

1 **Root membrane ubiquitinome under short-term osmotic stress**

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8

9 **Author contributions**

10 NB performed the plant culture, protein extraction, immunopurification and LC-MS/MS analyses; VD
11 analyzed the quantitative proteomics data and performed statistical analysis; SH conducted the
12 MaxQuant analysis; VR contributed to the LC-MS/MS analyses; LS collected the in silico interactomics
13 data; GK supervised and analyzed the interactomics data; AC and PN gathered the root phenotyping
14 data; LV characterized the role of K3 ubiquitination; and VS conceived the project, designed the
15 experiments, analyzed the data, and wrote the article.

16

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20

21 **Keywords**

22 aquaporin, mass spectrometry, osmotic stress, ubiquitination

23

24 **Abbreviations**

25 K-Ub: ubiquitinated lysine

26 LC-MS/MS: liquid chromatography-tandem mass spectrometry

27 L_p : root hydraulic conductivity

28 PIP: plasma membrane intrinsic protein

29 Ub: ubiquitin

30 Ubi-peptides: ubiquitinated peptides

31

32 **Short title**

33 Root membrane ubiquitinome

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35

36 **Abstract**

37 Osmotic stress can be detrimental to plants, whose survival relies heavily on proteomic plasticity.
38 Protein ubiquitination is a central post-translational modification in osmotic mediated stress. Plants
39 use the ubiquitin (Ub) proteasome system to modulate protein content, and a role for Ub in
40 mediating endocytosis and trafficking plant plasma membrane proteins has recently emerged. In this
41 study, we used the K-ε-GG antibody enrichment method integrated with high-resolution mass
42 spectrometry to compile a list of 719 ubiquitinated lysine (K-Ub) residues from 450 *Arabidopsis* root
43 membrane proteins (58% of which are transmembrane proteins), thereby adding to the database of
44 ubiquitinated substrates in plants. Although no Ub motifs could be identified, the presence of acidic
45 residues close to K-Ub was revealed. Our ubiquitinome analysis pointed to a broad role of
46 ubiquitination in the internalization and sorting of cargo proteins. Moreover, the simultaneous
47 proteome and ubiquitinome quantification showed that ubiquitination is mostly not involved in
48 membrane protein degradation in response to short osmotic treatment, but putatively in protein
49 internalization as described for the aquaporin PIP2;1. Our in silico analysis of ubiquitinated proteins
50 shows that two E2 Ub ligases, UBC32 and UBC34, putatively target membrane proteins under
51 osmotic stress. Finally, we revealed a positive role for UBC32 and UBC34 in primary root growth
52 under osmotic stress.

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54

55 **Introduction**

56 Plants are exposed to different types of abiotic stress conditions such as drought or salinity that
57 result in diminished plant growth and crop productivity (Lobell et al., 2011). Most of these conditions
58 impose osmotic stress on plants by reducing the water potential of the environment. The
59 consequences of osmotic stress manifest as inhibited cell elongation, stomata closure, reduced
60 photosynthetic activity, the translocation of assimilates, changes in various metabolic processes, and
61 disturbances in water and ion uptake.

62 The ability of plants to survive these abiotic stresses relies heavily on their proteomic plasticity.
63 Protein stability, activity, localization, and interactions with partners have all been widely described
64 as governed by ubiquitination (Mukhopadhyay and Riezman, 2007; Nelson and Millar, 2015).
65 Ubiquitin (Ub) is a 76-amino acid polypeptide that is highly conserved in eukaryotes and is
66 ubiquitously found in tissues. It is linked to either target proteins or itself through the sequential
67 action of three enzyme classes: Ub-activating enzymes (E1s), Ub-conjugating enzymes (E2s), and Ub
68 ligases (E3s) (Callis, 2014). The activities of these enzymes ultimately result in the covalent
69 attachment of Ub to a lysine (K) residue in the target protein. Ubiquitination can result in the

70 conjugation of a single moiety (mono-ubiquitination), multiple Ub molecules that are individually
71 attached (multi-mono-ubiquitination), or in the form of a chain (poly-ubiquitination) attached to a
72 specific substrate. Poly-Ub chains are formed by the further attachment of Ub moieties linked
73 together by one of the seven lysine residues present in a Ub molecule (K6, K11, K27, K29, K33, K48,
74 and K63), or by the N-terminal methionine in the form of head-tail linear repeats (Pickart and Eddins,
75 2004). Poly-Ub chains exhibit different topologies and are associated with diverse biological functions
76 (Walsh and Sadanandom, 2014). As an example, poly-ubiquitination involving residue K48 from
77 ubiquitin (K48-Ub linkage) triggers the degradation of target proteins by the 26S proteasome (Pickart
78 and Eddins, 2004). Much less is known about the other poly-ubiquitination chain linkages (Pickart
79 and Eddins, 2004; Walsh and Sadanandom, 2014). The K63-Ub linkage has been widely studied in
80 yeast and mammals and more recently in plants, and includes roles in the endocytosis of plasma
81 membrane proteins, DNA damage responses and, to a lesser extent, autophagy and signaling
82 (Komander and Rape, 2012; Romero-Barrios et al., 2020).

83 A role for ubiquitination in abiotic stress tolerance has emerged from the study of transgenic plants
84 overexpressing Ub genes that become more tolerant to multiple abiotic stresses (Guo et al., 2008;
85 Kang et al., 2016), and from the expression analysis of E2s and E3s. The *Arabidopsis* genome contains
86 over 1,400 genes encoding E3s, 37 canonical E2s, and seven E2 variant proteins (Stone, 2018). The
87 number of E3s associated with abiotic stress has increased dramatically over the last decade (Stone,
88 2018). In particular, exposure to stress (such as osmotic stress) increases abscisic acid levels;
89 moreover, the number of E3s associated with regulating abscisic acid production, signaling, and
90 response continues to grow, now including at least 25 different E3s (Stone, 2018). In addition to E3s,
91 it appears that E2s are not only utilized as Ub-transferring components, but also play an active role in
92 regulating the ubiquitination pathway (Zhou et al., 2010; Cui et al., 2012; Chen et al., 2016).
93 Furthermore, E2 enzymes can interact directly with their targets (Liu et al., 2012; Pan et al., 2020).
94 Indeed, recent studies have suggested that E2s regulate the specificity of target ubiquitination (Turek
95 et al., 2018; Romero-Barrios et al., 2020).

96 The low stoichiometry and short lifespans of ubiquitinated proteins present obstacles to the
97 identification of ubiquitinated proteins. K- ϵ -GG (DiGly, the remnant from ubiquitinated proteins
98 following trypsin digestion) antibody affinity enrichment provides an efficient method to gain
99 proteome-wide insight into ubiquitination processes, by capturing and concentrating this remnant of
100 ubiquitinated proteins treated with trypsin prior to MS/MS (Xie et al., 2015; Guo et al., 2017; Zhang
101 et al., 2017; Wang et al., 2019; He et al., 2020; Grubb et al., 2021). In the present study, we used a
102 high-affinity K- ϵ -GG antibody enrichment technique combined with high-accuracy MS/MS analyses to
103 generate quantitative ubiquitinome and proteome profiles of root membrane proteins from plants
104 treated with a short-term osmotic treatment. Altogether, we provide an extensive inventory of K-Ub

105 residues in a membrane protein fraction, highlight the presence of acidic residues in the vicinity of
106 the K-Ub residue, reveal a role for ubiquitination outside of the membrane protein degradation
107 process in response to short-term osmotic treatment, and demonstrate that the E2 ubiquitin ligases
108 UBC32 and UBC34 are positive regulators of primary root growth during osmotic stress.

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112 **Results**

113 The ubiquitinome response to osmotic stress was investigated by treating plants with 200 mM
114 mannitol for 1 h, followed by a combined quantitative analysis of the proteome and ubiquitinome of
115 a microsomal fraction, according to the proteomic workflow described in Figure 1.

116

117 **Differentially accumulated proteins in response to mannitol treatment**

118 A total of 6,081 proteins were identified based on an identification with at least 2 peptides (Table
119 S1), of which 26% were transmembrane proteins (Table S1). Using GO analysis of cell component
120 terms, we showed that a majority of GO terms were associated with membrane proteins, even when
121 the extrinsic proteome (*i.e.* proteins without any transmembrane domain) was exclusively considered
122 (Figure S1). These results show that the microsomal fraction is enriched in membrane proteins.
123 Treating plants with 200 mM mannitol for 1 h resulted in differentially accumulated proteins (DAPs),
124 which were identified through a quantitative label-free approach. Among the 226 DAPs that were
125 identified, 132 were up-accumulated (average increase: 1.51x), and 94 were down-accumulated
126 (average decrease: 0.61x) (Table S2). In addition, 1 protein appeared upon mannitol treatment, while
127 10 proteins disappeared (Table S2). DAPs were classified according to the GO functional categories of
128 “biological process”, “molecular function”, and “cellular component” (Figure S2). Interestingly,
129 enriched functions mostly concerned ATPase activities (Figure S2), in agreement with observations
130 showing the tight regulation of plasma membrane H⁺-ATPase in response to several biotic and abiotic
131 stress responses (Falhof et al., 2016).

132

133 **Characterization of the root membrane ubiquitinome**

134 To identify ubiquitinated proteins in *Arabidopsis* roots, we combined immunoaffinity enrichment
135 (using a high quality anti-K-ε-GG antibody; PTM biolabs) and high-resolution mass spectrometry.
136 Ubiquitinated peptides (Ubi-peptides) were considered as long as they had a score > 40 and if they
137 were identified in at least two independent samples. 719 Ubi-peptides harboring a total of 786 K-Ub
138 residues belonging to 450 proteins were identified, 264 of which contained at least one
139 transmembrane domain (Table S3, Table S4). Our GO enrichment analysis showed that ubiquitinated
140 proteins were enriched in transporters, in proteins involved in the regulation of intracellular pH, and
141 in cellular trafficking processes that were characterized by the GO terms “vesicle budding from
142 membrane”, “clathrin-dependent endocytosis” and “membrane invagination” (Figure 2) and included
143 15 proteins (Table S3). These observations suggest that membrane proteins, as well as the proteins
144 that drive their trafficking are ubiquitinated.

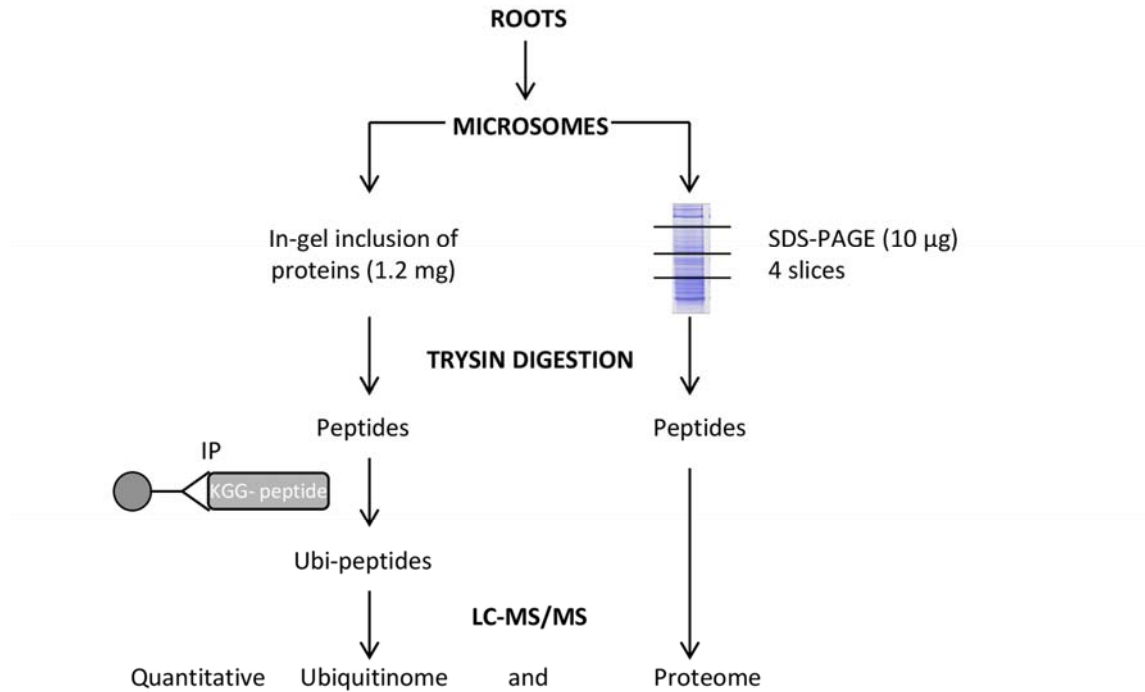


Figure 1. Workflow for quantitative profiling of the proteome and the ubiquitinome in Arabidopsis root membrane proteins upon mannitol treatment.

LC-MS/MS: liquid chromatography-tandem mass spectrometry.

IP: immunopurification.

145 Consensus peptide motifs for K-Ub residues were extracted using p-logo (O'Shea et al., 2013). In
146 total, 643 unique ubiquitinated sites were unable to highlight one unique motif (Table S5, Figure 3A-
147 B). However, the presence of an acidic amino acid close to K-Ub was observed in all motifs except
148 one. Although the nature of the poly-Ub linkage determines the role of ubiquitination, this
149 information is not available in a bottom-up proteomics strategy in which the use of trypsin induces
150 Ub proteolysis from ubiquitinated proteins. However, ubiquitinated peptides arising from the Ub
151 protein itself can provide information about poly-Ub linkages that occur in a protein sample. Seven K-

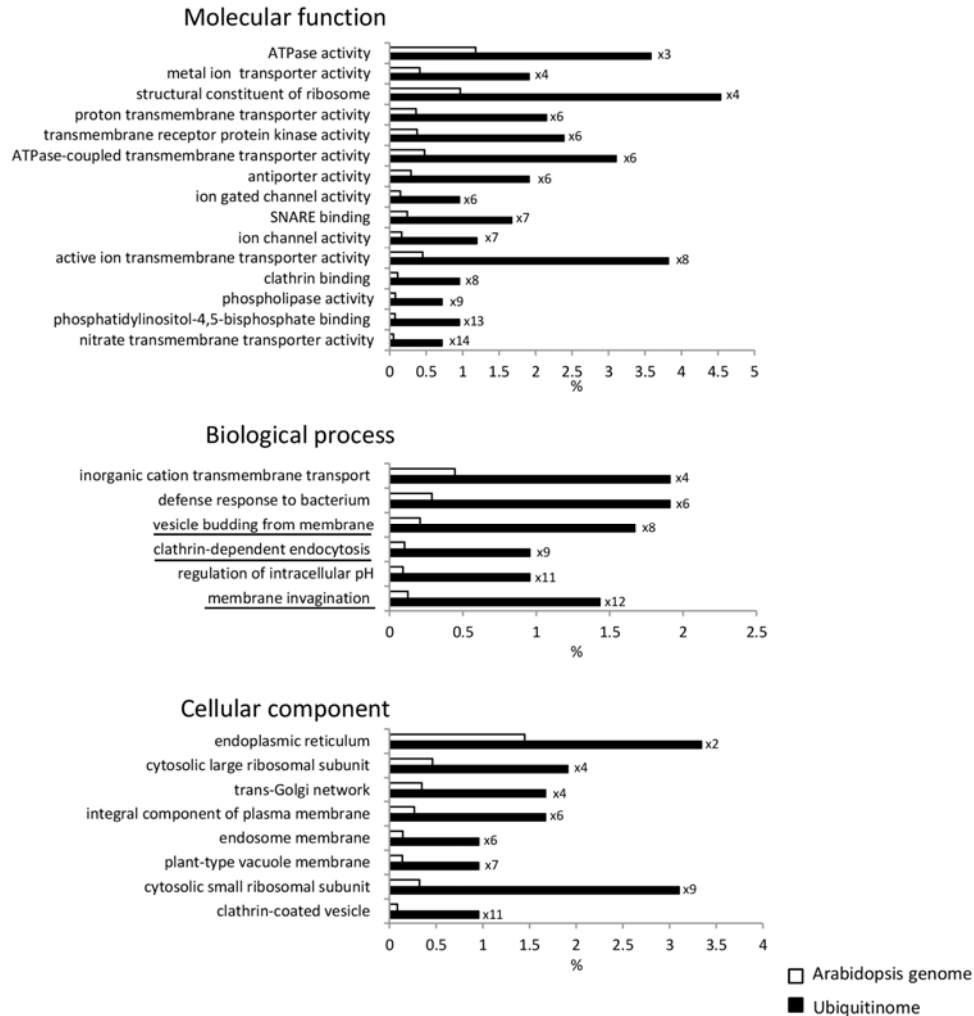


Figure 2. Functional enrichment analysis of ubiquitinated root microsomal proteins.

The percentage is calculated with regard to the number of identified ubiquitinated proteins (black) and the total number of Arabidopsis proteins (white). Numbers indicate the fold enrichment by comparison to the Arabidopsis genome. Underlined biological processes concern proteins involved in intracellular trafficking, and include 15 genes (Table S3).

152 Ubs were thus identified (K6, K11, K27, K29, K33, K48, K63) within Ub (Figure S3), showing multiple
 153 Ub-linkages within membrane proteins. Although peptide intensity is not indicative of the absolute
 154 quantity of each Ubi-peptide, the K48- and K63-Ub linkages appeared to predominate the poly-Ub
 155 linkages (Figure S3). One way to confirm these observations would be with a targeted proteomics
 156 approach that can provide absolute quantification of peptides (Tsuchiya et al., 2013). Nevertheless,
 157 our results reveal that mannitol treatment does not significantly modify the proportion of each poly-
 158 Ub linkage (Figure S3).

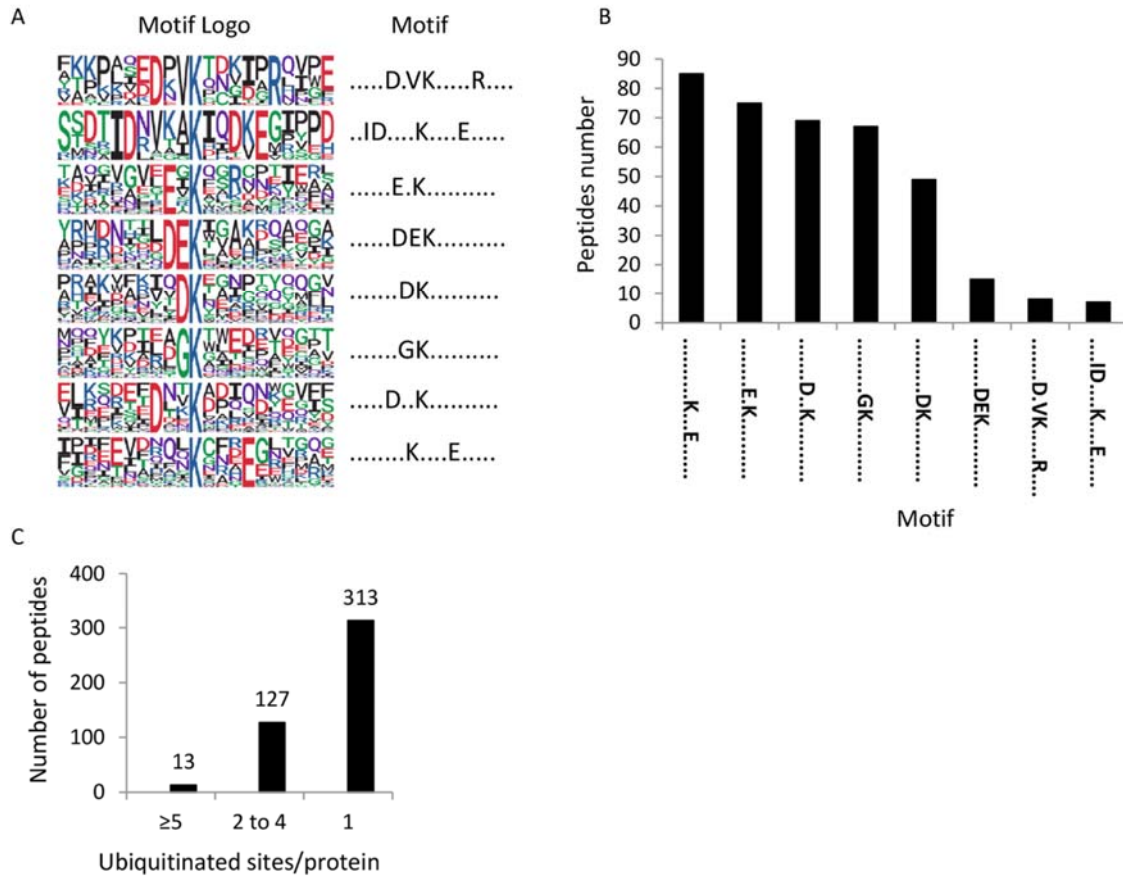


Figure 3. Motif analysis of identified K-Ub residues in root microsomes.

A. Ubiquitination motifs and the conservation of K-Ub residues. The height of each letter corresponds to the frequency of the amino acid residue in that position. The central K refers to the K-Ub residue. B. The number of identified peptides containing a K-Ub residue in each motif. C. The number of K-Ub sites per protein.

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160 Differentially accumulated ubiquitinated proteins in response to mannitol treatment

161 Out of 374 quantified Ubi-peptides, 82 showed quantitative variations in which 54 Ubi-peptides were
 162 up-accumulated and 28 were down-accumulated (Table S6). Enrichment-based clustering analyses
 163 showed that the ubiquitination of proteins altered by mannitol treatment mainly concerns ATPase,
 164 transporters and SNARE binding activities (Figure S4). An inverse quantitative relationship between a
 165 protein's abundance and its ubiquitinated form could be indicative of a role for ubiquitination in
 166 protein degradation. However, none of the 43 up-accumulated Ubi-peptides were affiliated with a
 167 decreased abundance in the corresponding protein (Table 1). In addition, among 24 down-
 168 accumulated Ubi-peptides, only 6 of them corresponded to accumulated proteins including CARK1,

169 HIR2, NRT2;1, PIRL5, AT1G48210.2 and AT3G47210.1 (Table 1). Thus, a role for ubiquitination in
170 degrading these protein could be considered. By contrast, for a majority of the proteins, the absence
171 of any inverse quantitative relationship between the protein and its ubiquitinated form suggests that
172 upon short-term osmotic treatment, ubiquitination could interfere with protein function or cellular
173 localization rather than with protein stability.

174

175 **Ubiquitination of PIP aquaporins**

176 Aquaporins define a large family of ubiquitous integral membrane proteins that mediate the
177 transport of water and small neutral solutes across membranes (Maurel et al., 2015). Plant
178 aquaporins show a large variety of isoforms. Indeed, 35 homologs within four homology subclasses
179 have been identified in *Arabidopsis*. The plasma membrane intrinsic proteins (PIPs) consist of 13
180 isoforms further subdivided into the PIP1 and PIP2 subgroups, and are the most abundant
181 aquaporins in the plasma membrane (Johanson and Gustavsson, 2002; Quigley et al., 2002). All 13
182 members of the PIP family were identified in this study (Table S1), and 9 of them exhibited
183 ubiquitinated residues in their N- and/or C-terminus (Table S3, Figure S5). Since only 3 lysine residues
184 are described in the literature as ubiquitinated (Chen et al., 2021; Grubb et al., 2021), the present
185 work greatly increases our knowledge regarding the ubiquitination of PIPs. Increased ubiquitination
186 of K3 and K276 was observed in PIP2;1 upon short-term mannitol treatment (Table 1). K276
187 ubiquitination was recently shown to mediate PIP2;1 degradation upon long-term drought (Chen et
188 al., 2021). However, since we observed that PIP2;1 cellular abundance was unchanged (Table 1), the
189 increased ubiquitination of K276 observed when plants are subjected to a 1-h mannitol treatment
190 cannot be involved in PIP2;1 degradation.

191 Next, we checked if there is a role for K3 ubiquitination using a simplified system of *Arabidopsis*
192 suspension cells with a low basal level of endogenous PIPs. We overexpressed PIP2;1 either wild-type
193 or carrying point mutations at K3 in alanine (K3A) and in arginine (K3R) (Santoni et al., 2006) with the
194 aim of preventing ubiquitination at that site. We previously showed by western blot analysis of total
195 protein extracts using an anti-PIP2;1 peptide antibody that there is a significantly strong
196 overexpression of PIP2;1 in these cells, as compared to untransformed cells or cells transformed with
197 an empty vector (PG) (Santoni et al., 2006). Here, using an ELISA assay, we observed a significant
198 accumulation of PIP2;1 in suspension cells overexpressing PIP2;1-K3A and PIP2;1-K3R as compared to
199 PIP2;1-WT (Figure 4), suggesting that K3 could be a ubiquitinable residue participating in the
200 degradation of PIP2;1. However, in roots placed under short-term osmotic treatment, increased K3
201 ubiquitination did not correlate with decreased PIP2;1 abundance, suggesting (as for K276) an
202 additional role for K3 ubiquitination outside of PIP2;1 degradation.

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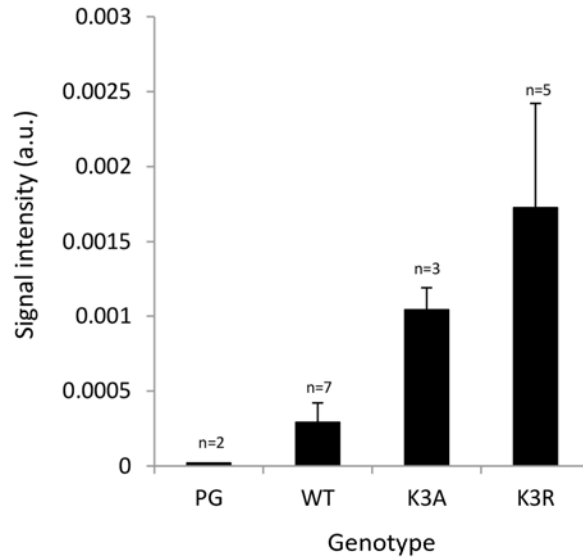


Figure 4. Relative abundance of PIP2 isoforms in Arabidopsis suspension cells overexpressing PIP2;1 WT and carrying a point mutation of K3 in alanine (K3A) and in arginine (K3R). ELISA assays were performed with total protein extracts and anti-PIP2 antibody (Santoni et al., 2006). The number of independent stable cell lines is indicated. Data were from three individual ELISA assays per cell line. Standard error is shown.

204 **The interactome of ubiquitinated proteins**

205 For additional insight into the extent of the role of ubiquitination, we next constructed a network for
206 the ubiquitinated proteins identified in this work and their interactants identified in previous yeast-
207 two hybrid (Braun et al., 2011) and Split-Ub global approaches (Chen et al., 2012; Jones et al., 2014).
208 This final network consisted of 1,011 proteins (Table S7a, Figure 5). Transport and trafficking
209 functions were enriched in such interactome and, interestingly, the most enriched process concerns
210 ubiquitination, with the GO term “protein K63 linked ubiquitination” showing a 37-fold enrichment
211 (Figure S6). Five E2s (UBC26, UBC32, UBC34, UBC35, UEVD1) and one E3 (UPL6) were identified in
212 this network (Table S7a). We investigated the extent of interaction of these enzymes with the
213 ubiquitinated proteins identified in the present work, and identified two E2s, UBC32 and UBC34,
214 which putatively interact with 15 and 31 ubiquitinated transmembrane proteins involved in water
215 and ion transport, respectively (Figure 5; Table S7b-c).

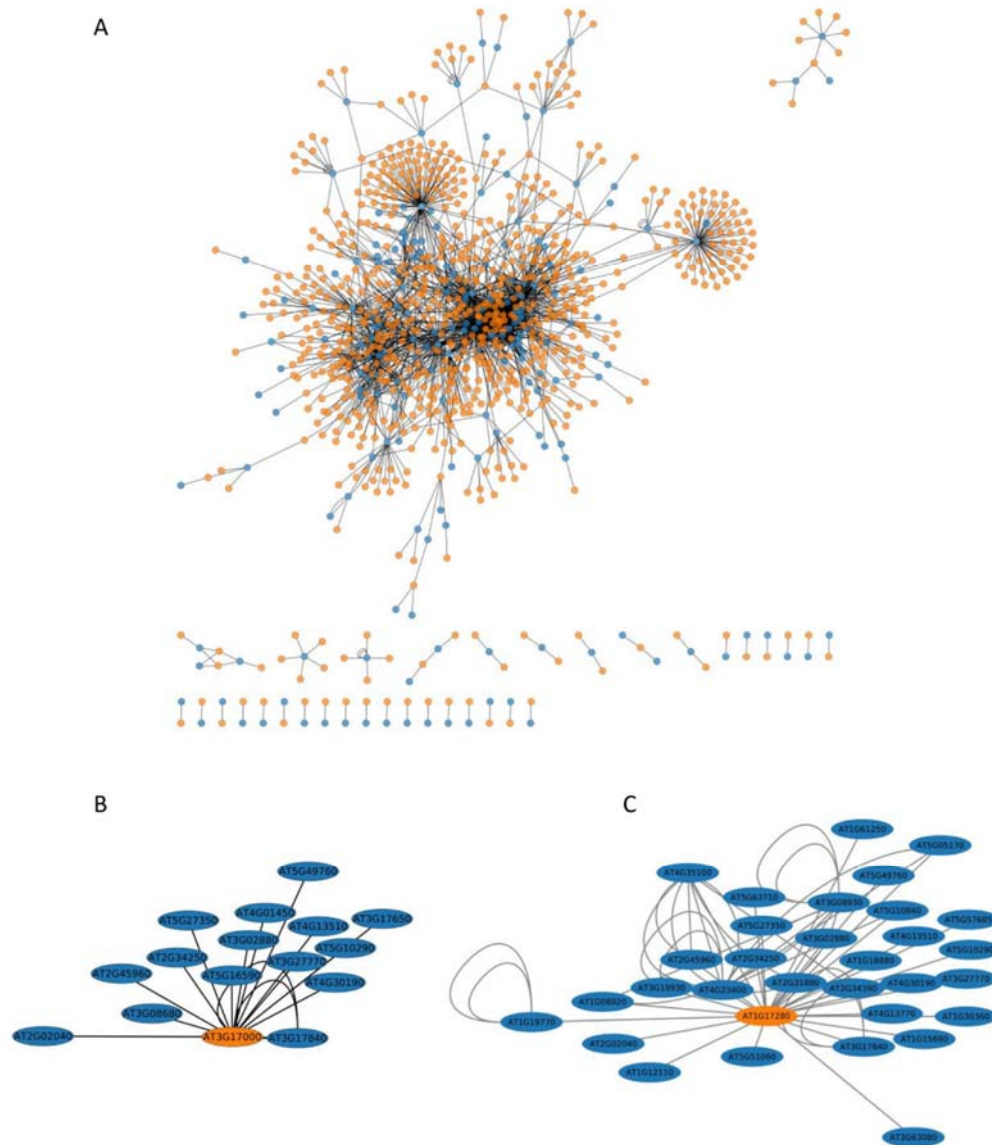


Figure 5. The interaction network of ubiquitinated proteins.

Interactants from a Y2H approach (Braun et al., 2011) and Split-Ub approach (Chen et al., 2012; Jones et al., 2014) were considered, and the network was visualized by Cytoscape (version 3.7.2). A. The network includes ubiquitinated proteins (blue) together with their reported interactants (orange) (Table S7a). B. The UBC32 subnetwork (see Table S7b). C. The UBC34 subnetwork (see Table S7c).

216 We then used a highly similar approach that focused specifically on DAUPs and their interactants,
217 which made it possible to build another network of 326 proteins enriched in transporters and
218 components of endocytic trafficking (Table S7d, Figure S7-S8). UBC32 and UBC34 appeared again as
219 E2s targeting 9 proteins whose ubiquitination changed with mannitol treatment (Figure S7, Table 1).
220 These proteins include NRT1;1-PTR8.3, NRT1-PTR6.3, two LRR-Receptor like kinases, a nodulin MtN21

221 like transporter, AHA2, the secretory carrier-associated membrane protein SCAMP3, the peroxidase
222 GPX5, and an unknown protein.

223

224 **The osmotic phenotype of *ubc32*, *ubc33* and *ubc34* mutants**

225 Root responses to osmotic stress involve high plasticity in root growth and architecture, which is
226 partly determined by primary root (PR) growth. To reinforce the roles of UBC32 and UBC34 in the
227 adaptive root response to osmotic stress, we studied the PR growth phenotype of corresponding
228 mutants in control conditions and upon osmotic treatment. Since *AtUBC32* and *AtUBC34* belong to a
229 small gene subfamily including *UBC33* (Ahn et al., 2018), we also studied the *ubc33* mutant and a
230 triple knockout mutant line, *ubc32xubc33xubc34*, due to the putative redundancy between the 3
231 genes. Five-day-old plants were transferred to either control MS medium or MS medium
232 supplemented with 0.2 M mannitol, and PR length was monitored for up to 5 days after transfer
233 (Figure 6, Figure S9, Table S8). The root growth in control WT plants (Col) was inhibited by 14% one
234 day after transfer, and by up to 43%, 5 days later (Figure 6). The *ubc32* and *ubc34* single mutants and
235 the triple mutant showed a significantly higher PR growth inhibition ranging from 25 % at day 1 after
236 transfer up to 50 % at day 5 after transfer (Figure 6). Thus, suppression of *AtUBC32* and *AtUBC34*
237 favored PR growth inhibition under mannitol treatment, suggesting that these genes contribute to PR
238 root growth under osmotic stress. This result is observable after only one day of treatment,
239 suggesting that these genes play an early role in the response to osmotic stress.

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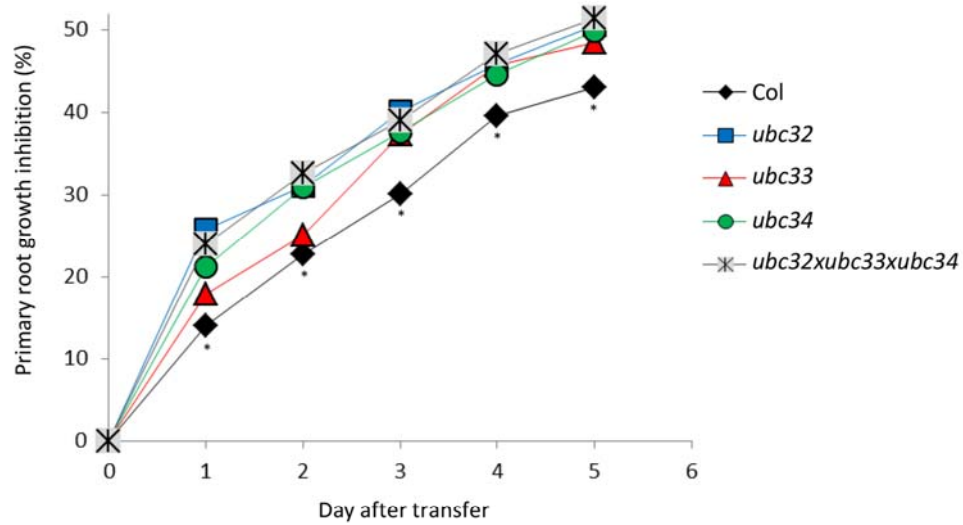


Figure 6. Inhibition of primary root length by mannitol in *Arabidopsis thaliana* WT plants (Col) and *ubc32*, *ubc33*, *ubc34* and *ubc32xubc33xubc34* mutants.

15 plants per condition were grown for 5 days in MS medium and then transferred to MS medium and MS medium supplemented with 200 mM mannitol. Primary root length was monitored up to 5 days after transfer (Figure S9). Asterisks means that the WT is significantly different from at least one mutant in a one-way ANOVA test combined with a Tukey test (p-value between 0.01 and 0.001 (Table S8)).

246 Discussion

247 A resource of ubiquitinated membrane proteins

248 Our study included 450 ubiquitinated proteins, significantly increasing the database size of
249 ubiquitinated membrane protein substrates in plants. In comparison to other large-scale *Arabidopsis*
250 ubiquitinomes (Maor et al., 2007; Manzano et al., 2008; Kim et al., 2013; Svozil et al., 2014; Walton
251 et al., 2016; Zhang et al., 2019; Romero-Barrios et al., 2020; Grubb et al., 2021), 207 novel proteins,
252 90% of which are transmembrane proteins, were identified as ubiquitinated (Table S9). We also
253 observed that 20% of Ubi-peptides can be additionally modified by oxidation, phosphorylation and
254 acetylation (Table 1, Table S4), adding complexity to cellular signaling. The major proton pump AHA2
255 harbors 21 K-Ub sites (Tables S3), four of them showing quantitative variations upon mannitol
256 treatment (Table 1). K888 in particular is ubiquitinated, and this site is located close to T881, whose
257 phosphorylation leads to pump activation (Fuglsang et al., 2014). Moreover, the doubly modified
258 peptide (*i.e.* by phosphorylation and ubiquitination) was accumulated upon mannitol treatment.

259 Recent studies have highlighted the importance of crosstalk between phosphorylation and
260 ubiquitination in several plant signaling pathways (Vu et al., 2018), and the presence of such a K-Ub
261 near a critical phospho-residue questions the role of this ubiquitination in the modulation of ATPase
262 function.

263 Even though no ubiquitin motif could be described, the K-Ub residue appeared to be preferentially
264 surrounded by an acidic residue (Figure 3). Similar observations can be obtained from ubiquitinome
265 studies in petunia flower and rice embryo (Guo et al., 2017; He et al., 2020). By contrast, previous
266 studies in rice leaf, wheat seedling and maize leaf have reported that alanine is enriched around K-Ub
267 (Xie et al., 2015; Zhang et al., 2017; Wang et al., 2019). Thus, although no Ub motif could be
268 identified, the presence of an acidic residue appears to be a common feature between 3 different
269 tissues (root, flower, embryo), whereas the close presence of an alanine residue appears to be more
270 specific for ubiquitinated residues in leaf proteins.

271 Our ubiquitinome study revealed that one-third of ubiquitinated proteins (151 proteins) harbor more
272 than one ubiquitination site. In two recent studies, a total of 422 proteins were identified as carrying
273 a K63-Ub linkage (Johnson and Vert, 2016; Romero-Barríos et al., 2020). Seventy-four of these
274 proteins were identified in the present study, 29 of which were identified as carrying a unique K-Ub
275 site that can be preferentially accounted for a K63-Ub linkage (Table S10). However, 44 proteins were
276 also described in the present study as harboring at least 2 K-Ub sites (Table S10). This suggests that
277 either all K-Ub residues are involved in K63-Ub linkage, or that different types of Ub linkages coexist
278 within the same protein, including K63-Ub. This type of coexistence has already been shown in
279 several studies; notably, the traffic and degradation of a particular receptor tyrosine kinase is likely to
280 be regulated by different K-48 and K-63 poly-Ub editing mechanisms (Marx et al., 2010).
281 Furthermore, the human nuclear protein Twist regulates a variety of cellular functions controlled by
282 gene transcription events and can be ubiquitinated through K48-Ub linkage, which induces
283 proteasome-dependent proteolysis and K63-Ub linkage for localization to the nucleus (Lee et al.,
284 2016). Finally, in plants, the ubiquitination pattern of oleosin includes K48-Ub linkage that induces
285 the proteasomal protein degradation, as well K63-Ub linkage which role remains unknown
286 (Deruyffelaere et al., 2015). Thus, the specific fate of proteins can be dictated by specific coexisting
287 poly-Ub linkages.

288 In addition to transporters and channels, we reported that vesicle transport-related proteins
289 including clathrin assembly proteins, the AP-2 complex subunit, v-SNARE proteins, vesicle associated
290 proteins and syntaxins were overrepresented in the ubiquitinome (Table S3). Endocytosis and
291 endosomal trafficking are essential processes in cells for controlling the dynamics and turnover of
292 plasma membrane proteins (Valencia et al., 2016). The recruitment of cargo into endocytic vesicles
293 (e.g. clathrin-coated pits) involves the 'endosomal sorting complex required for transport' (ESCRT)

294 multi-subunit complex, and requires adaptor proteins to eventually associate with clathrin (Valencia
295 et al., 2016). The vesicles fuse with the acceptor organelle in a process mediated by factors such as
296 SNAREs and small GTPases (Valencia et al., 2016). Here, we unexpectedly observed a concomitant
297 ubiquitination of cargos and proteins from the endocytic machinery (Table S3). While a role for the
298 ubiquitination of cargos in their endocytosis has recently emerged in plants (Romero-Barrios and
299 Vert, 2018), the ubiquitination of proteins involved in endocytosis is rarely documented. In animals,
300 several proteins involved in EGFR endocytosis were shown to be ubiquitinated after EGF stimulation
301 (Haglund et al., 2002). In yeast, ubiquitination was recently shown to function as a recycling signal for
302 sorting a SNARE into COPI vesicles in a non-degradative pathway (Xu et al., 2017). Therefore, our
303 results suggest a role for ubiquitination in regulating the function of proteins involved in endocytic
304 trafficking, highlighting a broad role for ubiquitin in internalizing and sorting cargo proteins.

305

306 **The role of ubiquitination in response to short-term osmotic treatment**

307 Because ubiquitination can induce protein degradation, we looked for an inverse relationship
308 between the abundance of proteins and their ubiquitinated form that could indicate a role for
309 ubiquitination in protein degradation. Caution must be taken with this assumption, since it is not
310 until the ubiquitin chain is assembled that it may act as a degradation signal (Clague et al., 2015). If a
311 large proportion of ubiquitin is likely to be attached as mono-ubiquitin, this might skew the inverse
312 relationship between the abundance of a protein and its ubiquitinated form. Although we may have
313 overestimated the number of concerned proteins, only 10% of them (n=6) exhibited this inverse
314 relationship, whereas 90% showed ubiquitination changes without any change in protein abundance
315 (Table 1). Thus, for a majority of these membrane proteins, ubiquitination is not involved in protein
316 degradation in response to short-term osmotic treatment. In particular, PIP2;1 abundance was
317 unchanged upon short-term mannitol treatment, while its ubiquitination increased at K3 and K276
318 (Table 1). This osmotic treatment was shown to induce maximal L_p inhibition by 60%, which can be
319 accounted for by a decrease in aquaporin function (Di Pietro et al., 2013) and not PIP degradation,
320 since the abundance of all PIPs was unchanged (Table S2). Thus, even though ubiquitination at K3
321 and K276 can be involved in PIP2;1 degradation (Figure 5, (Chen et al., 2021)), we assume that
322 ubiquitination induces different consequences upon short-term mannitol treatment. Indeed, short-
323 term osmotic treatment induces PIP2;1 selective endocytosis (Martiniere et al., 2019). In addition,
324 PIP2;1 is ubiquitinated by K63-Ub linkage (Johnson and Vert, 2016), a poly-Ub linkage that plays a
325 general function in the sorting of endocytosed cargos by the endosomal sorting complex required for
326 transport (Romero-Barrios and Vert, 2018). Thus, we hypothesize that the increase in PIP2;1
327 ubiquitination induced by short-term treatment should participate in internalizing PIP2;1 and not in
328 degradation of the protein. This result contrasts with the PIP2;1 degradation observed under long-

329 term drought treatment, due to the simultaneous activity of UBC32 and the E3 ligase Rma1 in
330 ubiquitinating PIP2;1 at K276 (Chen et al., 2021). The pairing of E3s with different E2s is dynamic and
331 changes in response to external stimuli (Turek et al., 2018). Thus, under short-term osmotic stress, a
332 specific E2/E3 combination that differs from UBC32/Rma1 could regulate PIP2;1 internalization.
333 However, this hypothesis will require additional experiments.

334

335 **UBC32 and UBC34 contribute to primary root growth under osmotic stress**

336 E2s have recently emerged as key mediators of chain assembly, in particular by dictating the K
337 residue within ubiquitin used to link the moieties in a chain (Turek et al., 2018). Our protein-protein
338 network analysis identified UBC32 and UBC34 as E2s that putatively interact with proteins whose
339 ubiquitination changed upon short mannitol treatment (Table 1, Figure S7). Recent expression
340 studies have suggested that E2s participate in abiotic stress responses (Zhou et al., 2010; Zhiguo et
341 al., 2015; Sharma and Bhatt, 2018). Surprisingly, the role of *UBC32* appears contradictory, since it has
342 been described as playing both negative and positive roles in response to long-term drought in (Ahn
343 et al., 2018) and (Chen et al., 2021), respectively. In particular, the survival rate of 3-week-old *ubc32*
344 plants after 12-20 days of withholding water has been reported to be both higher and lower than WT
345 plants in (Ahn et al., 2018) and (Chen et al., 2021), respectively. Our study reveals that, in the context
346 of short-term osmotic treatment, UBC32 and UBC34 positively regulate PR growth, thus playing a
347 positive role in osmotic stress tolerance. *UBC32*, *UBC33* and *UBC34* are all reported to participate in
348 the endoplasmic reticulum-associated degradation (ERAD) pathway in *Arabidopsis*, which is a major
349 degradation system involved in removing misfolded or unfolded proteins retained in the ER (Cui et
350 al., 2012; Chen et al., 2016; Chen et al., 2017; Zheng et al., 2019; Chen et al., 2021; Zhang et al.,
351 2021). The involvement of ERAD components suggests that short-term osmotic stress may also result
352 in ER/protein stress, which engages ERAD to control the secretion of plasma membrane proteins.
353 However, upon 1-h mannitol treatment, proteins that putatively interact with UBC32 and UBC34 and
354 show increased ubiquitination did not display any decreased cellular abundance (Table 1). We thus
355 speculate that UBC32 and UBC34 are not simply ERAD components, but that they also participate in
356 the ubiquitination process in other subcellular organelles such as the plasma membrane in response
357 to a short-term osmotic stress. In particular, an internalization of the aquaporin PIP2;1 (Chen et al.,
358 2021) was observed upon a short-term osmotic stress (Martiniere et al., 2019). We thus speculate
359 that, upon a short-term osmotic stress, a rapid coordinated internalization of aquaporins and of ions
360 transporters involved in plant mineral nutrition could contribute to maintain a minimal root growth.
361 Conversely, absence of internalization would impair root development. Soil is extremely
362 heterogeneous and root growth maintenance under unfavorable local environment could allow the

363 root tip to reach a more favorable environment and then to maintain on a longer term root foraging
364 and plant survival.

365

366

367 **Conclusion**

368 Our data present the ubiquitinome of root membrane proteins and its variation under osmotic
369 stress. Importantly, the results highlight specific post-translational modification patterns and suggest
370 approaches for exploring the physiological role of lysine ubiquitination in plants under osmotic stress.

371 Our results open new perspectives in the involvement of ubiquitination and trafficking of root plasma
372 membrane transporters in response to changes in local environment and future studies exploring the
373 function of these ubiquitination in PIP2;1 and ion transporters will be necessary to delineate its role
374 in the root nutrient foraging.

375

376 **Material and methods**

377 **Plant materials and growth conditions**

378 *Arabidopsis thaliana* ecotype Columbia (Col-8) was used as the control wild type (WT) plant. The
379 *atubc32* (SALK_092817), *atubc33* (*GABI_105_D10*), *atubc34* (SAIL_1249_C08) single T-DNA insertion
380 mutant alleles and the triple mutant *atubc32xatubc33xatubc34* were obtained from (Ahn et al.,
381 2018). Homozygosity of these mutants was verified using primers described in (Ahn et al., 2018). For
382 proteome and ubiquitinome studies, WT seeds were surface-sterilized and sown in 0.2-mL tubes
383 containing 0.8% agar prepared in pH 5.5 Hoagland-based solution (0.5 mM KH₂PO₄, 1.25 mM KNO₃,
384 0.75 mM MgSO₄, 1.5 mM CaNO₃, 50 μM H₃BO₃, 0.7 μM CuSO₄, 1 μM ZnSO₄, 12 μM MnSO₄, 0.24 μM
385 Na₂MoO₄, 50 μM Fe³⁺-EDTA). After 7 days in the growth chamber, the bottoms of the tubes were cut
386 off prior to transfer in 2.5-L opaque recipients in the same medium. Plants were grown for 8 weeks
387 under short-day conditions (8 h/16 h day/night; 23°C/20°C day/night) at a light intensity of 160
388 μmol.m⁻².s⁻¹ and 65% humidity. Plants were treated with 0.2 M mannitol for 1 h. Roots were
389 harvested and stored at -80°C until analysis.

390

391 **Microsome extraction**

392 Roots were crushed with a PULVERISETTE 2 Mortar Grinder (Fritsch) in liquid nitrogen and
393 microsomal proteins were extracted according to (Di Pietro et al., 2013), except that the grinding
394 buffer contained 10 mM N-ethylmaleimide and that the pellets were resuspended with a potter in
395 200 μL of Laemmli 1X buffer (65 mM Tris-HCl, pH 7.5, 5% glycerol, 2% SDS) (Laemmli, 1970). Proteins
396 were quantified using a detergent compatible with the Bradford assay kit (Thermo Scientific).

397

398 **Protein digestion**

399 For proteome analysis, three independent biological replicates from the control condition and
400 mannitol-treated plants were used. 10 μg of microsomes were fractionated using 10% precast SDS-
401 PAGE gel electrophoresis (Bio-Rad). After staining with Coomassie blue (R250, Bio-Rad), the gel was
402 rinsed with acetic acid/methanol (Destain, Bio-Rad). Each lane was cut into 4 bands. For the
403 ubiquitinome study, 2 independent biological replicates from the control condition and mannitol-
404 treated plants were used, and microsomal fractions (1.2 mg) were subjected to an in-tube acrylamide
405 inclusion (13% acrylamide/bis-acrylamide, 0.6% ammonium persulfate, 2.5% TEMED) adapted from
406 (Balliau et al., 2018). For proteome and ubiquitinome analyses, gel slices were treated according to
407 (Chen et al., 2019), with the exception that proteins were alkylated with 50 mM chloroacetamide for
408 ubiquitinome experiments. Proteins were digested with trypsin (Sequencing Grade Modified Trypsin,
409 Promega, Madison, WI, USA) at a 1:50 (trypsin:protein) ratio at 37°C overnight. Peptides were
410 extracted according to (Chen et al., 2019).

411

412 **Enrichment of ubiquitinated peptides**

413 Tryptic peptides were filtered through a C18 cartridge (Sep-Pack Classic, Waters) equilibrated with
414 0.1% TFA. After loading on the column, the peptides were washed twice with 0.1% TFA and then with
415 0.1% TFA and 5% ACN. Peptides were eluted with 0.1% TFA and 40% ACN, pooled, frozen overnight
416 at -80°C, and finally evaporated. Immunoprecipitation experiments were performed with 15 µL of
417 Pan anti-glycine lysine antibody conjugated to agarose beads (PTM Biolabs, Chicago, IL, USA)
418 according to the manufacturer's instructions. Briefly, tryptic peptides were dissolved in 300 µL of
419 WASH I (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, 0.25% n-Dodecyl β-D-maltoside, pH 8.0),
420 incubated 4 h at room temperature on a rotary shaker, and then sequentially washed 3 times with
421 WASH I, 3 times with WASH II (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0), and 3 times with
422 HPLC-grade water. The elution was performed 3 times with 100 µL of 0.1% TFA. For the LC-MS/MS
423 experiment, 300 µL of pooled eluates were dried under vacuum centrifuge and resuspended in 2%
424 FA.

425

426 **LC-MS/MS analysis**

427 The LC-MS/MS experiments were performed using a NCS 3500RS-ProFlow nano system (Thermo
428 Fisher Scientific Inc., Waltham, MA, USA) interfaced online with a nano easy ion source and a Q-
429 Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). The
430 samples were analyzed in a data-dependent acquisition mode. For total proteome and ubiquitinome
431 experiments, 2 µL and 6 µL of peptides were injected, respectively. Peptides were first loaded onto a
432 pre-column (Thermo Scientific PepMap 100 C18, 5 µm particle size, 100 Å pore size, 300 µm i.d. x 5
433 mm length) from the Ultimate 3000 autosampler with 0.05% TFA in water at a flow rate of 10
434 µL/min. The peptides were separated by reverse-phase column (Thermo Scientific PepMap C18, 3 µm
435 particle size, 100 Å pore size, 75 µm i.d. x 50 cm length) at a flow rate of 300 nL/min. After a 3-min
436 loading period, the column valve was switched to allow elution of peptides from the pre-column onto
437 the analytical column. The loading buffer (solvent A) consisted of 0.1% FA in water, and the elution
438 buffer (solvent B) was 0.1% FA in 80% ACN. The employed 3-step gradient consisted of 4-25% of
439 solvent B until 50 min for ubiquitinome (103 min for total proteome), then 25-40% of solvent B from
440 50 to 60 min for ubiquitinome (from 103 to 123 min for total proteome), and finally 40-90% of
441 solvent B from 60 to 62 min (123 to 125 min for total proteome). The total run time was 90 min for
442 ubiquitinome (150 min for total proteome), including a high organic wash step and a re-equilibration
443 step. Peptides were transferred to the gaseous phase with positive ion electrospray ionization at 1.8
444 kV. In the data-dependent acquisition procedure, the top 10 precursors were acquired between 375
445 and 1,500 m/z with a 2 Th (Thomson) selection window, a dynamic exclusion of 40 s, a normalized

446 collision energy of 27, and resolutions of 70,000 for MS and 17,500 for MS2. Spectra were recorded
447 with Xcalibur software (4.3.31.9) (Thermo Fisher Scientific). The mass spectrometry proteomics data
448 were deposited at the ProteomeXchange Consortium *via* the PRIDE partner repository with the
449 dataset identifier PXD022249. The reviewer account details are: Username:
450 reviewer_pxd022249@ebi.ac.uk, Password: B1h58X9Q.

451

452 **Identification and quantification of whole proteome and ubiquitinome**

453 For the proteome and the ubiquitinome, the resulting MS/MS data were processed using MaxQuant
454 with an integrated Andromeda search engine (version 1.6.6.0). Tandem mass spectra were searched
455 against the TAIR10 database (35,417 entries). The minimal peptide length was set to 6. The criteria
456 “Trypsin/P” was chosen as the digestion enzyme. Carbamidomethylation of cysteine was selected as
457 fixed modification and methionine oxidation, N-terminal acetylation and phosphorylation (S/T/Y)
458 were systematically selected as variable modifications. Up to 4 missed cleavages were systematically
459 allowed.

460 For proteome analysis, the mass tolerance of the precursor was 20 and 4.5 ppm for the first and
461 main searches, respectively, and was 20 ppm for the fragment ions. The function “match between
462 run” was used. Proteins were identified provided that they contained one unique trypsin peptide.
463 The rates of false peptide sequence assignment and false protein identification were fixed to be
464 lower than 1%. Quantification was performed with at least 2 peptides per protein, one of them
465 unique to the protein. To investigate differentially expressed proteins, Student’s t-test was
466 performed using protein Label-Free Quantification (LFQ) intensity values when present in at least 2
467 replicates and in at least 2 biological replicates per condition.

468 For ubiquitinome analysis, “GlyGly” on K residue was additionally specified as a variable modification.
469 The function “match between run” was not applied. The minimum score for peptides was set to 40.
470 The intensity of each peptide from the “evidence” table was normalized to the sum of all peptide
471 intensities in each sample, and a t-test was performed to investigate differentially-expressed
472 peptides. The ubiquitinated peptides with consistent fold changes in two replicates were counted,
473 and the significance of the abundance change among samples was evaluated as differentially
474 expressed by a Student’s t-test. A p-value < 0.05 was considered statistically significant. The
475 appearance/disappearance of peptides was considered on condition of their presence in two
476 biological replicates and the corresponding absence from the two other biological replicates. We
477 defined “absence” as no razor or unique peptide in any biological condition replicate, and
478 “presence” as the identification of at least one unique peptide in all replicates of a biological
479 condition.

480

481 **Bioinformatic analyses**

482 Gene Ontology (GO) term association and enrichment analyses were performed using Panther
483 (<http://www.pantherdb.org/>) (Mi et al., 2013). Fold enrichments were calculated based on the
484 frequency of proteins annotated to the term compared with their frequency in the *Arabidopsis*
485 proteome. The p-value combined with the false discovery rate (FDR) correction was used as criteria
486 of significant enrichment for GO catalogs, whereas a p-value < 0.05 was considered to be enriched
487 for GO terms. The most specific subclasses were considered. The GO annotation was classified based
488 on the “biological processes”, “molecular functions” and “cellular components” categories. GO terms
489 were reduced with rrvgo (<https://ssayols.github.io/rrvgo/>). The number of transmembrane domains
490 was estimated with Aramemnon (<http://aramemnon.botanik.uni-koeln.de/>). The p-logo software
491 (O'Shea et al., 2013) (<https://plogo.uconn.edu/>) was used to analyze the models of the sequences
492 with amino acids in specific positions of ubiquitin-21-mers (10 amino acids upstream and
493 downstream of the K-Ub site) in all of the protein sequences. In addition, the *Arabidopsis* proteome
494 was used as the background database, and the other parameters were set to default values. Protein-
495 protein interaction data were obtained from plant interactome databases, including results from a
496 yeast two-hybrid approach (Braun et al., 2011) and from Split-ubiquitin approaches (Chen et al.,
497 2012; Jones et al., 2014), in order to build a network including these ubiquitinated proteins together
498 with their reported interactants. Protein-protein interaction networks were visualized using
499 Cytoscape version 3.7.2 (Shannon et al., 2003).

500

501 **Ectopic expression of PIP2;1 in suspension cells**

502 Mutated PIP2;1 cDNAs were constructed according to (Santoni et al., 2006). Biolistic transformation
503 of 5-day-old suspension cells was performed as described in (Santoni et al., 2006), and transformed
504 cells were selected on 50 mg/L of hygromycin. Briefly, independent transformed cells were isolated
505 and further cultured on 40 mg/L of hygromycin. Stable insertion of the T-DNA was checked by PCR,
506 and the expression of PIP was detected by western blot as described in (Santoni et al., 2006).
507 Extraction of total proteins from suspension cells and ELISA measurements of PIP2 abundance were
508 performed as described in (Santoni et al., 2006).

509

510 **Root architecture analyses**

511 Plants were stratified for 2 days at 4°C and grown vertically on agar plates containing half-strength
512 Murashige and Skoog (MS) medium supplemented with 1% (w/v) sucrose and 2.5 mM MES-KOH pH
513 6, in a self-contained imaging unit equipped with a 16M pixel linear camera, a telecentric objective
514 and collimated LED backlight. Plants were grown in the imaging automat dedicated growth chamber

515 at 23°C in a 16-h light/8-h dark cycle with 70% relative humidity and a light intensity of 185 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$
516 (Vegeled Floodlight, Colasse Seraing, Belgium). Plates were imaged every day for 5 days.
517
518

519 **Table 1.** Variations in the ubiquitinated peptide and the corresponding protein in response to
520 mannitol treatment

AGI	Description	Ubi-peptide sequence	K-Ub	Ubi-peptide ratio	Protein ratio	UBC 32	UBC 34
proteins with decreased ubiquitination							
AT1G01580.1	FRO2	IEAFITRDNDAGDEA <u>K</u> AGK	528	DISP	INV		
AT1G08090.1	NRT2;1	ATLE <u>K</u> AGEVAKDKFGK	259	DISP	1.92 ($p = 0.03$)		
AT1G13480.1	Protein of unknown function	LDSELTSLG <u>K</u> SIEIGK	211	DISP	INV		
AT1G32450.1	NRT1;5	S _{ac} CLEIYN <u>K</u> DTM _{ox} <u>K</u> K	9 or 13 or 14	DISP	INV		
AT1G48210.2	Protein kinase superfamily protein	LSEDKV <u>K</u> QCV DAR	300	DISP	1.22 ($p = 0.03$)		
AT1G58030.1	CAT2	DGLLPFI SFDIN <u>K</u> R	369	DISP	INV		
AT2G17440.1	PIRL5, ras group-related	DITE <u>K</u> GAQAVVQYMNDLVEAR	484	DISP	1.51 ($p = 0.01$)		
AT2G23200.1	receptor-like protein kinase	S <u>K</u> GTIDEILDPSLIGQIETNSLKK	710	DISP	INV		
AT2G31610.1	40S ribosomal protein S3-1	TQNVLG <u>E</u> KGRR	62	0.33 ($p = 0.01$)	INV		
AT3G01290.1	HIR2	AEGEAES <u>K</u> YLSGLGIAR	196	DISP	1.23 ($p = 0.02$)		
AT3G04840.1	40S ribosomal protein S3a-1	NVG <u>K</u> TLVSR	45	DISP	INV		
AT3G17410.1	CARK1	LSEDKV <u>K</u> QCV DAR	301	DISP	1.27 ($p = 0.03$)		
AT3G47210.1	Protein of unknown function	<u>K</u> YIISYINEQVELDSR	62	DISP	1.28 ($p = 0.03$)		
AT3G51550.1	Feronia	VLGVGGFG <u>K</u> VYR	549	DISP	INV		
AT3G53480.1	ABC transporter ABCG37, PDR9	STLLDDGDESM _{ox} TE <u>K</u> GR	88	DISP	INV		
AT3G63080.1	glutathione peroxidase	DSS <u>G</u> KEVDLSVYQGK	25	DISP	INV		yes
AT4G08620.1	SULTR1;1	DF <u>G</u> GQTPAK	55	DISP	INV		
AT4G33360.1	Farnesol deshydrogenase	NVLEAV <u>K</u> ETKTVQ <u>K</u>	112 or 115 or 119	DISP	INV		
AT4G37060.1	PATATIN-like protein 5	IDDDTLEGDASTLDLST <u>K</u> SNLENLIK	340	DISP	INV		
AT5G14040.1	Mitochondrial phosphate carrier	FI <u>K</u> SEGYGGLYK	222	DISP	INV		
AT5G56010.1	HSP 90-3	APFDLFD <u>T</u> KK	326 or 327	DISP	INV		
AT5G65380.1	MATE efflux family protein	VANELGAGNG <u>K</u> GAR	334	DISP	INV		
proteins with increased ubiquitination							
AT1G01580.1	FRO2	DNDAGDEA <u>K</u> AG <u>K</u> IK	528 and 531	APP	INV		
AT1G02520.1	ABC transporter ABCB11, PGP11	<u>K</u> QCEGPIKDGIK	919	APP	INV		
AT1G08930.2	ERD6	DTIDM _{ox} TENGGET <u>K</u> MSELFQR	281	APP	INV		
AT1G08930.2	ERD6	DTIDM _{ox} TENGGET <u>K</u> M _{ox} SELFQR	281	APP	INV		
AT1G11680.1	Sterol 14-demethylase	SG <u>K</u> TENDM _{ox} LQCFIESK	253	APP	INV		
AT1G12110.1	NRT1, NPF6.3	<u>K</u> LELPADPSYLYDVDDIIAAEGS _{ph} M _{ox} <u>K</u> G <u>K</u>	267 or 291 or 293	APP	INV		yes
AT1G44170.3	ALDH3H1	LS <u>K</u> LLDEK	242	APP	INV		
AT1G55450.1	methyltransferase	A _{ac} ALSD <u>K</u> LADAYQNR	6	2.3 ($p = 0.01$)	INV		
AT1G59870.1	ABC transporter ABCB11, PDR8	EVDVT <u>K</u> LGDGEDRQK	94	APP	INV		
AT1G61250.2	Secretory carrier-associated membrane protein	ELQA <u>K</u> EAEK	71	APP	INV		yes
AT1G61670.1	Two-component response regulator	NELLFLPDDVEEG <u>K</u> RE	511	APP	INV		
AT2G02040.1	NRT1, NPF 8.3	AAVISEEES <u>K</u> SGDYSNSWR	325	APP	INV	yes	yes
AT2G24720.1	GLR2.2, glutamate receptor 2.2	DLW <u>K</u> EFLK	864	APP	INV		
AT2G32270.1	Zinc transporter 3	VSDGET _{ph} GESSVDSE <u>K</u> VQILR	177	APP	INV		
AT2G38360.1	PRA1.B4, prenylated RAB acceptor 1.B4	SALS <u>K</u> PESISDAAVR	68	1.72 ($p = 0.02$)	INV		
AT2G47000.1	ABC transporter ABCB4, PGP4	A _{ac} SESGLNGDPNILEEVSET <u>K</u> R	21	APP	INV		

AT3G04840.1	40S ribosomal protein S3a-1	IASEGL <u>K</u> HR	62	APP	INV		
AT3G08680.2	Probable inactive receptor kinase	AYYFS <u>K</u> DE <u>K</u>	407 or 410	APP	INV	yes	
AT3G27770.2	HUP53, HYPOXIA RESPONSE PROTEIN 53	SPLIDGDNM _{ox} VSFE <u>K</u> R	125	APP	INV	yes	yes
AT3G29310.1	BAG1	FVQYVDDCV <u>V</u> KR	230	APP	INV		
AT3G45710.1	NRT1, NPF2.5	DEDYHQYGLGKEAK	272	APP	INV		
AT3G51550.1	Feronia	AAT <u>K</u> NFDESR	534	APP	INV		
AT3G53420.2	PIP2;1	ASGS <u>K</u> SLGS _{ph} FR	276	1.79 (p = 0.01)	INV	yes*	
AT3G53420.2	PIP2;1	A _{ox} <u>K</u> DVEAVPGEGFQTR	3	1.69 (p = 0.015)	INV	yes*	
AT3G60330.2	AHA7	TQHGLTGT <u>G</u> KPVYER	903	APP	1.26 (p = 0.01)		
AT3G62250.1	40S ribosomal protein S27a-3	MQIFVKT <u>L</u> TG <u>K</u> TITLEVESSDTIDNVK	11	APP	INV		
AT4G01440.1	nodulin MtN21 EamA-like	FNEDDQEEDDDEQY <u>K</u> K	354 or 355	APP	INV		
AT4G09000.1	GRF1	AVD <u>K</u> DELTV _{ox} EEER	42	APP	INV		
AT4G25090.1	RBOHG	<u>K</u> ELSDM _{ox} LTESLKPTR	267	APP	INV		
AT4G30190.1	AHA2	AWLNLFEN <u>K</u>	857	APP	1.44 (p = 0.01)	yes	yes
AT4G30190.1	AHA2	WSEQEAAILVPGDIVSI <u>K</u>	157	APP	1.44 (p = 0.01)	yes	yes
AT4G30190.1	AHA2	T _{ph} LHGLQP <u>K</u> EAVNIFPEK	888	APP	1.44 (p = 0.01)	yes	yes
AT4G30190.1	AHA2	S _{ac} SLEDI <u>K</u> NETVDLEK	8	3.00 (p = 0.02)	1.44 (p = 0.01)	yes	yes
AT5G25930.1	LRR protein kinase family protein	LLVYEY <u>L</u> E <u>K</u> R	767	APP	INV		
AT5G35200.1	putative clathrin assembly protein	EAPLAAGV <u>K</u> K	310	APP	INV		
AT5G39510.1	v-SNARE 11	<u>K</u> ILTD _{ox} TR	184	APP	INV		
AT5G47910.1	RBOHD	N <u>K</u> LNLPN <u>F</u> LK	541	APP	INV		
AT5G59970.1	Histone superfamily protein	DNIQGIT <u>K</u> PAIR	32	1.57 (p = 0.02)	INV		
AT5G62300.2	40S ribosomal protein S20-1	A _{ox} TAYQPM <u>K</u> PG <u>K</u> AGLEEPLEQIHK	9 and 12	APP	INV		
AT5G62390.1	BAG7	AIAAAEAE <u>K</u> K	195	APP	INV		
AT5G62390.1	BAG7	LEPEYPL <u>K</u> YLCDR	90	3.02 (p = 0.03)	INV		
AT5G62390.1	BAG7	RLEPEYPL <u>K</u> YLCDR	90	2.09 (p = 0.01)	INV		

521 The table describes proteins for which protein and ubiquitin peptide quantification data are available

522 Columns 1 and 2: AGI and protein name.

523 Column 3: the ubiquitinated residue is underlined. ox: oxidation; ac: acetylation; ph: phosphorylation.

525 Column 4: K-Ub position in the protein.

526 Column 5: the quantitative ratio of Ubi-peptide between mannitol and control experiments, with the associated p-value. APP: appearance; DISP: disappearance.

528 Column 6: the protein quantitative ratio between mannitol and control experiments, with the associated p-value. INV: invariant protein.

530 Columns 7 and 8: protein interaction with UBC32 and UBC34 (Braun et al., 2011; Chen et al., 2012, Jones et al., 2014). *: Chen et al. 2021

532

533

534

535 **Supplemental material**

- 536 Supplemental Figure S1. Characterization of the microsomal fraction.
- 537 Supplemental Figure S2. Functional enrichment analysis of differentially accumulated proteins (DAPs)
538 in response to mannitol.
- 539 Supplemental Figure S3. Types of Ub linkages.
- 540 Supplemental Figure S4. Functional enrichment analysis of differentially accumulated ubiquitinated
541 proteins (DAUPs) in response to mannitol.
- 542 Supplemental Figure S5: K-Ub residues in PIP aquaporins.
- 543 Supplemental Figure S6: Functional enrichment analysis of the interactome of ubiquitinated proteins
544 (corresponding to Figure 6).
- 545 Supplemental Figure S7: Interaction network of DAUPs.
- 546 Supplemental Figure S8: Functional enrichment analysis of the DAUP interactome.
- 547 Supplemental Figure S9: Root growth phenotype of WT plants and *ubc* mutants in 3-day control and
548 0.2 M mannitol conditions.
- 549
- 550 Supplemental Table S1: The root microsomal proteome of plants in the control condition and upon
551 mannitol treatment.
- 552 Supplemental Table S2: Proteins with quantitative variations upon mannitol treatment.
- 553 Supplemental Table S3: Inventory of ubiquitinated proteins in a root microsomal fraction.
- 554 Supplemental Table S4: Inventory of Ubi-peptides in root microsomes.
- 555 Supplemental Table S5: Determination of the ubiquitination motif.
- 556 Supplemental Table S6: Peptide quantification after immunopurification with the anti-KGG antibody.
- 557 Supplemental Table S7a: Interaction network of ubiquitinated proteins.
- 558 Supplemental Table S7b: Interaction network of UBC32.
- 559 Supplemental Table S7c: Interaction network of UBC34.
- 560 Supplemental Table S7d: Interaction network of DAUPs.
- 561 Supplemental Table S8a: One-way analysis of variance of root growth inhibition upon 1-day
562 treatment with 0.2 M mannitol (corresponding to Figure 6).
- 563 Supplemental Table S8b: One-way analysis of variance of root growth inhibition upon 2-day
564 treatment with 0.2 M mannitol (corresponding to Figure 6).
- 565 Supplemental Table S8c: One-way analysis of variance of root growth inhibition upon 3-day
566 treatment with 0.2 M mannitol (corresponding to Figure 6).
- 567 Supplemental Table S8d: One-way analysis of variance of root growth inhibition upon 4-day
568 treatment with 0.2 M mannitol (corresponding to Figure 6).

569 Supplemental Table S8e: One-way analysis of variance of root growth inhibition upon 5-day
570 treatment with 0.2 M mannitol (corresponding to Figure 6).
571 Supplemental Table S9a: Ubiquitinated proteins described in other Arabidopsis ubiquitome studies.
572 Table S9b: Novel ubiquitinated proteins identified in the present work.
573 Supplemental Table S10: Ubiquitinated proteins identified in the present work and described in the
574 literature as containing a K63-Ub linkage.

575

576

577

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584 *Conflict of interest statement.* The authors declare no conflicts of interest.

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587 **Data availability statement**

588 The mass spectrometry proteomics data were deposited at the ProteomeXchange Consortium via the
589 PRIDE partner repository with the dataset identifier PXD022249.

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