the case for PCaP1, providing information about

603 structure and conformational dynamics of proteins over a wide range of time scales and
604 degrees of order. NMR experiments require samples at high protein concentration (typically >
605 100 μM), for which U(VI) exposure triggers precipitation of PCaP1 at pH 6, but not at pH 7.5.
606 Thus, all NMR experiments were performed at pH 7.5 with U(VI):protein ratios in the range
607 between 0:1 to 5:1.

608 The 1D ^1H NMR spectrum of PCaP1 shows a few backbone amide (HN) and side-chain methyl
609 (CH_3) NMR resonances outside the typical random-coil region, indicative of the presence of
610 well-folded protein segments (Supplementary Figure S8B). In addition, a more quantitative
611 assessment of the degree of tertiary structure in PCaP1 using the HETSOFast approach
612 (Schanda et al., 2006) confirmed the presence of both well-folded domains and flexible protein
613 regions (Supplementary Figure S8C).

614 In order to obtain site-specific information on the structural and dynamic properties of
615 recombinant PCaP1, we produced uniformly ^{13}C , ^{15}N -labeled PCaP1 and performed a set of
616 3D NMR assignment experiments (Best-TROSY HNC0, HNCACO, HNCA, HNCOCA,
617 HNCACB and HNCOCACB) (Favier and Brutscher, 2011; Solyom et al., 2013). Based on this
618 data set, 85% of the non-proline PCaP1 residues were unambiguously assigned (Figure 4A;
619 Supplementary Figure S9A). The assigned chemical shifts revealed the presence of a N-
620 terminal structured domain (residues 29-129), mainly formed by α -helices, and a highly flexible
621 (intrinsically disordered) C-terminal domain (residues 132-225) (Figure 4B; Supplementary
622 Figure S9A). We did not intend an atomic-resolution structure determination by NMR, but
623 rather computed a structural model with AlphaFold2, a DeepMind AI-based computational tool
624 that was proven to produce accurate structural models for the majority of the protein targets
625 tested (Jumper et al., 2021). The resulting AlphaFold2 structural model (Figure 4C) presents
626 all the secondary structural features (α -helical structure) that have been derived from our NMR
627 data, providing an independent validation of this computational model.

628 The 2D ^1H - ^{15}N correlation spectra of PCaP1 recorded in the presence of increasing amounts
629 of U(VI) resulted in a progressive decrease of peak intensities for residues located in the
630 structured N-terminal part, while no such intensity loss was observed for the highly flexible C-
631 terminal segment (Figure 5A). In contrary, some amide sites in this flexible part even gained
632 in NMR signal intensity. Interestingly, no changes in peak positions were observed during this
633 NMR titration experiment. These findings suggest that the structured N-terminal part of PCaP1
634 oligomerizes upon U(VI) binding. In order to obtain a more quantitative idea about the average
635 particle size in PCaP1:U(VI) mixed samples, we performed NMR-based translational diffusion
636 experiments. The intensity decay of observed ^1H signals, mainly corresponding to the flexible
637 C-terminal domain, as a function of the applied gradient-field strength are plotted in Figure 5B
638 for U(VI):PCaP1 ratios of 0:1; 1:1 and 5:1. The translational diffusion constants extracted from
639 these curves are 21% (1:1) and 42% (5:1) smaller in the presence of U(VI) as compared to the

640 diffusion of the free PCaP1 protein. This apparent slower diffusion corresponds to an average
641 particle size that is increased 2 and 5-fold for the 1:1 and 5:1 mixtures, respectively.
642 In the case of a 1:1 stoichiometric ratio, we observed a decrease in signal intensity in the ^1H -
643 ^{15}N spectrum by a factor 2 for the structured part of the protein (Figure 5C). In view of our
644 translational diffusion results, this reduced signal intensity may originate either from the
645 presence of U(VI)-bound PCaP1 dimers, or free PCaP1 in slow exchange (high binding affinity)
646 with larger PCaP1 oligomers that are invisible by NMR. In order to discriminate the two
647 scenarios, we performed ^{15}N spin relaxation experiments that provide quantitative information
648 about changes in rotational tumbling correlation times upon interaction with U(VI). Site-specific
649 ^{15}N relaxation rate constants T_2 and T_1 , measured for free PCaP1 and a 1:1 U(VI):PCaP1
650 mixture (Figure 5C), did not show any significant difference for the structured N-terminal part.
651 This observation is in agreement with a model of coexisting monomeric and oligomeric species
652 with only the free PCaP1 monomers contributing to the detected NMR signal. In an attempt to
653 detect additional NMR signals in the ^1H - ^{15}N correlation spectra, arising from U(VI)-bound
654 PCaP1 oligomers, we produced ^2H , ^{15}N -labeled PCaP1 protein (deuterated to about 70%) to
655 reduce spin-relaxation-induced NMR signal loss. However, even under these isotope-labeling
656 conditions favorable for the detection of more slowly tumbling molecules, no additional peaks
657 could be observed in an overnight experiment, strongly suggesting that the undetected species
658 correspond to relatively large oligomers (at least trimers or tetramers).
659 Surprisingly, for some peptide regions in the flexible C-terminal part of PCaP1, notably around
660 residue 160, we observed a significant increase in NMR signal intensity upon uranyl binding
661 (Figures 5A, C). Note that the corresponding ^1H - ^{15}N correlation peaks for these residues were
662 of (very) low intensity in the spectra of free PCaP1, most likely due to conformational
663 exchange-induced line broadening effects (dynamics on the μs – ms time scale). ^{15}N relaxation
664 rates (T_1 and T_2) indicated only minor changes in the local dynamics on the fast (sub-ns) time
665 scale. In particular, the high T_2 and low T_1 values confirmed that this domain remains highly
666 flexible in the presence of uranyl. The observed increase in signal intensity upon uranyl binding
667 for the peptide region around residue 160 can thus be explained either by a side effect of U(VI)-
668 binding to the structured N-terminal part, altering the dynamic properties of this peptide region,
669 or by additional binding site(s) in the C-terminal part of PCaP1, again inducing a change in the
670 local dynamics of the peptide chain.

671

672 3.4. *U(VI) binds to the structured N-terminal and disordered C-terminal domains of* 673 *PCaP1*

674 In an attempt to better understand the interaction of U(VI) with the structured and disordered
675 parts of the protein, we produced two truncated forms of PCaP1, deleted from all or part of the
676 flexible region of the protein (Supplementary Figure S2B, C). The first one was a minor splicing

677 variant of PCaP1, formed by cryptic intron splicing within exon 4 (herein named PCaP1v), that
678 was isolated in the course of PCR-cloning of the full-length ORF (this form represented 1/10th
679 of the clones obtained by PCR cloning). This cDNA encodes a PCaP1 protein form lacking
680 residues 172-212 (Supplementary Figure S9B). The second PCaP1 form produced (herein
681 named PCaP1 Δ) was designed to remove the whole C-terminal flexible domain (residues 143-
682 225). The structural integrity of the PCaP1 Δ construct was checked by NMR spectroscopy,
683 with no (significant) spectral changes in the ¹H-¹⁵N correlation spectrum of PCaP1 Δ with
684 respect to N-terminal residue signals detected in the spectrum of the full-length protein
685 (Supplementary Figure S10A). No degradation of recombinant proteins was also noted, as
686 judged by SDS-PAGE analysis and mass spectrometry in denaturing conditions
687 (Supplementary Figure S2).

688 The stoichiometry of U(VI) complexation to both PCaP1v and PCaP1 Δ forms was analyzed by
689 MicroSpin G-25 chromatography coupled to ICP-MS, at pH 6 and 7.5 under the same
690 conditions as for full-length PCaP1 (Supplementary Table S3). Data obtained for PCaP1v were
691 very close to those observed for PCaP1, (*i.e.* 0.5 U(VI)/monomer at pH 6 and ~2
692 U(VI)/monomer at pH 7.5), demonstrating full U(VI)-binding capacity of this truncated protein.
693 Concerning PCaP1 Δ , data obtained at pH 6 were also similar to those measured for the other
694 two forms (0.56 U(VI)/monomer). At pH 7.5, however, the U(VI)-binding capacity of PCaP1 Δ
695 was at least reduced by 2 times (≤ 1 U(VI)/monomer), as compared to that of the other forms.
696 Trp-fluorescence titration of PCaP1v by up to 2 U(VI) equivalents at pH 6 gave quenching
697 spectra profiles similar to those of the full-length PCaP1 protein (Supplementary Figure S11A),
698 yielding a calculated conditional stability constant for U(VI) complex (UO₂(P₂) model) $\log\beta_{12} =$
699 14.2 ± 0.7 , close to that measured for the full-length PCaP1 protein (Supplementary Table S5).
700 In contrast, the fluorescence spectrum of PCaP1 Δ was altered and fluorescence intensities
701 were too low to determine a conditional stability constant (Supplementary Figure S11B;
702 Supplementary Table S5).

703 NMR titration experiments adding increasing amounts of U(VI) to a sample of PCaP1 Δ showed
704 a shift in binding stoichiometry with respect to full-length PCaP1 (Supplementary Figure S10B,
705 C). At sub-stoichiometric amounts of U(VI) the NMR signal intensity decreased, without
706 changing the translational diffusion properties of the NMR-observable species (similar to full
707 length PCaP1), while no NMR signal was detected for U(VI):PCaP1 Δ ratios as soon as uranyl
708 was provided in excess. A SEC analysis of PCaP1 Δ protein exposed to a 5-fold excess U(VI),
709 using a Superdex 200 10_30 Increase GL column, showed that protein completely eluted as
710 large oligomers under these conditions, while the free protein behaved as a monomer
711 (Supplementary Figure S12). The NMR signal was partly recovered by adding EDTA (5 mM),

712 suggesting that U(VI) induced PCaP1 Δ oligomerization can be reversed by U(VI)-EDTA
713 complexation (not shown).

714

715 3.5. U(VI) translocation in Arabidopsis shoots is affected in a *pcap1* null-mutant

716 Our data showed that PCaP1 has the capacity to bind U(VI) with high affinity at various sites,
717 *in vitro*. In order to determine a possible involvement of PCaP1 on the fate of U(VI) *in planta*,
718 we obtained Arabidopsis seeds of an homozygous T-DNA insertion line of PCaP1 (*mdp25*; Li
719 et al. (2011); also known as *pcap1-4*; Nagata et al. (2016); herein named *knock-out (ko)*
720 mutant). Also, we generated transgenic lines expressing the full-length PCaP1 under the
721 control of a 35S promoter in the *ko* mutant line background (*ko/35S::PCaP1* lines). Three
722 independent homozygous transgenic lines carrying the *35S::PCaP1* construct, among the 9
723 obtained, were selected for subsequent U(VI) uptake and translocation analyses (#2; #10 and
724 #30 lines). A western blot analysis of total shoot proteins using a PCaP1 antibody showed no
725 detectable PCaP1 protein in the *ko* mutant, confirming that it is a genuine null-mutant (Figure
726 6A; Nagata et al. (2016)). Transgenic lines exhibited nearly wild-type levels of PCaP1
727 expression. To estimate the importance of PCaP1 synthesis on the accumulation of U(VI),
728 plants were grown for 4 weeks in a complete standard Gre medium, then transferred in a Gre
729 medium depleted of Pi and exposed to 20 μ M uranyl nitrate for short (1 or 2 days) and long
730 (11 days) periods of time. No obvious difference was observed on plant growth between the
731 different lines, for the same U(VI) exposure time (not shown). We used the photosynthetic
732 parameter F_v/F_m (measurement of photosystem II efficiency in dark-adapted leaves) as an
733 estimate of plant fitness, and found it was unchanged in all cases (comprised between $0.79 \pm$
734 0.01 and 0.81 ± 0.01). Root and shoot U(VI) content was determined by ICP-MS following
735 careful washing of plant tissues with sodium carbonate and deionized water to eliminate the
736 loosely-bound radionuclide. In agreement with previous observations made under comparable
737 conditions (Berthet et al., 2018; Doustaly et al., 2014; Saenen et al., 2014; Sarthou et al., 2022;
738 Vanhoudt et al., 2008), the amount of U(VI) in shoots was low compared to roots in wild-type
739 (Col-0) control plants, with an highest translocation factor (shoot to root ratio) of 6×10^{-3} ,
740 illustrating the low mobility of U(VI) in Arabidopsis (Figure 6B, C). Data presented in Figure 6B
741 showed that U(VI) biosorption in roots was poorly affected in *pcap1 ko* plants or in transgenic
742 lines as compared to Col-0 control plants, throughout the experiment. In contrast, U(VI)
743 translocation in Arabidopsis shoots was significantly affected in *ko* plants after 11 days of U(VI)
744 exposure, as compared to Col-0 controls, showing a 2 to 3-fold decrease in U(VI) translocation
745 (Figure 6C). No difference was observed for short periods of U(VI) exposure (days 1 or 2).
746 Finally, ectopic expression of PCaP1 cDNA in *pcap1 ko* plants (#2, #10, #30 transgenic lines)

747 restored wild-type levels of U(VI) translocation in shoots, in line with PCaP1 protein expression
748 levels.

749

750 **4. Discussion and conclusions**

751 The cellular toxicity of U(VI) in prokaryotic and eukaryotic organisms is not yet fully described
752 but it likely results from its ability to bind strongly to biomolecules, mainly proteins that contain
753 numerous uranyl-binding groups. Over the past few years, significant progress has been made
754 in identification of UraBPs from various organisms, including the hyperthermophilic archaea
755 *Pyrococcus furiosus* (Cvetkovic et al., 2010), the crayfish *Procambarus clarkii* (Xu et al., 2014),
756 the zebrafish *Danio rerio* (Bucher et al., 2014; Eb-Levadoux et al., 2017), rat (Frelon et al.,
757 2009) and humans (Basset et al., 2008; Dedieu et al., 2009; Huynh et al., 2015; Vidaud et al.,
758 2005; Vidaud et al., 2019). These proteins were identified by a combination of non-denaturing
759 electrophoretic and/or chromatographic approaches combined with proteomic analyses,
760 allowing to isolate proteins with affinity for U(VI) either *in vitro*, *in cellulo* or both.

761 Despite U(VI) is known to be taken up by plants and to have cellular toxic effects, very few of
762 the proteins involved in these processes have been identified so far (Berthet et al., 2018;
763 Doustaly et al., 2014; Laurette et al., 2012; Misson et al., 2009; Serre et al., 2019; Vanhoudt
764 et al., 2011). Recently, several evidences were provided that U(VI) is taken up by the roots of
765 Arabidopsis plants through MCA1 and ANN1 Ca²⁺-permeable cation channels (Sarhou et al.,
766 2022). However, the fate of U(VI) within plant cells is still largely unknown. In particular,
767 identification and characterization of UraBPs have not been done so far in higher plants or any
768 photosynthetic organism. Here, in order to identify UraBPs in Arabidopsis, we used an
769 immobilized metal affinity chromatography (IMAC) method, based on the cation-exchange
770 properties of aminophosphonate groups for U(VI) binding (Basset et al., 2008). Such a
771 technique was developed because uranyl cations have singular physico-chemical
772 characteristics that prevent them from being immobilized on commercially available metal
773 chelating supports. This IMAC technique was a powerful tool for capturing UraBPs *in vitro*,
774 from human serum (Basset et al., 2008), kidney-2 cell extracts (Dedieu et al., 2009) and
775 dopaminergic SH-SY5Y cells (Vidaud et al., 2019). Adapted to plant soluble protein extracts,
776 this technique allowed us to capture and identify 38 UraBPs from Arabidopsis shoots and/or
777 roots. Interestingly, several proteins identified in our study are homologous to UraBPs
778 identified in other organisms. For example, the glycolytic enzymes fructose-bis phosphate
779 aldolases 2 and 1 (UraBP7 and 13, in this study) are homologous to several aldolases
780 identified in human urano proteomes (Dedieu et al., 2009; Vidaud et al., 2019). Also,
781 peroxiredoxin Q (UraBP29), the plasma-membrane associated cation-binding protein, PCaP1
782 (UraBP25) and the glycine-rich RNA-binding protein 7 (UraBP33) are related to peroxiredoxin
783 1, the numerous actin network regulatory proteins, and various ribonucleoproteins, identified

784 in the human urano proteomes, respectively (Dedieu et al., 2009; Vidaud et al., 2019). Finally,
785 the beta carbonic anhydrase 2 (UraBP18) and the glutathione S-transferase F2 and F7
786 (UraBP15 and 35) are homologous to carbonic anhydrase and glutathione S-transferases,
787 respectively, identified as UraBPs in zebrafish and the crayfish (Bucher et al., 2016; Eb-
788 Levadoux et al., 2017; Xu et al., 2014).

789 Among the 38 UraBPs identified in this study, we focused on PCaP1 for a detailed
790 characterization. PCaP1 was previously shown to bind calcium possibly at a unique site and
791 copper at multiple sites, both *in vitro* and *in planta* (Ide et al., 2007; Nagasaki-Takeuchi et al.,
792 2008; Nagasaki et al., 2008; Nagata et al., 2016; Qin et al., 2014). Here, we established that
793 recombinant PCaP1 was indeed able to bind copper but also ferric ion to multiple sites. The
794 large number of glutamate residues in the protein sequence and particularly in the intrinsically
795 unstructured region probably accounted for this high-capacity metal binding. We also
796 demonstrated that PCaP1 was able to bind U(VI) with high affinity, and that calcium somewhat
797 competed with U(VI) for binding. At pH 6, PCaP1 displayed high U(VI)-binding affinity with a
798 conditional dissociation constant in the vicinity of 10 nM. By comparison, binding constants for
799 U(VI) of the same order (from the sub-nanomolar to the sub-micromolar range) were reported
800 for peptides or proteins with native or artificial metal-binding sites, using similar experimental
801 approaches (Acharya and Blindauer, 2016; Basset et al., 2013; Gallois et al., 2021; Laporte et
802 al., 2019; Le Clainche and Vita, 2006; Lebrun et al., 2014; Michon et al., 2010; Pardoux et al.,
803 2012; Qi et al., 2014; Sauge-Merle et al., 2017; Starck et al., 2017; Starck et al., 2015; Wegner
804 et al., 2009), and also reviewed by Lin (2020).

805 In a more singular way, our results are consistent with U(VI) exposure inducing PCaP1
806 oligomerization. Structural impacts of U(VI) binding to proteins reported in the literature vary
807 from slight secondary structural to large conformational changes, but to our knowledge, no
808 study describes a change of protein oligomeric state (Acharya and Blindauer, 2016; Bal et al.,
809 2013; Basset et al., 2013; Gallois et al., 2021; Hoarau et al., 2019; Lin et al., 2011; Pardoux et
810 al., 2012; Qi et al., 2014; Sun et al., 2014; Vidaud et al., 2007; Wan et al., 2012; Zhou et al.,
811 2014).

812 Structural evidences indicate that the presence of two oxo groups in uranyl cation promotes
813 the coordination of four to six additional ligands to U(VI) in its equatorial plane perpendicular
814 to the O-U-O axis (Garai and Delangle, 2020; Van Horn and Huang, 2006). In this context,
815 based on the results obtained on the full-length and truncated versions of PCaP1 by
816 fluorescence titration, U(VI) quantification by ICP-MS, solution-state NMR and SEC analyses,
817 we can suggest structural models for the interaction with uranyl (Figure 7). At pH 6, our data
818 are consistent with the occurrence of one U(VI)-binding site at the interface of two monomers
819 and coordinated by residues from the two monomers, yielding a stoichiometry of the complex
820 of 0.5 U(VI)/monomer (in line with the 1:2 model fitting the fluorescence titration data) (Figure

7A). Such a stoichiometry in U(VI)-PCaP1 complex was found in all the protein forms analyzed (PCaP1, PCaP1v and PCaP1 Δ) (Supplementary Table S3), suggesting that the U(VI)-binding site is located at the surface of the structured domain. At pH 7.5, the situation is more complex. According to our data, the well-folded N-terminal domain of PCaP1 possesses (at least) two high-affinity uranyl binding sites. Uranyl binding occurs at the interface between two monomers, thus allowing the formation of oligomers of different size and shape. The average stoichiometry U(VI):PCaP1 of such large oligomers is 1:1 (Figure 7B). The disordered C-terminal domain also possesses one or several uranyl binding sites of similar affinity than the N-terminal domain. Our study highlights the peptide segment around residue 160 to be involved in uranyl binding, as PCaP1v, retains the uranyl binding features of the full-length protein, and NMR shows altered conformational dynamics in this region upon uranyl binding. In the full-length protein, the flexible C-terminus is responsible for scavenging part of the available U(VI), which explains our results showing that about twice as much U(VI) is required to observe the complete disappearance of NMR signals from the well-folded monomeric N-terminal part of the full-length PCaP1 protein than of the isolated N-terminal domain. Uranyl-binding by the disordered C-terminal domain most likely induces the formation of a highly dynamic mesh of peptide chains, which contributes to the larger apparent particle size of U(VI)-PCaP1 complexes (Figure 5B). Such a configuration would be consistent, as observed, with a mean global U(VI) content for large complexes close to or higher than 2 U(VI)/monomer of full-length protein (about 1 U(VI) equivalent per structured domain and 1 U(VI) equivalent per unstructured domain) (Figure 7 B).

The capacity of PCaP1 to bind U(VI) *in vitro* does not provide insight into the functional, physiological and developmental consequences of protein-metal interactions *in planta*. PCaP1 is located on the inner side of the plasma membrane *via* N-myristoylation of the glycine 2 residue (Ide et al., 2007; Nagasaki et al., 2008; Nagata et al., 2016). At first glance, one would expect PCaP1 to prevent the diffusion of U(VI) into the cells and protect the plant from its toxic effects. This prediction is strengthened by the fact that PCaP1 is poly-phosphorylated *in vivo* (Supplementary Table S2; (Mattei et al., 2016; Rayapuram et al., 2014)), a situation known to amplify the affinity of proteins for U(VI) (Laporte et al., 2019; Qi et al., 2014; Starck et al., 2017; Starck et al., 2015). Such scavenging of U by a high-affinity protein to prevent its toxic outcomes would resemble that described for the UipA protein in U(VI)-tolerant *Microbacterium* species. UipA is a single-pass transmembrane protein exposed to the external phase of the plasma membrane; it is induced by U(VI) and protects *Microbacterium* from U(VI) entry thanks to the high affinity of its large soluble domain for U(VI) (Gallois et al., 2021). However, the role of PCaP1 does not resemble that of UipA. Indeed, the absence of PCaP1 in *pcap1-null* mutants has no impact on U(VI) accumulation in roots as compared with wild-type plants. On the contrary, the presence of wild-type levels of PCaP1 seems to promote translocation of U(VI)

858 from roots to aerial parts (Figure 6). In Arabidopsis, PCaP1 was found to regulate the actin
859 (severing activity) and the microtubule (depolymerizing activity) cytoskeleton in a calcium-
860 dependent manner (Li et al., 2011; Qin et al., 2014). PCaP1 was also proposed to play a role
861 in calcium signaling through its binding to calmodulin and phosphatidylinositol phosphates
862 (Kato et al., 2010; Nagasaki et al., 2008). In connection with these activities, PCaP1 negatively
863 regulates hypocotyl elongation (Li et al., 2011), pollen-tube growth (Qin et al., 2014), and the
864 root hydrotropic response (Tanaka-Takada et al., 2019). Moreover, it is involved in
865 plasmodesmata aperture associated to plant immunity (Cheng et al., 2020; Giovannoni et al.,
866 2021; Vijayapalani et al., 2012) and stomatal closure (Nagata et al., 2016). These structures
867 have a key role in the transport of small molecules and ion for cell-to-cell communication
868 (Roeder et al., 2022) and in water and metabolites fluxes into the xylem through the driving
869 force of transpiration (Hetherington and Woodward, 2003). It is conceivable that perturbation
870 of these mechanisms by inactivation of the *PCaP1* gene might be responsible for the observed
871 defect in U(VI) translocation. Further work is needed to determine precisely by which
872 mechanism PCaP1 favors U(VI) translocation to shoots and if the peculiar U(VI)-binding
873 properties of the protein is involved in this process, particularly given the apparent much higher
874 affinity of the protein for U(VI) than for calcium at the slightly alkaline pH of plant cell cytosol.

875

876 **Statement of environmental implication**

877 Uranium (U) is a harmful radionuclide that may be absorbed by plants from soil and,
878 consequently, contaminate the food chain, with human and animals health hazards. The
879 cellular toxicity and detoxification mechanisms of U are still poorly described but likely result
880 from its ability to bind strongly to biomolecules, mainly proteins. Our study describes for the
881 first time the large-scale identification of U-binding proteins from plant tissues and the further
882 characterization of one of them both *in vitro* and *in planta*. Our findings represent an important
883 milestone to advance knowledge in the fields of radionuclide toxicology and phytoremediation.

884

885 **Data availability**

886 All data supporting the findings of this study are included in this published article (and its
887 Supplementary Information files), or are available from the corresponding authors upon
888 reasonable request. NMR chemical shifts of PCaP1 have been deposited with the BMRB
889 (<http://www.bmrwisc.edu/>) under accession number 51607.

890 Sequence data from this article can be found in the GenBank/EMBL, Arabidopsis Genome
891 Initiative and/or UniProt databases under the following accession numbers: PCaP1
892 (At4g20260; Q96262); PCaP1v (splicing variant; ON209435).

893 The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE
894 (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD033354 and

895 10.6019/PXD033354. [The dataset is available with the Username:
896 reviewer_pxd033354@ebi.ac.uk and Password: 62dQFqjg].

897

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915

916 **Declaration of Competing Interest**

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

919

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930

931

932 **Figure legends**

933 **Figure 1:** Strategy for the isolation and identification of uranyl-binding proteins from
934 *Arabidopsis thaliana*.

935 **Figure 2:** Purification of recombinant PCaP1 and determination of its oligomerization state. A-
936 Documentation of PCaP1 purification. Polypeptides were separated by SDS-PAGE 12% and
937 stained with Coomassie Brilliant Blue R250. Lane 1: soluble proteins (25 µg) from *E. coli*
938 Rosetta cells harboring pET28-PCaP1 construct grown in the presence of IPTG; Lane 2:
939 ammonium sulfate 50% of saturation precipitating fraction (25 µg); Lane 3: BioGel HTP
940 hydroxyapatite column pool (15 µg); Lane 4: EMD-DEAE column pool (10 µg); Superdex 75
941 column pool (8 µg); M, molecular mass markers. B- Apparent molecular mass estimation of
942 native recombinant PCaP1 by gel filtration. Purified protein (150 µg) was resolved by size
943 exclusion chromatography (SEC) onto a Superdex 200 Increase 10/300 GL column. Eluted
944 fractions were analyzed by SDS-PAGE. Standard proteins for column calibration (inset) were
945 ferritin (440 kDa), covalbumin (75 kDa), carbonic anhydrase (29 kDa), and ribonuclease A
946 (13.7 kDa). $K_{av} = (V_e - V_o)/(V_t - V_o)$; V_e , elution volume; V_o , void volume; V_t , total volume. C-
947 Native mass spectrum of recombinant PCaP1. 1mer (monomer) = 24453 Da; 2mer (dimer) =
948 48906 Da.

949 **Figure 3:** Fluorescence emission spectrum analysis of uranyl binding to PCaP1 at pH 6. A-
950 Fluorescence titration of recombinant PCaP1 (0.8 µM) with UO_2^{2+} (0 to 2.5 equivalents) at pH
951 6 in MES buffer 10 mM, NaCl 100 mM, in the presence of IDA 10 µM, with excitation at 277
952 nm. B- Variation of the intensity at the peak maximum (342 nm) upon UO_2^{2+} addition. Circles
953 represent experimental (Exp) values and the solid line the best fit with the program SPECFIT,
954 taking into consideration the known conditional stability constants of the uranyl complexes with
955 IDA. $n_{eq}(U)$, number of U(VI) equivalents ($[U(VI)]/[PCaP1]$).

956 **Figure 4:** NMR assignment and structural features of PCaP1. A- Amide 1H - ^{15}N correlation
957 spectrum of free PCaP1. Resolved cross peaks are annotated by their residue type (one letter
958 amino acid code) and residue number. Sample: PCaP1 300 µM in Tris-HCl 10 mM pH 7.5,
959 NaN_3 0.03%. B- Helical propensity along the PCaP1 backbone (only the structured part is
960 shown) computed from the measured ^{13}C chemical shifts using the TALOS-N server
961 (<https://spin.niddk.nih.gov/bax/software/TALOS-N/>). The helical elements derived from this
962 plot (red bars) are in agreement with the structure predicted by Alpha Fold v2.0
963 (<https://alphafold.ebi.ac.uk/>) shown in (C). Model confidence (pLDDT score) for the structured
964 domain was >90 (highly confident). The image was produced using PyMOL (DeLano Scientific,
965 San Carlos, CA, USA) as a ribbon model colored in the “chainbows” mode.

966 **Figure 5:** NMR characterization of PCaP1-U(VI) interaction. A- Overlay of 2D 1H - ^{15}N
967 correlation spectra of free ^{15}N - ^{13}C labelled PCaP1 (100 µM) alone (black) or in complex (100
968 µM) with U(VI) (500 µM) (red) in Tris-HCl 10 mM pH 7.5. Only correlation peaks in the random

969 coil ^1H spectral region (indicated by dashed lines) remain visible upon U(VI) binding. B-
970 Diffusion ordered spectroscopy (DOSY) measurements of PCaP1 in the absence or in the
971 presence of 1 or 5 U(VI) equivalents. The observed NMR signal intensity (integrated over the
972 1D ^1H spectrum) is plotted as a function of the applied gradient strength. Fitting of decay curves
973 yields the translational diffusion constant. Translational diffusion of PCaP1 decreases U(VI)
974 with increasing amounts of uranyl, indicating the formation of larger particles. C- ^{15}N spin
975 relaxation rate constants (T_1 and T_2) measured for free PCaP1 (black) and PCaP1 in the
976 presence of an equal amount of U(VI) (red). In addition, the intensity ratios of ^1H - ^{15}N correlation
977 peaks detected in the absence and presence of an equal amount of U(VI) are plotted (top
978 panel).

979 **Figure 6:** Uranium content in wild type, *pcap1*-null mutant and transgenic lines expressing
980 PCaP1. Wild type (Col-0), *pcap1*-null mutant (*ko*) and transgenic lines expressing PCaP1
981 under the control of a 35S promoter (#2; #10 and #30 lines) were grown hydroponically for 5
982 weeks in Gre medium, and then challenged with 20 μM uranyl nitrate for 1, 2 and 11 days in
983 Gre medium with no phosphate. A- Western blot analysis of total shoot proteins with a PCaP1
984 antibody. B- Accumulation of U in roots. C- Accumulation of U in shoots. Samples were
985 dehydrated, digested with HNO_3 , and ^{238}U was measured by ICP-MS. Data distribution is
986 displayed in Tukey's boxplots with the median as the solid line inside the box, and the first and
987 third quartiles as the bottom and top lines of the box, respectively. Outliers are shown as
988 individual dots. Each distribution in boxplots represents $n = 4$ samples for roots and $n = 4-8$
989 samples for shoots. Statistical significance determined using non-parametric comparisons with
990 Tukey's test is shown: *, $p < 0.05$; **, $p < 0.01$.

991 **Figure 7:** Models for *in vitro* U(VI)-PCaP1 complexes formation at pH 6 and pH 7.5.

992
993

994 **Appendix A. Supporting information**

995 Supplementary data associated with this article can be found in the online version.

996

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998

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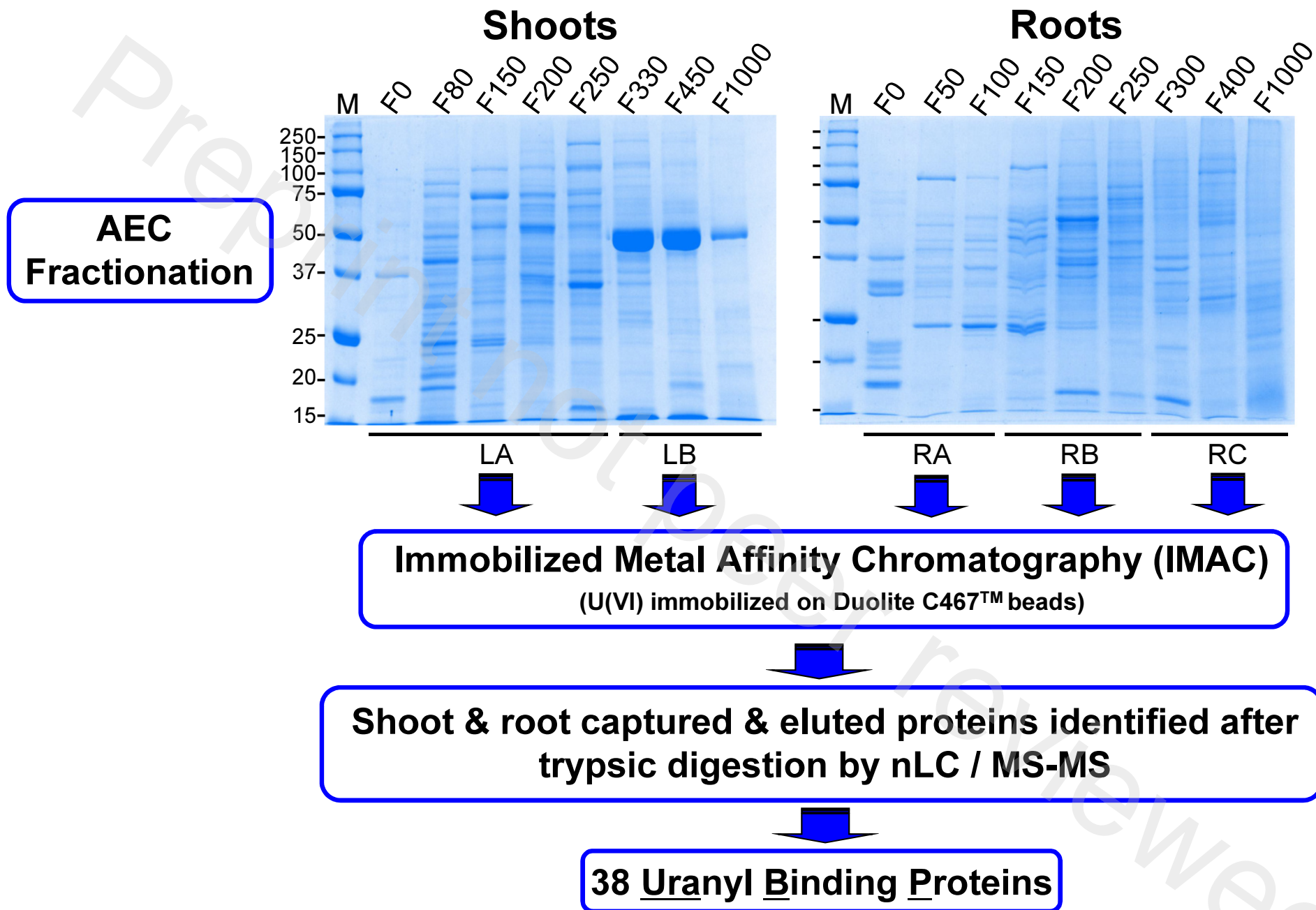


Figure 1: Strategy for the isolation and identification of uranyl-binding proteins from *Arabidopsis thaliana*.

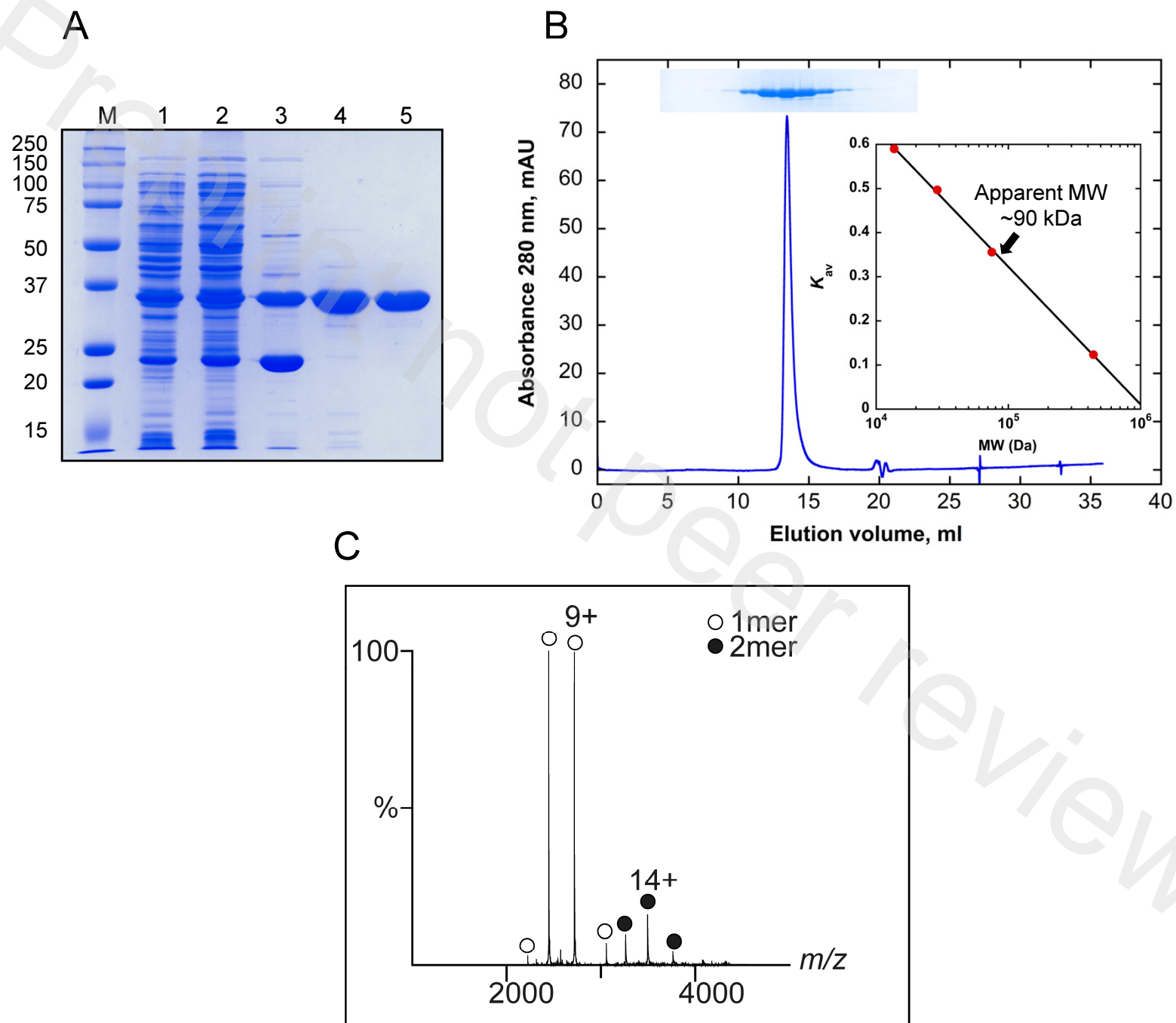


Figure 2 (legend on the next page)

Figure 2: Purification of recombinant PCaP1 and determination of its oligomerization state. A- Documentation of PCaP1 purification. Polypeptides were separated by SDS-PAGE 12% and stained with Coomassie Brilliant Blue R250. Lane 1: soluble proteins (25 µg) from *E. coli* Rosetta cells harboring pET28-PCaP1 construct grown in the presence of IPTG; Lane 2: ammonium sulfate 50% of saturation precipitating fraction (25 µg); Lane 3: BioGel HTP hydroxyapatite column pool (15 µg); Lane 4: EMD-DEAE column pool (10 µg); Superdex 75 column pool (8 µg); M, molecular mass markers. B- Apparent molecular mass estimation of native recombinant PCaP1 by gel filtration. Purified protein (150 µg) was resolved by size exclusion chromatography (SEC) onto a Superdex 200 Increase 10/300 GL column. Eluted fractions were analyzed by SDS-PAGE. Standard proteins for column calibration (inset) were ferritin (440 kDa), covalbumin (75 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa). $K_{av} = (V_e - V_o) / (V_t - V_o)$; V_e , elution volume; V_o , void volume; V_t , total volume. C- Native mass spectrum of recombinant PCaP1. 1mer (monomer) = 24453 Da; 2mer (dimer) = 48906 Da.

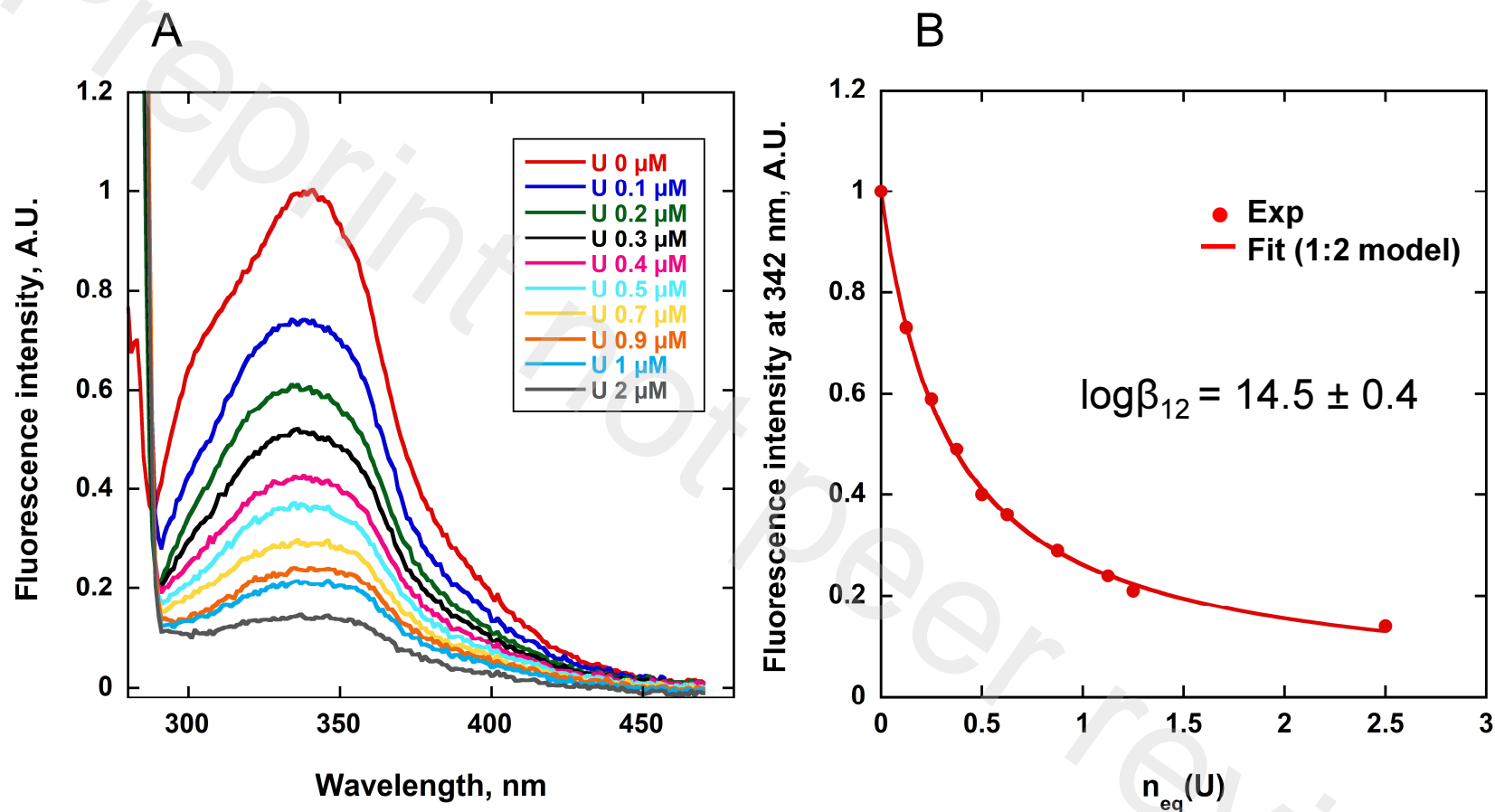


Figure 3: Fluorescence emission spectrum analysis of uranyl binding to PCaP1 at pH 6. A- Fluorescence titration of recombinant PCaP1 (0.8 μM) with UO_2^{2+} (0 to 2.5 equivalents) at pH 6 in MES buffer 10 mM, NaCl 100 mM, in the presence of IDA 10 μM, with excitation at 277 nm. B- Variation of the intensity at the peak maximum (342 nm) upon UO_2^{2+} addition. Circles represent experimental (Exp) values and the solid line the best fit with the program SPECFIT, taking into consideration the known conditional stability constants of the uranyl complexes with IDA. $n_{\text{eq}}(\text{U})$, number of U(VI) equivalents ($[\text{U(VI)}]/[\text{PCaP1}]$).

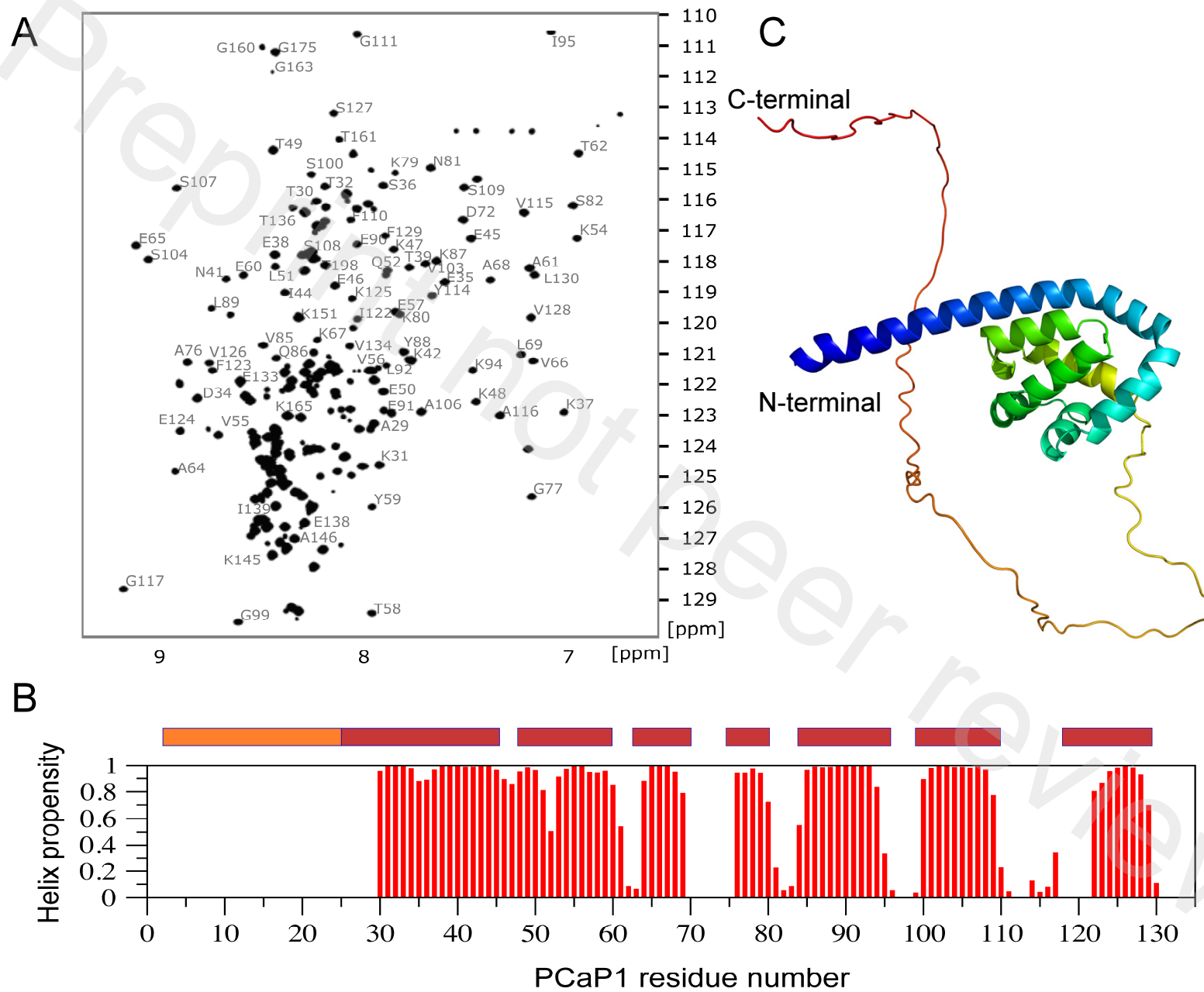


Figure 4 (legend on the next page)

Figure 4: NMR assignment and structural features of PCaP1. A- Amide ^1H - ^{15}N correlation spectrum of free PCaP1. Resolved cross peaks are annotated by their residue type (one letter amino acid code) and residue number. Sample: PCaP1 300 μM in Tris-HCl 10 mM pH 7.5, NaN_3 0.03%. B- Helical propensity along the PCaP1 backbone (only the structured part is shown) computed from the measured ^{13}C chemical shifts using the TALOS-N server (<https://spin.niddk.nih.gov/bax/software/TALOS-N/>). The helical elements derived from this plot (red bars) are in agreement with the structure predicted by Alpha Fold v2.0 (<https://alphafold.ebi.ac.uk/>) shown in (C). Model confidence (pLDDT score) for the structured domain was >90 (highly confident). The image was produced using PyMOL (DeLano Scientific, San Carlos, CA, USA) as a ribbon model colored in the “chainbows” mode.

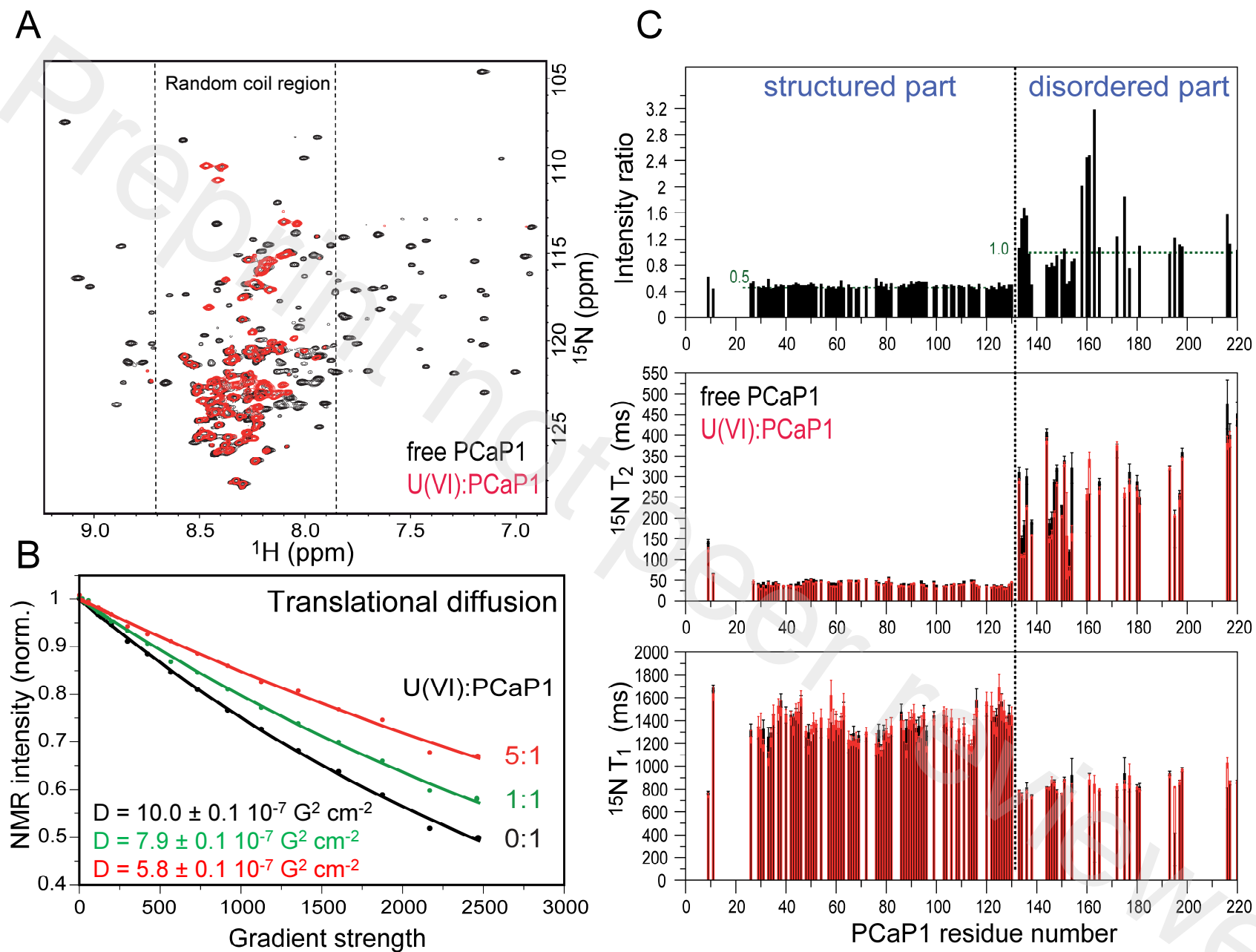


Figure 5 (legend on the next page)

Figure 5: NMR characterization of PCaP1-U(VI) interaction. A- Overlay of 2D ^1H - ^{15}N correlation spectra of free ^{15}N - ^{13}C labelled PCaP1 (100 μM) alone (black) or in complex (100 μM) with U(VI) (500 μM) (red) in Tris-HCl 10 mM pH 7.5. Only correlation peaks in the random coil ^1H spectral region (indicated by dashed lines) remain visible upon U(VI) binding. B- Diffusion ordered spectroscopy (DOSY) measurements of PCaP1 in the absence or in the presence of 1 or 5 U(VI) equivalents. The observed NMR signal intensity (integrated over the 1D ^1H spectrum) is plotted as a function of the applied gradient strength. Fitting of decay curves yields the translational diffusion constant. Translational diffusion of PCaP1 decreases U(VI) with increasing amounts of uranyl, indicating the formation of larger particles. C- ^{15}N spin relaxation rate constants (T_1 and T_2) measured for free PcaP1 (black) and PCaP1 in the presence of an equal amount of U(VI) (red). In addition, the intensity ratios of ^1H - ^{15}N correlation peaks detected in the absence and presence of an equal amount of U(VI) are plotted (top panel).

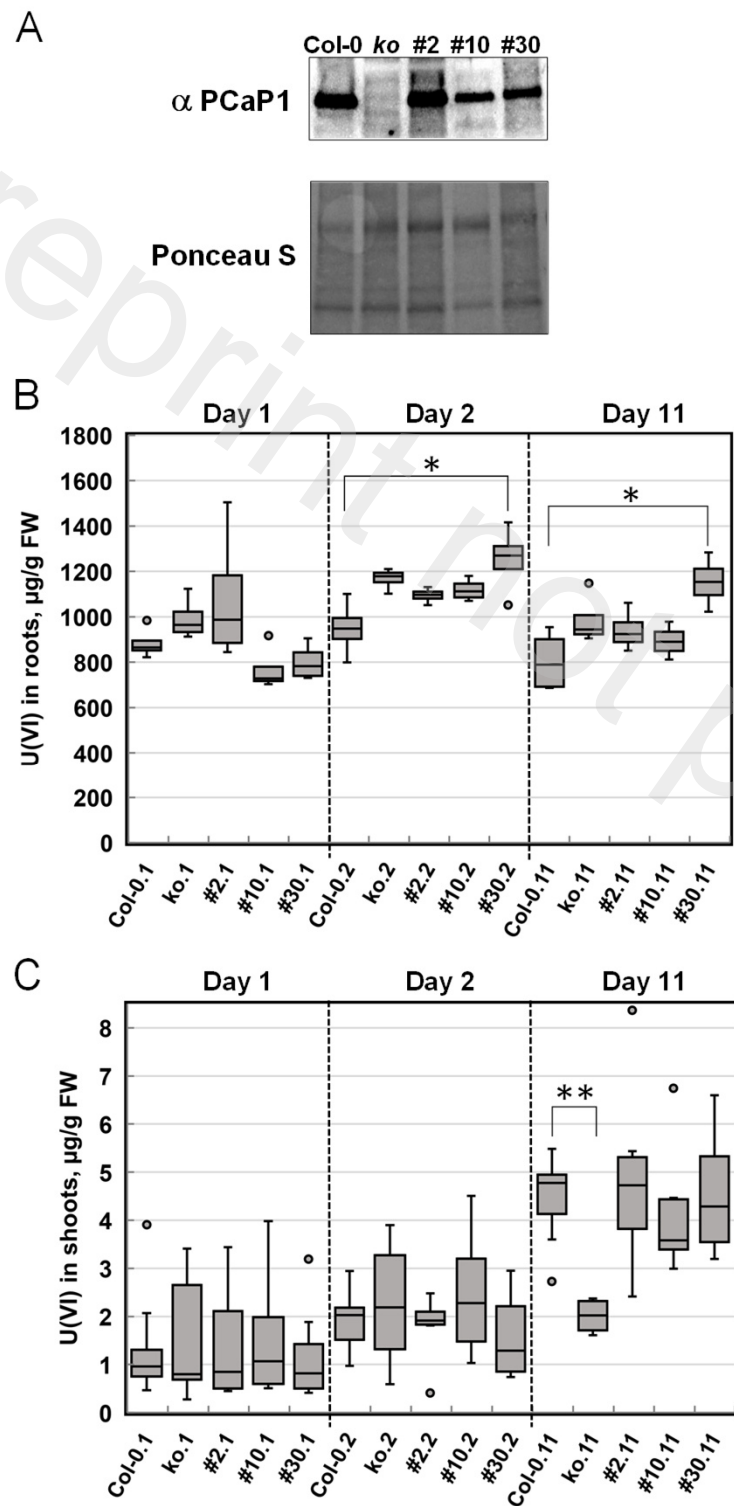


Figure 6: Uranium content in wild type, *pcap1*-null mutant and transgenic lines expressing PCaP1. Wild type (Col-0), *pcap1*-null mutant (*ko*) and transgenic lines expressing PCaP1 under the control of a 35S promoter (#2; #10 and #30 lines) were grown hydroponically for 5 weeks in Gre medium, and then challenged with 20 μ M uranyl nitrate for 1, 2 and 11 days in Gre medium with no phosphate. A- Western blot analysis of total shoot proteins with a PCaP1 antibody. B- Accumulation of U in roots. C- Accumulation of U in shoots. Samples were dehydrated, digested with HNO₃, and ²³⁸U was measured by ICP-MS. Data distribution is displayed in Tukey's boxplots with the median as the solid line inside the box, and the first and third quartiles as the bottom and top lines of the box, respectively. Outliers are shown as individual dots. Each distribution in boxplots represents $n = 4$ samples for roots and $n = 4-8$ samples for shoots. Statistical significance determined using non-parametric comparisons with Tukey's test is shown: *, $p < 0.05$; **, $p < 0.01$.

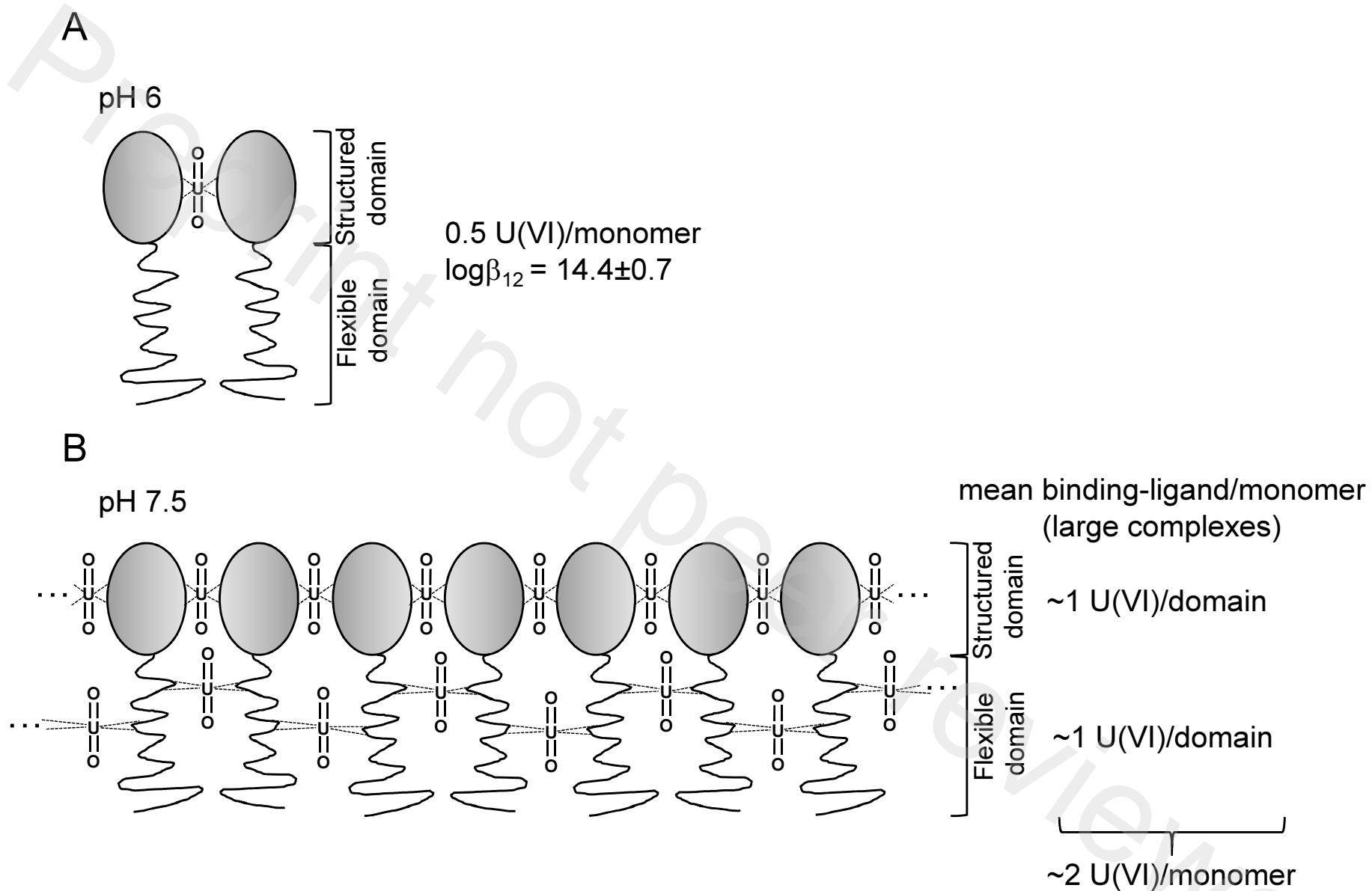


Figure 7: Models for *in vitro* U(VI)-PCaP1 complexes formation at pH 6 and pH 7.5.