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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

31 ¹ This work was supported by Swiss National Science Foundation (Schweizerische
32 Nationalfonds) grants 31003A-182462 and 31003A-159998 to Y.P.

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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 (H_2PO_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 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 Fe^{+2} to 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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160

161

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 3rd 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 μ 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).

195

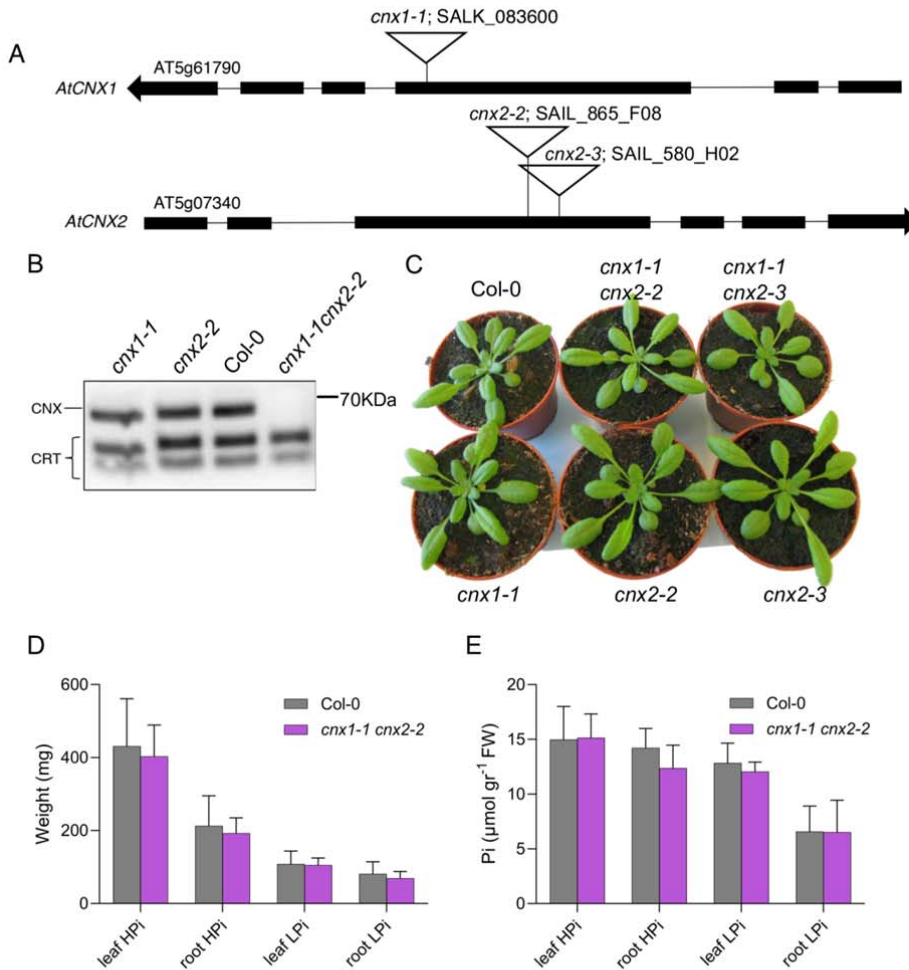


Figure 1. Phenotype of the *cnx1 cnx2* double mutant in soil. (A) Schematic diagram of the T-DNA insertions in the *CNX1* (At5g61790) and *CNX2* (At5g07340) genes in the *cnx* mutants. Exons are shown as black boxes. **(B)** Immunoblot analysis of CNX and CRT in whole protein extracts from seedlings. 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 μM 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

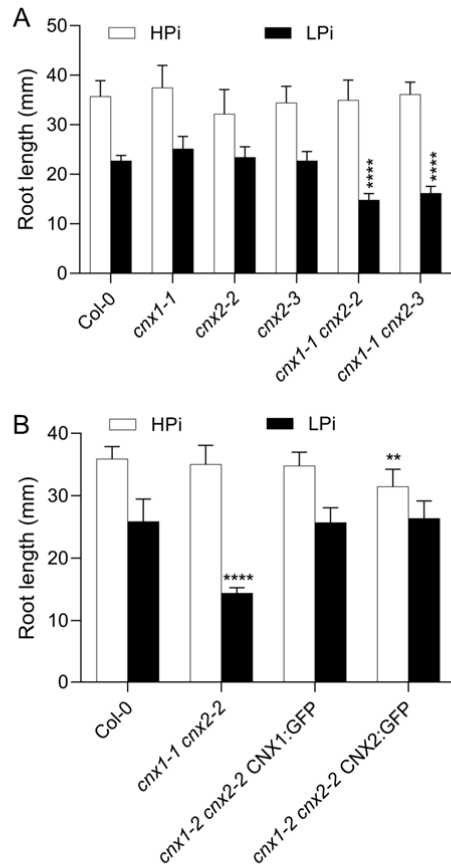


Figure 2. Primary root growth of the *cnx1 cnx2* double mutant under high and low Pi conditions. (A) Primary root length of Col-0 compared to *cnx1-1* and *cnx2-2* single and double mutants. **(B)** Complementation of the primary root phenotype of *cnx1-1 cnx2-2* plants transformed with the CNX1:GFP or CNX2:GFP construct. Plants were grown for 7 days on plates containing 1 mM Pi (HPi) or 75 μ 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 α 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 β -subunit of GCSII (Lu et

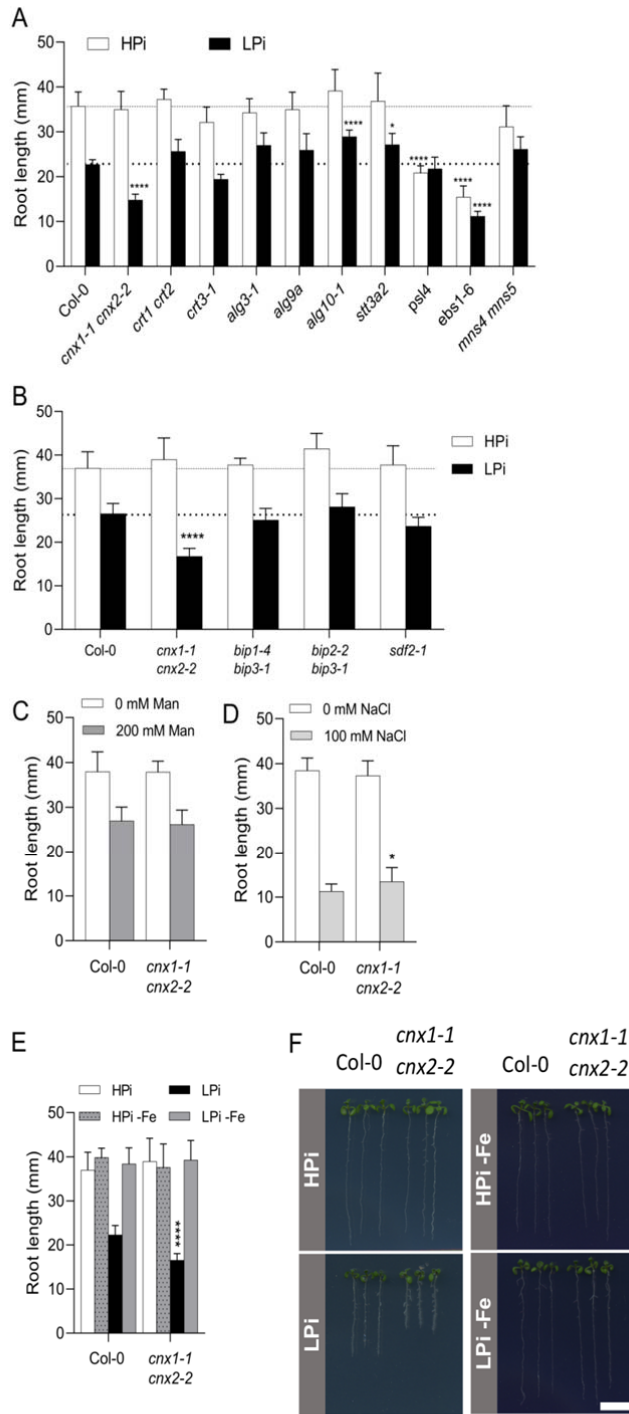


Figure 3. Primary root growth of mutants in genes involved in ER protein synthesis and quality control. (A-B) Plants were grown for 7 days on plates containing HPI or LPI before measuring primary root length. (C-D) Primary root length of *Col-0* and *cnx1-1 cnx2-2* plants after 7 days of growth on HPI 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 *ps14* 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 α -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).

227

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

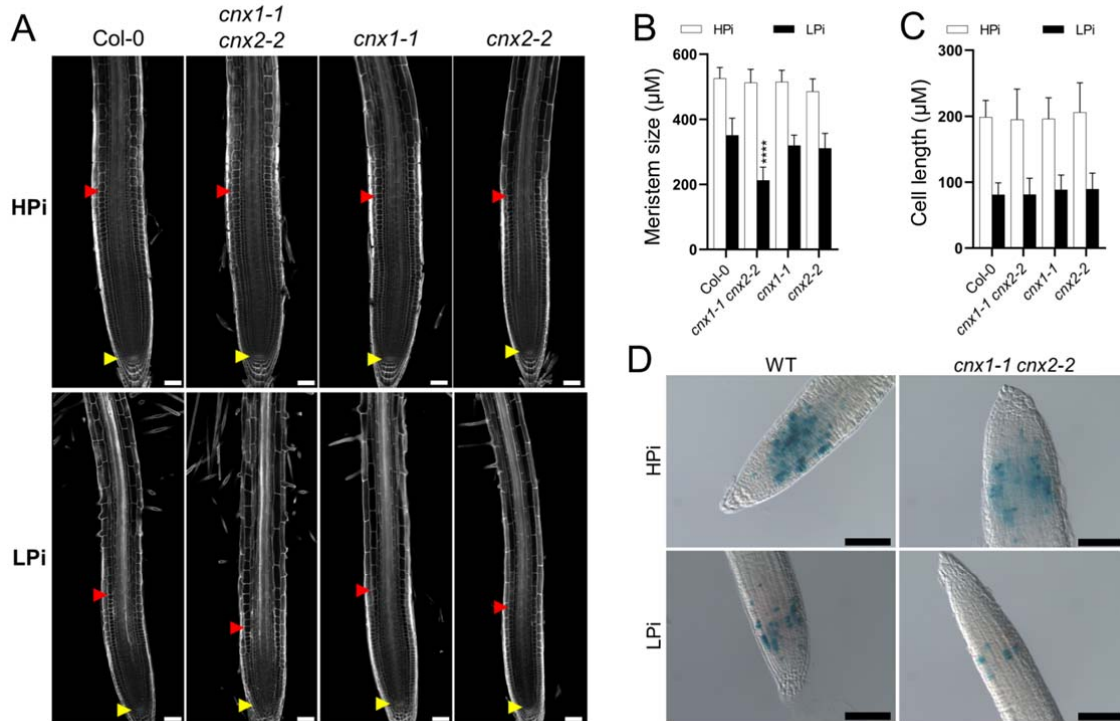


Figure 4. The *cnx1-1 cnx2-2* double mutant is affected in meristem activity. (A-C) Plants were grown for 7 days on plates containing HPI or LPi before measuring the length of the cell division zone in the meristem, defined in A by the red and red arrows (A, B) and cell length in the differentiation 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 β -glucuronidase activity. Bars represent 50 μ M in A and 100 μ 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).

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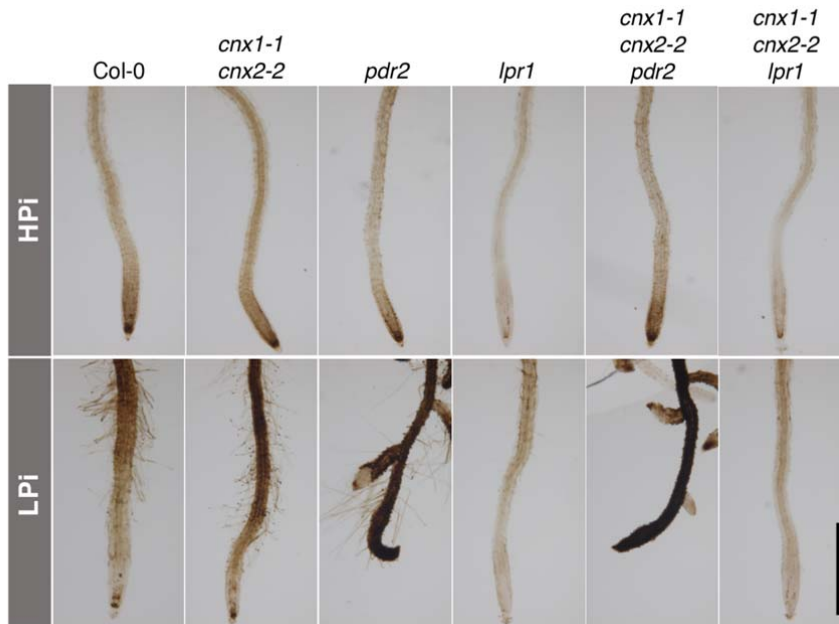


Figure 5. Fe accumulation and distribution in the roots of mutants grown under high and low Pi conditions. Plants were grown for 7 days on plates containing 1 mM or 75 μ M Pi and subjected to Perls/DAB staining for Fe visualization. Bar represents 1 mm.

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.

297

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

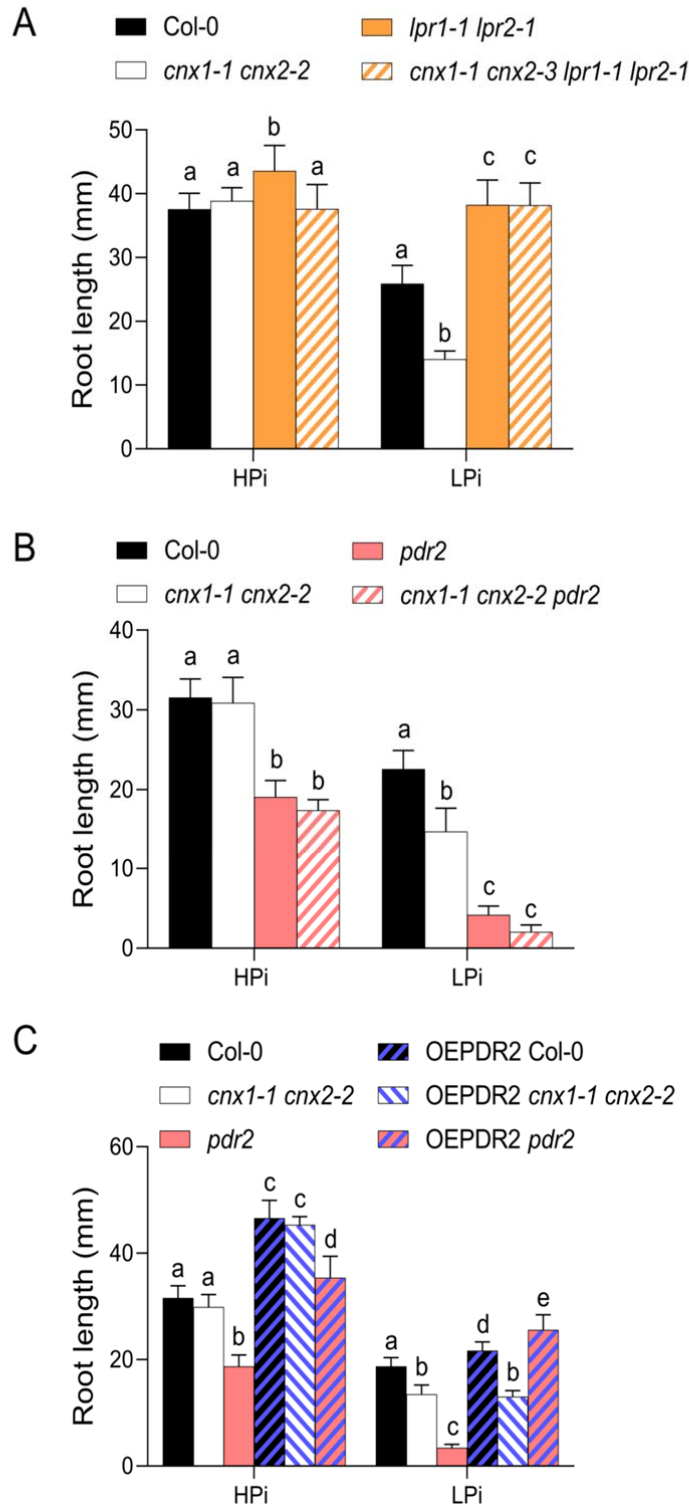


Figure 6. Epistatic interactions among *cnx1-1 cnx2-2*, *lpr1-1 lpr2-1*, and *pdr2*. Plants were grown for 7 days on HPi or LPi plates before recording primary root length. **(A)** Epistatic interaction between *cnx1-1 cnx2-2* and *lpr1-1 lpr2-1*. **(B)** Epistatic interaction between *cnx1-1 cnx2-2* 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.

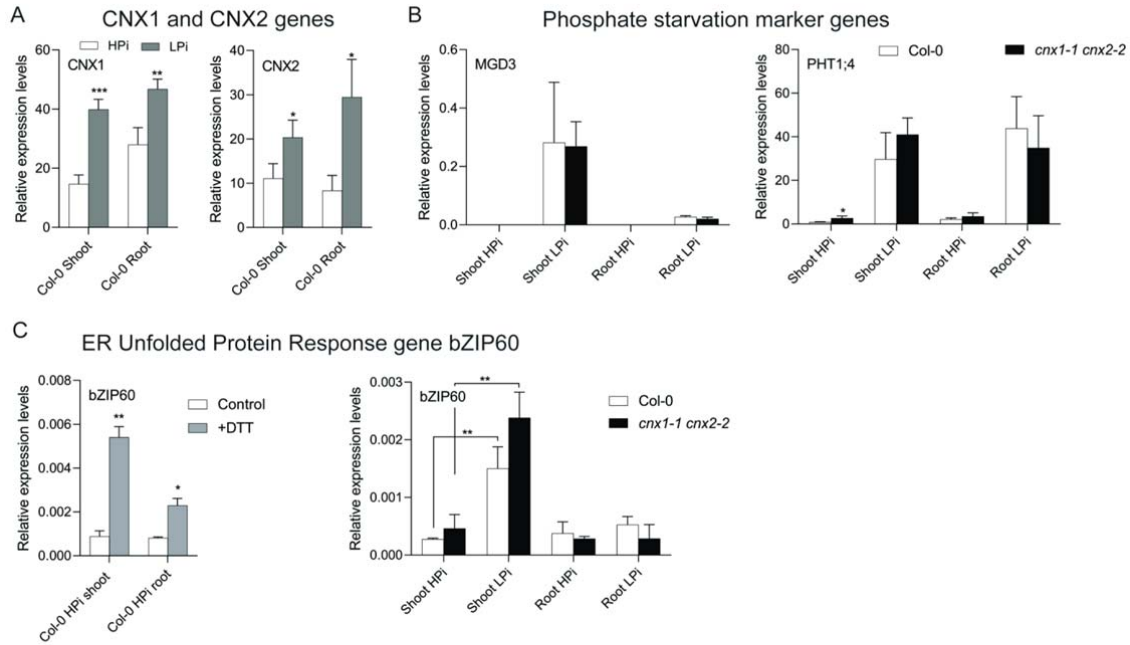


Figure 7. Impact of the *cnx1-1 cnx2-2* mutations on the expression of Pi-deficiency and unfolded protein response marker genes. (A) *CNX1* and *CNX2* expression in the shoots and roots of plants grown for 7 days in HPI or LPI medium. (B) Expression of the Pi-deficiency markers *MGD3* and *PHT1;4* in 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

323

324

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⁺² to Fe⁺³ (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 μM or 1 mM KH_2PO_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 μM . 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 KH_2PO_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)₁₅ 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 NaN₃ and 0.3% H₂O₂ 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) H₂O₂. 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 μ 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 μ 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 β -glucuronidase
555 activity. Bars represent 50 μ m in A and 100 μ 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 μ 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 μ 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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