

1 **Phosphoproteomics after nitrate treatments reveal an important role**
2 **for PIN2 phosphorylation in control of root system architecture.**

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16

17 **Abstract**

18 Nitrate is an important signaling molecule that commands genome-wide gene expression changes
19 that impact metabolism, physiology, plant growth and development. Although gene expression
20 responses to nitrate at the mRNA level have been characterized in great detail, the impact of nitrate
21 signaling at the proteome level has been much less explored. Most signaling pathways involve
22 post-translational modifications of key protein factors and chiefly among these modifications is
23 protein phosphorylation. In an effort to identify new components involved in nitrate responses in
24 plants, we performed analyses of the *Arabidopsis thaliana* root phosphoproteome in response to
25 nitrate treatments via liquid chromatography coupled to tandem mass spectrometry. We identified
26 268 phosphoproteins that show significant changes at 5 min or 20 min after nitrate treatments. The
27 large majority of these proteins (96%) are coded by genes that are not modulated at the expression
28 level in response to nitrate treatments in publicly available transcriptome data. Proteins identified
29 by 5 min include potential signaling-components such as kinases or transcription factors. In
30 contrast, by 20 min, proteins identified were associated with protein binding, transporter activity
31 or hormone metabolism functions. Interestingly, the phosphorylation profile of *NITRATE*
32 *TRANSPORTER 1.1* (*NRT1.1*) mutant plants in response to nitrate at 5 min was significantly
33 different (95%) as compared to wild-type plants. This result is consistent with the role of NRT1.1
34 as a key component of a nitrate signaling pathway that involves phosphoproteomic changes. Our
35 integrative bioinformatics analysis highlights auxin transport as an important mechanism
36 modulated by nitrate signaling at the post-translational level. We experimentally validated the role
37 of PIN2 phosphorylation in both primary and lateral root growth responses to nitrate. Our data
38 provide new insights into the phosphoproteome and identifies novel protein components that are
39 regulated post-translationally, such as PIN2, in nitrate responses in *Arabidopsis thaliana* roots.

40

41 Introduction

42 Nitrogen (N) is the mineral nutrient required in the greatest amounts by plants. N is often
43 scarce in natural and agricultural systems, constituting a major factor limiting plant growth and
44 agricultural yield. During the last 50 years, global demand for synthetic N fertilizers has
45 dramatically increased in response to growing agricultural demand. Depending on soil conditions
46 and plant species, less than 50% of the applied N fertilizer is taken up by crops. Excess N may
47 contaminate aquatic systems¹ or be released into the atmosphere as N-oxide gases^{2,3}, both leading
48 to detrimental effects on the environment and human health.

49 The relevance of N for plants is exemplified by its effects on leaf growth⁴, senescence⁵,
50 root system architecture^{6,7}, and flowering time^{8,9}. Due to its importance, plants have evolved
51 sophisticated mechanisms to adapt to fluctuating N availability. Furthermore, growth and
52 developmental processes can be regulated by varying the amount of N supplied to plants. For
53 instance, exogenous nitrate applications stimulate lateral root elongation, enabling root growth and
54 colonization in nitrate-rich soil patches^{10,11}. However, high nitrate concentrations reduce primary
55 and lateral root elongation under homogeneous growth-conditions¹². Nitrate is the main form of
56 inorganic N for plants in natural and agricultural soils^{13,14}. Besides its nutritional role, nitrate acts
57 as a signaling molecule that regulates several genes involved in a wide range of biological
58 processes^{15,16}. With advances in genomic technologies and system approaches, thousands of
59 nitrate-responsive genes have been identified in *Arabidopsis thaliana* roots and shoots¹⁷⁻²⁴. These
60 N-response genes include nitrate transporters, nitrate reductase (NR) and nitrite reductase (NiR),
61 putative transcription factors, and stress response genes, as well as genes whose products play roles
62 in glycolysis, N metabolism, and hormone pathways. Moreover, nitrate elicits local and systemic
63 signals to synchronize its availability with plant growth and development²⁵⁻²⁹. Although
64 transcriptional responses activated by nitrate have been described in great detail, it is clear that
65 regulation at the post-translational level is key for N-responses^{30,31}.

66 The role of protein phosphorylation in response to nitrate was initially identified in post-
67 translational modifications in N metabolism. The activity of NR, the enzyme that catalyzes the
68 first step of nitrate reduction, is modulated by protein phosphorylation and then inhibited by 14-3-
69 3 protein interaction^{32,33}. Studies in spinach leaves using ³²P labeling and kinase assays
70 demonstrated that the regulation of NR by light/dark and photosynthetic activity involves protein

71 phosphorylation^{34,35}. A subsequent study showed that a 14-3-3 family protein interacts with and
72 inactivates phosphorylated NR in the presence of covalent ions^{32,36}. Earlier experiments also
73 indicated that changes in gene expression in response to nitrate treatments require kinase and
74 phosphatase activities. In maize leaves for example, treatments with inhibitors of calmodulin-
75 dependent protein kinases repress nitrate induction of genes encoding nitrate assimilatory enzymes
76 such as NR, NiR, glutamine synthetase 2 (GS2) and ferredoxin glutamate synthase (Fd-
77 GOGAT)³⁷. Conversely, inhibition of protein phosphatases blocked the nitrate-response of NR,
78 NiR and GS2³⁷. In another study, pharmacological inhibitors of serine-threonine protein
79 phosphatase and tyrosine protein kinases repressed the nitrate-induced accumulation of transcripts
80 for NR and NiR in barley leaves³⁸. These early experiments suggested that changes in the status
81 of protein phosphorylation were important for the regulation of gene expression in response to
82 nitrate treatments.

83 The discovery that a kinase protein complex can directly phosphorylate the nitrate
84 transceptor NRT1.1/NPF6.3 demonstrated that phosphorylation plays an important role in nitrate
85 signaling. Under low-nitrate conditions, NRT1.1/NPF6.3 is phosphorylated in a threonine residue
86 (T101) by CIPK23-CBL9 complex (CIPK, CLB-interacting protein kinase; CBL, Calcineurin B-
87 like protein), shifting into a high-affinity nitrate transporter^{31,39}. In contrast, at high-nitrate levels,
88 NRT1.1/NPF6.3 is dephosphorylated at T101 and turns into a low-affinity transporter.
89 Experiments with a mutant mimicking the phosphorylated form of the transceptor showed the
90 importance of this phosphorylation for the regulation of gene expression at low nitrate
91 concentrations³⁹. Phosphorylation of NRT1.1/NPF6.3 also appears to play a role in the modulation
92 of auxin transport and repression of lateral root emergence under low-nitrate conditions^{40,41}.
93 Conversely, the dephosphorylated form of NRT1.1/NPF6.3 is critical for up-regulation of
94 *NITRATE TRANSPORTER 2.1* (*NRT2.1*) gene expression in response to nitrate^{39,40}. Bouguyon *et*
95 *al.* (2015)⁴⁰ studied different mutant alleles of NRT1.1/NPF6.3 (T101D, T101A and P492L
96 substitution), and proposed a different NRT1.1/NPF6.3-dependent signaling mechanism. The
97 short-term induction of *NRT2.1* at high nitrate is negatively affected by T101D substitutions but
98 not by P492L and T101A. Long-term regulation of *NRT2.1* transcripts at high nitrate and
99 repression of lateral root emergence at low nitrate showed an opposite pattern, where signaling
100 was suppressed by both T101A and P492L mutations but not affected by T101 substitution.
101 Another kinase involved in signaling is CIPK⁸⁴². In *cipk8* mutants, the rapid induction of genes or

102 primary nitrate response was strongly reduced (40-65% of WT levels), particularly in the low-
103 affinity phase⁴². Both CIPK8 and CIPK23 are rapidly induced by nitrate treatments and
104 downregulated in the *chl1-5* and *chl1-9* mutants, respectively^{39,42}. More recently, the calcium
105 sensor CBL1 and the protein phosphatase 2C (ABA-insensitive) ABI2 were also identified as
106 components of this signaling pathway, which regulates NRT1.1/NPF6.3 transport and sensing⁴³.
107 The calcium sensor CBL1 also interacts with CIPK23 and this complex was dephosphorylated by
108 ABI2⁴³.

109 In higher plants, CBL/CIPK complexes sense and decode Ca²⁺ signals, triggering specific
110 transduction pathways (reviewed by ⁴⁴). Recent studies have shown that Ca²⁺ plays a role in nitrate
111 signaling transduction and is important for the primary nitrate response in *Arabidopsis* roots⁴⁵.
112 Calcium is a key secondary messenger that triggers changes in signaling pathways, including
113 changes in phosphorylation levels^{46,47}. More recently, results published by Liu et al. (2017)³⁰ have
114 contributed to our understanding of the relationship between Ca²⁺ signaling and the first layer of
115 transcriptional regulators. They used the luciferase (LUC) reporter gene NIR-LUC, which exhibits
116 a physiological nitrate response in transgenic *Arabidopsis* plants, to identify three CPKs (CPK10,
117 CPK30, and CPK32) that activated the NIR-LUC reporter in an effective and synergistic manner.
118 Additionally, CPK10, CPK30, and CPK32 phosphorylated the transcription factor NIN-LIKE
119 PROTEIN 7 (NLP7) in a Ca²⁺ dependent manner³⁰, suggesting that CPK-NLP signaling is a key
120 regulator of primary nitrate responses⁴⁸. All these studies provide evidence that NRT1.1/NPF6.3,
121 calcium and phosphorylation of target proteins are key elements of a signaling pathway involved
122 in the nitrate response.

123 Global-scale proteomic analysis performed in *Arabidopsis* seedlings, mostly shoot organs,
124 showed that nitrogen starvation and resupply (nitrate or ammonium) modulates protein
125 phosphorylation over a time course of 30 min⁴⁹. In general, proteins such as receptor kinases and
126 transcription factors change their phosphorylation levels after nitrogen resupply at 5-10 min (fast
127 response). Proteins involved in protein synthesis and degradation, central and hormone metabolism
128 showed changes in their phosphorylation level after 10 min (late response). Another study showed
129 that nitrate deprivation affects both protein abundance and phosphorylation status⁵⁰. Nitrate
130 deprivation assays revealed that some proteins, mostly involved in transport, contain sites that are
131 dephosphorylated early in the response ⁵⁰.

132 In this study, we performed quantitative time-course analyses of the *Arabidopsis* root
133 phosphoproteome in response to nitrate via liquid chromatography coupled to tandem mass
134 spectrometry detection (HPLC-MS/MS). We chose to focus on root-phosphoproteomics profiling
135 in response to nitrate for several reasons: (i) phosphoproteomics and proteomics studies describe
136 phosphorylation levels as more dynamic and mainly independent of protein abundance^{51–53},
137 suggesting that many proteins are regulated by phosphorylation independent of their changes in
138 protein abundance. (ii) Previous global studies of N treatment focused on the proteome and
139 phosphoproteome in *Arabidopsis* seedlings, which interrogates mostly shoot tissues^{49,50}. In order
140 to search for new N-regulatory factors, our experimental approach focused on *Arabidopsis* roots
141 because early sensing and responses to N-supply occur in the roots. Several studies have shown
142 that HPLC-MS/MS provides accurate estimates of dynamic phosphorylation levels *in vivo*^{54–57}.

143 We used HPLC-MS/MS to identify phosphorylated proteins with differential profiles in
144 response to nitrate treatments at 0, 5 or 20 min. We found that the nature of these phosphorylated
145 proteins differed significantly from those encoded by genes implicated in nitrate via transcriptomic
146 studies. We found different types of phosphoproteins changing at 5 or 20 min after nitrate
147 treatments. Interestingly, the large majority of these changes depend on NRT1.1/NPF6.3. Kinases
148 and transcription factors were over-represented at 5 min, while proteins involved in protein binding
149 and transporter activity were common by 20 min of nitrate treatments. We found several
150 phosphoproteins involved in auxin transport, including the auxin efflux-carriers PIN2 and PIN4.
151 We validated the role of PIN2 and found dephosphorylation of PIN2 to be important for
152 modulation of root system architecture in response to nitrate. Our analysis reveals that the nitrate
153 signaling pathway mediated by NRT1.1/NPF6.3 leads to important changes in protein
154 phosphorylation patterns and proposes new players that participate in the developmental responses
155 to nitrate in plants.

156 **Results**

157 **Phosphoproteome analysis of *Arabidopsis* roots in response to nitrate treatments.**

158 In an effort to identify new components involved in nitrate responses in plants, we
159 performed large-scale mass spectrometry-based phosphoproteome experiments following nitrate
160 treatments in *Arabidopsis* roots. *A. thaliana* (*L.*) *Columbia-0* (Col-0) seedlings were grown
161 hydroponically, with a full nutrient solution (Murashige and Skoog basal medium without N)
162 containing 1 mM ammonium as the only N source for 14 days (time 0, see experimental
163 procedures). Two-week-old plants were treated with 5 mM KNO₃ or KCl, as control. We and other
164 laboratories have used this experimental setup in prior studies because it elicits robust primary
165 nitrate responses in *Arabidopsis* plants^{7,16,45,58,59}. A label-free high-performance liquid
166 chromatography-tandem mass spectrometry (HPLC-MS/MS) method was used to identify changes
167 in protein phosphorylation at 0, 5 or 20 minutes after nitrate treatments. We chose these time points
168 because they have shown to be effective in describing transient and persistent protein
169 phosphorylation responses^{49,55}. Moreover, this experimental design allows for comparison with
170 transcriptomics data obtained using the same experimental conditions^{7,45,58}. For phosphoproteome
171 analysis, we used a previously validated experimental pipeline^{52,53,60}. Briefly, phosphopeptides
172 were enriched using cerium oxide affinity capture and analyzed with a HPLC-MS/MS instrument
173 (Figure S1). The spectra were assigned to specific peptide sequences by the MASCOT search
174 engine (FDR < 0.1%). We quantified the relative abundance of each phosphoprotein using average
175 normalized spectral counts (SPCs) of the total number of spectral-peptide matches to protein
176 sequences in three independent biological replicates for each treatment condition. In total, we
177 identified and measured 6,560 unique phosphopeptides which unambiguously mapped to 2,048
178 phosphoprotein groups (Supplemental Table S1). The majority of identified phosphopeptides
179 (82%) were phosphorylated in a single residue (Figure S2A). The relative distribution of each
180 phosphorylated residue – 80% serine, 18% threonine, and 2% tyrosine (Figure S2B) – was
181 consistent with prior plant phosphoproteomic studies^{50,54,61}. The identified phosphopeptides were
182 mapped and grouped in phosphoprotein groups, where proteins that shared peptides were clustered
183 together. A group leader was assigned to each group, based on having the highest number of
184 peptide identifications; throughout the remainder of the article, “phosphoproteins” is synonymous
185 with “group leaders”. The majority of the identified phosphoproteins present one (50%), two
186 (25%) or three (12%) phosphorylated peptides (Figure S2C) with similar distributions of

187 phosphorylated residue (69% Ser, 28% Thr and 3% Tyr; Figure S2D). We recognized
188 phosphoproteins across several biological process, subcellular compartments and cellular
189 functions based on the Gene Ontology (GO) classification (Figure S3). No overrepresented GO
190 categories were observed when comparing against the *Arabidopsis* genome, showing that our
191 experimental strategy was unbiased with regards to annotated protein functions, subcellular
192 locations or biological processes and represents an unbiased *Arabidopsis* proteome sampling.

193 To identify nitrate-regulated phosphoproteins in *Arabidopsis* roots we performed
194 statistical analysis using analysis of variance (significance: $p < 0.05$). We identified 268
195 phosphoproteins that were significantly altered under our experimental conditions. We found 62
196 phosphoproteins regulated at 5 min after nitrate treatments, 40 of which were induced (Figure 1A).
197 We found 152 phosphoproteins differentially regulated at 20 min, 113 of which were induced by
198 the nitrate treatments. The large majority of phosphoproteins were found to be nitrate-regulated at
199 only one time-point, indicating that most changes in the phosphoproteome are transient with an
200 early (5 min) and late (20 min) component in response to nitrate treatments. Two previous studies
201 characterized the phosphoproteome in response to nitrate-depletion⁴⁹ or nitrate-resupply⁵⁰ (Figure
202 1C). Response to nitrate-resupply resulted in 10 common phosphoproteins and nitrate-deprivation
203 in 6 common proteins. These proteins were mostly associated with nitrate metabolism, such as
204 transporters (NRT2.1; ammonium transporter 1.3, AMT1.3), the *Arabidopsis* H⁺-ATPase 2
205 (AHA2) proton pump, and nitrate reductase 2 (NIA2). The comparison of the phosphoproteins
206 identified here with transcriptome data revealed that 96% of the phosphoproteins are encoded by
207 genes that do not change expression at the mRNA level (Figure S4). We compared our results with
208 an integrated analysis of available root microarray data under contrasting nitrate conditions (27
209 experimental datasets corresponding to 131 arrays⁶²). This study identified a group of 2286 nitrate-
210 responsive genes regulated at transcription levels. Only 11 of these genes were found in our
211 phosphoproteomic dataset.

212 Our approach identified new genes coding for phosphoproteins putatively involved in
213 nitrate responses. Changes in phosphorylation patterns improve our understanding of signaling
214 mechanisms that connect nitrate transporters/sensors with transcriptomic response and other
215 biological processes. The small overlap between the phosphoproteomic studies to date highlights
216 the importance of ours and additional future studies to address this important aspect of
217 posttranslational modifications in response to N-signals in plants.

218 **Functional enrichment in the phosphoproteome reveals distinctive signaling and regulatory**
219 **processes occurring in early and late responses to nitrate.**

220 To evaluate the biological significance of the phosphoproteome patterns observed in
221 response to nitrate, hierarchical clustering analysis was performed on the phosphoprotein dataset
222 at 5 and 20 min following nitrate treatments in Col-0 roots (Figure 2). In order to identify the most
223 prominent functional categories affected, we searched for overrepresented biological terms in each
224 cluster using the BioMaps program⁶³ and the PANTHER classification system^{64,65} (significance:
225 $p < 0.05$, corrected by FDR). This analysis highlighted several signaling, regulatory or metabolic
226 functions differentially associated with early (5 min) and late (20 min) components in response to
227 nitrate treatments. “DNA binding” and “Nucleic acid binding” categories were overrepresented in
228 cluster 6, containing phosphoproteins that increase their levels at 5 min and do not change at 20
229 min. This cluster also includes previously undescribed transcription factors (TFs) in the N-
230 response cascade in diverse transcriptomic studies^{21–24,66}. In contrast, cluster groups containing
231 mostly phosphoproteins that changed their levels at 20 min in response to nitrate were enriched in
232 the functional categories: “transport” and “nitrogen compounds metabolism” (clusters 2 and 8).
233 Consistent with this, increases in phosphoprotein levels in response to nitrate were observed in
234 NRT2.1, the lysine–histidine-like transporter 4 (LHT4), the UDP-Arabinofuranose Transporter 4,
235 AMT1-3 and the K⁺-transporter 8 (KUP8) only at 20 min following nitrate treatment. Increased
236 abundance of the phospho-peptide Ser-501, corresponding to NRT2.1, was observed at 20 min
237 after nitrate treatment. This site is localized in a C-terminal phosphorylation hotspot
238 (Phosphorylation site database and predictor Phosphat4.0^{67,68}) and was also identified in nitrate-
239 deprivation experiments with an opposite phosphorylation response⁵⁰. Also, phosphopeptides for
240 AMT1.3 with phosphorylation at Thr-464 and Ser-487 were identified as “up-regulated” by nitrate
241 treatments at 20 min. The phosphorylation of both sites inhibits transport function⁶⁹ and were also
242 identified as phosphopeptides in nitrate deprivation⁵⁰ and resupply⁴⁹ experiments. We also found
243 that nitrate strongly increased levels of phosphorylated nitrate reductase NIA2 at the highly
244 conserved and regulatory site Ser-534⁷⁰. These results are consistent with previous studies and
245 suggest overall regulation of N metabolism by phosphorylation of key players by 20 min after
246 nitrate treatments.

247 The final group of clusters showed enrichment in signaling and regulatory pathways, with
248 different profiles at 5 min and 20 min. Clusters 1, 5, and 7, contained phosphoproteins with
249 opposite regulation at 5 and 20 min, and showed proteins related to microRNA processing,
250 phosphoinositide and phosphatidylinositol binding functions. Phosphoinositides can act in
251 signaling pathways and serve as precursors for phospholipase C (PLC)-mediated signaling. A
252 previous study in our laboratory implicated a PLC-activity in the nitrate signaling pathway⁴⁵. Our
253 results are consistent with these results and show that PLC2, represented by the phosphopeptide
254 Ser-280, was identified as up-regulated in response to nitrate at 20 min (in Cluster 1). Cluster 10
255 is interesting for nitrate responses because it includes many components involved in classical
256 processes regulated by nitrate in *Arabidopsis* roots. For instance, auxin and lateral root
257 development are biological functions enriched in this group, which is consistent with auxin
258 pathways being modulated by nitrate^{16,71}. Several reports indicate that auxin acts as regulator of
259 root system architecture in response to nitrate availability^{7,71,72}.

260 Overall, our dataset captures the dynamic effects of N-signaling on phosphoproteome
261 profiles, which implicate a cascade of nonoverlapping processes in early and late responses. The
262 earliest steps in the N phospho-dynamics were involved in signal transduction and transcription
263 factor activity. In contrast, the later phosphoprotein data-set was enriched in metabolic, transport
264 and root developmental processes. These temporal mechanisms show a transition of
265 phosphorylation dynamics from phosphoproteins essential to signaling networks to proteins
266 associated with biological processes involved in nitrate response.

267 **NRT1.1/AtNPF6.3 is essential for transient phosphoprotein changes in response to nitrate** 268 **treatments**

269 The only nitrate sensor described to date is the nitrate transporter, NRT1.1/NPF6.3^{39,73}. In
270 order to understand the importance of NRT1.1/NPF6.3 for nitrate elicited changes in the
271 phosphoproteome observed, we analyzed the phosphoproteomic profile of roots treated with 5 mM
272 KNO₃ or KCl (control) in a *nrt1.1-null* background (mutant *chl1-5*), using the same experimental
273 conditions described above. 74% of phosphoproteins were detected in both datasets, yet only 5%
274 of the nitrate-phosphoproteome response observed in wild-type plants was maintained in the *chl1-*
275 *5* mutant (Figure 3). Moreover, 95% of phosphoprotein levels were altered in the *chl1-5* mutant
276 plants. This result indicates that this gene is important for modulating protein phosphorylation in

277 response to nitrate, in addition its established role as nitrate transceptor.

278 Our analysis indicates that NRT1.1/NPF6.3 is critical for maintaining the *Arabidopsis*
279 phosphoproteome in response to nitrate availability. It also denotes that NRT1.1/NPF6.3 function
280 is required for rapid changes in phosphorylation of key proteins in response to nitrate in
281 *Arabidopsis* roots. For example, phosphorylated peptides that map to proteins associated with
282 nitrogen metabolism (NRT2.1, AMT1-3 and NIA2) were identified in *chl1-5* mutant roots, but
283 their levels were not affected in response to nitrate.

284 **Network analysis reveals regulatory sub-networks connected to transcription factors and** 285 **potential kinases in response to nitrate.**

286 To uncover key biological processes modulated by changes in phosphorylation, we
287 performed a multinet network analysis with our phosphoproteomics data. We generated this network
288 by integrating different levels of information, including protein-protein interactions from
289 BioGRID⁷⁴, predicted protein-DNA interactions of *Arabidopsis* TFs (DapSeq)^{75,76}, *Arabidopsis*
290 metabolic pathways (KEGG), and miRNA-RNA, as described previously¹⁶. We also integrated
291 kinase-substrate predictions and identified the most significant phosphorylation motifs and their
292 predicted kinase families from our phosphoproteomic datasets using the Motif-X algorithm⁷⁷ and
293 the PhosPhAt Kinase-Target interactions database⁷⁸ (Figure S5). We used the Cytoscape⁷⁹
294 software to visualize the resulting network, wherein genes that encoded each phosphoprotein were
295 represented as nodes linked by edges that signify any of the functional relationships annotated in
296 the various databases indicated above. We generated a network of 206 nodes with 700 interactions
297 (Figure 4A). Although the majority of these genes are not regulated by nitrate at the mRNA level,
298 they form a highly interconnected network which includes potential regulatory transcription
299 factors and kinase components. They are connected by multiple edges, including protein-protein,
300 protein-DNA, and metabolic interactions. This result suggests that the products of these genes
301 form connected biological modules that are coordinately regulated at the post-translational level.
302 This network included several TFs with a high number of regulatory links. The most connected
303 were the Trihelix transcription factor 1 (GTL1), the WRKY DNA-binding protein 65 (WRKY65),
304 and the RELATED TO VERNALIZATION 1 (RTV1) transcription factors, which had not
305 previously been characterized in the context of nitrate response. Intriguingly, GTL1 regulates root
306 hair growth in *Arabidopsis*⁸⁰, which has recently been described as a biological process modulated

307 by nitrate treatments under the same experimental conditions⁸¹. A previous study indicated that
308 WRKY65 interacts at the protein level with the mitogen-activated protein kinase 10 (MPK10),
309 which binds with the lateral organ boundaries domain 16 (LBD16), LBD18, and LBD29
310 transcription factors^{82,83}. These LBDs are inducible by auxin and play a role in the formation of
311 lateral roots⁸³. In addition, MPK10 interacted with other genes involved in the auxin response⁸²,
312 while LBD29 regulated genes involved in auxin transport, including auxin efflux-carriers PIN1
313 and PIN2⁸³. This evidence suggests that WRKY65 could be involved in nitrate–auxin signaling
314 crosstalk. These three TFs appear to coordinate different subnetworks largely involved in auxin
315 transport and nitrogen metabolism. Consistent with this observation, analysis of over-represented
316 gene ontology annotations highlights the importance of auxin transport (Figure 4B). Other over-
317 represented biological functions in our network were mRNA binding and splicing, regulation of
318 translation and kinase activity (Figure 4B).

319 Overall, this network analysis highlights a potential role of multiples TFs in linking the N
320 signal and regulatory nitrate responses that show significant enrichment for key functions in
321 signaling pathways and validates the important role auxin plays in the nitrate-response of root
322 system architecture.

323 **PIN2 is important for modulation of root system architecture in response to nitrate** 324 **treatments**

325 Auxin is a key phytohormone in plants, involved in growth and developmental responses.
326 Several reports have shown that auxin mediates root developmental responses to nitrate
327 availability^{7,16,71,72,84–86}. Nitrate can regulate auxin biosynthesis, transport and accumulation. In
328 response to nitrate, several auxin-related modules are regulated, including the upregulation of
329 auxin receptor AUXIN SIGNALING F-BOX 3 (AFB3) and the feedback regulation by miR393^{7,71}.
330 This auxin-signaling component in response to nitrate is implicated in both primary and lateral
331 root growth⁷. Other important evidence comes from the analyses of the *Arabidopsis* transcriptomic
332 response upon nitrate treatments. They show that several genes involved in auxin transport are
333 affected, including auxin efflux-carriers PIN1, PIN4 and PIN7^{16,71}. Moreover, the nitrate
334 transceptor NRT1.1/NPF6.3 not only senses and transports nitrate but can also transports auxin, a
335 process that regulates auxin-localization patterns and lateral-root elongation⁸⁴. Consistent with
336 these prior observations, auxin transport was conspicuous throughout our entire phosphoproteomic

337 analysis (Figure 2 and 4). PIN phosphorylation has been shown to be essential for auxin transport
338 and distribution⁸⁷⁻⁹². PINs phosphorylation in conserved serine and/or threonine of the central loop
339 controls intracellular trafficking, recycling and polar membrane localization of PIN proteins
340 (Review by⁸⁷). Previous studies indicate that PIN polar localization explains auxin fluxes and
341 distribution patterns, which could mediate differential growth in diverse plant tissue such as
342 roots^{91,93}. To validate the relevance of phosphorylation in auxin transport and its connection with
343 nitrate response, we chose the auxin efflux-carrier PIN2 (identified in our experimental dataset)
344 due to its potential role in linking nitrate and changes in root system architecture (RSA). We found
345 an uncharacterized PIN2 phosphorylation site (Ser439) at the end of the hydrophilic cytoplasmic
346 loop (C-loop, Figure 5A). Its phosphopeptide levels decreased by close to 75% in response to
347 nitrate treatments by 5 min (Figure 5B). PIN2 belongs to the PIN-FORMED protein family of
348 auxin transporters and is the principal component mediating basipetal auxin transport in roots^{94,95}.
349 This polar auxin transport is essential for root gravitropism⁹⁵ and lateral root formation⁹⁶.
350 Intriguingly, we detected only one phosphorylated peptide for PIN2 in response to nitrate. Protein
351 sequence alignment indicated that this phosphosite is highly conserved in different plant species
352 representing gymnosperm, mono- and dicotyledonous plant lineages of seed plants (Figure 5A).
353 This phosphopeptide has also been described as down-regulated after auxin treatment but its
354 function remains an open question⁵⁷.

355 As a first step to understand the function of PIN2 phosphorylation in the nitrate response,
356 we performed a Phos-tag Western Blot analysis to confirm the changes in PIN2 phosphorylation
357 after nitrate treatment. We detected two, fast- and slow-mobility, PIN2 specific bands indicating
358 the presence of two phospho-populations in response to nitrate: one more and another less
359 phosphorylated PIN2. In contrast, only one slow-mobility band corresponding to the more
360 phosphorylated PIN2 subpopulation could be observed at time 0 (ammonium-supplied roots) or
361 under control conditions (KCl-treated roots) (Figure 5C). To assess whether these changes in PIN2
362 phosphorylation status are a result of a change in protein abundance, we analyzed PIN2-GFP
363 protein levels by Western Blot under our experimental conditions. We introgressed the construct
364 *PIN2::PIN2-GFP* (*PIN2^{wt}-GFP*) into the *pin2* loss-of-function mutant plant *eir1-1*⁹⁷. No
365 differences in protein levels were observed in roots treated with nitrate as compared to roots at
366 time 0 (Figure 5D). To understand the function of this specific PIN2 phosphosite Ser439, we also
367 analyzed the protein levels in *pin2* null mutant complemented with phospho-null

368 (*PIN2::PIN2^{S439A}-GFP*) or phospho-mimic (*PIN2::PIN2^{S439D}-GFP*) versions of PIN2-GFP.
369 Similarly, PIN2 protein concentrations were similar when our experimental conditions mimicked
370 PIN2 phospho-modifications (Figure 5D). Moreover, no regulation at the mRNA level was
371 observed in *PIN2* during nitrate responses (Figure 5E). These results demonstrated that nitrate
372 regulates PIN2 at the posttranslational level, causing de-phosphorylation of PIN2 at a specific
373 phosphosite.

374 Next, we evaluated the role of PIN2 in root system architecture (RSA) in response to nitrate
375 treatments. We grew wild-type (Col-0) and *pin2* mutant (*eir1-1*) plants for 2 weeks on ammonium
376 as sole N source (time 0) and evaluated RSA after nitrate treatments. We measured primary root
377 length 3 days after 5 mM KNO₃ or KCl treatments. As expected for this experimental set up, we
378 found that nitrate-treated wild-type plants developed shorter primary roots as compared to KCl-
379 treated plants, consistent with earlier results indicating that nitrate treatments inhibit primary root
380 elongation under these experimental conditions⁷ (Figure 6A). However, primary roots of *eir1-1*
381 plants were not significantly inhibited by nitrate treatments as compared to wild-type plants. We
382 also analyzed the density of lateral roots in response to nitrate treatments. In wild-type plants,
383 nitrate treatments increased the number of lateral roots (emerged and initiating) as compared with
384 KCl treatments (Figure 6B). In contrast, the lateral root response to nitrate treatment was altered
385 in the *eir1-1* mutant and the density of lateral roots was significantly reduced as compared with
386 wild-type plants (Figure 6B). These results show that PIN2 plays an important role in modulating
387 RSA in response to nitrate treatments.

388

389 **PIN2 de-phosphorylation of Ser439 plays a role in modulating the root system architecture** 390 **in response to nitrate treatments.**

391 To understand the function of the PIN2 phosphosite identified in this study, we analyzed
392 the *pin2* null mutant *eir1-1* complemented with phospho-null (S439A) or phospho-mimic (S439D)
393 versions of PIN2-GFP. As PIN2 plays an important role in root gravitropism, we evaluated the
394 gravitropic curvature in wild-type, *eir1-1* mutant plants and PIN2-GFP transgenic plants. All
395 seedlings were germinated and grown in MS media in the absence of nitrate for 7 days, and then
396 they were transferred to agar plates with or without nitrate. Agar plates were rotated 90° and root
397 curvature was measured at 24 hours. As expected, *pin2* mutant plants showed an agravitropic

398 phenotype. Both *PIN2^{S439D}-GFP* and *PIN2^{S439A}-GFP* were able to rescue the agravitropic
399 phenotype of *eir1-1* mutant plants. This result indicates that the phosphorylation status of PIN2 at
400 S439 is not relevant for root gravitropic responses in *Arabidopsis* (Figure S6). This result also
401 indicates that phosphorylation at S439 does not impair all PIN2 functions.

402 Next, we evaluated the RSA in response to nitrate treatments in the same phospho-
403 mimicking (*PIN2^{S439D}-GFP*) or -null (*PIN2^{S439A}-GFP*) genotypes and compared it to wild-type
404 plants. Plants were grown for 2 weeks on ammonium as sole N source and then treated with 5 mM
405 KNO₃ or KCl for three days. Complementation of the *eir1-1* mutant with a wild-type version of
406 PIN2 (*PIN2^{wt}*) restored normal RSA responses to nitrate (Figure 7). *eir1-1* mutant plants
407 complemented with *PIN2^{S439A}* showed RSA changes similar to wild-type plants in response to
408 nitrate treatments, albeit slightly less pronounced. Three days after nitrate treatments, primary root
409 growth was inhibited 42% by nitrate in the *PIN2^{S439A}-GFP* genotype as compared with nitrate-
410 treated wild-type plants (Figure 7A). Similarly, lateral root density in *PIN2^{S439A}-GFP* plants
411 increased 47% as compared to wild-type plants in response to nitrate treatments (Figure 7B). In
412 contrast at the end of the 3-day treatment, the primary root length and lateral root density did not
413 differ between nitrate or KCl treatment in *eir1-1* mutant plants complemented with *PIN2^{S439D}-*
414 *GFP*. This result, comparable to the response in *eir1-1* roots, indicates that regulation of PIN2
415 phosphorylation status at S439 is necessary for normal RSA modulation in response to nitrate
416 treatments.

417

418 **PIN2 phosphosite regulates polar plasma membrane localization in response to nitrate.**

419 To explore the impact of nitrate-regulated phosphorylation of PIN2 on cellular localization,
420 we examined the subcellular localization pattern in PIN2-GFP genotypes with phosphosite
421 substitutions. PIN2^{WT}-GFP proteins were accumulated at the plasma membrane of epidermal and
422 cortical cells, as previously described (Figure 8A)⁸⁶. PIN2^{WT}-GFP fluorescence signal increased
423 in epidermal and cortical cells 2 hours after nitrate treatments as compared to roots in control
424 conditions (roots without nitrate treatments, Figure 8A). Interestingly, mutations at S439 altered
425 this pattern. PIN2^{S439A}-GFP plants showed higher fluorescence in the plasma membrane even
426 without nitrate treatment as compared to PIN2^{wt}-GFP or PIN2^{S439D}-GFP (Figure 8A). The total
427 fluorescence in all experimental conditions analyzed here was similar (Figure 8B). In response to

428 nitrate, PIN2^{WT}-GFP and PIN2^{S439A}-GFP were accumulated at the plasma membrane of epidermal
429 and cortical cells at comparable levels (Figure 8C and 8D). On the contrary, PIN2^{S439D}-GFP plants
430 showed lower levels at epidermal and cortical cells in response to nitrate treatments as compared
431 to PIN2^{WT}-GFP or PIN2^{S439A}-GFP (Figure 8C and 8D).

432 These results indicate that PIN2 phosphorylation status at S439 is important for a correct
433 subcellular localization-pattern in response to nitrate treatments. Moreover, these results indicate
434 that post-translational control impinging upon PIN2 localization is required for RSA changes in
435 response to nitrate treatments.

436

437 Discussion

438 A key plant nutrient, N also acts as a signal that regulates a myriad of plant growth and
439 developmental processes. Nitrate, a main N-source in natural and agriculture soils, elicits genome-
440 wide changes in gene expression for thousands of genes involved in various biological functions.
441 Nitrate responses have been characterized in great detail at the transcriptome level. However, post-
442 translational modifications have not been characterized in detail. In this study, we evaluated
443 phosphoproteomic profiles in *Arabidopsis* roots in response to nitrate treatments. We focused on
444 characterizing early nitrate-elicited changes in protein phosphorylation. Protein phosphorylation
445 and dephosphorylation plays a central role in modulating protein function in plant signaling-
446 pathways involved in a wide range of processes relating to hormones, nutrients, and responses to
447 stress^{54-57,61,98}.

448 Our analysis demonstrated that early and late changes in phosphoprotein levels occur in
449 response to nitrate in roots. Furthermore, we identified candidates for nitrate-signaling and
450 biological functions underlying the nitrate response in roots. Interestingly, the majority of proteins
451 and corresponding genes identified in our analysis have not been previously associated with nitrate
452 responses. The phosphoproteomic profile was characteristic of each time-point, showing dynamic
453 changes in phosphorylation patterns in response to nitrate treatments. Early changes in
454 phosphorylation levels (5 min) mainly affected proteins associated with gene regulation, including
455 transcription factors and splicing process. These results are consistent with rapid and dynamic N-
456 responses observed at the mRNA level described in previous studies^{21-23,71}. Recent studies have
457 identified new transcription factors²¹⁻²³ involved in the control of gene expression by nitrate.
458 Interestingly, the majority of the transcription factors that we detected as differentially
459 phosphorylated had not been identified as part of the nitrate response. The phosphoproteomic
460 response to nitrate at 20 min revealed a group of different phosphoproteins, mostly involved in
461 protein binding and transport. In this dataset, we found proteins known to be involved in nitrogen
462 response as differentially phosphorylated, including the high-affinity nitrate transporter
463 NRT2.1^{49,50} and AMT1.3⁶⁹. In our experimental conditions, site T464 in AMT1.3 was
464 phosphorylated. Since phosphorylation of this site inhibits ammonium transport⁶⁹, the increased
465 phosphorylation status at this site may be related to the fact that our experimental conditions
466 focused on nitrate transport in *Arabidopsis* roots. We also identified phosphoproteins associated
467 with signaling pathways and transcription factors, uncovering regulatory networks linked to

468 transcriptomic changes occurring later in the response to nitrate. The corresponding genes have
469 not been characterized in the context of nitrate responses. For instance, one potentially novel
470 regulatory factor is ABA-responsive element binding protein 3 (AREB 3), whose phosphoprotein
471 levels are increased in response to nitrate treatments. This transcription factor is involved in ABA
472 signaling^{99,100}. Nitrate triggers ABA accumulation at the root tip¹⁰¹ and several studies indicate
473 that ABA plays a role in regulation of lateral root growth by nitrate¹⁰².

474 NRT1.1/NPF6.3 is the main nitrate sensor described to date^{31,39}. Interestingly, we observed
475 important differences in the phosphoproteome of *chl1-5* mutant as compared to wild-type plants
476 in response to nitrate treatments. These results emphasize that NRT1.1/NPF6.3, calcium and
477 kinases/phosphatases make-up the canonical nitrate-signaling pathway of *Arabidopsis* roots.
478 Recent evidence showed that NRT1.1/NPF6.3 and PLC activity are required for nitrate-induced
479 increases in cytoplasmic Ca²⁺ levels⁴⁵. Our results indicated that PLC2 phosphoprotein levels
480 increased in response to nitrate. Nitrate signaling pathways also involve CBL–CIPK complex and
481 CPK–NLP regulatory network (reviewed by ⁴⁸).

482 The transcriptomic nitrate-response was not mirrored at phosphoproteomic levels. This
483 lack of correlation between mRNA and protein or phosphoprotein levels has been documented in
484 other nitrogen-phosphoproteomic studies^{49,50}, plant responses to different stimulus^{52,53} and other
485 organisms¹⁰³. Furthermore, post-translational regulation does not require a change in gene
486 expression or *de novo* protein synthesis. Post-translational control is faster, allowing rapid
487 adaptation to environmental changes. Interestingly, the genes coding for nitrate-modulated
488 phosphoproteins identified in this study are highly co-expressed across many different
489 experimental conditions but not regulated by nitrate treatments¹⁰⁴. This finding suggests that this
490 group of genes is functionally related and regulated at the mRNA level in response to several
491 endogenous or exogenous cues. In the context of nitrate responses, the products of these genes are
492 regulated at the post-translational level, uncovering a new layer of control that enables signal
493 crosstalk and fine tuning. Our results highlight the need for integrated analysis and data sets at
494 different levels to decipher plant responses to environmental cues.

495 It is now clear that auxin plays a central role in the plant root response to changes in nitrate
496 availability. Nitrate regulates primary root growth, lateral root initiation and elongation. Auxin in
497 turn is key during root development⁹⁶, particularly in initiation and growth of lateral roots⁹³.

498 Several reports show that auxin signaling, biosynthesis, transport, and accumulation are affected
499 during nitrate responses^{72,84}, and transcriptomic analyses demonstrate that genes involved in auxin
500 response are controlled by nitrate^{7,16,71}. The main nitrate transporter NRT1.1/NPF6.3 can also
501 transport auxin^{84,86}. A recent study also showed that NRT1.1/NPF6.3 negatively regulates the
502 *TAR2* auxin-biosynthetic gene and the *LAX3* auxin-influx transport gene at low nitrate
503 concentrations, repressing lateral root development¹⁰⁵. These results suggest that an interplay
504 between nitrate signaling and auxin transport occurs at different levels^{106–108}. Consistent with these
505 findings, we found that the molecular function “auxin transport” was overrepresented in our cluster
506 and network analyses. We showed that dephosphorylation of PIN2 in a novel phosphosite is a part
507 of a regulatory mechanism for RSA responses triggered by nitrate. The
508 phosphorylation/dephosphorylation of PIN proteins at specific sites (serine or threonine) located
509 in their higher loops has been shown to play important roles in modulating PIN functions^{88,91,92,109},
510 including trafficking¹¹⁰. We showed that phosphorylation/dephosphorylation of PIN2 at S439 is
511 important for PIN2 plasma membrane localization in epidermal and cortical cells in response to
512 nitrate. Previous studies have shown that changes in PIN2 membrane localization and polarity
513 interfere with PIN2 function in auxin transport with an impact on RSA during alkaline stress¹¹¹ or
514 low phosphate¹¹². In addition, recent studies showed that kinase cascade modules 3’-
515 PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1 (PKC1)-D6 PROTEIN KINASEs
516 (D6PK) and PKC1- AGC1 kinase PROTEIN KINASE ASSOCIATED WITH BRX (PAX)
517 regulate auxin distribution through PIN phosphorylation^{113,114}. The phosphorylation of PIN affects
518 PIN-mediated auxin transport, which controls plant growth¹¹³ and other developmental
519 processes¹¹⁴. These results suggest that PIN phosphorylation is part of a regulatory switch that
520 strictly controls the directional transport of auxin and subsequent growth or developmental
521 processes.

522 Our results suggest a model (Figure 9) in which nitrate promotes dephosphorylation of
523 PIN2, which then impacts localization and auxin transport. Modulation of PIN2 function could
524 affect growth of primary and lateral roots for optimal nutrient uptake. Beyond this new regulatory
525 mechanism involving PIN2 protein, our phosphoproteomics results identify novel proteins, which
526 may be interesting targets for future studies or biotechnological developments for improved
527 nitrogen use-efficiency or crop yield.

528

529 **Acknowledgements**

530

531 This work was supported by Millennium Institute for Integrative Biology—iBio (Iniciativa
532 Científica Milenio-MINECON), Fondo de Desarrollo de Areas Prioritarias (FONDAP) Center for
533 Genome Regulation (15090007), Fondo Nacional de Desarrollo Científico y Tecnológico
534 (FONDECYT) 1141097 (to RAG) and 1171631 (to AV). We would like to thank Unidad de
535 Microscopía Avanzada UC (UMA UC).

536 **Methods**

537

538 **Plant material and growth conditions.**

539 *Arabidopsis thaliana* (L.) Heynh. Columbia-0 accession plants were used as wild type genotype in all
540 experiments. The transgenic lines *PIN2::PIN2^{wt}-GFP*, *PIN2::PIN2^{S439D}-GFP* and *PIN2::PIN2^{S439A}-GFP*
541 were introduced into *eir1-1* background (*pin2* null mutant plants), using strategies described previously.
542 *PIN2* lines were generated by Gibson assembly as described¹¹⁵ using pGREEN backbone vector¹¹⁶.
543 *PIN2::PIN2^{wt}-GFP* line was generated by insertion of *mGFP5* into *PIN2* coding sequence at nucleotide
544 1215 from ATG (between Thr405 and Arg406)^{94,117}. For *PIN2* promoter, 2178 bp upstream of the start
545 codon was used. For the generations of *PIN2::PIN2^{S439D}-GFP* and *PIN2::PIN2^{S439A}-GFP* lines Serine 439
546 was replaced by aspartate (*PIN2^{S439D}*) or alanine (*PIN2^{S439A}*) by site-directed mutagenesis using Gibson
547 Assembly.

548

549 Seeds were sterilized using 50% chlorine solution for 7 minutes and washed with sterile distilled water
550 three times. Then, 1,500 *Arabidopsis* seedlings were placed into a hydroponic system (Phytatrays) with
551 MS-modified basal salt media without N (Phytotechnology Laboratories, M531) supplemented with 1 mM
552 ammonium as the only N source. Plants were grown under long-day photoperiods (16h light/ 8h dark and
553 a temperature of 22 °C) for 14 days using a plant growth incubator (Percival Scientific, Inc.). At day 15,
554 plants were treated with 5 mM KNO₃ or 5 mM KCl for different time periods as indicated, using a protocol
555 described previously⁷. For the phenotypic study of root response to nitrate, plants were grown as described
556 above and were treated with 5 mM KNO₃ or 5 mM KCl for 3 days.

557

558 **Root architecture analysis.**

559 For root phenotyping, plants were scanned in plates using an Epson Perfection V700 Photo scanner, and
560 root length were measured using Fiji (v1.52). Initiating and emerging lateral roots were analyzed using DIC
561 optics on a Nikon Eclipse 80i microscope, as described⁷. The data were statistically analyzed in the Graph
562 Pad Prism 5 Program.

563

564 **Protein extraction, phosphopeptide enrichment and mass spectrometry analysis.**

565 Proteins were isolated from 1 g of frozen tissue per sample in each experimental condition (2-3 biological
566 replicates). Sample preparation and protein extraction were performed using previously described
567 methods^{52,53}. Phosphopeptide enrichment was performed using 1% (w/v) colloidal CeO₂ into an acidified
568 peptide solution at 1:10 (w/w). Mass spectrometry (MS) and peptide identification were based on protocols
569 described previously⁶⁰. Briefly, the generated spectra were analyzed on LTQ Velos linear ion trap tandem
570 mass spectrometer (Thermo Electron) and phosphorylation sites were identified into a specific amino acid
571 within a peptide by using the variable modification localization score in Agilent Spectrum Mill software¹¹⁸.
572 Proteins were grouped based on their shared, common phosphopeptides using principles of parsimony to
573 address redundancy in proteins. Proteins classified within the same group share the same subset of
574 phosphopeptides. Phosphoprotein levels were quantified using spectral counting, as described
575 previously^{52,53}. MS data were normalized using the total number of spectral counts (SPC) for each MS run.
576 Expressed phosphoproteins were defined by at least one SPC, after the application of quality score cutoff
577 in MS analysis, in minimum two of the tree biological replicates. To identify nitrate-regulated
578 phosphoproteins in *Arabidopsis* roots, raw data were log transformed and quantile normalized using R
579 studio (<https://rstudio.com>) and MEV (<http://mev.tm4.org/>) software. The statistical multifactor analysis of
580 variance (significance: $p < 0.05$) and *pos-hoc* analysis (significance: $p < 0.1$) were performed using R-
581 studio. Encoded genes for phosphoproteins showing a similar pattern were analyzed and visualized using
582 the average-linkage hierarchical clustering performed in Cluster 2.11 software, as described¹¹⁹.

583

584

585

586 **Gene network analysis**

587 *Arabidopsis* encoded genes from our phosphoproteomics data were used. The gene network was generated
588 by integrating different information, including protein-protein interactions from BioGRID⁷⁴, predicted
589 protein-DNA interactions of *Arabidopsis* TFs (DapSeq)^{75,76}, *Arabidopsis* metabolic pathways (KEGG), and
590 miRNA-RNA, as described previously¹⁶. This analysis also included predicted regulatory connections
591 between phosphoproteins that we detected and kinase families. A kinase-substrate analysis, identifying the
592 most significant phosphorylation motifs and their predicted kinases were performed using the Motif-X
593 algorithm⁷⁷ and PhosPhAt Kinase-Target interactions database^{78,120}. The resulting network was visualized
594 using CYTOSCAPE software⁷⁹. Cluster and Gene Ontology analysis into the network were achieved with
595 ClusterMaker¹²¹ and Bingo¹²¹ tools for biological networks in cytoscape, respectively.

597 **Phos-Tag PAGE and western immunoblotting**

598 Affinity-based SDS PAGE identification of phosphorylated PIN2 isoforms were performed based on the
599 protocols by Komis et al.¹²² and the protocol given by the Phos-tag manufacturer (FUJIFILM Waco
600 Chemicals) with slight modifications. *Arabidopsis* seedlings were grown on modified MS plates with
601 ammonium as the only N source for 7 days. Next, seedlings (n≥40) were transferred to 5 mM nitrate
602 amended agar plates and were incubated for 6 hours in light. Root samples were collected and homogenized
603 in liquid N₂ and then extracted with extraction buffer – 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5%
604 Triton X-100, 10 μM MG-132 and 0.1 mM PMSF - supplemented with protease and phosphatase inhibitor
605 cocktails (Roche). Buffer volumes were adjusted to fresh weight (100 μL/100 mg tissues). Homogenized
606 samples were centrifuged (4°C, 15 min, 14000 rpm) and the supernatant was aliquoted (50μg protein/20-
607 25 μL) and incubated at 45°C for 5 min in the presence of SDS loading buffer. Next, samples were loaded
608 onto an acrylamide, Bis-Tris/HCl gel containing 50 μmol/L Phos-tagTM (AAL-107) pendant and Zn²⁺ as
609 cation. Electrophoresis were run at 15 mA/gel for 5~ 6 hours or until the proteins are nicely separated
610 (usually until the 25 kDa prestained protein marker just exit the gel assembly). Next gels were incubated
611 for 30 minutes in transfer buffer containing 10 mM EDTA and were blotted to PVDF membranes using
612 Tris/Glycine transfer buffer (25 mM Tris, 192 mM Glycine, 5% methanol). After blocking with 5% milk
613 in TBST, the membrane was probed with α-PIN2 antibody (1:1000) for 2 h (RT) followed by α-rabbit IgG-
614 HRP (1:15000) (Amersham) for 1 h (RT). After treating the membrane with Supersignal West Femto
615 western chemiluminescent HRP substrate (Thermo Fisher Scientific), luminescent signals were detected
616 using a liquid nitrogen-cooled charge-coupled device camera (Biorad). Digital images were analyzed, and
617 signals were quantified using the Fiji software.

619 **Confocal microscopy and image analysis**

620 *eir1-1* mutants complemented with *PIN2::PIN2^{wt}-GFP*, *PIN2::PIN2^{S439A}-GFP*, *PIN2::PIN2^{S439D}-GFP* were
621 grown in modified MS plates with 1 mM ammonium as the only N source. 2 week-old plants were treated
622 with 5 mM nitrate or without treatment (control conditions) for 2 hours. Roots were stained with propidium
623 iodide (PI) and mounted on a slide for microscopic analysis. Images were acquired with Zeiss LSM880
624 confocal microscope with Airyscan equipped with a 40×Plan-Apochromat water immersion objective.
625 Fluorescence signals for GFP (excitation 488 nm, emission 507 nm) and PI (excitation 536 nm, emission
626 617 nm) were detected. Microscopy

628 For image quantification (PIN2-GFP fluorescence intensity measurements) maximum intensity projections
629 of confocal pictures were used in epidermis and cortex cells. Images were handled and analyzed with Zeiss
630 blue (v3.1) and Fiji (v1.52) software. The experiment was performed with 3 biological replicates and 3-4
631 roots per experimental conditions were analyzed in each replicate.

632
633
634
635
636

637 **Figure Legends**

638

639 **Figure 1. Characterization of the phosphoproteome profile in *Arabidopsis* roots in response to nitrate.**

640 Venn diagrams show the overlap of transient (5 min) and more persistent (20 min) changes in
641 phosphoprotein relative-abundance after 5 mM nitrate treatments compared with each control condition (5
642 mM KCl) in *Arabidopsis* roots. (A) Overlap of phosphoproteins that were “up-regulated” by nitrate at 5
643 min (dark yellow) and 20 min (light yellow) (B) Overlap of phosphoproteins that were “down-regulated”
644 by nitrate at 5 min (blue) and 20 min (light blue). (C) Identification of common phosphoproteins with
645 published phosphoproteome data-sets in response to nitrate-deprivation⁵⁰ or nitrate-resupply⁴⁹.

646

647 **Figure 2. Functional analysis of the phosphoproteome profile reveals signaling and regulatory**
648 **processes occurring in early and late response to nitrate.** The Figure showed a hierarchical clustering

649 of nitrate-phosphoproteins with differential abundance at 5- or 20-min in response to nitrate.
650 Phosphoproteins were normalized by Z-score and clustered using the Euclidean distance method with
651 average linkage. The resulting clusters are shown as a heat map, where vertical bars and numbers to the
652 right of the map denote the group (composed of all terminal nodes in the hierarchical tree) of
653 phosphoprotein (correlation > 0.9) with a selected profile in early (5-min) or late (20-min) nitrate-response.
654 Functional Gene Ontology (GO) categories significantly enriched (hypergeometric test with FDR, $p < 0.05$)
655 in each cluster are highlighted to the right of the group.

656

657

658 **Figure 3. Characterization of the phosphoproteome profile in response to nitrate in roots of**
659 ***Arabidopsis* (Col-0) or *chl1-5* mutant plants.** Percentage of nitrate-phosphoproteome responses at 5 min
660 in wild-type plants that were maintained in *chl1-5*-mutant plant roots.

661

662 **Figure 4. Gene network of nitrate-modulated phosphoproteins.** (A) A network for the phosphoproteins
663 (encoded genes) and putative kinases family were represented as nodes with color and shapes assigned
664 according to function (e.g., blue squares: metabolic encoded-genes, green triangle: transcription factors or
665 grey hexagon: kinase families). Edges connecting nodes represent functional interactions: transcription
666 factor/TARGET regulation or predicted kinase-substrate control. The size of the triangle or hexagon is
667 proportional to the number of targets of the TF or putative kinase family, respectively. (B) Over-represented
668 biological processes in the phosphoproteins gene-network in response to nitrate (hypergeometric test with
669 FDR, $p < 0.05$). The network was analyzed using BINGO and ClueGO tools in Cytoscape software. Over-
670 represented gene ontology terms are shown as a node connected by edges based on semantic relationships
671 as defined in the ontology.

672

673 **Figure 5. Nitrate regulates PIN2 phosphorylation levels.** (A) Schematic representation of the

674 phosphosite (Ser 439) identified in PIN2 in our study. PIN2 protein possesses three specific regions: a
675 region with five transmembrane segments (residues 1–163), a central hydrophilic loop extending from
676 residue 164 to 482 and a region with five additional transmembrane segments (residues 483–647). Protein
677 alignment with PIN2 or PIN-like proteins from different species indicates conservation of the serine residue
678 in eudicot, monocot, and gymnosperm species. The red arrow highlights the S439 in the central loop of
679 PIN2 proteins in different plants. The size of each protein in amino acids and the percentage of identity
680 against the *Arabidopsis* PIN2 are indicated. (B) Levels of PIN2 phosphopeptide in our experiments. Bars
681 represent the mean plus standard error of replicates. The asterisk indicates statistically significant
682 differences ($p < 0.05$). (C) Detection of phosphorylation PIN2 by Phos-tag Western blotting. *Arabidopsis*
683 plants (Col-0) were growth in ammonium as only nitrogen source, and treated with 5 mM KNO₃ or 5mM
684 KCl as control. Total protein from roots were analyzed in SDS PAGE using Phos-tag to detect changes in
685 phosphorylation status. Immunoblotting was performed with PIN2 antibody. Total proteins isolated from
686 *eir1.1* roots were used as a negative control. (D) Western blot against PIN2 protein comparing nitrate treated
687 (KNO₃) and control (0) condition in *Arabidopsis* roots for all genotypes (*eir1-1* mutant background was

688 complemented with PIN2::PIN2^{wt}-GFP (PIN2^{wt}), PIN2::PIN2^{S439D}-GFP (PIN2^{S439D} phospho-mimic point
689 mutation) or PIN2::PIN2^{S439A}-GFP (PIN2^{S439A}, phospho-null point mutation). (E) Time-course analysis of
690 PIN2 mRNA levels in response to nitrate treatments in *Arabidopsis* roots.

691
692

693 **Figure 6. PIN2 is essential for nitrate regulation of primary root growth and lateral root density.** (A)
694 Primary root length of Col-0 wild type plants or *eir1-1* mutant plants was measured using the ImageJ
695 program 3 days after 5 mM KNO₃ or KCl treatments. (B) Number of initiating and emerging lateral roots
696 of Col-0 or *eir1-1* mutant plants were counted using light microscopy 3 days after 5 mM KNO₃ or KCl
697 treatments. Bars represent mean plus standard error of replicate experiments. Asterisk indicates statistically
698 significant difference between means (** $p < 0.01$).

699

700 **Figure 7. PIN2 dephosphorylation at S439 is important for modulation of primary root growth and**
701 **lateral root density in response to nitrate treatments.** Four different genotypes were used in these
702 experiments: *Arabidopsis* Col-0, *eir1-1* mutant background complemented with PIN2::PIN2^{wt}-GFP
703 (PIN2^{wt}), PIN2::PIN2^{S439D}-GFP (PIN2^{S439D} phospho-mimic point mutation) or PIN2::PIN2^{S439A}-GFP
704 (PIN2^{S439A}, phospho-null point mutation). All genotypes were grown hydroponically as described in
705 Methods and treated with 5 mM nitrate or KCl (A) Primary root length of the different genotypes was
706 measured using the ImageJ program 3 days after 5 mM KNO₃ or KCl treatments (B) Number of initiating
707 and emerging lateral roots for all genotypes were counted using light microscopy 3 days after 5 mM KNO₃
708 or KCl treatments. Bars represent mean plus standard errors. Asterisk indicate statistically significant
709 difference between means (* $p < 0.05$, ** $p < 0.01$).

710

711 **Figure 8. PIN2 phosphorylation at S439 is important for modulation of protein localization in**
712 **response to nitrate treatments.** Three different genotypes were used in these experiments. *eir1-1* mutant
713 background complemented with PIN2::PIN2^{wt}-GFP (PIN2^{wt}), PIN2::PIN2^{S439D}-GFP (PIN2^{S439D} phospho-
714 mimic point mutation) or PIN2::PIN2^{S439A}-GFP (PIN2^{S439A}, phospho-null point mutation). All genotypes
715 were grown hydroponically as described in Methods and treated with 5 mM nitrate for 2 hours. (A) Confocal
716 microscopy using Zeiss Airyscan microscope and Zeiss-blue3.1 software was used to visualize PIN2 in
717 *Arabidopsis* roots. “e” denotes epidermis and “c” cortex. Scale bar = 20 μ m. Tuckey box plots show PIN2-
718 GFP fluorescence intensity quantification (number of roots analyzed per experimental conditions = 8-10;
719 arbitrary units, a.u.) at the total cell membrane in roots (B), and from epidermal (C) and cortex (D) plasma
720 membrane in the different genotypes. Letters denote statistically significant difference between means as
721 determined by ANOVA analysis ($p < 0.05$).

722

723 **Figure 9. Schematic model of the role of PIN2 and phosphorylation in root nitrate responses.** Nitrate
724 treatments increase cytosolic calcium levels which interact with calcium binding proteins such as kinases
725 in the nitrate signaling pathway. Kinases phosphorylate protein targets such as transcription factors that can
726 mediate changes in gene expression in response to nitrate treatments. Nitrate can also cause
727 dephosphorylation of specific proteins. We showed nitrate treatments promote PIN2 dephosphorylation at
728 S439 which impact membrane localization and polarity of the PIN2 protein. This post-translational
729 regulatory mechanism is important for modulation of primary root growth and lateral root density in
730 response to nitrate treatments. Arrows and bar-headed solid lines represent activation or inhibition in
731 response to nitrate, respectively. Dashed arrows indicate proposed connections.

732

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735

736 **Figure S1. An overview of the root phosphoproteomic experiment.** (A) Experimental design to identify
737 changes in phosphoproteins in response to nitrate treatments in *Arabidopsis thaliana* roots. (B)
738 Phosphoproteomic strategy for the enrichment, quantification and analysis of phosphopeptides.

739
740 **Figure S2. An overview of the root phosphoproteomic analysis.** (A) Distribution of the number of
741 phosphosites per peptide for the indicated experimental data set (0, 5 or 20 minutes after nitrate treatment).
742 (B) Relative distribution of phosphorylated residues in each peptide for indicated experimental data set. (C)
743 Distribution of the number of phospho-peptides per protein for indicated experimental data set. (D)
744 Percentage of phospho-proteins that present each phosphorylated residues for indicated data set.

745
746 **Figure S3.** Distribution of phosphoproteins across GO categories: biological process (A), cellular functions
747 (B) and sub cellular compartment (C) for each experimental data set.

748
749 **Figure S4. Comparison between phosphoproteomics and transcriptomic dataset in response to**
750 **nitrate.** The list of encoded genes in response to nitrate at transcriptomic (data-set 27 affymetrix experiment
751 Canales et al., 2014) and phosphoproteomic (our work) levels were represented using the SUNGEAR tool
752 (Poultney et al., 2007). The triangle shows each data set at the vertices: transcriptomic data-set,
753 phosphoproteomic data (5 min), phosphoproteomic data (20 min). The size of the circles inside the figure
754 are proportional with the number of genes in each data set, as indicated by the arrows around the circle.
755 The number of genes is indicated in each circle.

756
757 **Figure S5. Motif-X analysis of nitrate-responsive phosphopeptides.** Amino acid sequences from -5 to
758 +5 residues (the phosphorylated residue was 0) were scanned with the Motif-X algorithm. Motif list of
759 phosphopeptides that met the criteria for phosphoproteins up-regulated (A) and down-regulated (B) by
760 nitrate at 5 min. Motif list of phosphopeptides that met the criteria for phosphoproteins up-regulated (C)
761 and down-regulated (D) by nitrate at 20 min See table S1 for the complete set of nitrate-regulated
762 phosphoproteins.

763
764 **Figure S6. Phosphoprotein PIN2 (S439) in root gravitropic response.** *pin2* null mutant background
765 (*eir1-1*) complemented with non-modified or wild type version of PIN2 (PIN2^{wt}), phospho-null (S439A) or
766 phospho-mimic (S439D) versions of PIN2 (PIN2^{S439A} or PIN2^{S439D}, respectively) were grown in MS agar
767 plate supplemented with 0 mM (A) or 5 mM (B) nitrate. At day 7, plates were rotated 90° and root curvature
768 was measured at 24 hours with imageJ software. The figure shown a histogram of root gravitropic response
769 where the length of black bars indicates the relative frequency of plants with the corresponding classes of
770 angle. The average (μ) plus standard deviation of seedlings scored per line is indicated below each circle.
771 Asterisk indicate statistically significant difference between means (** $p < 0.01$).

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775 **References**
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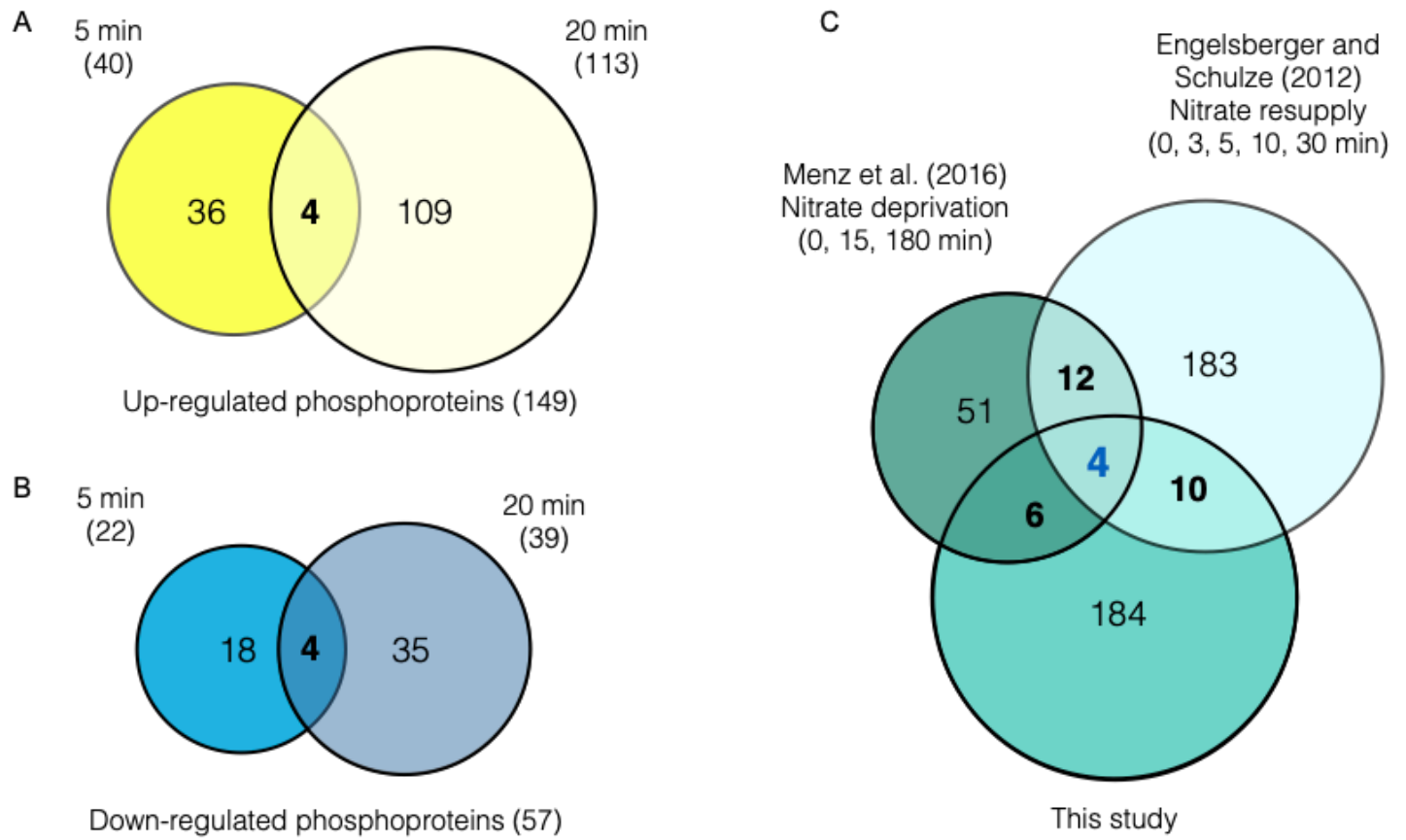


Figure 1

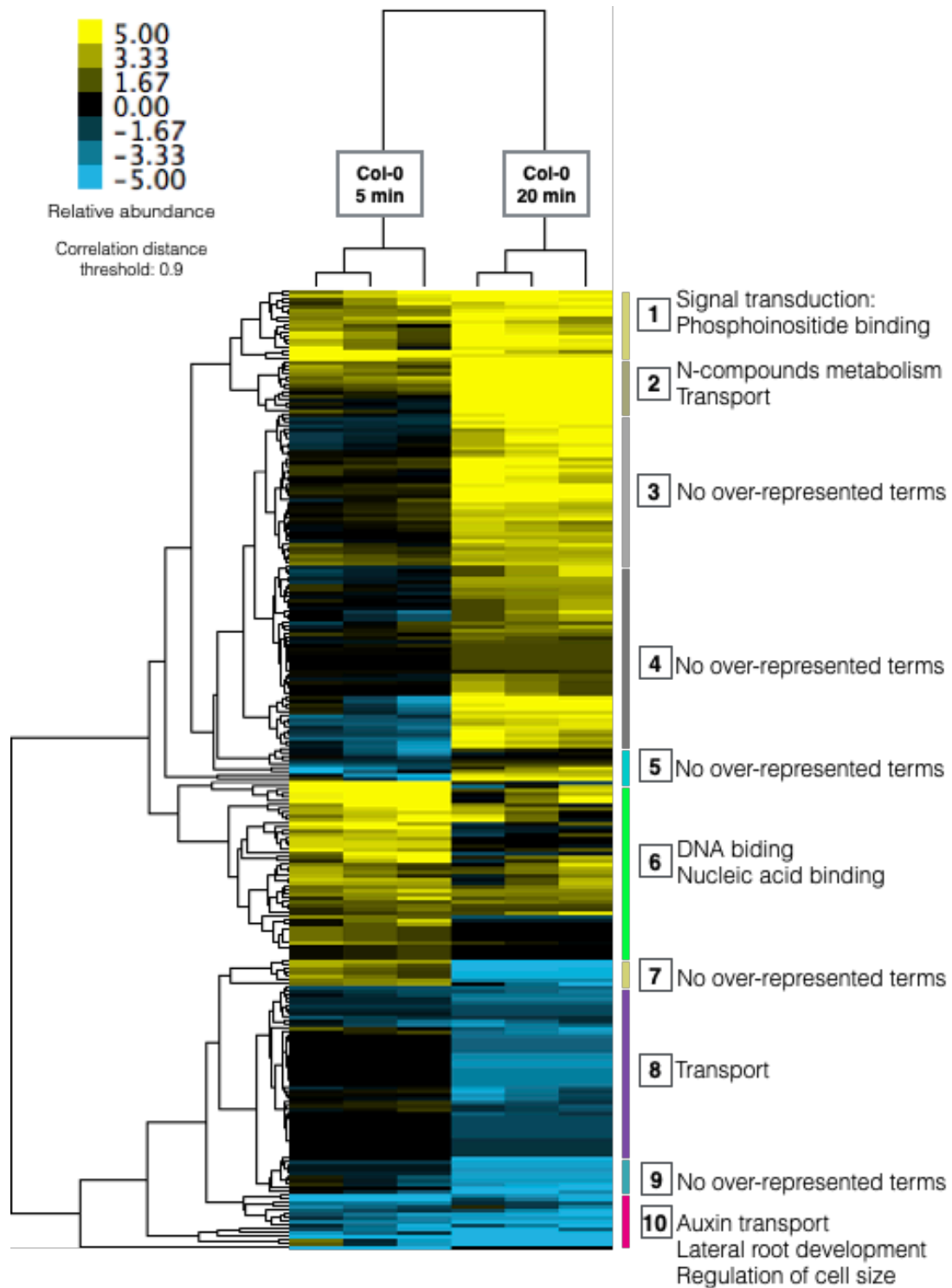


Figure 2

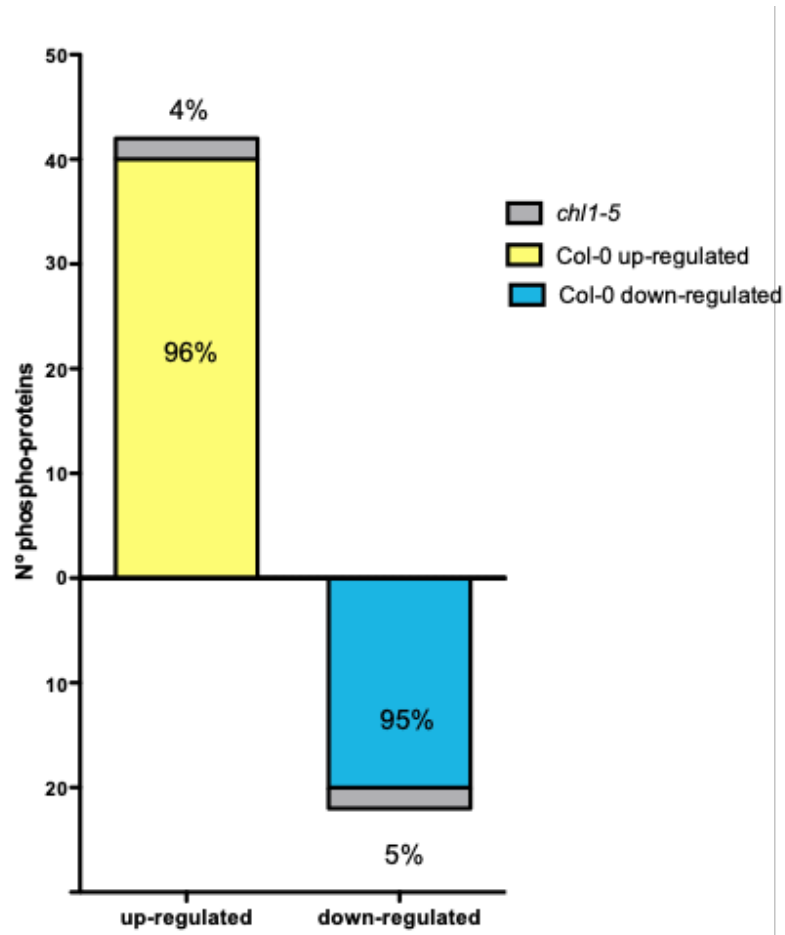
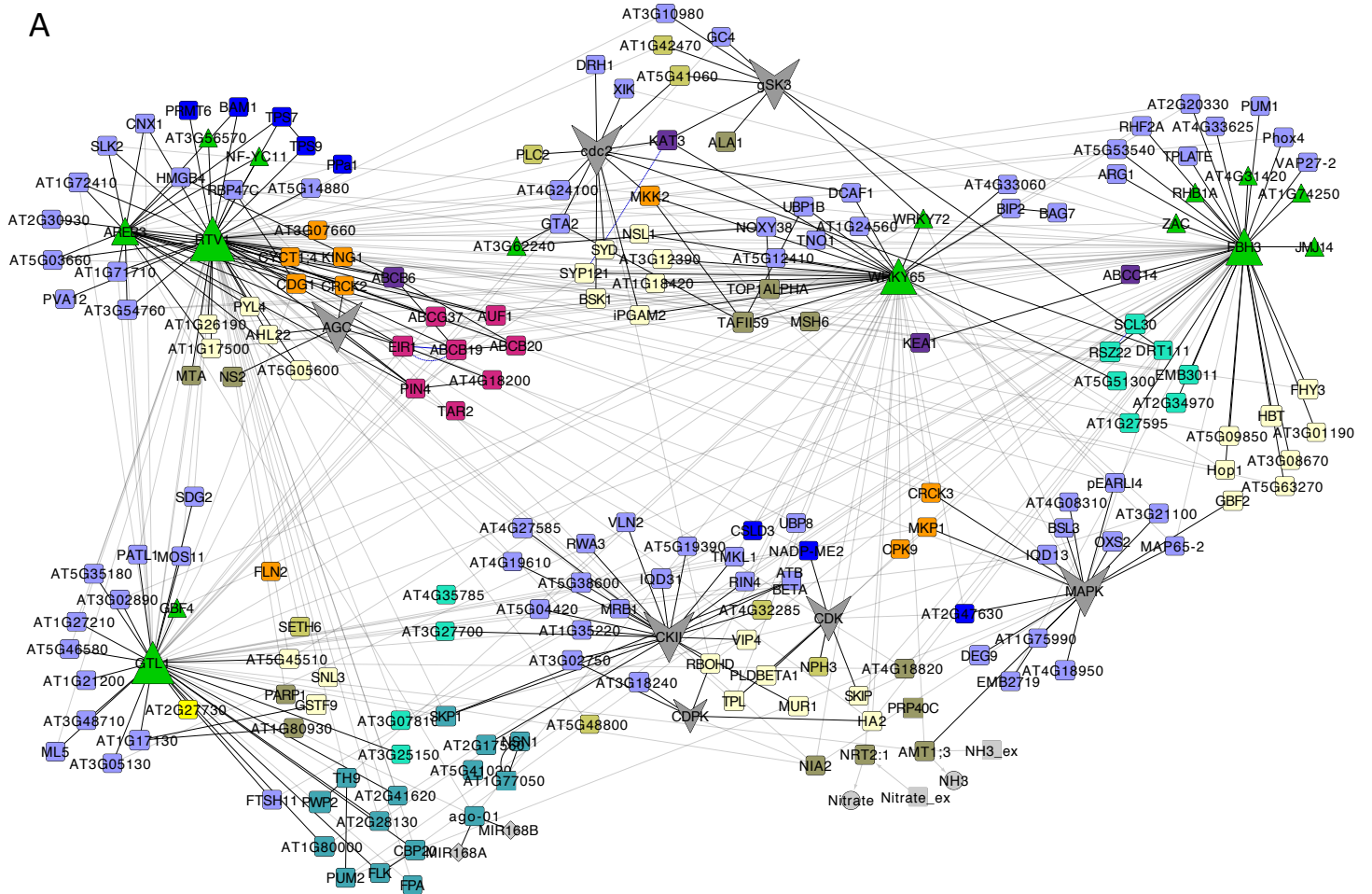


Figure 3

A



B

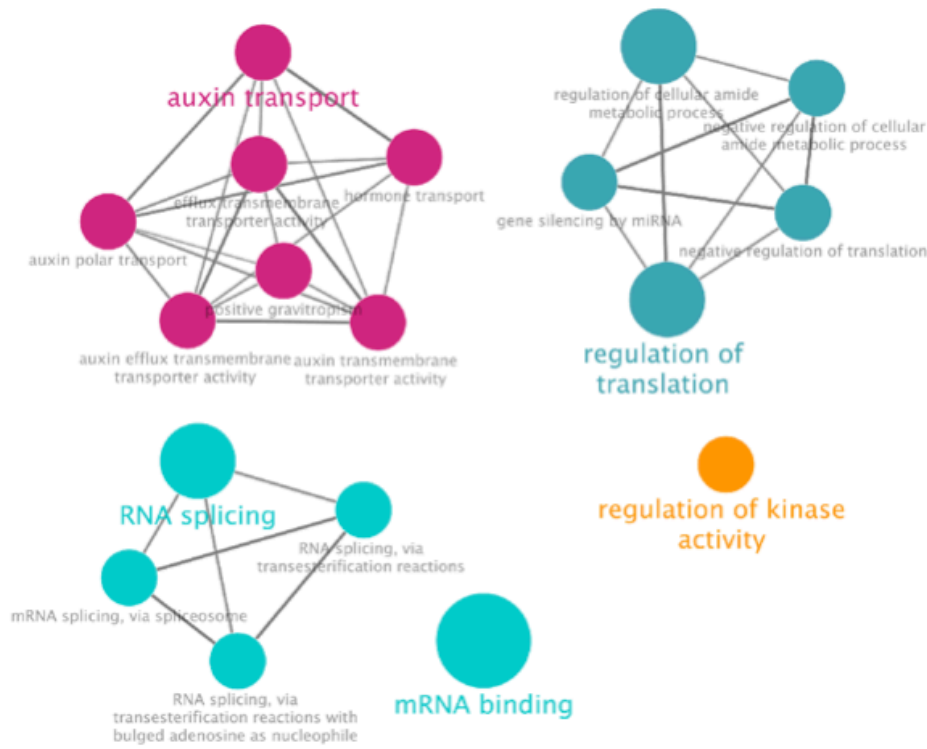


Figure 4

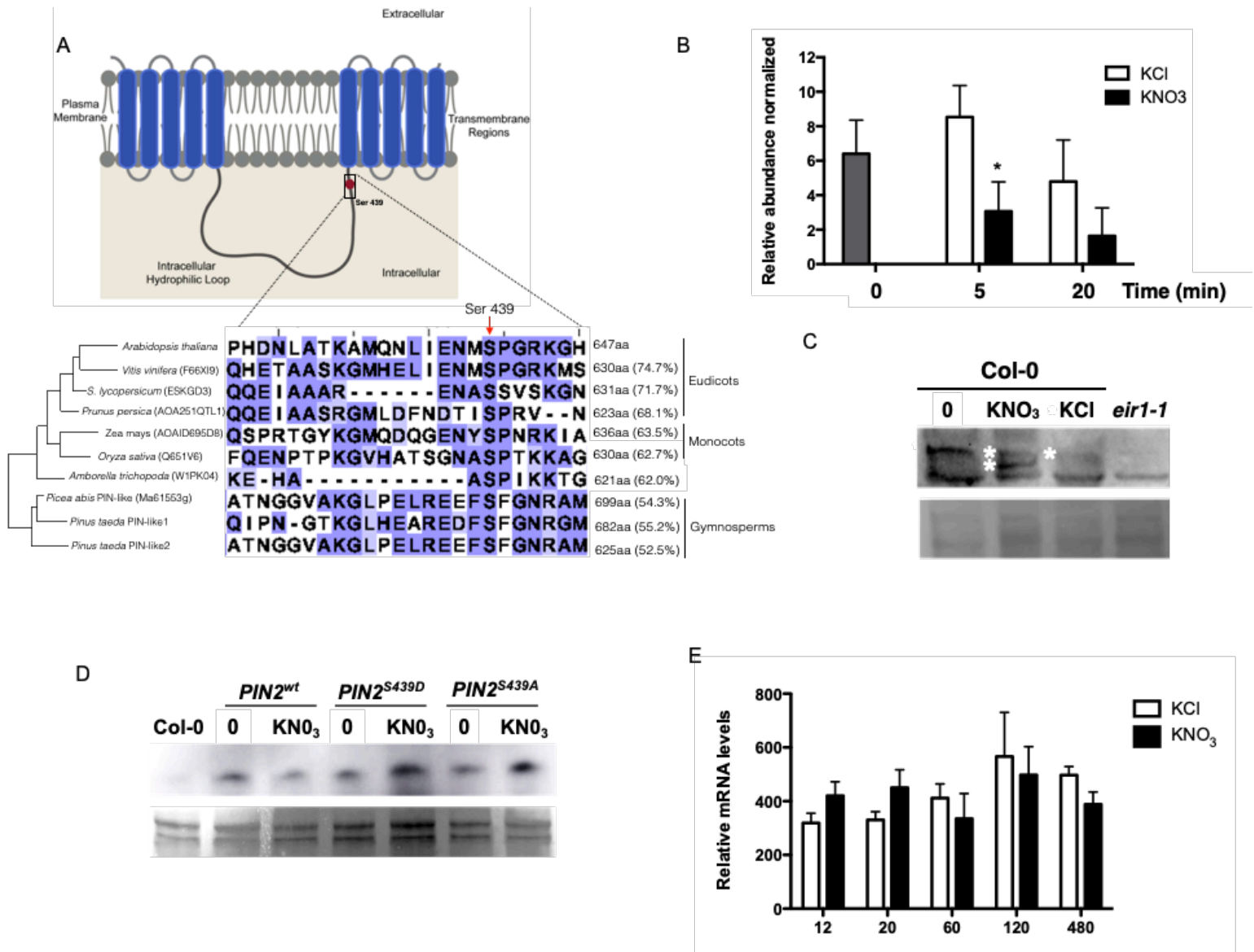
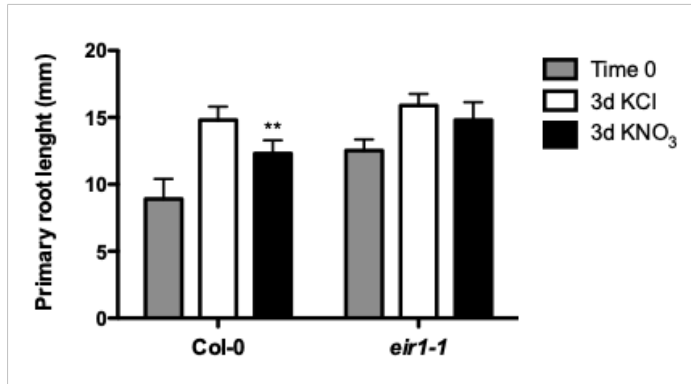


Figure 5

A



B

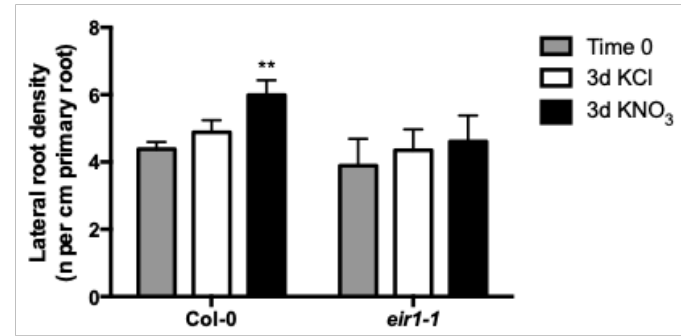


Figure 6

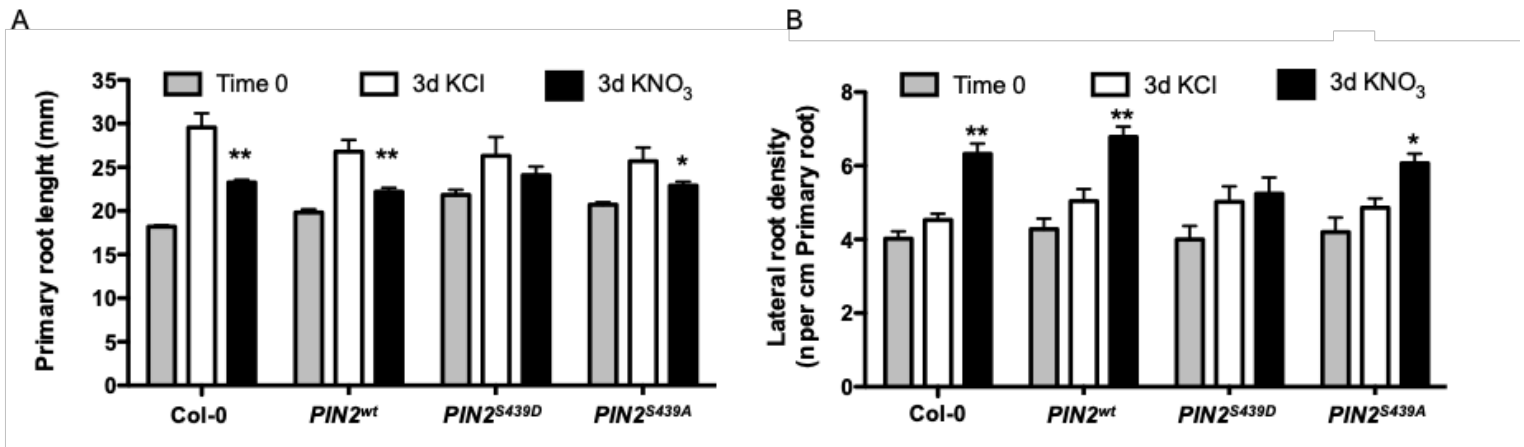
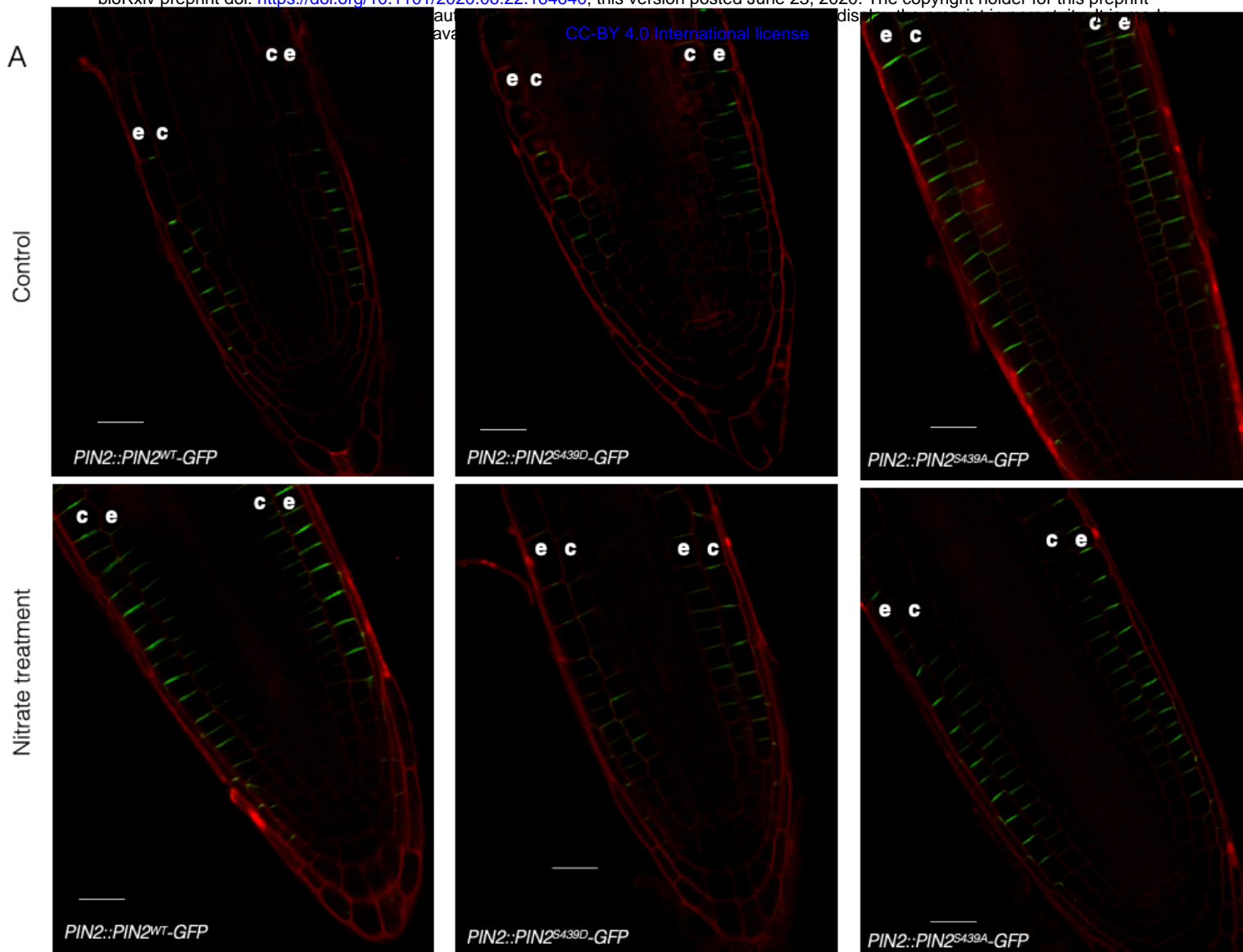
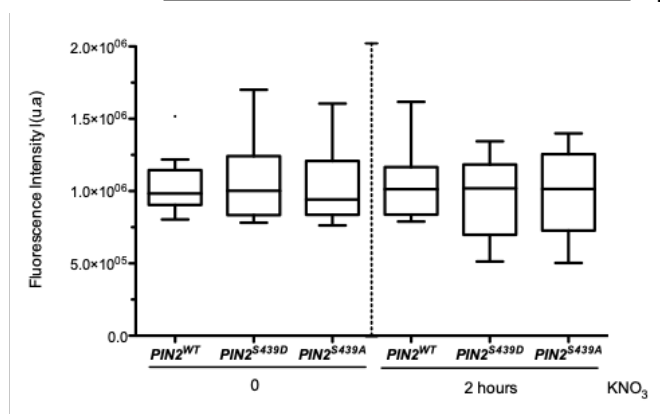


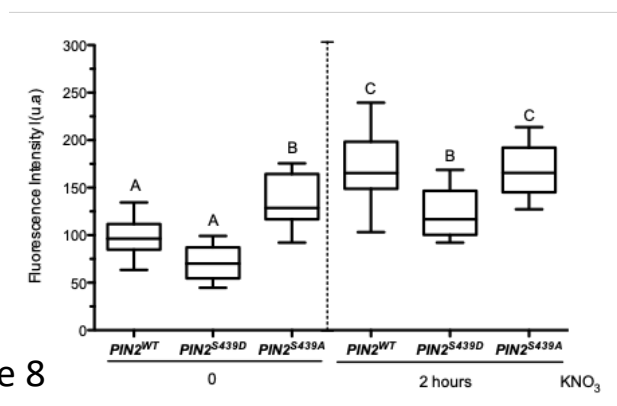
Figure 7



B



C



D

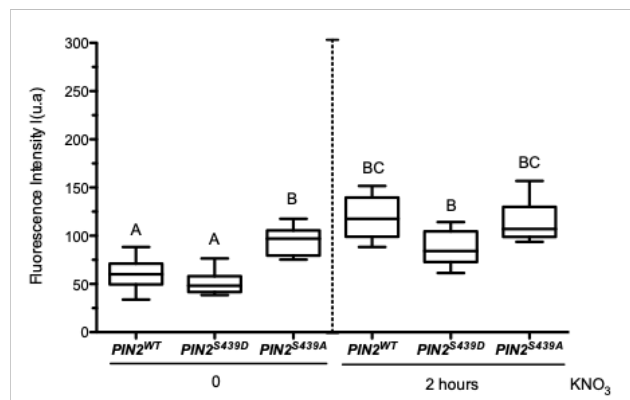


Figure 8

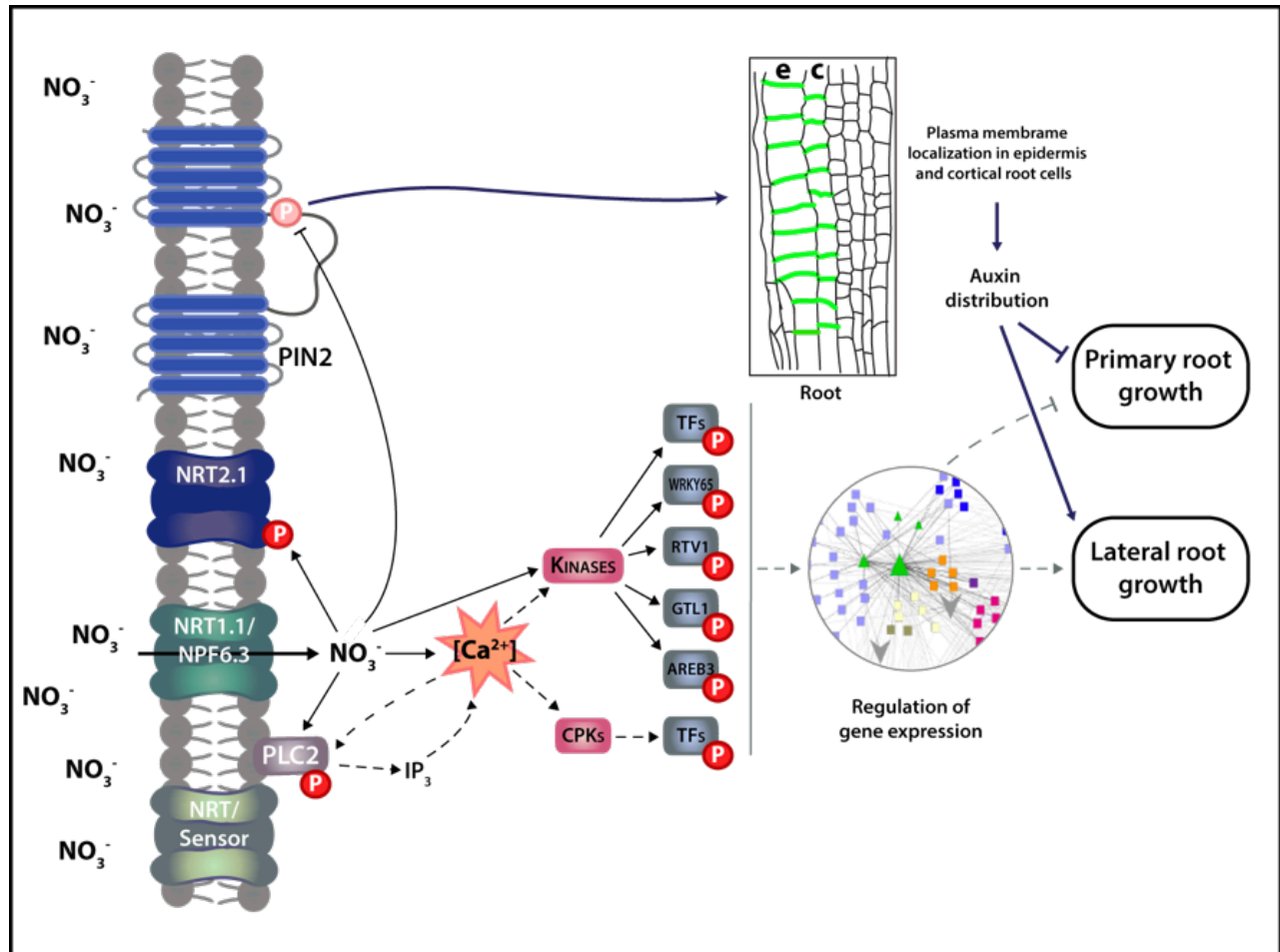


Figure 9

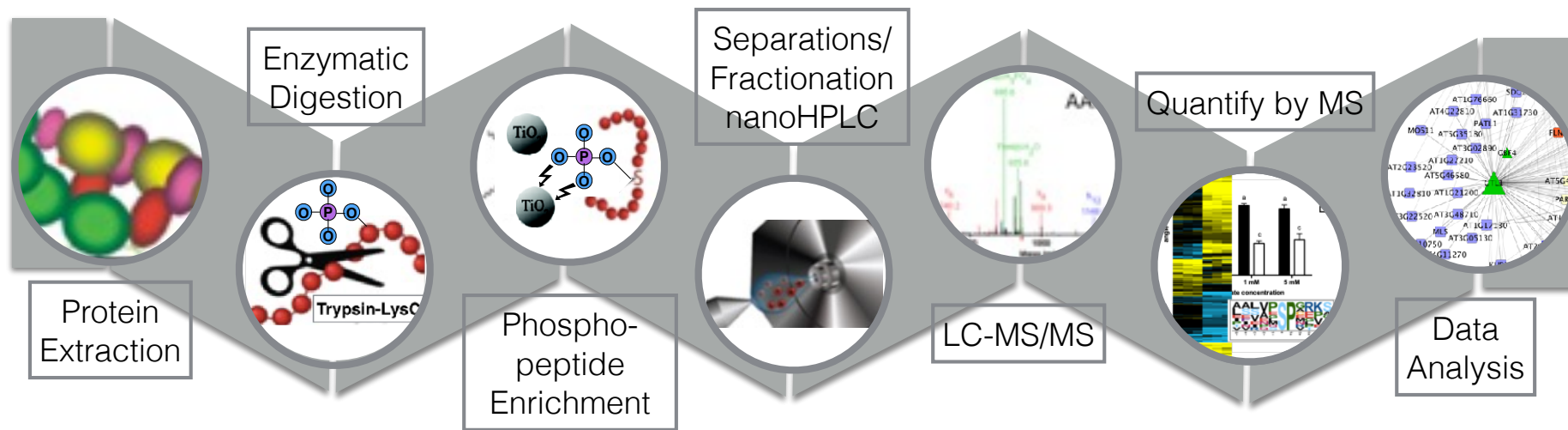
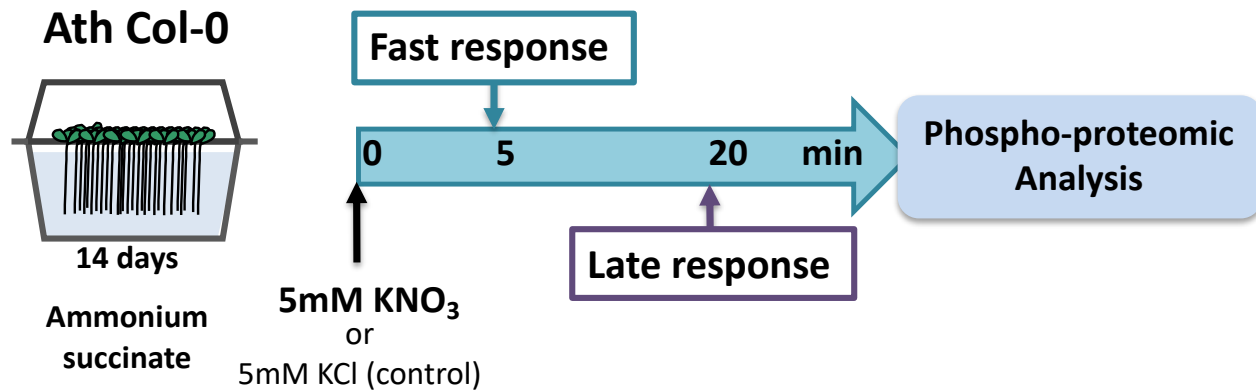


Figure S1

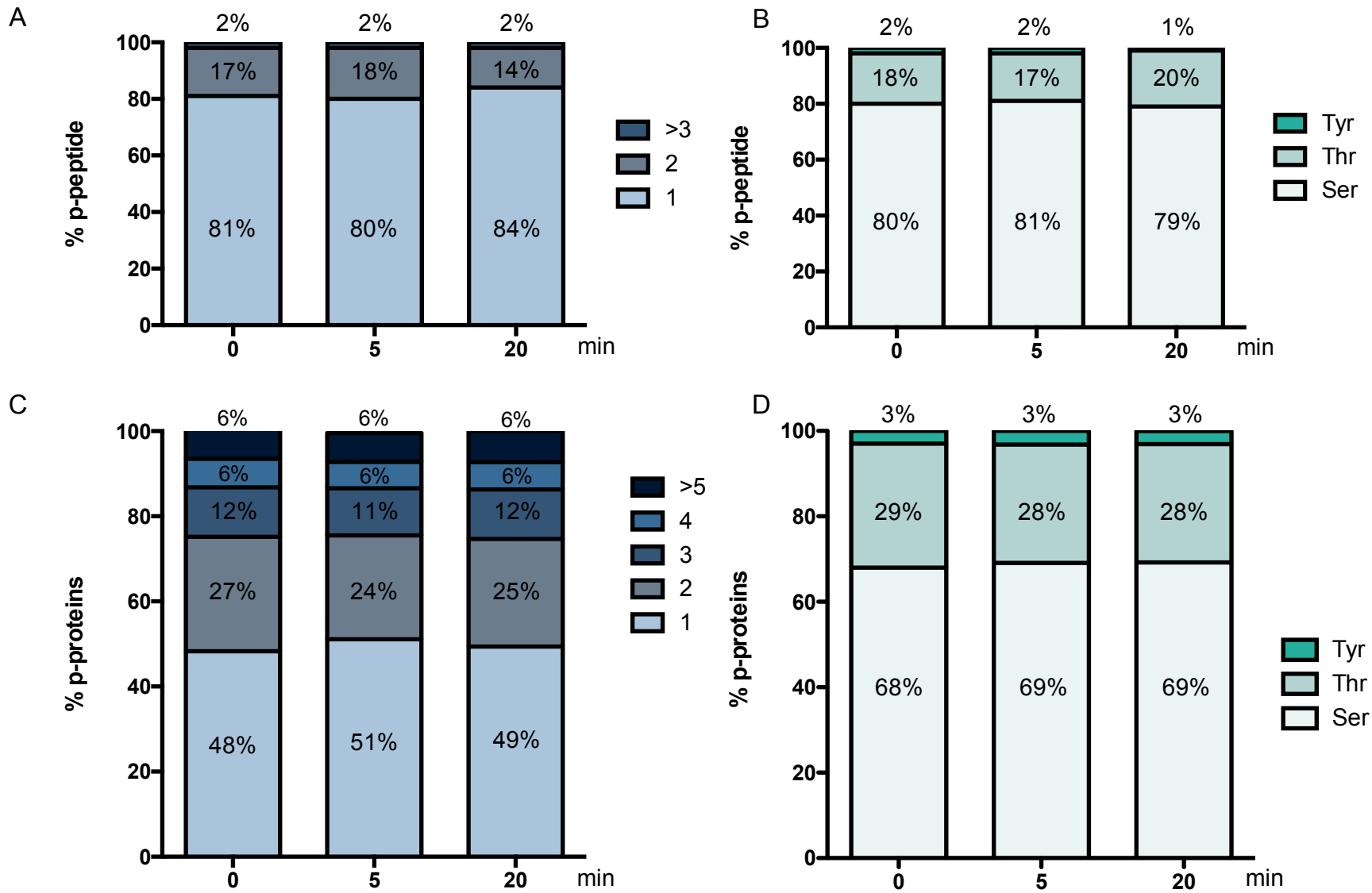


Figure S2.

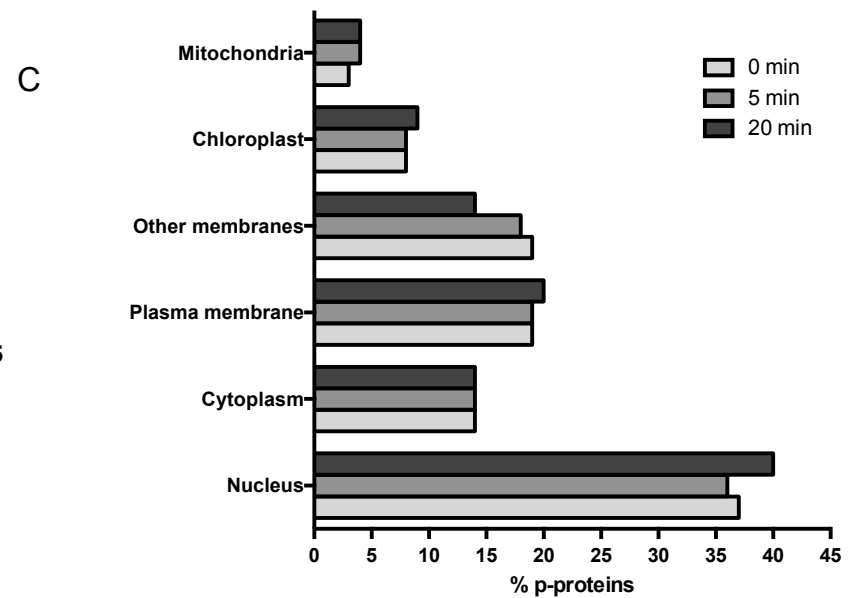
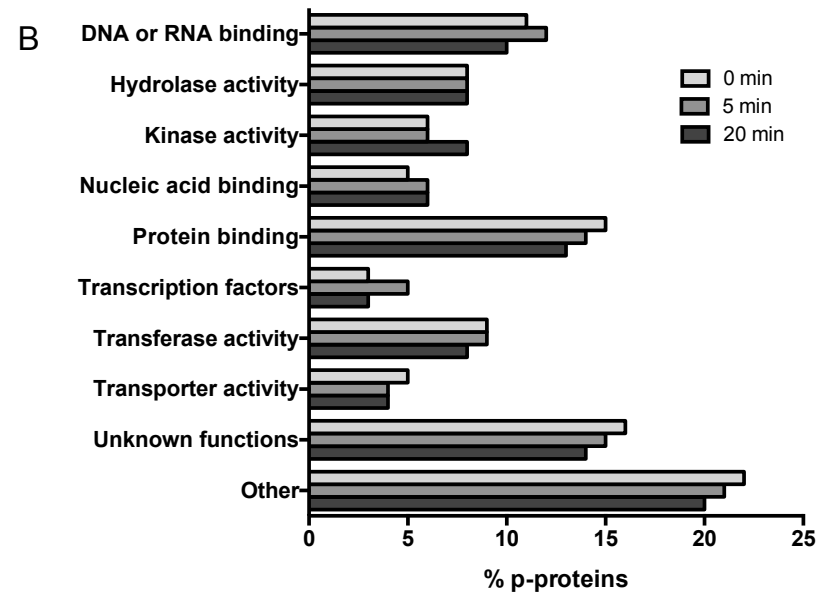
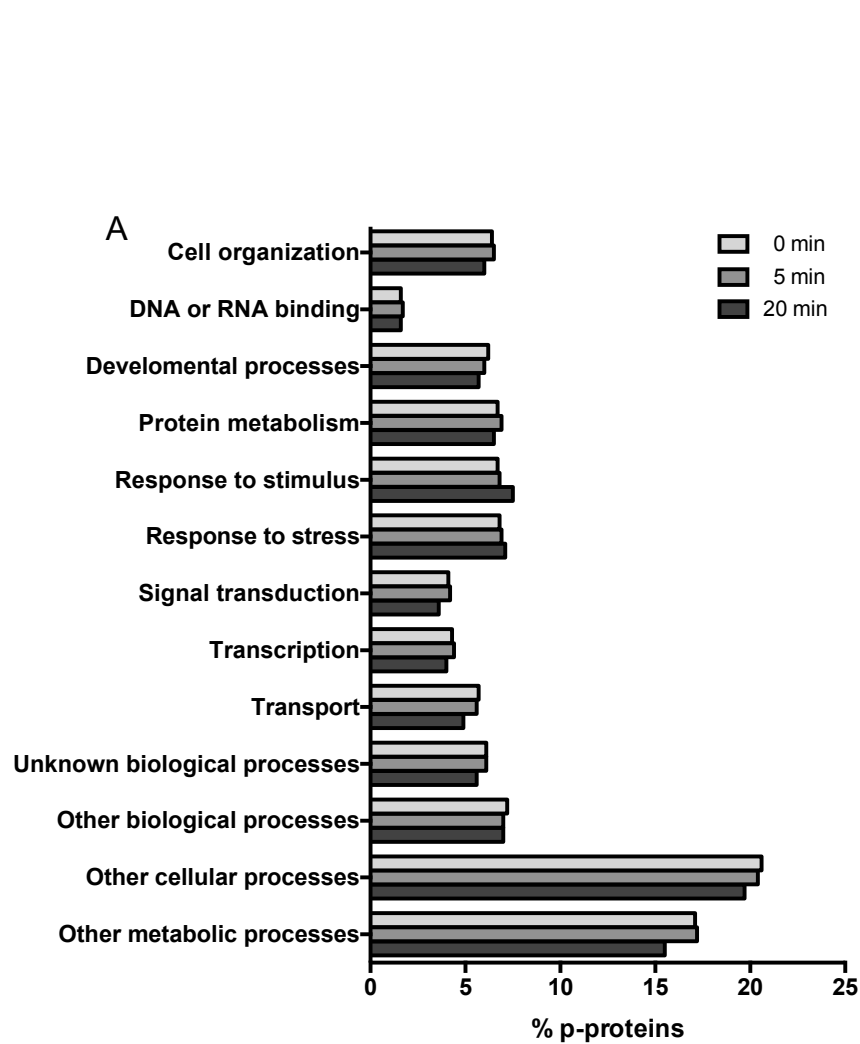


Figure S3.

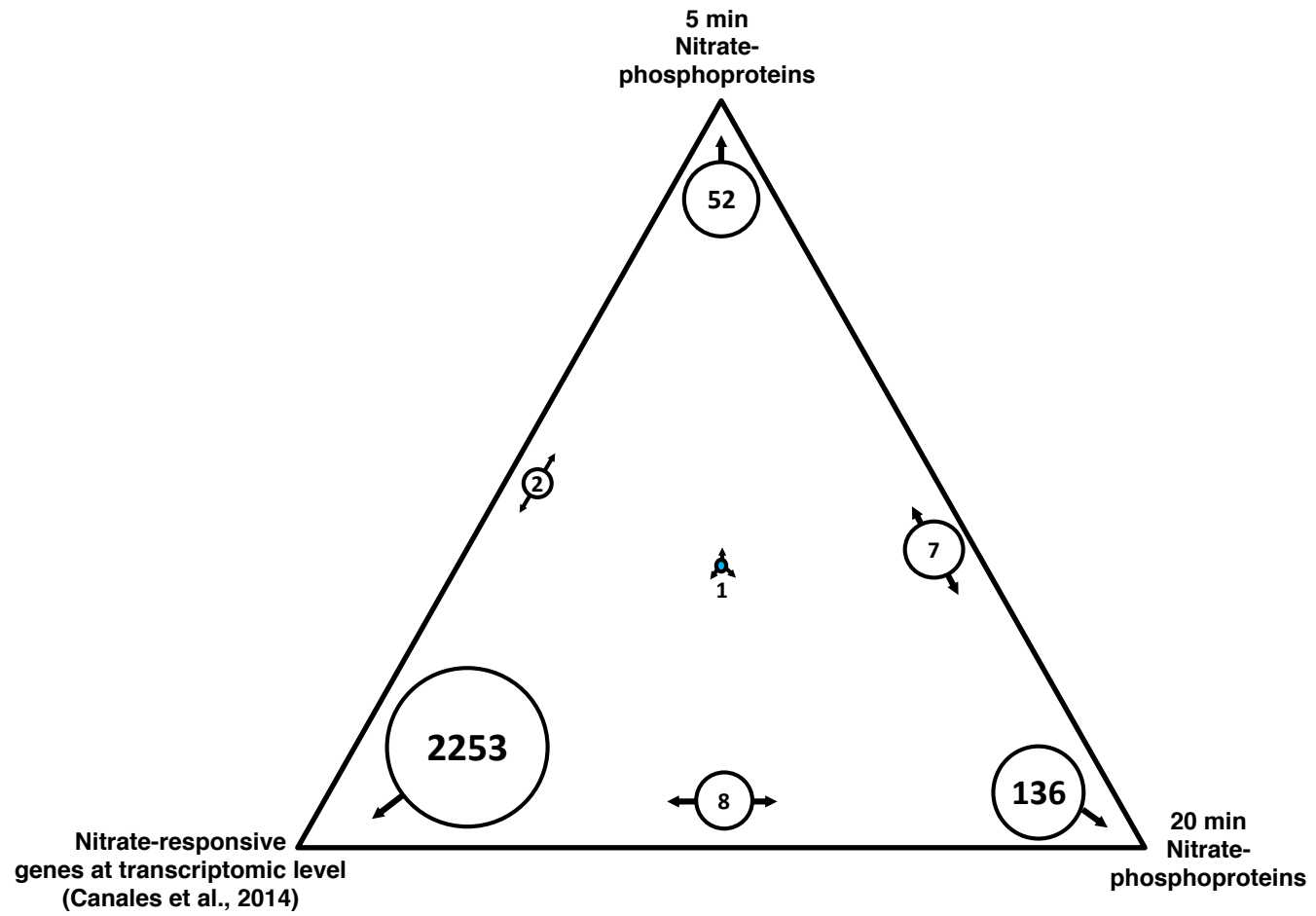
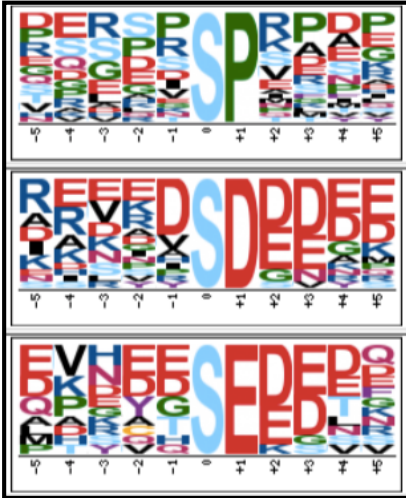


Figure S4.

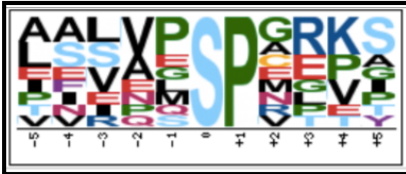
A

Motif Logo



B

Motif Logo



C

Motif Logo



D

Motif Logo

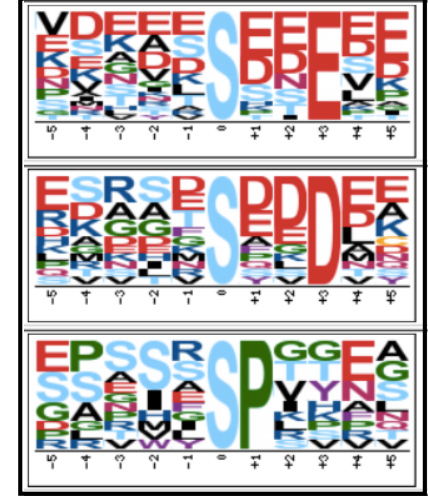


Figure S5

A

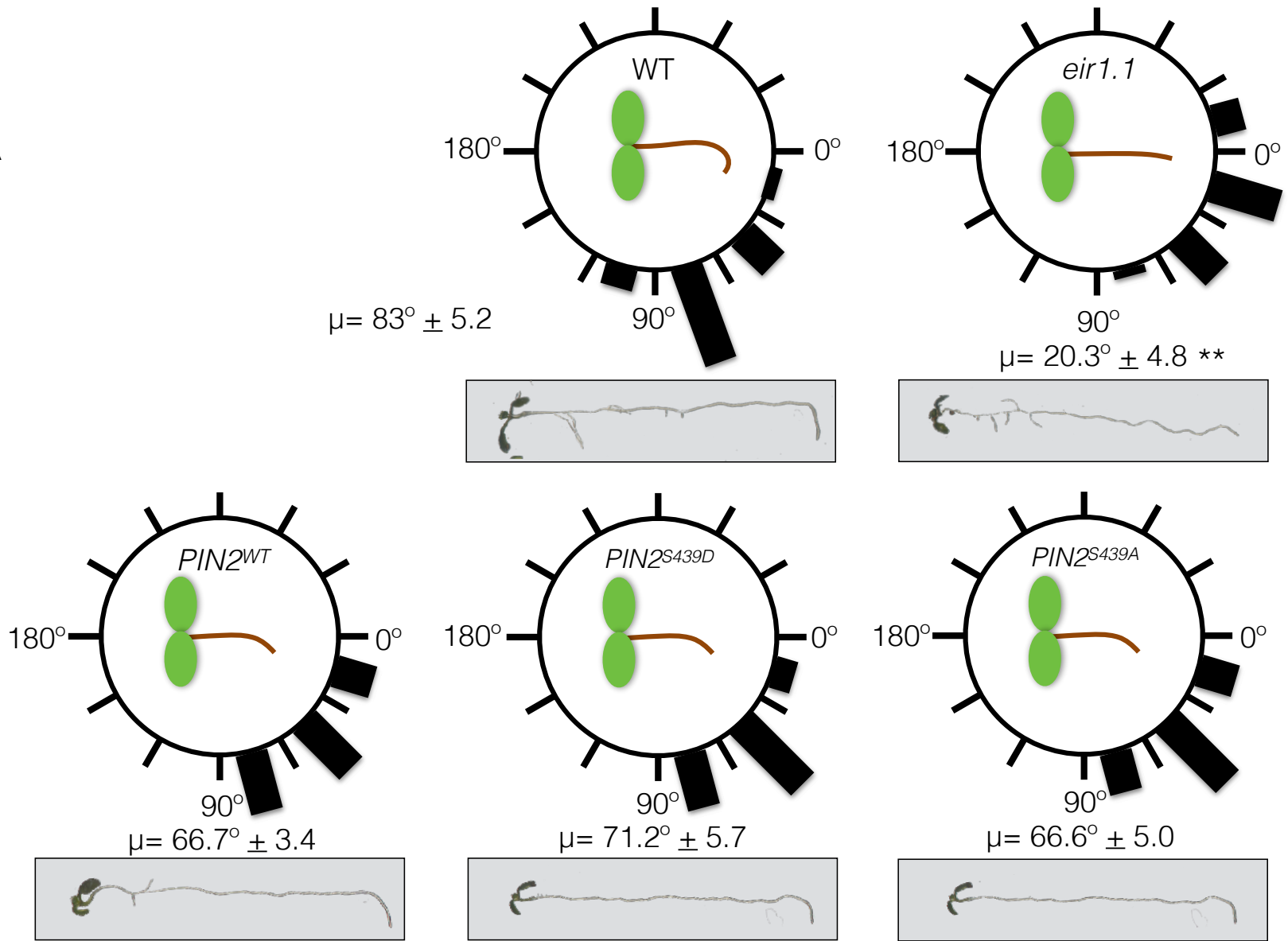


Figure S6.

B

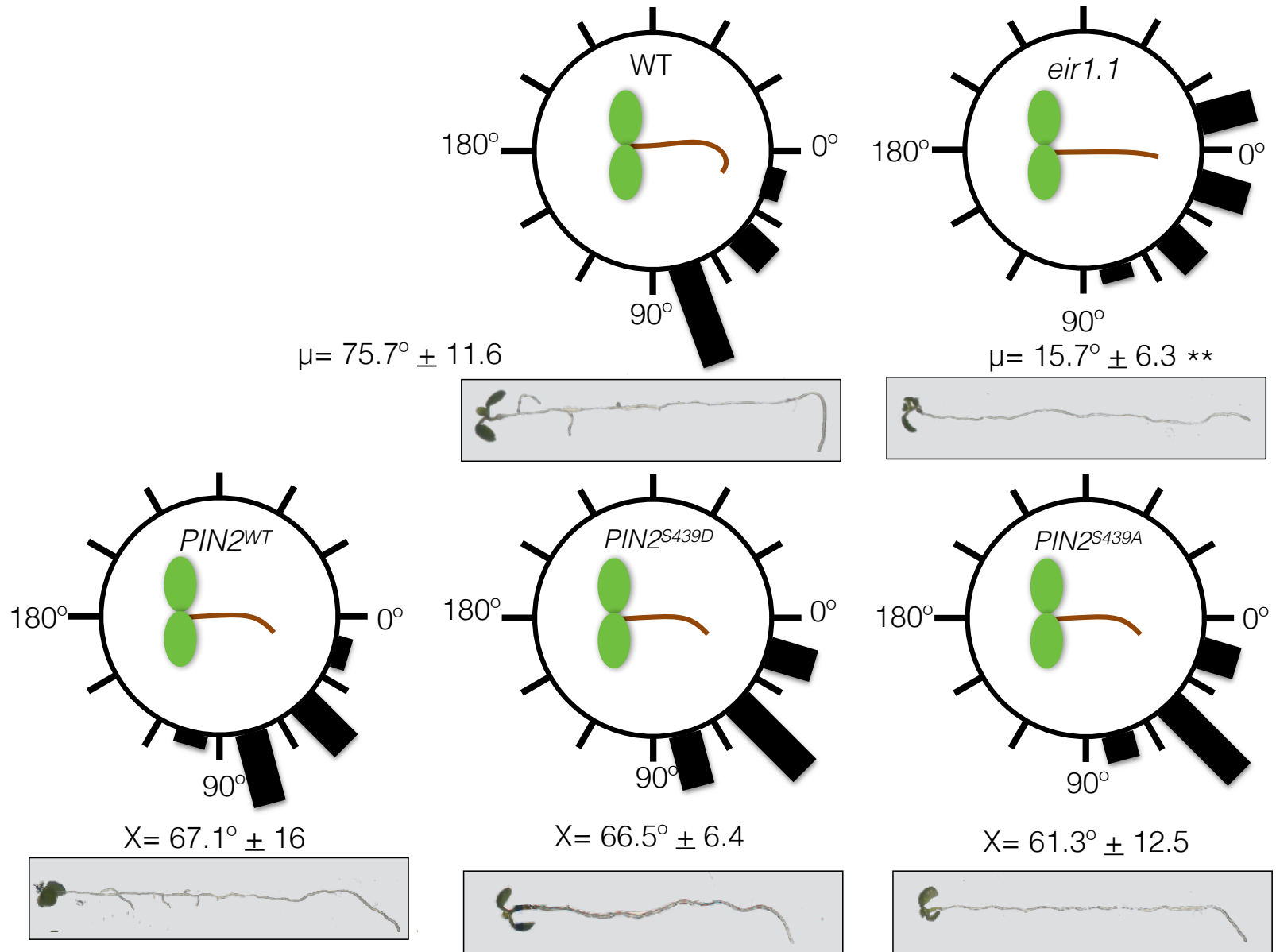


Figure S6.