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## **Endoplasmic reticulum calnexins participate in the primary root growth response to phosphate deficiency**

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**Short title:** Calnexin and phosphate deficiency

**One sentence summary:** Calnexin, a lectin chaperone engaged in the folding of N-glycosylated proteins in the ER, participates in primary root adaptation to low phosphate conditions.

29 **Footnotes**

30

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42 J Montpetit and YP conceived the project. JC contributed to gene expression analysis as well  
43 as confocal and optical microscopy, YFH and EV contributed to GUS and GFP analysis, and  
44 J Montpetit performed all other experiments. J Müller and SA helped with the analysis of Fe  
45 deposition, and RS helped generate several plant lines. YP and JC wrote the manuscript. All  
46 authors read and approved the final manuscript. YP agrees to serve as the author responsible  
47 for contact and ensures communication.

48

49 **Abstract**

50 Accumulation of incompletely folded proteins in the endoplasmic reticulum (ER) leads to ER  
51 stress, activates ER protein degradation pathways, and upregulates genes involved in protein  
52 folding (Unfolded Protein Response; UPR). ER stress has been associated with abiotic stress  
53 conditions that affect protein folding, including salt stress. However, the role of ER protein  
54 folding in plant responses to nutrient deficiencies is unclear. We analyzed several *Arabidopsis*  
55 *thaliana* mutants affected in ER protein quality control and established that both *CALNEXIN*  
56 (*CNX*) genes function in the primary root's response to phosphate (Pi) deficiency. *CNX* and  
57 calreticulin (CRT) are homologous ER lectins that bind to N-glycosylated proteins to promote  
58 their folding. Growth of *cnx1-1* and *cnx2-2* single mutants was similar to that of the wild type  
59 under high and low Pi conditions, but the *cnx1-1 cnx2-2* double mutant showed decreased  
60 primary root growth under low Pi conditions due to reduced meristematic cell division. This  
61 phenotype was specific to Pi deficiency; the double mutant responded normally to osmotic  
62 and salt stress. The root growth phenotype was Fe dependent and was associated with Fe  
63 accumulation in the root. Two genes involved in Fe-dependent inhibition of root growth  
64 under Pi deficiency, the ferroxidase gene *LPR1* and P5-type ATPase *PDR2*, are epistatic to  
65 *CNX1/CNX2*. Overexpressing *PDR2* failed to complement the *cnx1-1 cnx2-2* root phenotype.  
66 *cnx1-1 cnx2-2* showed no evidence of UPR activation, indicating a limited effect on ER  
67 protein folding. *CNX* might process a set of N-glycosylated proteins specifically involved in  
68 the response to Pi deficiency.

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72 **Introduction**

73

74 The endoplasmic reticulum (ER) serves as the major entry point for proteins into the secretory  
75 pathway as well as for proteins destined for the plasma membrane (PM). It is estimated that  
76 approximately one-third of cellular proteins pass through this organelle (Strasser, 2018). The  
77 ER is thus a major site for folding and quality control of proteins involved in numerous  
78 cellular processes, including cell wall synthesis, nutrient transport, and PM-based signal  
79 transduction (Brandizzi, 2021). The ER harbors two main pathways to assist in protein  
80 folding. The first pathway involves the general chaperones BiPs, which belong to the classical  
81 heat shock protein 70 (HSP70) family, the DNA J protein ERdj3 and its associated stromal-  
82 derived factor 2 (SDF2) protein, and protein disulfide isomerases (PDI), which promote the

83 formation of disulfide bonds (Strasser, 2018). The second pathway, a distinct ER folding  
84 pathway known as the calnexin-calreticulin cycle, is dedicated to N-glycosylated proteins.  
85 Calnexin (CNX) and calreticulin (CRT) are lectins that share a common architecture  
86 consisting of two major domains: a glycan binding domain and a long flexible P-domain  
87 involved in recruiting other co-chaperones such as PDIs. While CNX is anchored to the ER  
88 via a transmembrane domain, its homologue CRT is soluble within the ER matrix and harbors  
89 a luminal KDEL ER retrieval signal (Strasser, 2018; Kozlov and Gehring, 2020). *Arabidopsis*  
90 *thaliana* contains two CNX genes and three CRT genes (Persson et al., 2003; Liu et al.,  
91 2017).

92

93 In the CNX-CRT cycle, proteins entering the ER are first conjugated with a  
94  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  glycan on specific asparagines by the oligosaccharyltransferase (OST)  
95 complex. The N-linked glycans are then trimmed by two glucosidases (GCSI and GCSII) to  
96 generate a monoglucosylated protein, which specifically binds to CNX or CRT to promote  
97 protein folding and maturation. Removal of the terminal glucose by GCSII leads to the release  
98 of the glycoprotein from CNX/CRT. If the protein is inappropriately folded after release, the  
99 glucosyltransferase UDP-glucose:glycoprotein glucosyltransferase (UGGT) adds back a  
100 terminal glucose, enabling the re-association of the misfolded glycoprotein with CNX or CRT  
101 and thus initiating an additional round of folding (Liu and Howell, 2010; Strasser, 2018).

102

103 ER proteins that repeatedly fail to properly fold after several rounds of the CNX-CRT cycle  
104 are directed to become degraded. An important pathway for ER protein degradation involves  
105 the translocation of misfolded proteins to the cytosol for proteasomal degradation, a process  
106 termed ER-associated degradation (ERAD). Protein degradation through ERAD involves the  
107 recognition and transport of misfolded proteins across the ER membrane to the cytosol,  
108 followed by polyubiquitination and degradation via the 26S proteasome (Chen et al., 2020).  
109 The accumulation of misfolded proteins in the ER leads to ER stress and the activation of the  
110 unfolded protein response (UPR). In turn, the activation of the UPR results in the  
111 upregulation of genes involved in vesicular trafficking, ERAD, and protein folding, including  
112 *BiPs* and *PDIs* (Liu and Howell, 2016). The UPR signaling pathway has two branches. In the  
113 first branch, the ER-anchored RNA splicing factor IRE1 modifies the mRNA of the  
114 transcription factor bZIP60, yielding a form of bZIP60 that lacks a transmembrane domain  
115 and is targeted to the nucleus. The second branch of the UPR signaling pathway activates two  
116 other members of the bZIP family, bZIP17 and bZIP28, via protease processing in the Golgi

117 (Liu and Howell, 2016). Chronic ER stress that cannot be resolved by the activation of ERAD  
118 and the UPR can lead to programmed cell death as well as autophagy (Manghwar and Li,  
119 2022).

120

121 ER stress has been associated with numerous abiotic stress factors that are thought to lead to  
122 defects in protein folding in the ER, such as heat, drought, osmotic, salt, and metal stress. The  
123 link between the control of ER protein folding and abiotic stress has been demonstrated via  
124 the analysis of mutants as well as transgenic plants overexpressing genes encoding ER  
125 chaperones, such as *BiP*, *CNX*, and *PDI*s, as well as genes involved in the ERAD and UPR  
126 pathways, including *IRE1* and *bZIP28* (Gao et al., 2008; Deng et al., 2011; Kim et al., 2013;  
127 Joshi et al., 2019; Park and Park, 2019; Reyes-Impellizzeri and Moreno, 2021). However,  
128 whether the control of protein folding in the ER has a role in plant responses to nutrient  
129 deficiency has not been determined, although recent work has shown that autophagy may be  
130 implicated in such stress (Naumann et al., 2019; Stephani et al., 2020; Yoshitake et al., 2021).

131

132 Phosphorus is one of the most important nutrients affecting plant growth in both agricultural  
133 and natural ecosystems (Poirier et al., 2022). Plants acquire phosphorus almost exclusively  
134 via the transport of soluble inorganic phosphate ( $\text{H}_2\text{PO}_4^-$ ; Pi) into roots. Plants have evolved a  
135 series of metabolic and developmental responses to Pi deficiency that are aimed at  
136 maximizing Pi acquisition from the environment and optimizing its internal use for growth  
137 and reproduction (Dissanayaka et al., 2021; Poirier et al., 2022). One of the best-characterized  
138 responses of roots to phosphate deficiency is a decrease in primary root growth associated  
139 with reduced root meristem size (Crombez et al., 2019). This phenotype has been associated  
140 with the presence of  $\text{Fe}^{+3}$ -malate complexes in the root meristem, which generate reactive  
141 oxygen species (ROS), and in turn lead to changes in the cell wall structure and inhibition of  
142 cell-to-cell communication (Müller et al., 2015; Balzergue et al., 2017; Mora-Macias et al.,  
143 2017). Genetic screens for genes that contribute to changes in primary root growth under Pi  
144 deficiency identified *LPR1* and *LPR2*, encoding ferroxidases that convert  $\text{Fe}^{+2}$  to  $\text{Fe}^{+3}$ , and  
145 *PDR2*, encoding an ER-localized P5-type ATPase thought to negatively affect LPR activity  
146 via an unknown mechanism (Ticconi and Abel, 2004; Svistoonoff et al., 2007; Ticconi et al.,  
147 2009; Naumann et al., 2022). Additional proteins found to participate in this pathway include  
148 the malate and citrate efflux channel *ALMT1*; the *STOP1* transcription factor, which  
149 regulates *ALMT1* expression; *ALS3* and *STAR1*, which together form a tonoplast ABC  
150 transporter complex involved in plant tolerance to aluminum (although the nature of the

151 molecule that is transported remains to be defined); and the CLE14 peptide receptors CLV2  
152 and PEPR2 (Balzergue et al., 2017; Dong et al., 2017; Gutierrez-Alanis et al., 2017; Mora-  
153 Macias et al., 2017).

154

155 In the present study, we analyzed Arabidopsis mutants affected in components of ER protein  
156 folding and quality control for their response to phosphate deficiency. We determined that  
157 CNX proteins participate in the Fe-dependent inhibition of primary root growth in response to  
158 phosphate deficiency.

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162 **Results**

163 **The *cnx1 cnx2* double mutant shows reduced primary root growth under low Pi**  
164 **conditions**

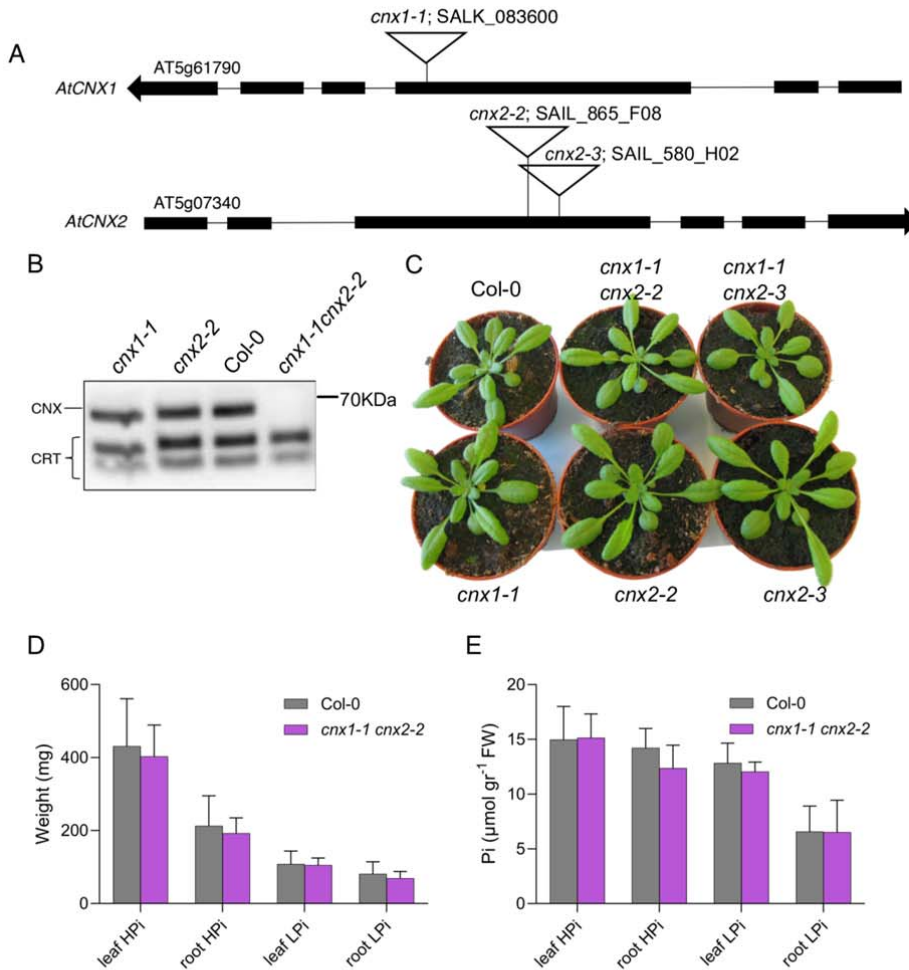
165 We crossed the Arabidopsis *cnx1-1* mutant (SALK\_083600), which has a T-DNA insertion in  
166 the 3<sup>rd</sup> exon of *CNX1* (At5g61790), with *cnx2-2* (SAIL\_865\_F08) and *cnx2-3*  
167 (SAIL\_580\_H02), which have T-DNA insertions in the third exon of *CNX2* (At5g07340), to  
168 create two independent double mutant combinations (Figure 1A). Immunoblot analysis of  
169 protein extracts from whole seedlings showed that CNX proteins were absent in the *cnx1-1*  
170 *cnx2-2* double mutant, indicating that these mutant alleles are likely null (Figure 1B). We  
171 grew the plants in fertilized soil and in clay irrigated with nutrient solution containing 1 mM  
172 Pi (high Pi; HPi) or 75  $\mu$ M Pi (low Pi; LPi) and found no significant differences between the  
173 single and double mutants compared to the wild type Col-0 in terms of fresh weight (Figure  
174 1C and D) or Pi content (Figure 1E) in roots or rosettes. By contrast, in seedlings grown on  
175 solid medium, primary root length was significantly reduced in the *cnx1-1 cnx2-2* and *cnx1-1*  
176 *cnx2-3* double mutants compared to Col-0 under LPi but not HPi conditions (Figure 2A). This  
177 phenotype was complemented by transforming the *cnx1-1 cnx2-2* double mutant with the  
178 *CNX1-GFP* or *CNX2-GFP* fusion construct driven by their respective endogenous promoters  
179 (Figure 2B). Confocal microscopy of roots of the complemented lines expressing CNX1-GFP  
180 or CNX2-GFP revealed localization of these fusion proteins in the ER (Supplemental Figure  
181 S1A). Co-localization of CNX1-GFP and CNX2-GFP with an ER marker (ER-RFP) was  
182 observed in transiently transfected tobacco (*Nicotiana benthamiana*) leaf cells (Supplemental  
183 Figure S1B).

184

185 **Mutants in other components of the CNX/CRT cycle and ER chaperone system do not**  
186 **reproduce the *cnx1 cnx2* root growth phenotype under low Pi**

187 In addition to CNX, ER protein quality control relies on numerous other proteins, including  
188 chaperones and enzymes involved in glycosylation and glycan modifications in the ER  
189 (Strasser, 2018). We therefore examined primary root growth of mutants in various  
190 components of the CNX/CRT cycle and ER protein quality control under LPi conditions.  
191 Arabidopsis CRTs are encoded by three genes, which are divided into two groups based on  
192 sequence homology and function: *CRT1/CRT2* and *CRT3* (Persson et al., 2003; Christensen et  
193 al., 2010). No significant differences were detected in the root growth of *crt1 crt2* or *crt3*  
194 mutants under HPi or LPi conditions compared to Col-0 (Figure 3A).

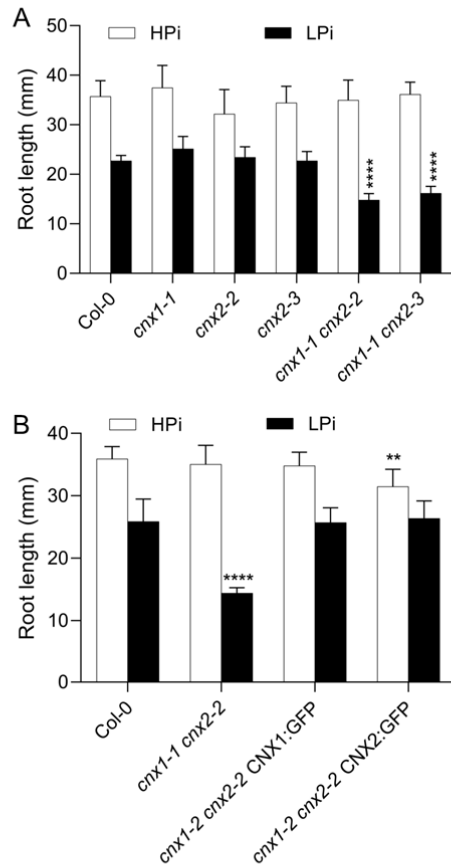
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**Figure 1. Phenotype of the *cnx1 cnx2* double mutant in soil. (A)** Schematic diagram of the T-DNA insertions in the *CNX1* (At5g61790) and *CNX2* (At5g07340) genes in the *cnx* mutants. Exons are shown as black boxes. **(B)** Immunoblot analysis of CNX and CRT in whole protein extracts from seedlings. The position of the 70 KDa molecular-weight marker is shown on the right. **(C)** Rosettes of 3.5-week-old plants grown in soil. **(D, E)** Fresh weight (D) and Pi content (E) in whole rosettes (leaf) and roots of plants grown for 4 weeks in clay irrigated with nutrient solution containing 1 mM Pi (HPi) or 75  $\mu\text{M}$  Pi (LPi). Statistical analysis was performed by Student's t test compared to the Col-0 control, error bars = SD, n = 8-10.

196 The synthesis of the core oligosaccharide unit  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  involves a series of ER  
 197 glycosyltransferases including the mannosyltransferases ALG3 and ALG9 and the  
 198 glucosyltransferase ALG10 (Kajiura et al., 2010; Farid et al., 2011; Hong et al., 2012).  
 199 Following its synthesis, the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  unit is added to ER proteins co-translationally



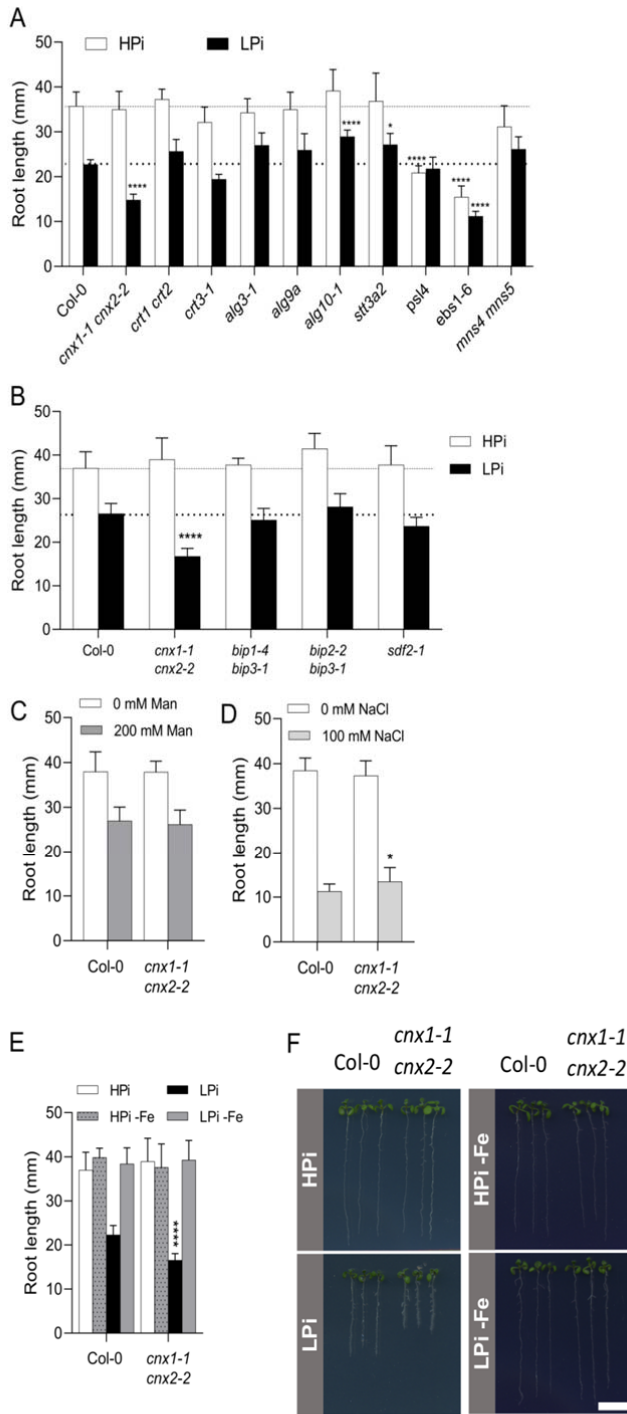


**Figure 2. Primary root growth of the *cnx1 cnx2* double mutant under high and low Pi conditions. (A)** Primary root length of Col-0 compared to *cnx1-1* and *cnx2-2* single and double mutants. **(B)** Complementation of the primary root phenotype of *cnx1-1 cnx2-2* plants transformed with the CNX1:GFP or CNX2:GFP construct. Plants were grown for 7 days on plates containing 1 mM Pi (HPi) or 75  $\mu$ M Pi (LPi) before measuring primary root length. Statistical analysis was performed by two-way ANOVA followed by a Tukey's test, and significant differences compared to Col-0 in each growth condition are shown: \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; error bars = SD;  $n \geq 9$ .

200 by the membrane-associated heteromeric OST complex, which includes the catalytic STT3  
 201 subunit encoded by *STT3A* in Arabidopsis (Koiwa et al., 2003). Primary root growth under  
 202 HPi and LPi conditions was not reduced in *alg3-1*, *alg9a*, *alg10-1*, or *stt3a2* mutants  
 203 compared to Col-0 (Figure 3A).

204

205 The presence of terminal  $\alpha$ 1,2-linked glucose residues, which facilitate the interaction  
 206 between CNX/CRT and N-glycosylated proteins, is regulated by the trimming action of  
 207 GCSII and the glucosylating action of UGGT. *PSLA* encodes the  $\beta$ -subunit of GCSII (Lu et



**Figure 3. Primary root growth of mutants in genes involved in ER protein synthesis and quality control.** (A-B) Plants were grown for 7 days on plates containing HPI or LPI before measuring primary root length. (C-D) Primary root length of *Col-0* and *cnx1-1 cnx2-2* plants after 7 days of growth on HPI plates (C) without or with 200 mM mannose or (D) without or with 100 mM NaCl. (E-F) Primary root length of *Col-0* and *cnx1-1 cnx2-2* after 7 days of growth on plates containing HPI or LPI half-strength MS medium, or the same medium with ferrozine to chelate Fe (HPI-Fe and LPI-Fe). Statistical analysis was performed by two-way ANOVA followed by a Tukey's test, and significant differences compared to *Col-0* in each growth condition are shown, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001, error bars = SD, *n* ≥ 5. Bar in F represents 1 cm.

208 al., 2009). The primary roots of the *psl4* mutant were shorter than *Col-0* when grown on HPI

209 medium, but there was no significant further reduction in their length when grown on LPi  
210 medium (Figure 3A). Primary root growth was severely compromised in the *ebs1-6/uggt1-1*  
211 mutant on HPi medium, and this effect was only slightly enhanced on LPi medium (Figure  
212 3A).

213

214 ER proteins that pass through the CNX/CRT cycle but remain inappropriately folded are  
215 degraded by ERAD. This process involves the trimming of mannosyl groups on the N-glycan  
216 chain by the  $\alpha$ -mannosidases MNS4 and MNS5 (Huttner et al., 2014). Primary root growth of  
217 the *mns4 mns5* double mutant was not significantly different from Col-0 on HPi or LPi  
218 medium (Figure 3A).

219

220 We also examined the role of the ER chaperone pathway involving BiP and SDF2 in the  
221 response of Arabidopsis roots to Pi deficiency. While SDF2 is encoded by a single gene in  
222 Arabidopsis (Nekrasov et al., 2009), three genes encode the ER BiP chaperones. *BIP1* and  
223 *BIP2* encode proteins that are 99% identical and are ubiquitously expressed, while the more  
224 divergent *BiP3* is expressed under ER stress (Maruyama et al., 2014). Root growth of the  
225 *bip1-4 bip3-1*, *bip2-2 bip3-1*, and *sdf2-1* mutants was similar to that of Col-0 on both HPi and  
226 LPi media (Figure 3B).

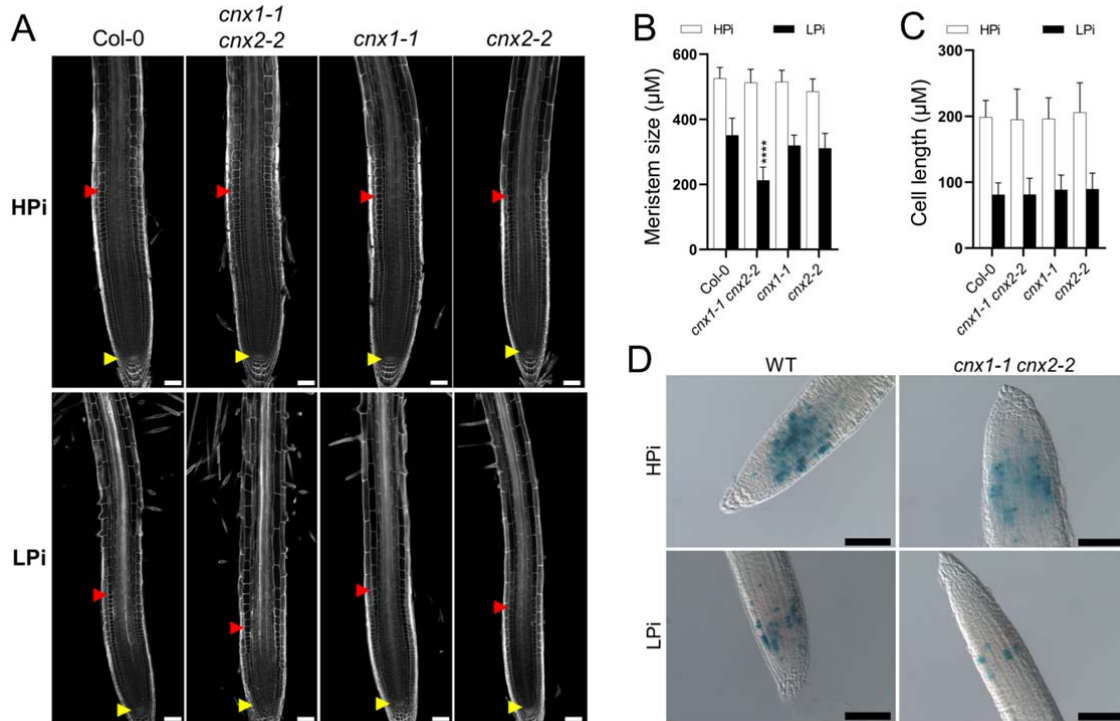
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228 Several mutants related to the CNX/CRT cycle and ER protein homeostasis, including *alg10*,  
229 *stt3a*, *mns4 mns5*, and *ebs1-6/uggt1*, exhibit strong root growth phenotypes under salt stress  
230 (Koiwa et al., 2003; Farid et al., 2011; Huttner et al., 2014; Blanco-Herrera et al., 2015). To  
231 investigate whether the reduced primary root length observed in *cnx1-1 cnx2-2* was specific to  
232 Pi deficiency stress, we examined root growth in this double mutant under two other abiotic  
233 stress conditions that reduced primary root growth: osmotic stress (200 mM mannitol) and  
234 salt stress (100 mM NaCl). Under both stress conditions, primary root growth was similar in  
235 the *cnx1-1 cnx2-2* double mutant and Col-0 (Figure 3C-D), indicating that the root growth  
236 phenotype of this double mutant is specific to Pi deficiency stress.

237

### 238 **The root phenotype of *cnx1 cnx2* is due to reduced root apical meristem activity**

239 Reduced primary root growth under stress conditions can be caused by reduced cell division  
240 within the meristem, reduced cell elongation, or both. Under LPi but not HPi conditions, the  
241 meristematic zone was smaller in *cnx1-1 cnx2-2* compared to Col-0 and the corresponding  
242 single mutants (Figure 4A, B). By contrast, the cell length in the elongation zone was not



**Figure 4. The *cnx1-1 cnx2-2* double mutant is affected in meristem activity. (A-C)** Plants were grown for 7 days on plates containing HPI or LPI before measuring the length of the cell division zone in the meristem, defined in A by the red and red arrows (A, B) and cell length in the differentiation zone (C). Statistical analysis (B, C) was performed by two-way ANOVA followed by a Tukey's test; significant differences compared to Col-0 under each growth condition are shown: \*\*\*\*,  $P < 0.0001$ ; error bars = SD;  $n \geq 5$  in (B) and 20 in (C). **(D)** Col-0 and *cnx1-1 cnx2-2* plants transformed with the *cylinB1:GUS* reporter gene construct were grown for 7 days on plates containing HPI or LPI medium and stained for  $\beta$ -glucuronidase activity. Bars represent 50  $\mu$ M in A and 100  $\mu$ M in D.

243 significantly different between the mutants and Col-0 under HPI or LPI conditions (Figure  
 244 4A, C). These data indicate that *cnx1-1 cnx2-2* is mainly affected in meristematic cell division  
 245 under LPI conditions. To further evaluate the contribution of cell division to the mutant  
 246 phenotype, we introduced into the *cnx1-1 cnx2-2* double mutant a reporter construct for cell  
 247 division consisting of labile GUS under the control of the cyclin B1 promoter (Colon-  
 248 Carmona et al., 1999). The number of dividing, GUS-expressing cells was similar in *cnx1-1*  
 249 *cnx2-2* vs. Col-0 roots under HPI conditions (Figure 4D). By contrast, a clear reduction in  
 250 GUS-expressing cells was observed in Col-0 roots grown under LPI, in accordance with the  
 251 known reduction in meristematic cell division under these conditions (Ticconi et al., 2004).  
 252 Importantly, a further reduction in GUS expression in roots was observed in the *cnx1-1 cnx2-2*  
 253 double mutant compared to Col-0 on LPI (Figure 4D). Altogether, these data indicate that

254 the altered primary root growth of *cnx1-1 cnx2-2* is primarily due to reduced meristematic cell  
255 division under LPi conditions.

256

257 **The root phenotype of *cnx1-1 cnx2-2* is dependent on Fe and associated with increased**  
258 **Fe deposition in the meristem**

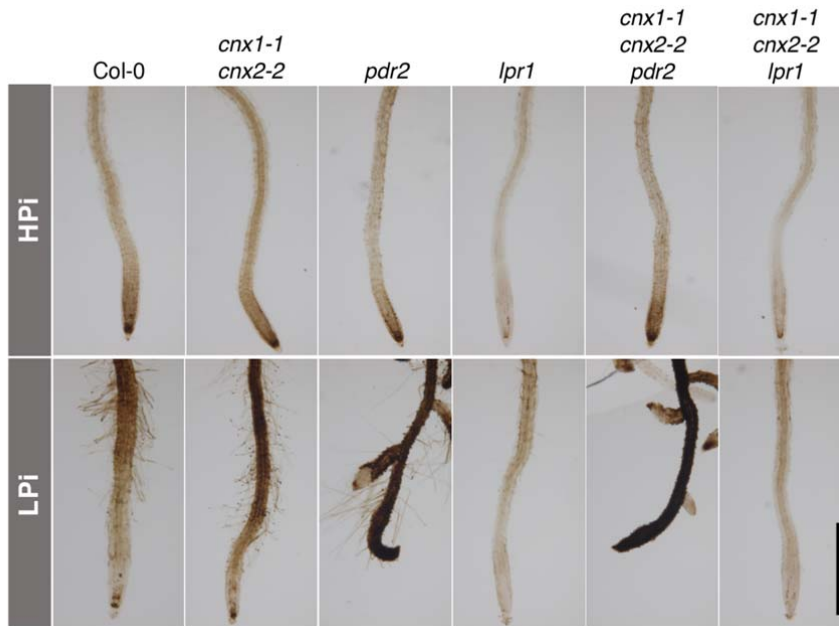
259 Several studies have shown that the reduced primary root growth of plants under low Pi in  
260 Col-0 and in various mutants with more severe root growth inhibition is dependent on the  
261 presence of Fe in the growth medium (Ticconi et al., 2009; Müller et al., 2015; Balzergue et  
262 al., 2017; Dong et al., 2017). Indeed, a comparison of root growth on HPi and LPi medium  
263 with and without Fe showed that the reduced primary root growth observed in *cnx1-1 cnx2-2*  
264 under LPi conditions was also dependent on the presence of Fe in the medium (Figure 3E-F).  
265 We used Perls-DAB staining to examine the distribution of apoplastic Fe in plants grown  
266 under HPi and LPi conditions. The *lpr1* mutant (which is insensitive to low Pi-induced root  
267 growth inhibition) and *pdr2* (which shows very strongly reduced primary root growth under  
268 low Pi conditions) were used as controls (Müller et al., 2015). In plants grown under HPi  
269 conditions, no significant differences were observed in Fe distribution in the root  
270 meristematic and elongation zones between Col-0 and *cnx1-1 cnx2-2* or *pdr2*, whereas *lpr1*  
271 showed substantially reduced Fe deposition (Figure 5, upper panels). Under LPi conditions,  
272 the *cnx1-1 cnx2-2* double mutant showed robust enhancement of Fe deposition in the root  
273 differentiation zone and more modest enhancement in the root differentiation and  
274 meristematic zones compared to Col-0, whereas *pdr2* roots showed extensive Fe deposition  
275 throughout the root, and *lpr1* showed minimal Fe deposition (Figure 5, lower panels).

276

277 ***pdr2* and *lpr1 lpr2* are epistatic to *cnx1-1 cnx2-2***

278 We examined the epistasis among *cnx1-1 cnx2-2*, *lpr1 lpr2*, and *pdr2* by generating triple and  
279 quadruple mutants. Primary root growth of *cnx1-1 cnx2-2 lpr1 lpr2* was insensitive to low Pi,  
280 as the primary root length of this quadruple mutant was identical to that of *lpr1 lpr2* and  
281 longer than that of Col-0 under LPi conditions (Figure 6A). The *pdr2* mutant showed reduced  
282 primary root growth in HPi; this phenotype remained unchanged in the *cnx1-1 cnx2-2 pdr2*  
283 triple mutant. On LPi medium, the *pdr2* mutant showed more strongly reduced primary root  
284 growth than *cnx1-1 cnx2-2*, and this phenotype was maintained in the *cnx1-1 cnx2-2 pdr2*  
285 triple mutant (Figure 6B). The epistatic action of *lpr1* and *pdr2* over *cnx1-1 cnx2-2* was also  
286 observed at the level of Fe accumulation for roots grown under HPi and LPi (Figure 5).

287



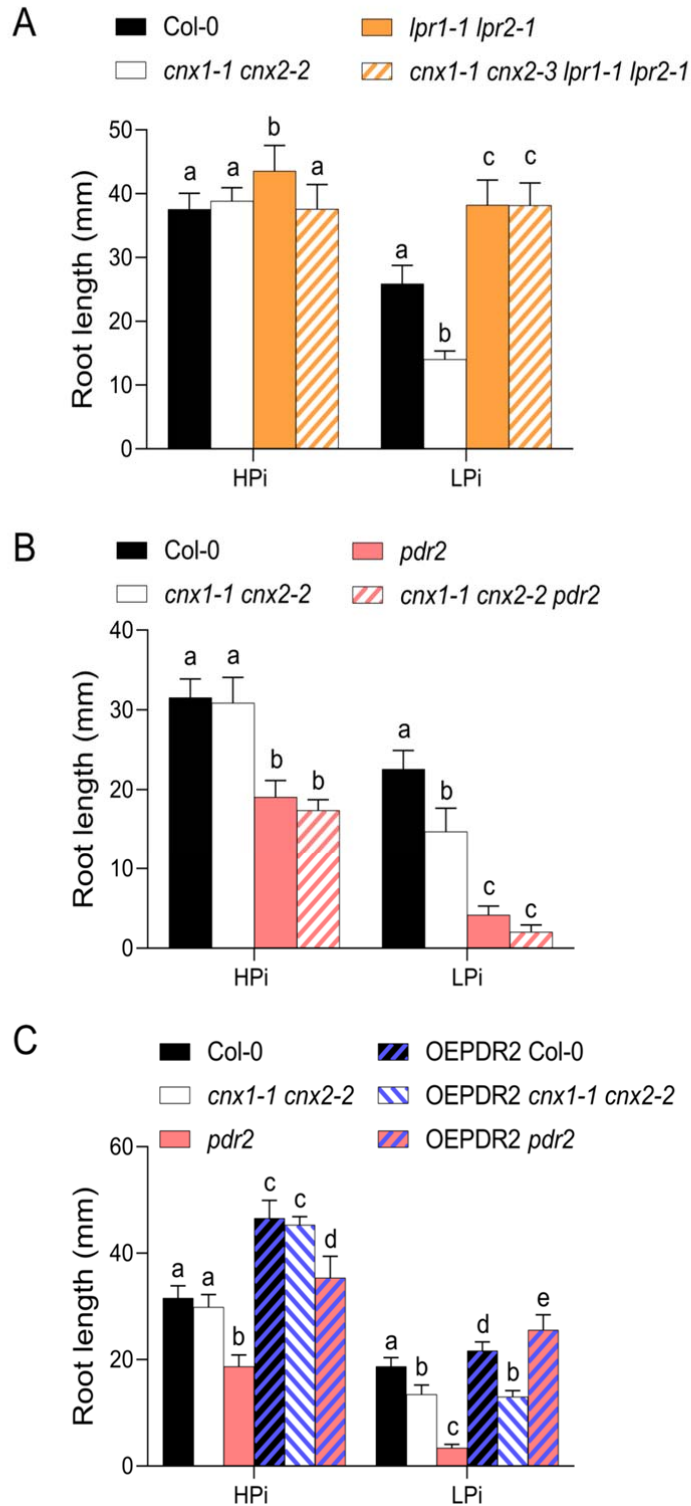
**Figure 5. Fe accumulation and distribution in the roots of mutants grown under high and low Pi conditions.** Plants were grown for 7 days on plates containing 1 mM or 75  $\mu$ M Pi and subjected to Perls/DAB staining for Fe visualization. Bar represents 1 mm.

288 We also examined the effect of overexpressing *PDR2* driven by the CaMV35S promoter. In  
 289 both Col-0 and *pdr2*, overexpression of *PDR2* led to significantly longer primary roots  
 290 compared to Col-0 plants on both HPi and LPi media. By contrast, while overexpressing  
 291 *PDR2* in the *cnx1-1 cnx2-2* double mutant background also resulted in longer primary roots  
 292 compared to Col-0 grown under HPi conditions, the same plants showed shorter primary roots  
 293 than Col-0 and comparable root length to the *cnx1-1 cnx2-2* double mutant when grown under  
 294 LPi conditions (Figure 6C). Overall, these data indicate that the primary root phenotypes of  
 295 *pdr2* and *lpr1 lpr2* are epistatic to *cnx1-1 cnx2-2* under LPi and that overexpressing *PDR2*  
 296 failed to rescue the short root phenotype of *cnx1-1 cnx2-2* under LPi.

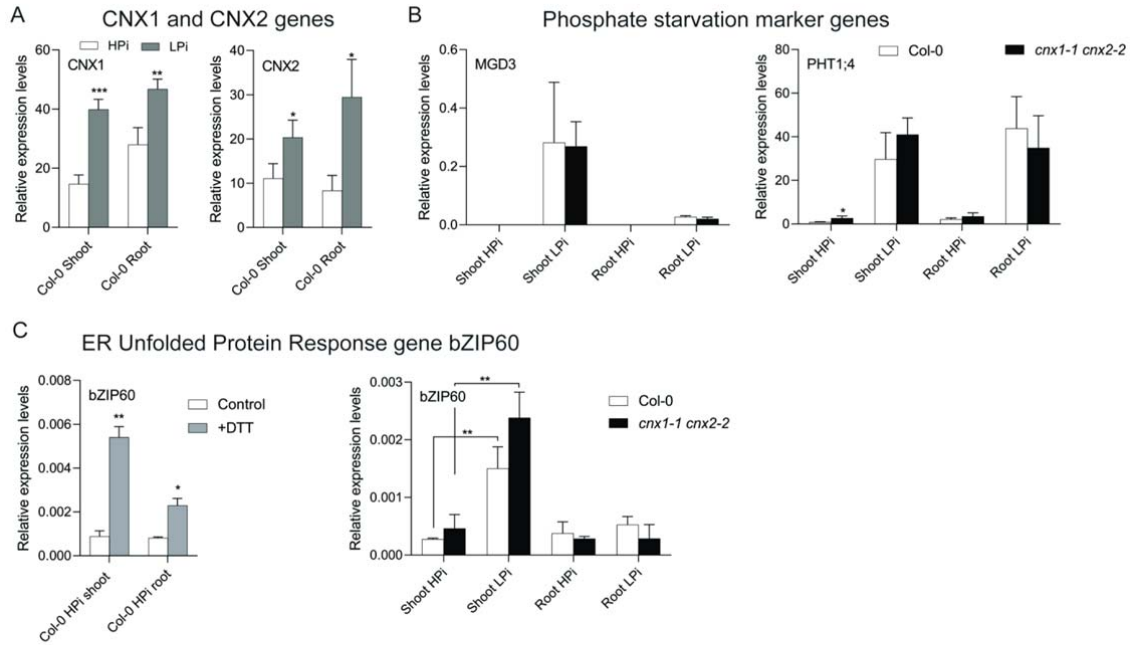
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### 298 **Pi deficiency induces CNX gene expression and ER stress**

299 We examined the expression of *CNX1* and *CNX2* in the shoots and roots of plants grown on  
 300 LPi and HPi media via quantitative RT-PCR. The expression of both *CNX1* and *CNX2*  
 301 significantly increased under Pi-deficient conditions (Figure 7A). However, the increase in



**Figure 6. Epistatic interactions among *cnx1-1 cnx2-2*, *lpr1-1 lpr2-1*, and *pdr2*.** Plants were grown for 7 days on HPi or LPi plates before recording primary root length. (A) Epistatic interaction between *cnx1-1 cnx2-2* and *lpr1-1 lpr2-1*. (B) Epistatic interaction between *cnx1-1 cnx2-2* and *pdr2*. (C) A T-DNA cassette for *PDR2* overexpression under the control of the CaMV35S promoter (OEPR2) was introgressed into Col-0, *cnx1-1 cnx2-2*, and *pdr2*. Statistical analysis was performed by two-way ANOVA followed by a Tukey's test, and significant differences within each growth condition are shown. Different lowercase letters (a, b, c or d) indicate a significant difference with a p-value <0.05, n ≥ 6.



**Figure 7. Impact of the *cnx1-1 cnx2-2* mutations on the expression of Pi-deficiency and unfolded protein response marker genes. (A) *CNX1* and *CNX2* expression in the shoots and roots of plants grown for 7 days in HPI or LPI medium. (B) Expression of the Pi-deficiency markers *MGD3* and *PHT1;4* in the shoots and roots of Col-0 and *cnx1-1 cnx2-2* grown for 7 days on HPI or LPI medium. (C) Induction of ER Unfolded Protein Response marker gene *bZIP60* in the shoots and roots of Col-0 at 24 h after the addition of 2 mM DTT and in the *cnx1-1 cnx2-2* double mutant compared to Col-0 grown under HPI or LPI conditions. Statistical analysis was performed by Student's t test comparing different treatments (HPI and LPI for A and C, Control and DTT for C) and Col-0 vs. *cnx1-1 cnx2-2* (B, C), with significant differences indicated by asterisks (\*), \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001. Error bars = SD, n = 3.**

303 genes, such as *MGD3* and *PHT1;4* (Figure 7B).

304

305 We investigated the transcriptional response of the *cnx1-1 cnx2-2* double mutant to Pi  
 306 deficiency conditions by examining *MGD3* and *PHT1;4* expression. The expression of both  
 307 genes in shoots and roots did not significantly differ between *cnx1-1 cnx2-2* and Col-0 on HPI  
 308 or LPI medium, except that *PHT1;4* was slightly upregulated in *cnx1-1 cnx2-2* shoots on HPI  
 309 medium (Figure 7B).

310

311 The accumulation of mis-folded proteins in the ER leads to ER stress and the increased  
 312 expression of the transcription factor gene *bZIP60* (Lu and Christopher, 2008). To determine  
 313 whether LPI treatment leads to ER stress and whether the *cnx1-1 cnx2-2* double mutant  
 314 exhibits greater signs of ER stress compared to Col-0 plants, we compared the expression of  
 315 *bZIP60* in *cnx1-1 cnx2-2* vs. Col-0 plants grown on HPI and LPI. When we treated plants with



316 the reducing agent dithiothreitol (DTT) to induce ER stress, *bZIP60* was upregulated, with a  
317 greater increase in shoots compared to roots (Figure 7C) (Lu and Christopher, 2008). Under  
318 LPi conditions, *bZIP60* expression significantly increased in shoots but not roots in both Col-  
319 0 and *cnx1-1 cnx2-2*, with no significant difference in *bZIP60* expression between these lines  
320 (Figure 7C). Thus, the removal of calnexin did not lead to an increase in ER stress compared  
321 to Col-0 under either HPI or LPi conditions.

322

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325

326 **Discussion**

327

328 Both CNX1 and CNX2 are localized to the ER in Arabidopsis, and the corresponding genes  
329 are broadly expressed in most tissues (except that only *CNX1* is significantly expressed in  
330 pollen) and throughout development in both shoots and roots (Liu et al., 2017). Previous  
331 analysis of higher-order Arabidopsis mutants of *CNX* and *CRT* revealed that while the *cnx1*  
332 *cnx2* double mutant had no phenotype under normal growth conditions, the *crt1 crt2* double  
333 mutant and the *crt1 crt2 crt3* triple mutant showed reduced rosette growth in soil and reduced  
334 hypocotyl elongation in the dark (Christensen et al., 2010; Kim et al., 2013; Vu et al., 2017).  
335 The *cnx1 crt1 crt2 crt3* quadruple mutant showed stronger defects in shoot and root  
336 developmental under normal conditions, as well as compromised fertility due to strongly  
337 reduced pollen viability and pollen tube growth. The inactivation of all *CNX* and *CRT* genes  
338 in the quintuple *cnx1 cnx2 crt1 crt2 crt3* mutant was lethal (Vu et al., 2017). Interestingly, the  
339 phenotype of the *cnx1 crt1 crt2 crt3* mutant was fully complemented by expressing either  
340 *CNX1* or *CNX2* under the control of the *CNX1* promoter, highlighting the functional  
341 redundancy of *CNX1* and *CNX2* (Vu et al., 2017).

342

343 In the present study, while the *cnx1-1* and *cnx2-2* single mutants showed no defect in primary  
344 root growth under HPi or LPi conditions, the *cnx1-1 cnx2-2* double mutant showed reduced  
345 primary root growth under LPi but not HPi conditions; this phenotype was complemented by  
346 the expression of either *CNX1* or *CNX2* driven by their native promoters. Thus, *CNX1* and  
347 *CNX2* are both required and play functionally redundant roles in the response of primary roots  
348 to Pi deficiency. Interestingly, no other mutant analyzed that is impaired in various aspects of  
349 N-glycan synthesis and the CNX-CRT cycle showed defects in primary root growth  
350 specifically under LPi conditions. These results could potentially reflect the presence of  
351 genetic redundancy or the induction of compensatory mechanisms in these mutants that do  
352 not function in the *cnx1-2 cnx2-2* mutant.

353

354 Several mutants tested for primary root growth under LPi conditions, including *alg10-1*,  
355 *stt3a2*, *ebs1-6*, and *mns4 mns5*, were previously shown to have altered root growth under salt  
356 stress (Koiwa et al., 2003; Farid et al., 2011; Huttner et al., 2014; Blanco-Herrera et al.,  
357 2015). Considering that the growth of *cnx1-1 cnx2-2* roots was comparable to Col-0 under salt  
358 stress and osmotic stress, it is likely that defects in different components of the CNX-CRT  
359 cycle affect distinct N-glycosylated proteins to different extents. That is, the proteins affected

360 in the *alg10-1*, *stt3a2*, *ebs1-6*, and *mns4 mns5* mutants are involved in the salt stress response,  
361 and those affected in *cnx1-1 cnx2-2* are involved in the Pi deficiency response.

362

363 The *cnx1 cnx2* mutant shares several features with the *pdr2*, *als3*, and *star1* mutants in terms  
364 of their responses to LPi conditions, including Fe-dependent reduced primary root growth  
365 associated with a reduction in root meristem size (Ticconi et al., 2004; Müller et al., 2015;  
366 Dong et al., 2017). However, the *pdr2*, *als3*, and *star1* mutants have additional root  
367 phenotypes under LPi conditions that are not observed in *cnx1-1 cnx2-2*, such as reduced cell  
368 length in the root elongation zone and a generally more distorted cellular organization in the  
369 root meristem. Furthermore, the apparent apoplastic Fe accumulation (as visualized by Perls-  
370 DAB staining) in *pdr2*, *als3*, and *star1* roots grown in LPi is higher in both the elongation and  
371 meristematic zones compared to *cnx1-1 cnx2-2* (Ticconi et al., 2004; Müller et al., 2015;  
372 Dong et al., 2017). Initial characterization of mutants such as *pdr2*, *lpr1*, *almt1*, and *als3*  
373 linked strong apoplastic Fe staining in the root meristematic and elongation zones with  
374 inhibited cell division and cell elongation. Fe accumulation in the meristem is associated with  
375 ROS production, which affects cell wall structure and meristem cell division via reduced  
376 mobility of SHORT-ROOT (SHR) in the stem cell niche (Müller et al., 2015; Balzergue et al.,  
377 2017). However, a more detailed analysis of dynamic changes in Fe accumulation and  
378 primary root growth over time revealed that the extent of primary root growth inhibition  
379 cannot simply be directly linked to the level of apoplastic Fe accumulation in the root  
380 meristem and elongation zone (Wang et al., 2019).

381

382 *PDR2* encodes a member of the eukaryotic type V subfamily (P5) of P-type ATPase (Ticconi  
383 et al., 2009). *PDR2* is abundant in the ER, but its mode of action and transport activity are  
384 largely unknown, although recent work has reported a role of the yeast P5A ATPase Spfl in  
385 protein translocation in the ER (McKenna et al., 2020). *PDR2* is thought to modulate the  
386 activity and/or abundance of the ferroxidase LPR1 in the apoplast, which is responsible for  
387 the oxidation of Fe<sup>+2</sup> to Fe<sup>+3</sup> (Müller et al., 2015; Naumann et al., 2022). Consequently, the  
388 *lpr1* phenotypes (in terms of both Fe deposition and reduced primary root growth under LPi  
389 conditions) are epistatic to *pdr2* (Ticconi et al., 2009). The *lpr1* phenotypes are also epistatic  
390 to *cnx1-1 cnx2-2*. It is unknown if *PDR2* is N-glycosylated and if it enters the CNX-CTR  
391 cycle. However, considering the milder phenotypes of *cnx1-1 cnx2-2* compared to *pdr2* and  
392 the finding that overexpressing *PDR2* did not influence the reduced primary root growth of

393 *cnx1 cnx2* on LPi medium, it is unlikely that the root growth phenotype of *cnx1-1 cnx2-2* is  
394 mediated by reduced PDR2 activity.

395

396 The lack of calnexin leads to a range of phenotypes in fungi and animals, from lethality in the  
397 yeast *Schizosaccharomyces pombe* to developmental and neurological abnormalities in  
398 zebrafish, mouse, and *Drosophila* (Parlati et al., 1995; Kraus et al., 2010; Hung et al., 2013;  
399 Xiao et al., 2017). The current study highlights a novel role for calnexin in the response of  
400 primary root growth to Pi deficiency. Phosphate deficiency has been associated with an  
401 increase in autophagy in root tips and leaves as well as an increase in *CNX1* and *BiP2*  
402 expression (Naumann et al., 2019; Yoshitake et al., 2021). Here, Pi deficiency resulted in the  
403 increased expression of *CNX1* and *CNX2* in both roots and shoots as well as *bZIP60* in shoots.  
404 Collectively, these data reveal that Pi deficiency is associated with an increase in ER stress.  
405 Yet, the absence of a significant difference in *bZIP60* expression between Col-0 and the *cnx1-*  
406 *1 cnx2-2* double mutant indicates that the absence of calnexin in Arabidopsis does not lead to  
407 a systematic increase in ER stress, at least under HPi or LPi conditions. This implies that the  
408 folding and activity of a restricted number of N-glycosylated proteins are likely affected by  
409 the absence of calnexin; one or a few of these proteins likely contribute to the reduced  
410 primary root growth under LPi conditions.

411

412 A study of leucine-rich repeat receptor kinases involved in innate immunity revealed  
413 markedly different impacts of N-glycosylation on homologous receptor activity. For example,  
414 while the activity of ERF (involved in binding the bacterial elongation factor EF-Tu) was  
415 compromised by mutation of only a few of its N-glycosylation sites, the activity of the  
416 homologous protein FLS2 (a flagellin receptor) was not disrupted by mutation of several of  
417 its N-glycosylation sites (Sun et al., 2012). In accordance with these results, the activity of  
418 ERF but not of FLS2 was compromised in several mutants of genes involved in the CNX-  
419 CRT cycle, including *CRT3*, *SDF2*, *PSL4*, *PSL5*, *EBS1*, and *OST3/6* (Li et al., 2009; Lu et al.,  
420 2009; Nekrasov et al., 2009; von Numers et al., 2010; Farid et al., 2013). Therefore, it is  
421 unlikely that bioinformatics tools that predict the presence of N-glycosylated proteins in roots  
422 will be sufficient to identify the client N-glycosylated proteins that contribute to the Fe-  
423 dependent reduction in primary root growth under LPi conditions. Instead, a more promising  
424 approach would be a proteomic analysis aimed at experimentally detecting proteins adversely  
425 affected by the absence of calnexin.

426

427

428

## 429 **Material and Methods**

### 430 **Plant lines and growth conditions**

431 *Arabidopsis thaliana* seeds were surface sterilized and grown for 7 days on plates containing  
432 half-strength Murashige and Skoog (MS) medium without phosphate (Caisson Laboratories)  
433 supplemented with 75  $\mu\text{M}$  or 1 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 5.8), 1% (w/v) sucrose, 0.7% (w/v)  
434 agarose, and 500 mg/L 2-(N-morpholino) ethanesulfonic acid (final pH 5.8). To induce  
435 different levels of phosphate and iron deficiency, ferrozine was added to the medium at a final  
436 concentration of 100  $\mu\text{M}$ . Plants were grown vertically on plates at 22°C under a continuous  
437 light intensity of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

438

439 Plants were also grown in soil or in a clay-based substrate (Seramis) irrigated with phosphate-  
440 free half-strength MS supplemented with  $\text{KH}_2\text{PO}_4$  buffer, pH 5.8. The growth chamber  
441 conditions were 22°C and 60% relative humidity with a 16-h-light/8-h-dark photoperiod with  
442 100  $\mu\text{E/m}^2$  per s of white light.

443

444 All *Arabidopsis* lines used in this study are in the Col-0 background. A single *cnx1*  
445 (SALK\_083600C) allele and two *cnx2* (SAIL\_865\_F08 and SAIL\_580\_H02) mutant alleles  
446 were identified from T-DNA insertional lines obtained from the European *Arabidopsis* Stock  
447 Center (NASC) (<http://arabidopsis.info>). Supplemental Table S1 lists the sources of all other  
448 lines used in this study. Plants overexpressing *PDR2* under the control of the CaMV35S  
449 promoter (Ticconi et al., 2009) as well as plants expressing the reporter construct  
450 *cycB1::GUS* (Colon-Carmona et al., 1999) were described previously.

451

### 452 **Phosphate quantification**

453 Quantification of Pi was performed as previously described (Ames, 1966). Shoot or root  
454 material was placed in pure water, and at least three freeze-thaw cycles were applied to  
455 release the inorganic Pi, which was quantified via a molybdate assay using a standard curve.

456

### 457 **DNA constructs and gene expression analysis**

458 PCR-generated fragments of the *CNX1* and *CNX2* genomic regions lacking stop codons and  
459 including the 1-kbp promoter regions were obtained using Phusion HF DNA polymerase  
460 (New England Biolabs), inserted into pENTR-2B, and recombined in pMDC107 to generate  
461 the GFP-tagged construct using Gateway technology. The binary vectors were introduced into  
462 *Arabidopsis* plants via *Agrobacterium tumefaciens*-mediated transformation using the floral  
463 dip method (Clough and Bent, 1998).

464

465 Total RNA was extracted from roots using an RNA Purification kit as described by the  
466 manufacturer (Promega), followed by DNase I treatment. cDNA was synthesized from 1 µg  
467 of RNA using M-MLV Reverse Transcriptase (Promega) and oligo d(T)<sub>15</sub> following the  
468 manufacturer's instructions. qPCR analysis was performed using SYBR Select Master Mix  
469 (Applied Biosystems) with primer pairs specific to genes of interest; *ACT2* was used for data  
470 normalization. The primer sequences are listed in Supplemental Table S2.

471

#### 472 **Root measurements, microscopy, and staining procedures**

473 Root length was measured using seedlings grown on vertically oriented plates. The plates  
474 were scanned on a flatbed scanner to produce image files suitable for quantitative analysis  
475 using ImageJ software (v1.44p).

476

477 Confocal microscopy was performed using a Zeiss LSM 880 confocal laser scanning  
478 microscope. Plant roots were treated with Clearsee solution and stained with calcofluor white  
479 (Ursache et al., 2018) to visualize cell walls. A line expressing the *cycB1::GUS* reporter was  
480 used to introgress the construct into the *cnx1-1 cnx2-2* double mutant background. Roots were  
481 stained for GUS activity as previously described (Lagarde et al., 1996). The tissues were  
482 vacuum infiltrated to enhance tissue penetration. Stained tissues were cleared in chloral  
483 hydrate solution (2.7 g/mL in 30% glycerol) and analyzed using a Leica DM5000B bright-  
484 field microscope.

485

486 Iron accumulation in seedlings was assayed by Perls-DAB staining as previously described  
487 (Müller et al., 2015). Briefly, seedlings were incubated in 4 mL of 2% (v/v) HCl and 2%  
488 (w/v) potassium ferrocyanide for 30 min. The samples were washed with water and incubated  
489 for 45 min in 4 mL of 10 mM NaN<sub>3</sub> and 0.3% H<sub>2</sub>O<sub>2</sub> in methanol. The samples were then  
490 washed with 100 mM Na-phosphate buffer (pH 7.4) and incubated for 30 min in the same

491 buffer containing 0.025% (w/v) DAB and 0.005% (v/v) H<sub>2</sub>O<sub>2</sub>. Finally, the samples were  
492 washed twice with water, cleared with chloral hydrate (1 g/mL, 15% glycerol), and analyzed  
493 using an optical microscope.

494

#### 495 **Immunoblot analysis**

496 Proteins were extracted from homogenized plant material at 4°C in extraction buffer  
497 containing 10 mM phosphate buffer, pH 7.4, 300 mM sucrose, 150 mM NaCl, 5 mM EDTA,  
498 5 mM EGTA, 1 mM DTT, 20 mM NaF, and 1× protease inhibitor (Roche EDTA Free  
499 Complete Mini Tablet) and sonicated for 10 min in an ice-cold water bath. Fifty micrograms  
500 of proteins were separated by SDS-PAGE and transferred to an Amersham Hybond-P PVDF  
501 membrane (GE Healthcare). The membrane was probed with rabbit polyclonal antibodies  
502 against maize calreticulin, which cross-reacts with both Arabidopsis calnexin and calreticulin  
503 (Persson et al., 2003), and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) using  
504 Western Bright Sirius HRP substrate (Advansta). Signal intensity was measured using a GE  
505 Healthcare ImageQuant RT ECL Imager.

506

#### 507 **Acknowledgments**

508 The authors are grateful to Shuh-ichi Nishikawa (Niigata University, Japan) and Cyril Zipfel  
509 (University of Zurich, Switzerland) for seeds of the *bip* and *sdf2* mutants, respectively.

510

#### 511 **Competing interests**

512 None

513

514

#### 515 **Figure legends**

516

517 **Figure 1. Phenotype of the *cnx1 cnx2* double mutant in soil.** (A) Schematic diagram of the  
518 T-DNA insertions in the *CNX1* (At5g61790) and *CNX2* (At5g07340) genes in the *cnx*  
519 mutants. Exons are shown as black boxes. (B) Immunoblot analysis of CNX and CRT in  
520 whole protein extracts from seedlings. The position of the 70 KDa molecular weight marker is  
521 shown on the right. (C) Rosettes of 3.5-week-old plants grown in soil. (D, E) Fresh weight  
522 (D) and Pi content (E) in whole rosettes (leaf) and roots of plants grown for 4 weeks in clay

523 irrigated with nutrient solution containing 1 mM Pi (HPi) or 75  $\mu$ M Pi (LPi). Statistical  
524 analysis was performed by Student's *t*-test compared to the Col-0 control, error bars = SD, n  
525 = 8–10.

526

527 **Figure 2. Primary root growth of the *cnx1 cnx2* double mutant under high and low Pi**  
528 **conditions. (A)** Primary root length of Col-0 compared to *cnx1-1* and *cnx2-2* single and  
529 double mutants. **(B)** Complementation of the primary root phenotype of *cnx1-1 cnx2-2* plants  
530 transformed with the CNX1:GFP or CNX2:GFP construct. Plants were grown for 7 days on  
531 plates containing 1 mM Pi (HPi) or 75  $\mu$ M Pi (LPi) before measuring primary root length.  
532 Statistical analysis was performed by two-way ANOVA followed by a Tukey's test, and  
533 significant differences compared to Col-0 in each growth condition are shown: \*\*, P < 0.01;  
534 \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001; error bars = SD; n  $\geq$  9.

535

536 **Figure 3. Primary root growth of mutants in genes involved in ER protein synthesis and**  
537 **quality control. (A-B)** Plants were grown for 7 days on plates containing HPi or LPi before  
538 measuring primary root length. **(C-D)** Primary root length of Col-0 and *cnx1-1 cnx2-2* plants  
539 after 7 days of growth on HPi plates (C) without or with 200 mM mannose or (D) without or  
540 with 100 mM NaCl. **(E-F)** Primary root length of Col-0 and *cnx1-1 cnx2-2* after 7 days of  
541 growth on plates containing HPi or LPi half-strength MS medium or the same medium with  
542 ferrozine to chelate Fe (HPi -Fe and LPi -Fe). Statistical analysis was performed by two-way  
543 ANOVA followed by a Tukey's test, and significant differences compared to Col-0 in each  
544 growth condition are shown, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001, error bars  
545 = SD, n  $\geq$  5. Bar represents 1 cm in F.

546

547 **Figure 4. The *cnx1-1 cnx2-2* double mutant is affected in meristem activity. (A-C)** Plants  
548 were grown for 7 days on plates containing HPi or LPi before measuring the length of the cell  
549 division zone in the meristem, defined in A by the red and red arrows (A, B) and cell length in  
550 the differentiation zone (C). Statistical analysis (B, C) was performed by two-way ANOVA  
551 followed by a Tukey's test; significant differences compared to Col-0 under each growth  
552 condition are shown: \*\*\*\*, P < 0.0001; error bars = SD; n  $\geq$  5 in (B) and 20 in (C). **(D)** Col-0  
553 and *cnx1-1 cnx2-2* plants transformed with the *cylinB1:GUS* reporter gene construct were  
554 grown for 7 days on plates containing HPi or LPi medium and stained for  $\beta$ -glucuronidase  
555 activity. Bars represent 50  $\mu$ m in A and 100  $\mu$ m in D.

556



557 **Figure 5. Fe accumulation and distribution in the roots of mutants grown under high**  
558 **and low Pi conditions.** Plants were grown for 7 days on plates containing 1 mM or 75  $\mu$ M Pi  
559 and subjected to Perls-DAB staining for Fe visualization. Bar represents 1 mm.

560

561 **Figure 6. Epistatic interactions among *cnx1-1 cnx2-2*, *lpr1-1 lpr2-1*, and *pdr2*.** Plants were  
562 grown for 7 days on HPi or LPi plates before recording primary root length. **(A)** Epistatic  
563 interaction between *cnx1-1 cnx2-2* and *lpr1-1 lpr2-1*. **(B)** Epistatic interaction between *cnx1-1*  
564 *cnx2-2* and *pdr2*. **(C)** A T-DNA cassette for *PDR2* overexpression under the control of the  
565 CaMV35S promoter (OEPDR2) was introgressed into Col-0, *cnx1-1 cnx2-2*, and *pdr2*.  
566 Statistical analysis was performed by two-way ANOVA followed by a Tukey's test, and  
567 significant differences within each growth condition are shown. Different lowercase letters (a,  
568 b, c, or d) indicate a significant difference with a P-value < 0.05, n  $\geq$  6.

569

570 **Figure 7. Impact of the *cnx1-1 cnx2-2* mutations on the expression of Pi deficiency and**  
571 **unfolded protein response marker genes.** **(A)** *CNX1* and *CNX2* expression in the shoots and  
572 roots of plants grown for 7 days in HPi or LPi medium. **(B)** Expression of the Pi deficiency  
573 markers *MGD3* and *PHT1;4* in the shoots and roots of Col-0 and *cnx1-1 cnx2-2* grown for 7  
574 days on HPi or LPi medium. **(C)** Induction of ER Unfolded Protein Response marker gene  
575 *bZIP60* in the shoots and roots of Col-0 at 24 h after the addition of 2 mM DTT and in the  
576 *cnx1-1 cnx2-2* double mutant compared to Col-0 grown under HPi or LPi conditions.  
577 Statistical analysis was performed by Student's *t*-test comparing different treatments (HPi and  
578 LPi for A and C, Control and DTT for C) and Col-0 vs. *cnx1-1 cnx2-2* (B, C), with significant  
579 differences indicated by asterisks:\*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001. Error bars = SD, n  
580 = 3.

581

582

583 **Supplemental data**

584

585 **Supplemental Figures**

586

587 **Figure S1. Localization of *CNX1::CNX1-GFP* and *CNX2::CNX2-GFP* in the ER.** **(A)**  
588 Expression of *CNX1::CNX1-GFP* and *CNX2::CNX2-GFP* in roots tips of transgenic *cnx1-1*  
589 *cnx2-2* plants. Bars = 10  $\mu$ m. **(B)** Transient co-expression of *CaMV35S::CNX1-GFP* and  
590 *CaMV35S::CNX2-GFP* with the ER marker ER-RFP in tobacco leaves.

591

592

593 **Supplemental Tables**

594

595 **Table S1.** List of mutants used in this study.

596 **Table S2.** Primer list.

597

598

599

600

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